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Correspondence Address
Trakya Üniversitesi Fen Bilimleri Enstitüsü Binası, Balkan Yerleşkesi – 22030 Edirne / TÜRKİYE
e-mail: tujns@trakya.edu.tr
Tel: +90 284 2358230
Fax: +90 284 2358237

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Research Article

Identification of benthic habitat types of the Çanakkale Strait coast using the European Nature Information System and the Barcelona Convention habitat classification schemes

Zeynep Tekeli [1*], Herdem Aslan [2]

[1] Çanakkale Onsekiz Mart University, School of Graduate Studies, Department of Biology, Çanakkale, TÜRKİYE
[2] Çanakkale Onsekiz Mart University, Faculty of Science, Department of Biology, Çanakkale, TÜRKİYE

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Abstract: The Çanakkale Strait is important in terms of its location within the Turkish Straits System and its biodiversity. Since it is a Special Environmental Protection Area (SEPA), it is important to determine the existing habitat types. Although the use of European Nature Information System (EUNIS) and Barcelona Convention (BC) habitat classification systems in marine habitat studies has just begun in Türkiye, studies using these systems have been carried out in numerous countries over many years. This study aimed to identify habitat types in the supra-, medio- and upper infralittoral (down to 0.5 m depth) zones of the Çanakkale Strait, in accordance with the EUNIS and the BC classifications. The SACFOR abundance scale was applied to zoo- and phytobenthic species in determining these habitat types. Field studies were carried out in soft and hard substrate areas at 16 stations between May and August 2019. Fourteen marine benthic habitat types have been determined using EUNIS and 12 using the BC systems from rock, biogenic, coarse and sand sediment types. No statistically significant difference ($p > 0.05$) was found between the benthic marine habitat types determined for the supra- and upper infralittoral zones according to BC and EUNIS. On the contrary, in the mediolittoral zone, a statistically low significant difference was found between habitat types for both habitat classification systems (R = 0.318, $p = 0.004$ for EUNIS; R = 0.514, $p = 0.001$ for BC). In this study, the differences and similarities of habitat types in defined EUNIS and BC systems are discussed. The EUNIS habitat classification system was found to be more representative than BC of the studied area, but both classification systems were found to be insufficient for the Eastern Mediterranean littoral communities and a new habitat hierarchy is needed. We present this study as a paradigm for future application to marine habitat studies to be carried out in Turkish waters.

Özet: Çanakkale Boğazı, Türk Boğazlar Sistemi içerisindeki konumu ve biyolojik çeşitliliği açısından önem taşımaktadır. Özel Çevre Koruma Bölgesi olması nedeniyle mevcut habitat tiplerinin belirlenmesi önemlidir. Türkiye'de deniz habitatı çalışmalarında Avrupa Doğa Bilgi Sistemi (EUNIS) ve Barselona Sözleşmesi (BC) habitat sınıflandırma sistemlerinin kullanımına yeni başlanmış olmasına rağmen, birçok ülkede bu sistemlerin kullanıldığı çalışmalar uzun yıllardan beri yürütülmektedir. Bu çalışma, Çanakkale Boğazı'nın supra, medio- ve üst infralittoral (0,5 m derinliğe kadar) bölgelerindeki habitat tiplerinin EUNIS ve BC sınıflandırmalarına uygun olarak belirlenmesini amaçlamıştır. Bu habitat tiplerinin belirlenmesinde zoo- ve fitobentik türlere SACFOR bolluk ölçeği uygulanmıştır. Mayıs ve Ağustos 2019 tarihleri arasında 16 istasyonda yumuşak ve sert substrat alanlarında saha çalışmaları gerçekleştirilmiştir. Kaya, biyojenik, kaba ve kum sediment tiplerinden EUNIS kullanılarak 14, BC sistemleri kullanılarak 12 deniz bentik habitat tipi belirlendi. BC ve EUNIS'e göre supra- ve üst infralittoral bölgeler için belirlenen bentik denizel habitat tipleri arasında istatistiksel olarak anlamlı bir fark bulunmamıştır ($p > 0.05$). Aksine, mediolittoral bölgede her iki habitat sınıflandırma sistemi için de habitat tipleri arasında istatistiksel olarak düşük anlamlı bir fark bulunmuştur (EUNIS için R = 0,318, $p = 0,004$; BC için R = 0,514, $p = 0,001$). Bu çalışmada EUNIS ve BC'deki habitat tiplerinin farklılıkları ve benzerlikleri tartışılmaktadır. EUNIS habitat sınıflandırma sisteminin çalışılan alanı BC'den daha iyi temsil ettiği görülmüştür, ancak her iki sınıflandırma sisteminin de Doğu Akdeniz kıyı toplulukları için yetersiz olduğu ve yeni bir habitat hiyerarşisine ihtiyaç duyulduğu tespit edilmiştir. Bu çalışmayı, Türkiye sularında gerçekleştirilecek deniz habitatı çalışmalarına gelecekte uygulanabilecek bir örnek olarak sunuyoruz.

Edited by:
Graham Saunders

Corresponding Author:
Zeynep Tekeli
zeynep.tekeli571@gmail.com

ORCID iDs of the authors:
ZT. 0000-0002-3539-6805
HA. 0000-0002-0872-2919

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Introduction

The Çanakkale Strait, which is part of the Turkish Straits System (TSS), connects the Aegean Sea, the Mediterranean Sea and the Black Sea. The length of the Strait is approximately 70 km (Aslan-Cihangir & Pancucci-Papadopoulou 2011), with the narrowest point at Nara Cape (Oğuz & Sur 1989) and an average depth of 55 m (Aslan-Cihangir & Pancucci-Papadopoulou 2011). The Strait has a reverse two-layer current system (Oğuz & Sur 1989). In this system, the less saline upper layer water from the Black Sea flows through the Çanakkale Strait into the Aegean Sea, while the saltier lower layer water originating from the Mediterranean Sea moves through the Çanakkale Strait into the Black Sea. The Çanakkale Strait is a biological corridor that limits the passage of some species (Öztürk & Öztürk 1996, Aslan-Cihangir *et al*. 2009) as well as an important waterway that allows some pollutants to pass to the Aegean Sea (Aslan *et al*. 2021).

The Çanakkale Strait, the Marmara Sea and the İstanbul Strait are enclosed within the Special Environmental Protection Area (SEPA) boundary (T.C. Çevre, Şehircilik ve İklim Değişikliği Bakanlığı 2021). In addition, the Strait is subject to intense ship traffic due to its geographical location, and there is also significant urbanization in the coastal areas. In this study, we focused on the supra-, medio- and upper infralittoral zones of the Çanakkale Strait. The sediments of the supralittoral zone have a high level of water retention (Gili *et al.* 2014) and are situated directly adjacent to the terrestrial area. The mediolittoral zone is tidal, with water periodically retreating and returning, and species living here are physiologically tolerant of this phenomenon (Gili *et al.* 2014). The infralittoral zone is permanently immersed in water, extending to the depths where marine spermatophytes and photophilic algae can be seen (Gili *et al.* 2014). The Mediterranean Sea is, however, generally acknowledged to have little or no tidal movement in its coastal waters (McElderry 1963). It has been estimated that 50% of mediolitoral habitats and 27% of infralittoral habitats are vulnerable and 37% of infralittoral habitats are endangered in the EU28 countries bordering the Mediterranean according an the IUCN Red List assessment (Gubbay *et al.* 2016).

The coastal region constitutes a complex ecosystem (Dethier & Harper 2011) in which benthic marine habitats exhibit high ecological variation (Sokołowski *et al.* 2021) and support productive areas where a large range of biota perform vital activities (Henseler *et al.* 2019). However, these habitats are affected by human-induced effects as well as environmental factors such as currents and waves. An effective way to monitor and maintain biodiversity is to evaluate spatial change in the mapped extent and distribution of benthic marine habitats, together with the associated changes in the community composition of the marine invertebrates, algae and spermatophyte species that characterize these habitats. These maps are required, not only for national authorities, but also for the statutory reporting obligations set out in European Union (EU) Directives such as the Habitats Directive (HD) (Council Directive 92/43/EEC) (European Council 1992) and the Marine Strategy Framework Directive (MSFD) (Directive 2008/56/EC) (European Union 2008). A number of habitat classification systems have been developed for marine protection purposes, such as CORINE (Devillers *et al.* 1991), JNCC (Connor *et al.* 2004), HELCOM HUB (Wijkmark *et al.* 2015). However, these classification systems broadly only apply to marine habitats on a regional scale and are not intended to operate over larger scales. For this reason, the European Nature Information System (EUNIS), a collective pan-European classification system covering all habitats including terrestrial, freshwater and marine, has been developed (Davies *et al.* 2004). Apart from the EUNIS classification system, a separate system developed under the Barcelona Convention and adopted in 1998 (Montefalcone *et al.* 2021), constitutes the first classification system specifically for benthic marine habitat types in the Mediterranean Sea. Montefalcone *et al.* (2021) has subsequently produced a revised version of the Barcelona Convention (BC) habitat classification system.

The EUNIS habitat classification is divided by region into Atlantic (ATL), Arctic (ARC), Baltic (BAL), Black Sea (BLS), Mediterranean (MED) and all seas (all). At the coarsest level marine benthic habitat types in the EUNIS classification system are defined by substrate: rock (M1), biogenic (M2), coarse (M3), mixed (M4), sand (M5) and mud (M6) (Table 1) (European Environment Agency 2022). There are six levels in EUNIS (M: first level, MA2: second level, MA22: third level, MA227: fourth level, MA2271: fifth level and MA22711: sixth level). As in the EUNIS classification system, the BC habitat classification system varies according to vertical zones, comprising littoral (A) (supra- and midlittoral), infralittoral (B), circalittoral (C) and beyond, together with substrate types such as rock (M1), biogenic (M2), coarse (M3), mixed (M4), sand (M5) and mud (M6) (Table 1) (Montefalcone *et al.* 2021). There are five levels in the BC classification system (M: first level, MA1: second level, MA1.5: third level, MA1.53: fourth level, and MA1.532: fifth level).

Table 1. Habitat codes in the EUNIS habitat classification system and in the BC habitat classification system according to substrate types.

Zones/Substrate types	Rock	Biogenic habitat	Coarse	Mixed	Sand	Mud
Littoral	MA1	MA2	MA3	MA4	MA5	MA6
Infralittoral	MB1	MB2	MB3	MB4	MB5	MB6

Marine habitat mapping studies have been undertaken along the coasts of other seas worldwide for many years (Riedl 1959, Bakran-Petricioli *et al.* 2006, Barberá *et al.* 2012, Monteiro *et al.* 2013, Galparsoro *et al.* 2015, Henriques *et al.* 2015, Rolet *et al.* 2015, Sokołowski *et al.* 2021, Vasquez *et al.* 2023, etc.). Nevertheless, Gubbay *et al.* (2016) point out that there remains a lack of data on 49% of all Mediterranean habitats within the national boundaries of EU28 countries, extending from the mediolittoral to the circalittoral zone, further noting that the majority of Mediterranean marine habitats and zones have been under-reported and poorly studied. Marine studies incorporating a habitat mapping approach have, however, recently begun in Türkiye, but there are presently only very few studies that have utilized the EUNIS habitat classification system (Topaloğlu *et al.* 2016, Aslan *et al.* 2018, 2019, Kaboğlu *et al.* 2022) in contrast to the greater number of terrestrial and freshwater studies (Mergen & Karacaoğlu 2015, Çakmak & Aytaç 2020, 2021, Demir *et al.* 2022, etc.).

The aims of this study were to undertake fauna and flora sampling at stations located in the supra-, medio- and upper infralittoral zones of the Çanakkale Strait, implementing the use of the SACFOR abundance scale to zoo- and phytobenthic species to: (i) determine the habitat types present according to EUNIS and BC habitat classification systems using zone, sediment type and zoo- and phytobenthic species information and (ii) undertake a critical comparison of identified EUNIS and BC habitat types.

Materials and Methods

Field Study

The study was carried out in three different zones under known marine influence (supra-, medio- and upper infralittoral zones) at a total of 16 stations in the Çanakkale Strait (Fig. 1, Table 2) in 2019. Data on the abundance of bivalve species in the medio and upper infralittoral are given by Tekeli & Aslan (2020). The sampling method and qualitative and quantitative properties of assemblages have been previously reported by Tekeli & Aslan (2023). For this study, the methods for standardizing species abundance and thus providing the basis for defining habitat types was achieved using the SACFOR scale (explained below). The taxonomy used follows WoRMS (2022).

SACFOR abundance criteria (Table 3) were used for the identified zoo- and phytobenthic species (Hiscock 1990). Among these criteria, the coverage criteria of some species in a marine habitat varies according to the substrate colonization pattern of the species (e.g. 'crust/meadow' or 'massive/turf') and the individual body size (<1 cm, 1–3 cm, 3–15 cm, >15 cm). According to these criteria, six abundance categories are defined: Super-Abundant (S), Abundant (A), Common (C), Frequent (F), Occasional (O), Rare (R), and Present (P) (Hiscock 1990).

Table 2. Codes, names and coordinates of studied stations (Tekeli & Aslan 2023).

Stations		Coordinates	
		Latitude	Longitude
CA	Cardak	40° 23′ 09″ N	26° 42′ 28″ E
SC	Suluca	40° 17′ 36″ N	26° 37′ 10″ E
KL	Kemiklialan	40° 16′ 54″ N	26° 36′ 01″ E
YL	Yapıldak	40° 13′ 51″ N	26° 32′ 17″ E
MB	Mega Beach	40° 08′ 27″ N	26° 23′ 58″ E
KP	Kepez	40° 05′ 31″ N	26° 21′ 53″ E
GZ	Güzelyalı	40° 02′ 02″ N	26° 20′ 18″ E
KM	Kumkale	40° 00′ 02″ N	26° 15′ 38″ E
GL	Gelibolu	40° 24′ 54″ N	26° 40′ 46″ E
ST	Sütlüce	40° 20′ 18″ N	26° 36′ 19″ E
BR	Burhanlı	40° 18′ 20″ N	26° 33′ 42″ E
AK	Akbaş	40° 13′ 48″ N	26° 26′ 03″ E
KY	Kilye	40° 12′ 12″ N	26° 21′ 29″ E
HZ	Havuzlar	40° 07′ 54″ N	26° 21′ 21″ E
SN	Soğanlıdere	40° 06′ 11″ N	26° 19′ 10″ E
MT	Morto	40° 03′ 02″ N	26° 12′ 54″ E

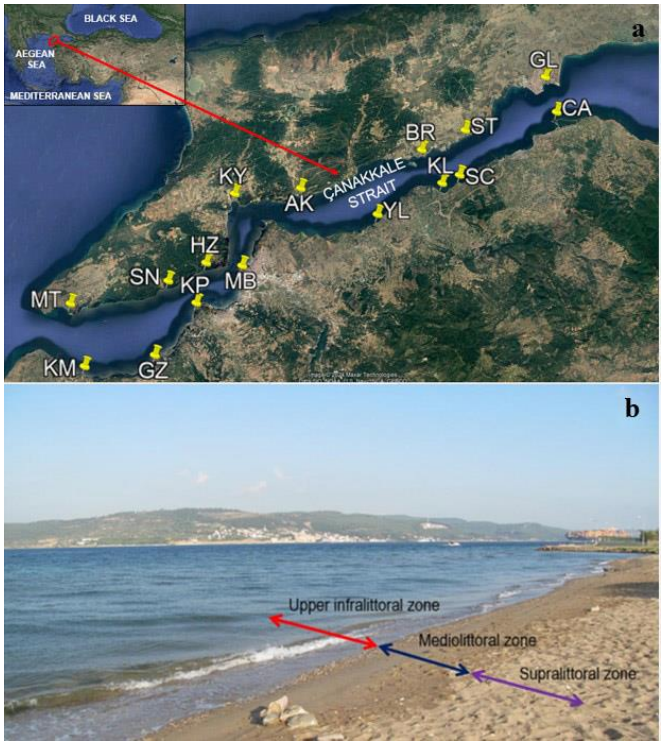

Fig. 1a. Map of stations studied in the Çanakkale Strait, Türkiye, **b.** an example of supra-, medio- and upper infralittoral zones at Burhanlı Station.

Table 3. SACFOR abundance scale: Super-Abundant (S), Abundant (A), Common (C), Frequent (F), Occasional (O), Rare (R), and Present (P) (Joint Nature Conservation Committee 2013).

% Cover scale	Growth form		Size of individuals/colonies				Density scale	
	Crust/Meadow	Massive/Turf	< 1 cm	1-3 cm	3-15 cm	> 15 cm		
> 80%	S		S				>1/0.001 m^2 (1 × 1 cm)	>10,000/m^2
40-79%	A	S	A	S			1-9/0.001 m^2	1000-9999/m^2
20-39%	C	A	C	A	S		1-9/0.01 m^2 (10 × 10 cm)	100-999/m^2
10-19%	F	C	F	C	A	S	1-9/0.1 m^2	10-99/m^2
5-9%	O	F	O	F	C	A	1-9/ m^2	
1-5% or density	R	O	R	O	F	C	1-9/10 m^2 (3.16 × 3.16 m)	
< 1% or density		R		R	O	F	1-9/100 m^2 (10 × 10 m)	
					R	O	1-9/1000m^2 (31.6 × 31.6m)	
						R	<1/1000 m^2	

Statistical analyses were undertaken using the SACFOR abundance values of zoo- and phytobenthic species, with values assigned to each of the SACFOR abundance categories (see Table 3). Non-metric MultiDimensional Scaling (n-MDS) and one-way ANOSIM analysis (to determine whether there are significant differences among EUNIS or BC) were performed for statistical interpretation of zoo- and phytobenthic species at the stations. In addition, one-way ANOSIM analysis was conducted separately for habitat types assigned to either the EUNIS or BC systems. These analyses were performed using the PRIMER version 7 statistical package program (Clarke & Gorley 2015).

Habitat types for the zones of Çanakkale Strait stations were simultaneously determined according to the habitat classification criteria established for the EUNIS and BC systems. In determining the habitat types, the general appearance of the area, zone differences, sediment types and the dominance of zoo- and phytobenthic species were taken into account along with expert knowledge. The identified zonal habitat types assigned to EUNIS and BC categories were mapped to their locations on the Çanakkale Strait coast using ArcGIS version 10.5.

Results

Overall, a total of 14 EUNIS and 12 BC habitat types were found to be present across all survey stations. These are shown in Table 4.

Their occurence at specific stations is outlined in the following.

Table 4. EUNIS and BC habitat types assigned to shore locations in the present study with their descriptions.

EUNIS	BC
Supralittoral Zone	
MA151-Biocenosis of Mediterranean supralittoral rock	**MA1.513-**Facies with Gastropoda and/or with Chthamalidae
MA25-Mediterranean littoral biogenic habitat	**MA2.54-**Banks of dead leaves of macrophytes (banquettes)
MA351-Assemblages of the slowly drying wracks biocenosis in Mediterranean supralittoral coarse sediment	**MA3.51b-**Beaches with slowly-drying wracks
MA551-Biocenosis of Mediterranean supralittoral sands	**MA5.51-**Supralittoral sand
Mediolittoral Zone	
MA153-Biocenosis of Mediterranean upper mediolittoral rock	**MA1.53-**Upper midlittoral rock
MA256-Assemblages of the mediolittoral detritus biocenosis characterized by temporal biogenic substrates	
MA2561-Facies of banks of dead leaves of *Posidonia oceanica*	**MA2.54-**Banks of dead leaves of macrophytes (banquettes)
MA352-Biocenosis of Mediterranean mediolittoral coarse detritus	**MA3.52-**Midlittoral coarse sediment
MA552-Biocenosis of Mediterranean mediolittoral sands	**MA5.52-**Midlittoral sand
Upper Infralittoral Zone	
MB151-Biocenosis of Mediterranean infralittoral algae	**MB1.51a-**Well illuminated infralittoral rock, exposed
MB35-Mediterranean infralittoral coarse sediment	**MB3.5-**Infralittoral coarse sediment
MB353-Biocenosis of Mediterranean infralittoral pebbles	**MB3.53-**Infralittoral pebbles
MB55-Mediterranean infralittoral sand	**MB5.5-**Infralittoral sand
MB551-Biocenosis of Mediterranean fine surface sands	**MB5.51-**Fine sand in very shallow waters

Table 5. Zoo- and phytobenthic species at stations in the supralittoral zone numbered using the SACFOR abundance scale (Super-abundant (S): 6, Abundant (A): 5, Common (C): 4, Frequent (F): 3, Occasional (O): 2, Rare (R) and Present (P): 1).

	Stations															
	CA	**SC**	**KL**	**YL**	**MB**	**KP**	**GZ**	**KM**	**GL**	**ST**	**BR**	**AK**	**KY**	**HZ**	**SN**	**MT**
PHYTOBENTHIC SPECIES																
OCHROPHYTA																
Cladosiphon sp.	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Colpomenia sp.	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cystoseira sp.	4	0	2	0	0	0	3	0	0	0	0	0	0	0	3	0
RHODOPHYTA																
Ceramium sp.	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
Gracilaria sp.	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
Polysiphonia sp.	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
CHLOROPHYTA																
Cladophora sp.	4	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
Ulva rigida C. Agardh, 1823	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0
Ulva sp.	1	0	0	0	0	0	3	0	0	0	2	0	0	0	0	0
TRACHEOPHYTA																
Cymodocea nodosa (Ucria) Ascherson, 1870	2	0	0	0	0	0	2	0	0	2	5	0	1	0	0	0
Posidonia oceanica (Linnaeus) Delile, 1813	0	0	0	0	0	0	3	0	0	0	1	0	0	0	0	0
Zostera marina Linnaeus, 1753	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Nanozostera noltei (Hornemann) Tomlinson&Posluszny, 2001	2	0	0	0	0	0	2	0	0	2	2	0	2	0	0	2
Unidentified spermatophytes	4	0	0	0	0	0	2	0	0	4	4	0	2	0	0	5
ZOOBENTHIC SPECIES																
MOLLUSCA																
Bittium reticulatum (da Costa, 1778)	0	0	0	0	4	0	0	4	0	0	0	0	0	3	0	0
Mangelia pontica Milaschewitsch, 1908	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0
Melarhaphe neritoides (Linnaeus, 1758)	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0
Pusillina radiata (R.A. Philippi, 1836)	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0
Rissoa splendida Eichwald, 1830	0	0	0	4	0	0	0	0	0	0	3	0	0	0	0	0
Tricolia sp.	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0
Truncatella subcylindrica (Linnaeus, 1767)	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0
Mytilus galloprovincialis Lamarck, 1819	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0
ANNELIDA																
Polychaeta sp.	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Capitella sp.	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0
Capitellidae sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
Orbiniidae sp.	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Oligochaeta sp.	4	3	5	4	0	6	6	0	0	6	3	5	5	5	0	6
ARTHROPODA																
Acaridae sp.	0	0	0	0	0	5	0	0	0	0	0	3	0	0	0	0
Ampithoe ramondi Audouin, 1826	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0
Armadillidium sp.	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0
Armadilloniscus ellipticus (Harger, 1878)	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0
Bodotria scorpioides (Montagu, 1804)	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
Caprella sp.	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0
Cryptorchestia cavimana (Heller, 1865)	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0
Halophiloscia couchii (Kinahan, 1858)	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0
Microdeutopus sp.	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Orchestia montagui Audouin, 1826	0	0	0	0	0	0	4	0	0	5	0	0	0	0	0	0
Orchestia sp.	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Speziorchestia stephenseni (Cecchini, 1928)	0	0	0	0	0	0	6	0	0	6	0	0	0	0	0	0
Talitrus saltator (Montagu, 1808)	0	0	4	0	0	4	0	0	0	0	0	0	0	0	0	0
Tylos latreillii Audouin, 1826	0	4	4	4	0	3	5	0	0	0	0	0	4	4	3	4
Chilopoda sp.	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0
Insecta spp.	3	4	5	0	0	5	4	0	0	0	2	4	0	0	3	0

Supralittoral Zone

Medium sand dominates the sediment samples taken in the supralittoral zones of the stations (Fig. 2). Since the supralittoral zone of Station GL has a hard bottom, no sediment samples could be taken from this station.

The SACFOR abundance values for a total of 42 species (29 zoobenthic and 13 phytobenthic) found in the supralittoral zone are provided in Table 5.

In the supralittoral zone, four habitat types were identified and assigned to both EUNIS and BC classifications.

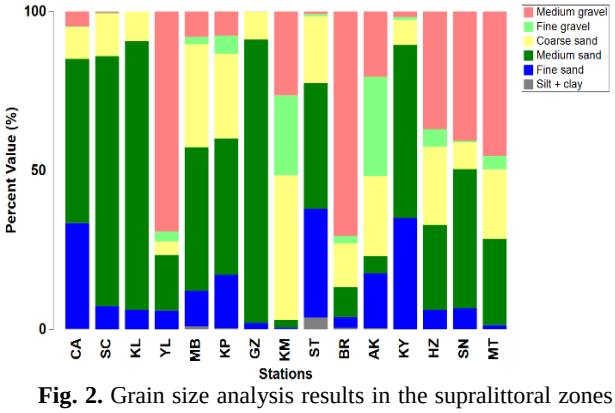

Fig. 2. Grain size analysis results in the supralittoral zones of the stations (%) (Modified from Tekeli & Aslan 2023).

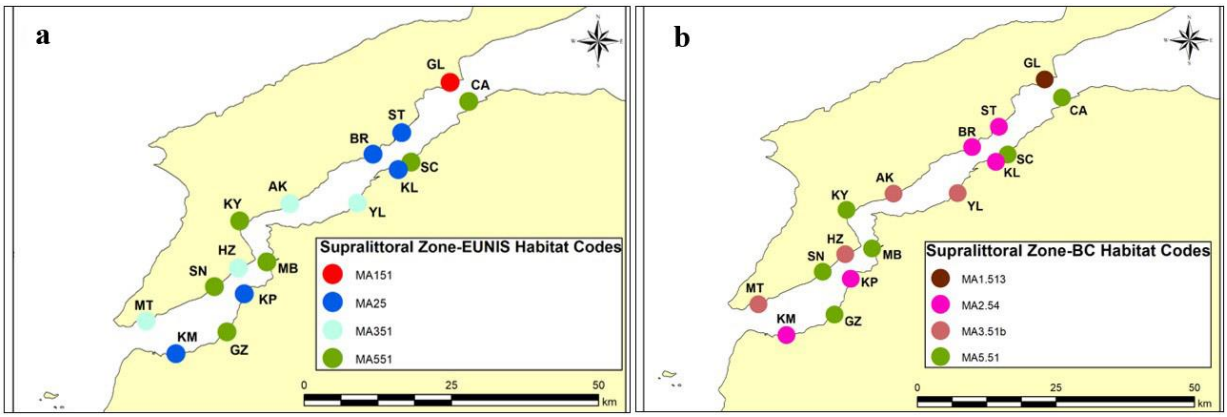

Fig. 3. Map representation of **a.** EUNIS, **b.** BC habitat types in the supralittoral zones of the stations (EUNIS Habitat Types present: MA151, MA25, MA351, MA551. BC Habitat Types present: MA1.513, MA2.54, MA3.51b, MA5.51). See Table 4 for habitat code definitions.

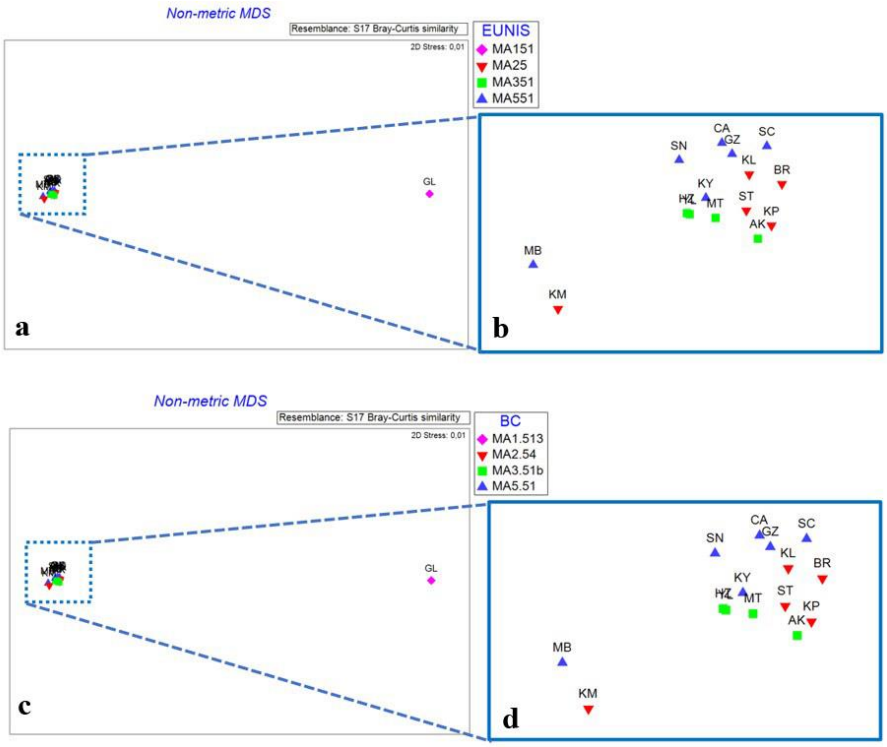

Fig. 4. n-MDS ordination as assigned to **a-b.** EUNIS, **c-d.** BC habitat types in the supralittoral zone (GL station is omitted in **b** and **d** section) (EUNIS Habitat Types: MA151-Biocenosis of Mediterranean supralittoral rock, MA25-Mediterranean littoral biogenic habitat, MA351-Assemblages of the slowly drying wracks biocenosis in Mediterranean supralittoral coarse sediment, MA551-Biocenosis of Mediterranean supralittoral sands; BC Habitat Types: MA1.513-Facies with Gastropoda and/or with Chthamalidae, MA2.54-Banks of dead leaves of macrophytes (banquettes), MA3.51b-Beaches with slowly-drying wracks, MA5.51-Supralittoral sand).

While the 3rd (MA25) and 4th (MA151, MA351 and MA551) level habitat types were determined in EUNIS (Fig. 3a), the 4th (MA2.54, MA3.51b and MA5.51) and 5th (MA1.513) level habitat types were also determined at lower hierarchical levels in the BC classification (Fig. 3b).

According to the n-MDS results in the supralittoral, the Station GL is statistically remote from all other stations due to its rocky nature both in the EUNIS (Figs 4a-b) and in the BC (Figs 4c-d) systems. For this reason, Station GL was omitted from further analyses to allow greater discrimination when considering the remaining stations (Figs 4b, 4d).

The results of the one-way ANOSIM analysis in the supralittoral, indicate that no statistically significant differences were present between the assigned sample zones in terms of both EUNIS (R = 0.039, p = 0.333) and BC (R = 0.039, p = 0.323) habitat types.

Mediolittoral Zone

Coarse sand (SC, KL, KP and GZ Stations), medium gravel (YL, BR, HZ Stations), medium sand (CA, MB, ST, KY, SN Stations), and fine sand (AK Station) dominate at some stations in the mediolittoral zones (Fig. 5). Sediment samples could not be taken from Stations GL and KM due to a hard bottom structure and a dense spermatophyte accumulation, respectively.

A total of 86 species (57 zoobenthic and 29 phytobenthic), were found in the mediolittoral zone and quantified using the SACFOR abundance scale. These are are shown in Table 6.

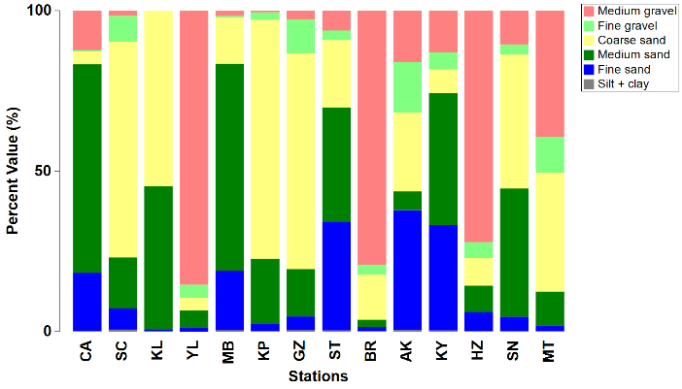

Fig. 5. Granulometric (%) analysis results for the mediolittoral zones of the stations (Modified from Tekeli & Aslan 2023).

Table 6. Zoo- and phytobenthic species at stations in the mediolittoral zone quantified according to the SACFOR abundance scale (Super-abundant: 6, Abundant: 5, Common: 4, Frequent: 3, Occasional: 2, Rare and Present: 1).

PHYTOBENTHIC SPECIES	Stations															
	CA	SC	KL	YL	MB	KP	GZ	KM	GL	ST	BR	AK	KY	HZ	SN	MT
OCHROPHYTA																
Cladosiphon sp.	2	0	1	4	0	0	0	0	0	0	0	0	0	0	0	0
Colpomenia sp.	3	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0
Cystoseira sp.	4	3	4	4	0	0	4	3	0	0	1	0	0	0	0	0
Dictyota sp.	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
Padina pavonica (Linnaeus) Thivy, 1960	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
RHODOPHYTA																
Ceramium virgatum Roth, 1797	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0
Ceramium sp.	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0
Corallina officinalis Linnaeus, 1758	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
Gracilaria sp.	0	0	1	0	0	0	3	2	0	1	3	0	0	0	0	0
Laurencia obtusa (Hudson) J.V.Lamouroux, 1813	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
Gelidium corneum (Hudson) J.V.Lamouroux, 1813	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0
Gelidium sp.	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0
Hypnea musciformis (Wulfen) J.V.Lamouroux, 1813	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
Jania rubens (Linnaeus) J.V.Lamouroux, 1816	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0
Leptosiphonia brodiei (Dillwyn) A.M.Savoie & G.W. Saunders, 2019	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0
Palisada perforata (Bory) K.W.Nam, 2007	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Polysiphonia sp.	4	3	1	3	0	0	3	0	1	0	0	1	0	0	0	0
CHLOROPHYTA																
Caulerpa cylindracea Sonder, 1845	0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0
Chaetomorpha sp.	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Cladophora sericea (Hudson) Kützing, 1843	0	0	0	0	0	0	0	0	4	0	0	2	0	0	0	0
Cladophora sp.	1	0	2	4	0	0	0	0	0	0	3	1	1	0	0	0
Codium fragile (Suringar) Hariot, 1889	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0
Ulva intestinalis Linnaeus, 1753	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0
Ulva rigida C.Agardh, 1823	0	0	0	0	0	5	0	0	4	0	0	0	0	0	0	0
Ulva sp.	4	4	0	0	0	0	4	3	2	0	2	1	0	0	0	0
TRACHEOPHYTA																
Cymodocea nodosa (Ucria) Ascherson, 1870	2	0	2	6	0	0	2	6	1	6	5	0	2	2	0	0
Zostera marina Linnaeus, 1753	2	0	2	5	0	0	0	0	0	2	0	0	0	0	0	0
Nanozostera noltei (Hornemann) Tomlinson & Posluszny, 2001	2	0	2	5	0	0	2	3	1	5	2	0	2	0	0	0
Posidonia oceanica (Linnaeus) Delile, 1813	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0
Unidentified spermatophytes	4	2	2	2	0	0	2	6	0	6	5	2	5	1	0	0

Table 6. Zoo- and phytobenthic species at stations in the mediolittoral zone quantified according to the SACFOR abundance scale (Super-abundant: 6, Abundant: 5, Common: 4, Frequent: 3, Occasional: 2, Rare and Present: 1) (Continued).

ZOOBENTHIC SPECIES	Stations															
	CA	SC	KL	YL	MB	KP	GZ	KM	GL	ST	BR	AK	KY	HZ	SN	MT
MOLLUSCA																
Acanthochitona crinita (Pennant, 1777)	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0
Alvania discors (T. Brown, 1818)	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	4
Gibbula turbinoides (Deshayes, 1835)	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
Melarhaphe neritoides (Linnaeus, 1758)	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0
Myosotella myosotis (Draparnaud, 1801)	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0
Ocenebra edwardsii (Payraudeau, 1826)	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
Pusia granum (Forbes, 1844)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
Pusia tricolor (Gmelin, 1791)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Pusillina marginata (Michaud, 1830)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Pusillina radiata (R. A. Philippi, 1836)	0	0	0	0	0	0	5	3	0	0	0	0	0	0	0	0
Rissoa decorata R. A. Philippi, 1846	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0
Rissoa guerinii Récluz, 1843	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0
Rissoa splendida Eichwald, 1830	0	0	0	0	0	0	3	4	0	0	3	4	0	3	0	3
Tricolia sp.	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0
Tritia neritea (Linnaeus, 1758)	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0
Truncatella subcylindrica (Linnaeus, 1767)	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0
Donacilla cornea (Poli, 1791)	0	0	4	0	0	0	0	0	0	0	0	3	0	0	0	3
Hiatella arctica (Linnaeus, 1767)	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
Mytilaster lineatus (Gmelin, 1791)	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0
Mytilus galloprovincialis Lamarck, 1819	4	6	0	4	0	5	0	0	4	0	0	0	0	0	0	0
ANNELIDA																
Polychaeta sp.	0	0	0	3	0	0	4	0	0	0	0	0	0	0	0	0
Capitella sp.	0	0	0	0	0	0	4	0	0	0	3	3	0	0	0	3
Naineris laevigata (Grube, 1855)	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	4
Nereididae sp.	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	3
Namanereis pontica (Bobretzky, 1872)	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	5
Perinereis cultrifera (Grube, 1840)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Platynereis dumerilii (Audouin & Milne Edwards, 1833)	0	0	0	0	0	0	0	0	0	0	3	3	0	0	0	0
Saccocirrus papillocercus Bobretzky, 1872	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0
Syllis amica Quatrefages, 1866	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0
Syllis beneliahuae (Campoy, 1982)	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
Syllis sp.	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0
Oligochaeta sp.	4	6	4	6	4	6	6	6	4	5	5	4	5	3	0	4
NEMERTEA																
Nemertea sp.	0	0	0	0	0	0	4	0	5	0	4	6	0	0	5	5
ARTHROPODA																
Acaridae sp.	0	0	0	4	0	5	0	4	0	0	0	0	0	0	0	3
Apohyale crassipes (Heller, 1866)	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0
Apohyale perieri (Lucas, 1846)	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0
Armadilloniscus ellipticus (Harger, 1878)	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0
Bopyrus crangorum (Fabricius, 1798)	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0
Carcinus aestuarii Nardo, 1847	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0
Chondrochelia savignyi (Kroyer, 1842)	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0
Cryptorchestia cavimana (Heller, 1865)	0	0	4	0	0	3	0	0	0	0	0	0	0	0	0	0
Elasmopus brasiliensis (Dana, 1853)	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0
Eurydice affinis Hansen, 1905	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0
Halophiloscia couchii (Kinahan, 1858)	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0
Idotea balthica (Pallas, 1772)	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0
Orchestia montagui Audouin, 1826	0	0	4	6	0	0	0	4	0	0	0	0	0	0	0	0
Orchestia sp.	4	0	0	0	0	0	0	0	0	0	4	0	0	2	0	0
Pectenogammarus olivii (H. Milne Edwards, 1830)	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0
Protohyale (Protohyale) schmidtii (Heller, 1866)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
Speziorchestia stephenseni (Cecchini, 1928)	0	0	4	6	0	3	5	6	0	4	0	0	0	0	0	0
Sphaeroma serratum (J. C. Fabricius, 1787)	0	0	0	0	0	0	0	0	0	0	0	3	0	5	0	4
Stenothoe tergestina (Nebeski, 1881)	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0
Talitrus saltator (Montagu, 1808)	0	5	0	0	0	5	0	0	0	0	0	0	0	0	0	2
Tanais dulongii (Audouin, 1826)	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	2
Tylos latreillii Audouin, 1826	0	0	0	0	0	0	0	5	0	0	0	0	4	0	0	4
Insecta spp.	4	5	5	5	3	5	3	5	3	0	3	0	0	0	0	4
ECHINODERMATA																
Asterias rubens Linnaeus, 1758	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0

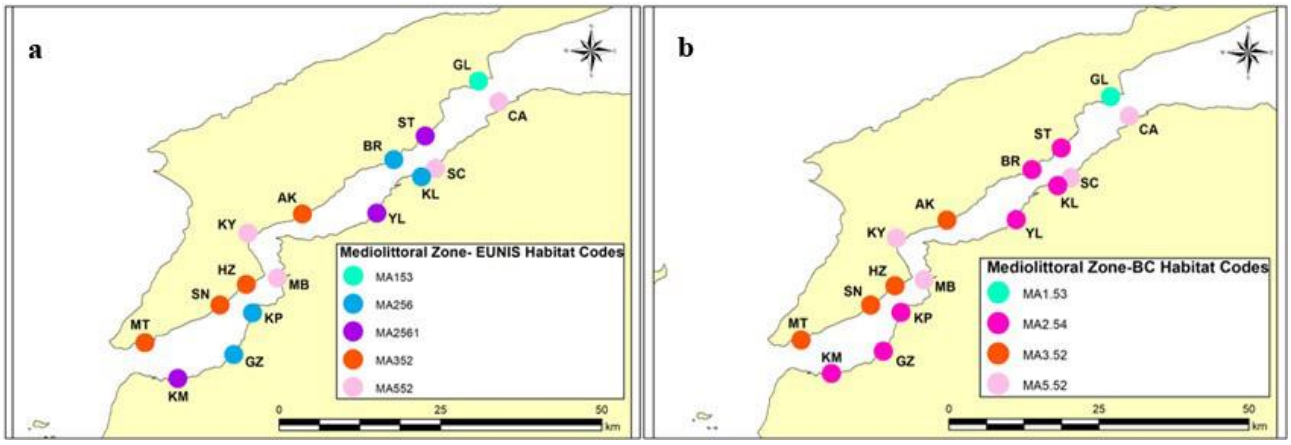

Fig. 6. Mapped locations of **a.** EUNIS, **b.** BC habitat types in the mediolittoral zones of the stations (EUNIS Habitat Types present: MA153, MA256, MA2561, MA352, MA552. BC Habitat Types present: MA1.53, MA2.54, MA3.52, MA5.52). See Table 4 for habitat code definitions.

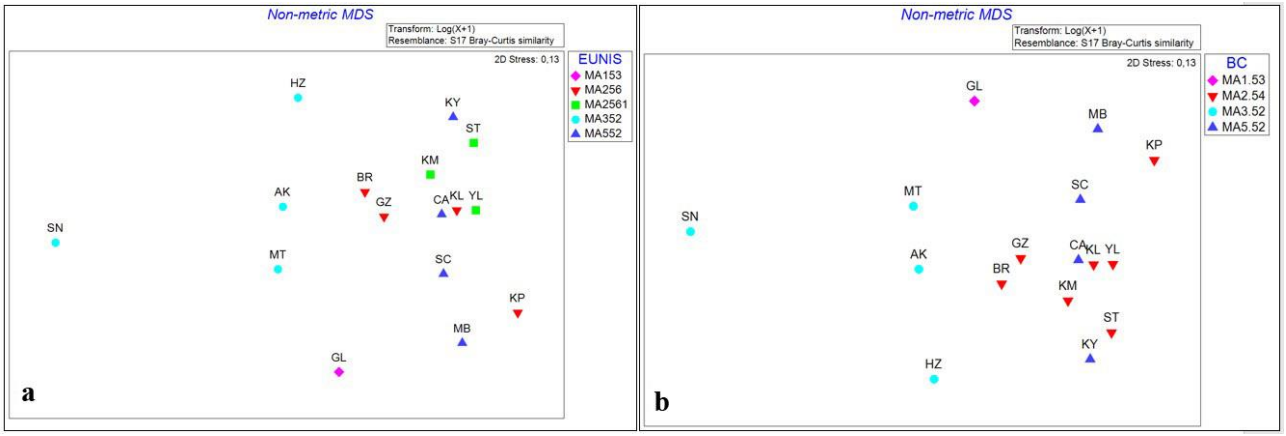

Fig. 7. n-MDS plot of communities assigned to **a.** EUNIS, **b.** BC habitat types in the mediolittoral zone (EUNIS Habitat Types: MA153-Biocenosis of Mediterranean upper mediolittoral rock, MA256-Assemblages of the mediolittoral detritus biocenosis characterised by temporal biogenic substrates, MA2561-Facies of banks of dead leaves of *Posidonia oceanica*, MA352-Biocenosis of Mediterranean mediolittoral coarse detritus, MA552-Biocenosis of Mediterranean mediolittoral sands; BC Habitat Types: MA1.53-Upper midlittoral rock, MA2.54-Banks of dead leaves of macrophytes (banquettes), MA3.52-Midlittoral coarse sediment, MA5.52-Midlittoral sand).

Five habitat types according to EUNIS and four according to the BC system were recorded in the mediolittoral zone. While lower hierarchical 4th (MA153, MA256, MA352 and MA552) and 5th (MA2561) level habitat types were determined using the EUNIS classification system (Fig. 6a), only 4th (MA1.53, MA2.54, MA3.52 and MA5.52) level habitat types were identified using the BC system (Fig. 6b).

The results of n-MDS analysis of the mediolittoral communities indicate that there is a clustering of stations aggregated according to assigned habitat types both in the EUNIS (Fig. 7a) and BC (Fig. 7b) systems.

The results of one-way ANOSIM analysis in the mediolittoral, indicate that there is a statistically significant difference between assemblages assigned to habitat types, both in terms of EUNIS (R = 0.318, p = 0.004) and BC (R = 0.514, p = 0.001), but the level of dissimilarity is low. Pairwise analysis results are given in Table 7 (for only those that are statistically significant).

Table 7. Pairwise analysis results for EUNIS and BC habitat types.

	R statistic	*p*-value
EUNIS		
MA552-MA352	0.573	0.029
MA256-MA352	0.323	0.029
MA2561-MA352	0.565	0.029
BC		
MA5.52-MA3.52	0.573	0.029
MA2.54-MA3.52	0.679	0.003

Upper Infralittoral Zone

Medium gravel dominates the samples taken in the upper infralittoral zone stations (Fig. 8). The upper infralittoral zones of Stations SC, KP and GL are characterised by hard substrata and therefore sediment samples could not be obtained from these three stations.

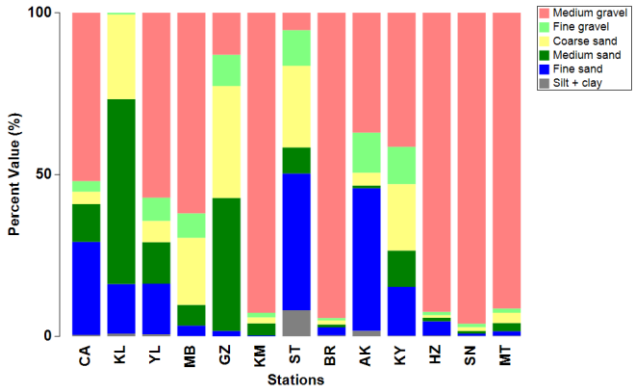

Fig. 8. Granulometric (%) analysis results in the upper infralittoral zones of the stations (Modified from Tekeli & Aslan 2023).

A total of 132 zoobenthic and 27 phytobenthic taxa, comprising 159 individual species were recorded using the SACFOR abundance scale in the upper infralittoral zone (Table 8).

Five EUNIS and five BC habitat types were identified in the upper infralittoral zone. For the EUNIS habitats (Fig. 9a), 3rd (MB35 and MB55) and 4th (MB151, MB353 and MB551) level habitat types were determined, while for the BC (Fig. 9b), 3rd (MB3.5, MB5.5) and 4th (MB1.51a, MB3.53 and MB5.51) level habitat types were identified.

The n-MDS analysis of the species abundance data confirms the community similarities with both the assigned EUNIS (Fig. 10a) and BC (Fig. 10b) habitats types forming discrete aggregations.

Table 8. Zoo- and phytobenthic species at stations in the upper infralittoral zone quantified according to the SACFOR abundance scale (Super-abundant: 6, Abundant: 5, Common: 4, Frequent: 3, Occasional: 2, Rare and Present: 1).

PHYTOBENTHIC SPECIES	Stations															
	CA	SC	KL	YL	MB	KP	GZ	KM	GL	ST	BR	AK	KY	HZ	SN	MT
OCHROPHYTA																
Cystoseira sp.	0	3	4	0	0	0	1	1	0	0	1	0	0	0	0	0
Padina pavonica (Linnaeus) Thivy, 1960	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0
Dictyota dichotoma (Hudson) J.V.Lamouroux, 1809	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0
Treptacantha barbata (Stackhouse) Orellana&Sansón, 2019	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0
RHODOPHYTA																
Ceramium virgatum Roth, 1797	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ceramium sp.	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Corallina officinalis Linnaeus, 1758	0	0	0	0	0	0	1	0	4	0	0	0	0	0	0	0
Dasya sp.	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
Gelidium corneum (Hudson) J.V.Lamouroux, 1813	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0
Gelidium sp.	0	0	0	0	0	0	1	0	3	0	0	0	0	0	0	0
Gracilaria sp.	0	1	0	0	0	0	0	2	0	0	0	0	0	0	0	0
Hypnea musciformis (Wulfen) J.V.Lamouroux, 1813	0	0	0	0	0	0	0	0	0	0	0	0	0	3	1	0
Laurencia obtusa (Hudson) J.V.Lamouroux, 1813	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
Leptosiphonia brodiei (Dillwyn) Savoie & Saunders, 2019	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0
Palisada perforata (Bory) K.W.Nam, 2007	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0
Polysiphonia sp.	0	0	0	2	0	0	1	0	2	0	0	0	0	0	1	0
CHLOROPHYTA																
Caulerpa cylindracea Sonder, 1845	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
Cladophora glomerata (Linnaeus) Kützing, 1843	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Cladophora laetevirens (Dillwyn) Kützing, 1843	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0
Cladophora sp.	0	0	0	0	0	0	2	1	2	0	3	0	0	0	0	3
Ulva intestinalis Linnaeus, 1753	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ulva prolifera O.F.Müller, 1778	0	0	0	0	0	0	2	0	2	0	0	0	0	0	0	0
Ulva rigida C.Agardh, 1823	0	4	0	0	0	3	3	0	0	0	0	0	0	0	0	0
Ulva sp.	0	1	0	0	0	0	0	2	2	0	0	0	0	3	0	1
TRACHEOPHYTA																
Cymodocea nodosa (Ucria) Ascherson, 1870	0	0	0	5	0	0	2	1	0	0	2	0	0	0	0	0
Nanozostera noltei (Hornemann) Tomlinson&Posluszny,2001	0	0	0	0	0	0	1	2	0	0	2	0	0	0	0	0
Posidonia oceanica (Linnaeus) Delile, 1813	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
Unidentified spermatophytes	0	0	1	0	0	0	1	4	1	0	2	0	0	0	0	0

Table 8. Zoo- and phytobenthic species at stations in the upper infralittoral zone quantified according to the SACFOR abundance scale (Super-abundant: 6, Abundant: 5, Common: 4, Frequent: 3, Occasional: 2, Rare and Present: 1) (Continued).

ZOOBENTHIC SPECIES	Stations															
	CA	**SC**	**KL**	**YL**	**MB**	**KP**	**GZ**	**KM**	**GL**	**ST**	**BR**	**AK**	**KY**	**HZ**	**SN**	**MT**
CNIDARIA																
Actinia equina (Linnaeus, 1758)	0	0	0	0	0	2	0	0	0	0	0	0	0	0	2	0
MOLLUSCA																
Acanthochitona crinita (Pennant, 1777)	0	0	0	3	0	0	0	0	2	0	0	0	0	0	0	0
Acanthochitona fascicularis (Linnaeus, 1767)	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Lepidochitona caprearum (Scacchi, 1836)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Alvania discors (T. Brown, 1818)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
Bittium latreillii (Payraudeau, 1826)	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
Cerithium vulgatum Bruguière, 1792	2	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0
Enginella leucozona (R. A. Philippi, 1844)	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0
Gibbula sp.	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0
Gibbula turbinoides (Deshayes, 1835)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Steromphala adansonii (Payraudeau, 1826)	0	0	0	2	0	0	0	0	0	0	0	0	0	4	0	0
Tornus subcarinatus (Montagu, 1803)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Tricolia pullus (Linnaeus, 1758)	0	0	0	0	0	0	0	0	2	0	2	0	0	0	0	0
Tricolia sp.	0	4	0	2	0	3	0	0	0	0	0	0	0	0	0	0
Tritia neritea (Linnaeus, 1758)	0	0	0	2	0	0	0	0	0	0	0	0	0	0	2	0
Tritia reticulata (Linnaeus, 1758)	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Truncatella subcylindrica (Linnaeus, 1767)	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	2
Pusillina lineolata (Michaud, 1830)	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
Pusillina radiata (R. A. Philippi, 1836)	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0
Rissoa similis Scacchi, 1836	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0
Rissoa splendida Eichwald, 1830	0	0	0	0	0	0	0	4	0	3	0	0	3	0	3	3
Arcuatula senhousia (W. H. Benson, 1842)	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0
Donacilla cornea (Poli, 1791)	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0
Hiatella arctica (Linnaeus, 1767)	0	0	0	0	0	2	0	0	3	0	0	0	0	0	0	0
Irus irus (Linnaeus, 1758)	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Musculus costulatus (Risso, 1826)	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0
Mytilaster lineatus (Gmelin, 1791)	0	4	0	0	0	4	2	0	5	0	0	0	0	0	0	3
Mytilus galloprovincialis Lamarck, 1819	0	6	0	3	3	6	0	0	4	0	0	0	0	0	0	0
Polititapes aureus (Gmelin, 1791)	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0
Ruditapes philippinarum (A. Adams & Reeve, 1850)	0	0	0	2	0	0	2	0	0	0	0	0	0	0	0	2
ANNELIDA																
Polychaeta sp.	2	3	4	3	0	3	2	0	3	2	4	0	0	2	2	4
Amphiglena mediterranea (Leydig, 1851)	0	2	0	2	0	3	2	0	0	0	0	0	0	0	0	2
Aphelochaeta sp.	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0
Brania sp.	0	2	5	0	0	2	0	0	0	0	0	0	0	2	2	3
Capitella sp.	0	0	3	4	0	2	3	0	0	3	4	3	3	2	2	4
Capitellidae sp.	0	0	0	3	0	0	0	0	0	0	0	0	0	2	0	3
Cirratulidae sp.	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	4
Cirratulus sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
Cirrophorus sp.	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Exogone dispar (Webster, 1879)	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
Exogone sp.	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	2
Malacoceros sp.	0	0	0	2	0	2	0	0	0	0	0	2	0	0	0	0
Naineris laevigata (Grube, 1855)	3	0	0	4	0	0	0	0	0	0	3	0	4	0	0	5
Nereididae sp.	0	2	0	4	0	3	2	0	3	0	4	0	0	3	0	4

Table 8. Zoo- and phytobenthic species at stations in the upper infralittoral zone quantified according to the SACFOR abundance scale (Super-abundant: 6, Abundant: 5, Common: 4, Frequent: 3, Occasional: 2, Rare and Present: 1) (Continued).

ZOOBENTHIC SPECIES	Stations															
	CA	SC	KL	YL	MB	KP	GZ	KM	GL	ST	BR	AK	KY	HZ	SN	MT
ANNELIDA																
Nereis sp.	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Notomastus latericeus Sars, 1851	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0
Notomastus lineatus Claparède, 1869	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Notomastus sp.	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Orbiniidae sp.	0	4	0	2	0	3	0	0	0	0	0	0	0	0	0	4
Paraonidae sp.	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0
Parexogone caribensis (San Martín, 1991)	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Perinereis cultrifera (Grube, 1840)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	4
Platynereis dumerilii (Audouin & Milne Edwards, 1833)	0	4	0	4	0	4	2	0	3	0	0	0	0	0	0	0
Prionospio sp.	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
Protoaricia oerstedii (Claparède, 1864)	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	3
Sabellidae sp.	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0
Saccocirrus papillocercus Bobretzky, 1872	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0
Salvatoria sp.	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	2
Scolelepis sp.	0	0	4	0	0	0	0	0	0	3	0	0	0	0	0	0
Spio decorata Bobretzky, 1870	0	0	0	4	0	0	2	0	0	0	0	0	0	0	0	0
Spio sp.	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0
Spionidae sp.	0	2	3	2	0	0	0	0	0	0	0	0	2	0	0	0
Sthenelais boa (Johnston, 1833)	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
Syllidae sp.	0	0	5	2	0	2	0	0	2	0	0	0	2	0	0	3
Syllides sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Syllis amica Quatrefages, 1866	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
Syllis beneliahuae (Campoy, 1982)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
Syllis krohnii Ehlers, 1864	0	2	0	0	0	0	0	0	0	0	0	0	0	0	2	2
Syllis sp.	0	0	0	2	0	2	0	0	3	0	0	0	0	0	0	0
Oligochaeta sp.	3	2	6	4	0	3	4	2	2	0	3	0	3	0	2	4
NEMERTEA																
Nemertea sp.	0	4	0	5	5	0	0	0	0	0	0	0	3	0	0	0
ARTHROPODA																
Acaridae sp.	0	2	0	0	0	2	0	0	0	0	2	0	0	0	0	0
Ampelisca diadema (A. Costa, 1853)	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0
Ampelisca sp.	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Amphipoda sp.	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0
Ampithoe ramondi Audouin, 1826	0	5	0	3	0	4	0	0	3	0	3	0	0	2	2	0
Anoplodactylus petiolatus (Krøyer, 1844)	0	3	0	0	0	0	0	0	3	0	0	0	0	0	0	0
Apherusa sp.	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Apocorophium acutum (Chevreux, 1908)	0	3	0	0	0	3	0	0	0	0	0	0	0	0	0	4
Apohyale crassipes (Heller, 1866)	0	3	0	0	0	0	0	2	3	0	0	0	0	0	0	0
Callianassa sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
Caprella mitis Mayer, 1890	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0
Caprella rapax Mayer, 1890	0	3	0	3	0	4	0	0	0	0	0	0	0	0	0	0
Carcinus aestuarii Nardo, 1847	0	4	0	4	0	4	0	0	0	0	0	0	0	0	0	3
Chondrochelia savignyi (Kroyer, 1842)	0	2	0	3	0	0	0	0	0	0	2	0	0	0	0	0
Clibanarius erythropus (Latreille, 1818)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Cumacea sp.	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Dexamine spinosa (Montagu, 1813)	0	0	0	3	0	3	0	0	0	0	2	0	0	0	0	0

Table 8. Zoo- and phytobenthic species at stations in the upper infralittoral zone quantified according to the SACFOR abundance scale (Super-abundant: 6, Abundant: 5, Common: 4, Frequent: 3, Occasional: 2, Rare and Present: 1) (Continued).

ZOOBENTHIC SPECIES	Stations															
	CA	**SC**	**KL**	**YL**	**MB**	**KP**	**GZ**	**KM**	**GL**	**ST**	**BR**	**AK**	**KY**	**HZ**	**SN**	**MT**
ARTHROPODA																
Diogenes pugilator (Roux, 1829)	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Diogenidae sp.	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
Dynamene bicolor (Rathke, 1836)	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0
Echinogammarus incertae sedis dahli (Stock, 1968)	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0
Pectenogammarus foxi (Schellenberg, 1928)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0
Echinogammarus sp.	0	3	0	0	0	0	0	2	0	0	0	0	0	0	0	0
Elasmopus brasiliensis (Dana, 1853)	0	4	0	0	0	0	0	0	0	0	0	0	0	0	2	0
Elasmopus pectenicrus (Spence Bate, 1863)	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0
Elasmopus pocillimanus (Spence Bate, 1863)	0	4	0	0	0	0	0	0	4	0	0	0	0	3	0	0
Ericthonius difformis H. Milne Edwards, 1830	0	3	0	3	0	0	0	0	0	0	0	0	0	0	0	0
Ericthonius sp.	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0
Gammaropsis sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
Gammarus subtypicus Stock, 1966	0	4	0	0	0	4	0	0	0	0	0	0	0	0	0	0
Harpacticoida sp.	0	2	3	2	0	3	0	0	0	0	0	0	0	0	0	3
Hyale pontica Rathke, 1836	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0
Idotea balthica (Pallas, 1772)	0	0	4	2	0	3	0	0	0	0	0	0	0	0	0	0
Idotea metallica Bosc, 1801	0	0	3	3	0	4	0	0	0	0	0	0	0	0	0	0
Janira sp.	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0
Jassa marmorata Holmes, 1905	0	0	0	3	0	3	0	0	0	0	0	0	0	0	0	0
Joeropsis brevicornis brevicornis Koehler, 1885	0	3	0	0	0	2	0	0	0	0	0	0	0	0	0	0
Lysianassa caesarea Ruffo, 1987	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0
Melita hergensis Reid, 1939	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0
Melita palmata (Montagu, 1804)	0	4	0	4	0	0	0	0	0	0	0	0	3	2	3	3
Microdeutopus anomalus (Rathke, 1843)	0	4	0	4	0	4	0	0	0	0	0	0	0	0	0	0
Microdeutopus bifidus Myers, 1977	0	3	0	4	0	0	0	0	0	0	0	0	0	3	0	4
Microdeutopus sp.	0	0	0	4	0	2	0	0	0	0	0	0	0	0	0	3
Monocorophium sextonae (Crawford, 1937)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
Pachygrapsus marmoratus (Fabricius, 1787)	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0
Pagurus sp.	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	2
Paraniphargus valesi (Karaman, 1955)	0	0	0	3	0	4	0	0	0	0	0	0	0	0	0	0
Perioculodes sp.	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pirimela denticulata (Montagu, 1808)	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
Protohyale (Protohyale) schmidtii (Heller, 1866)	0	4	0	0	0	0	0	0	0	0	0	0	0	0	4	0
Stenosoma capito (Rathke, 1836)	0	4	0	0	0	3	0	0	0	0	0	0	0	0	0	0
Stenothoe elachista Krapp-Schickel, 1975	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
Stenothoe monoculoides (Montagu, 1813)	0	4	0	3	0	4	0	0	3	0	0	0	0	0	0	0
Stenothoe tergestina (Nebeski, 1881)	0	4	0	0	0	2	0	0	4	0	0	0	0	0	0	0
Tanais dulongii (Audouin, 1826)	0	0	0	0	0	0	0	0	4	0	0	0	0	2	0	0
Urothoe poseidonis Reibish, 1905	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Xantho poressa (Olivi, 1792)	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0
Insecta spp.	0	0	0	3	3	4	0	0	3	2	4	0	0	4	4	5
ECHINODERMATA																
Amphipholis squamata (Delle Chiaje, 1828)	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
Asterias rubens Linnaeus, 1758	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0
Paracentrotus lividus (Lamarck, 1816)	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0

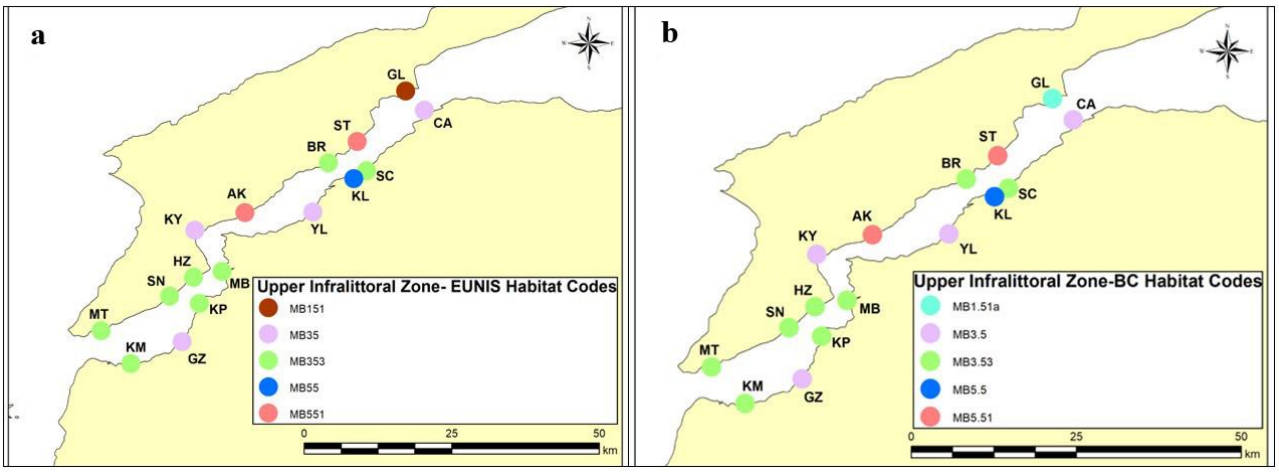

Fig. 9. Map representation of assigned **a.** EUNIS, **b.** BC habitat types in the upper infralittoral zones of the surveyed stations (EUNIS Habitat Types present: MB151, MB35, MB353, MB55, MB551. BC Habitat Types present: MB1.51a, MB3.5, MB3.53, MB5.5, MB5.51). See Table 4 for habitat code definitions.

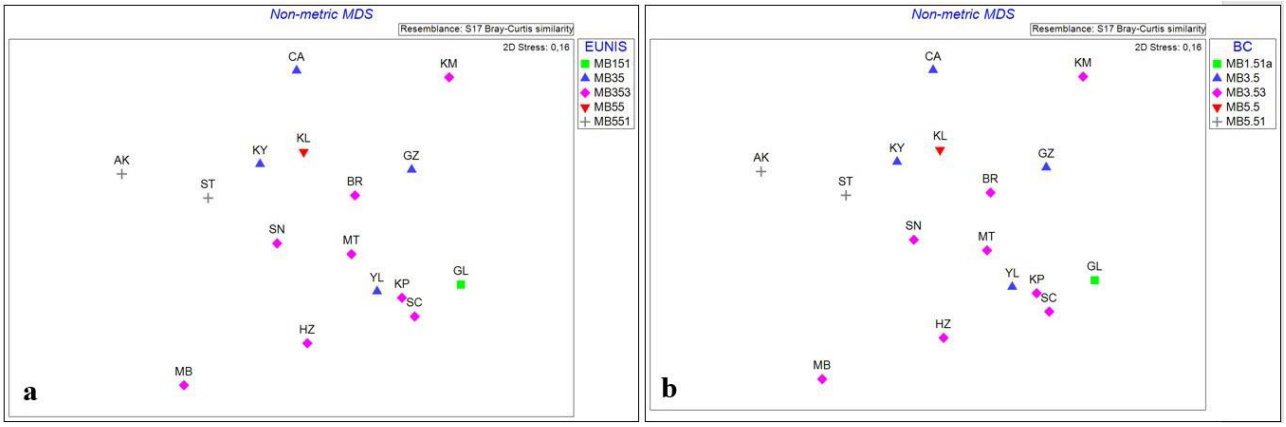

Fig. 10. n-MDS graph according to **a.** EUNIS, **b.** BC habitat types in the upper infralittoral zone (EUNIS Habitat Types: MB151-Biocenosis of Mediterranean infralittoral algae, MB35-Mediterranean infralittoral coarse sediment, MB353-Biocenosis of Mediterranean infralittoral pebbles, MB55-Mediterranean infralittoral sand, MB551-Biocenosis of Mediterranean fine surface sands; BC Habitat Types: MB1.51a-Well illuminated infralittoral rock, exposed, MB3.5-Infralittoral coarse sediment, MB3.53-Infralittoral pebbles, MB5.5-Infralittoral sand, MB5.51-Fine sand in very shallow waters).

One-way ANOSIM analysis of the the upper infralittoral samples, however, was unable to detect a statistically significant difference in the communities, either in terms of EUNIS ($R = 0.151$, $p = 0.165$) or BC ($R = 0.151$, $p = 0.182$).

Discussion

In this study we have identified 14 habitat types that broadly align to the EUNIS and 12 to the BC habitat classification systems for the supra-, medio- and upper infralittoral zone (Table 4).

Supralittoral zone

- Habitat type MA151 in EUNIS refers in general terms to supralittoral rock substrata, while the BC habitat type MA1.513 introduces a biota element and specifically emphasizes the dominance of the Gastropoda, either in combination with, or replaced by, chthamaloid barnacle groups. Hard substratum was recorded at a single station (Station GL), where gastropods and barnacle species were dominant. The gastropod species observed in this habitat type was *Melarhaphe neritoides*, a locally common species.

- The EUNIS habitat type MA25 relates to biogenic habitats, either formed by live organisms or their remains, while the corresponding BC habitat type, MA2.54, specifically refers to the accumulation of macrophyte leaves. There are five stations that qualified as biogenic habitat types. These habitat types were assigned due to the observed establishment of spermatophytes in the supralittoral zone at stations KL, KM, ST and BR, and the accumulation of various dead algae and spermatophyte in the supralittoral zone at station KP. Abundant or super-abundant Oligochaeta sp. and abundant Insecta spp. were observed to characterize this habitat type.

- The two EUNIS habitat types MA351 and MA551 are almost directly equivalent to the BC types MA3.51b and MA5.51. Four stations had coarse sediment and six stations comprised sand habitat types. Sand and gravel ratios were decisive in allocating these coarse habitat types to the supralittoral zones at Stations YL, AK, HZ

and MT. Of the species recorded in this habitat type, Oligochaeta sp. varied from super-abundant to common, while the isopod *Tylos latreillei* was common. Sand habitat types were recorded where there was a predominance of sand content in the supralittoral zones of Stations CA, SC, MB, GZ, KY and SN. Here, Oligochaeta sp. also varied from super-abundant to common, while *Tylos latreillei* was common or abundant and Insecta spp. were common.

Mediolittoral zone

- The EUNIS MA153 and BC MA1.53 habitat types are essentially identical. Station GL, which was the only one to have hard substrata, is a suitable fit for this habitat type. Species detected within this habitat type included *Mytilaster lineatus* (super-abundant), Nemertea sp. (abundant) and *Tanais dulongii* (abundant).

- While the MA256 habitat type in EUNIS does not have an exact equivalent in BC, the MA2561 habitat type in EUNIS corresponds closely to the BC MA2.54 habitat type. There are seven stations that qualify as biogenic habitat types. For biogenic habitat types, the EUNIS and BC hierachical levels differ. The observed spermatophyte and algae accumulation was critical in assigning the EUNIS habitat type MA256 to the mediolittoral zones of Stations KL, KP, GZ and BR. The species observed in this habitat type were: Insecta spp. (abundant) and Oligochaeta sp. which varied from super-abundant to common. *Speziorchestia stephenseni* was common or abundant. Conversely, due to the high accumulation of marine spermatophytes (*Cymodocea nodosa*, *Nanozostera noltei* and Unidentified spermatophytes), the MA2561 habitat type (EUNIS), which is one level below the MA256 habitat type, was allocated to the mediolittoral zones of Stations YL, KM and ST. The species recorded in this habitat type were: *Nanozostera noltei* (abundant), Acaridae sp. (common), Unidentified spermatophytes (super-abundant), Insecta spp. (abundant), and Oligochaeta sp. (abundant or super-abundant). *Orchestia montagui* and *Speziorchestia stephenseni* are common or super-abundant, while *Cymodocea nodosa* was super-abundant. The BC, MA2.54 habitat type was assigned to the mediolittoral of Stations KL, YL, KP, GZ, KM, ST and BR. The species observed in this habitat type were: Insecta spp. (abundant), Oligochaeta sp. and *Speziorchestia stephenseni* (both varying between super-abundant and common. *Cymodocea nodosa* and Unidentified spermatophytes were abundant or super-abundant.

- EUNIS habitat types MA352 and MA552 are equivalent to BC habitat types MA3.52 and MA5.52. Mediolittoral zones in four stations incorporate coarse sediment habitat types and four stations with sand habitat types. Coarse sediment habitat types (MA352, MA3.52) were assigned due to the dominance of gravel and sand substrate in the mediolittoral zones of Stations AK, HZ, SN and MT. The species recorded in this habitat type were: Nemertea sp. (abundant or super-abundant), Oligochaeta sp. (common), *Rissoa splendida* (common) and *Sphaeroma serratum* (common to abundant). The mediolittoral zones of Stations CA, SC, MB and KY were characterized by sand, qualifying them as MA552 and MA5.52 typologies. The species recorded in these habitats were: Oligochaeta sp. (varying between super-abundant to common) and Insecta spp. (common or abundant).

Upper infralittoral zone

- While the MB151 habitat type of EUNIS emphasizes the dominance of the infralittoral algae, the BC habitat type MB1.51 is broadly similar, but deviates significantly by explicitly mentioning rock substratum and wave exposure. The upper infralittoral zone of Station GL comprises a rock habitat type. This habitat allocation was influenced by the presence of a hard bottom and an abundance of bivalves (abundant *Mytilaster lineatus*) and algae.

- The four EUNIS habitat types (MB35, MB353, MB55, MB551) and the four respectively aligned BC habitat types (MB3.5, MB3.53, MB5.5, MB5.51) are almost identical in terms of content. Twelve of the survey stations incorporate infralittoral coarse substrate types and three stations contain sand habitat types. In the upper infralittoral of Stations CA, GZ, YL and KY, the MB35 and MB3.5 types were selected because of the presence of coarse sediment. Species recorded in this habitat type were: *Capitella* sp., *Nainereis laevigata* and Oligochaeta sp., all with an abundance evaluated as common. Habitat types MB353 and MB3.53 were appropriate to the upper infralittoral zones of Stations SC, MB, KP, KM, BR, HZ, SN and MT, where there were significant deposits of pebble-sized stones. The species recorded in this habitat type were restricted to common or abundant Insecta spp. The upper infralittoral zone of station KL was sandy, and thus habitats MB55 and MB5.5 were considered appropriate. The biota was dominated by super abundant Oligochaeta sp., with abundant *Brania* sp. and Syllidae sp. Fine sand was found to be present in the shallow upper infralittoral of Stations ST and AK, leading to their assignment to the habitat types MB551 and MB5.51. *Capitella* sp. was recorded as frequent in this habitat type.

The EUNIS and BC habitat classification systems are intrinsically similar, but differences have emerged as each system has evolved through various updates. These differences are examined below in the context of our study.

i. Firstly, in the EUNIS system, the zone where biota are immersed in water only part of the time is referred to as the mediolittoral, while the BC classification system refers to this zone as the midlittoral. Thus the EUNIS and the BC habitat classification systems use different terms for the same zone.

ii. The BC habitat type MA2.54 was selected for either the supra- and mediolittoral at various stations, but different codes are required for this habitat type depending on the supra- and mediolittoral zones.

iii. In both the EUNIS and the BC classification systems, the mediolittoral zone is segregated into the two components, upper and lower. However, due to the small tidal range in the study area and the narrow area of the

mediolittoral zones even with modest shore inclines, it is difficult to distinguish these.

iv. While spermatophytes are an explicit component of the EUNIS biogenic habitat type MA2561, the BC classification system incorporates references to a wider group of marine macrophytes integrated into multiple different habitat types (MA1.51b, MA2.54, MA3.51, MA4.51, MA5.51). This causes differences between the two habitat classification systems which are difficult to resolve.

Dead mussel accumulations were a noticeable feature in the supra-, medio- and infralittoral zones at AK Station during the sampling. This is a consequence of mussel farming carried out in the vicinity of Station AK in the years before this survey. This accumulation is likely to persist in the long term and presently constitutes a specific attractant to marine biota. Thus these areas can be considered a type of littoral biogenic habitat that is currently not recognized in either the EUNIS or BC classification systems.

ANOSIM analysis results performed after applying EUNIS or BC habitat categorisation in the supra- and upper infralittoral zones of the stations show that there is no statistically significant difference in terms of EUNIS and BC habitat classifications. It is possible that the level of discrimination may currently be diminished because these habitat types have been largely developed and applied in the EU states west of Türkiye and neither classification system yet fully recognizes marine assemblages occurring exclusively within the Eastern Mediterranean.

The conservation of animal and plant species together with the protection of their wider environment is ultimately only possible by understanding the characteristics of the habitats that support these species and by conducting long-term monitoring activities to determine if any change is occurring, whether natural or anthropogenic. Habitat-targeted studies for environmental management or protection purposes have been undertaken in Türkiye, but these have been largely restricted to terrestrial and freshwater environments, with far fewer studies applied in marine areas. Topaloğlu *et al.* (2016) identified a total of 15 coastal and marine habitat types in the littoral part of Şile in the Western Black Sea, while Aslan *et al.* (2018) undertook sublittoral surveys to a depth of 30 m around the island of Gökçeada, recording substate and species abundance data in order to initiate the mapping of EUNIS habitat types in Turkish waters. Subsequently, Kaboğlu *et al.* (2022) identified 15 marine habitat types at a depth of 0-50 m in Foça SEPA, according to the EUNIS classification.

While still in its infancy in Türkiye, the development and use of habitat classification systems as descriptive ecological "units" has been occuring across the Mediterranean region for some time. One of the earliest efforts can be traced back to Riedl (1959), cited in the articles of Fraschetti *et al.* (2008), which described work on the classification of marine habitats on hard substrata in the Mediterranean, while Bakran-Petricioli *et al.* (2006) applied habitat classes to the mapping of marine habitats along the Croatian coast. Barberá *et al.* (2012) studied marine habitat types as defined by EUNIS and the Barcelona Convention in the Menorca Channel. Henriques *et al.* (2015) conducted a study on benthic habitat types according to EUNIS on the southwestern coast of Portugal. Galparsoro *et al.* (2015) proposed 13 new EUNIS habitat classes following surveys along the Spanish coast of the Bay of Biscay.

Beyond the Mediterranean, the use of marine habitat classification continues to expand across the realms of marine research, management and conservation. During the undertaking of the MeshAtlantic marine mapping project, the observations of Monteiro *et al.* (2013) resulted in a proposed 45 new EUNIS habitat types for the Atlantic coast. Rolet *et al.* (2015) applied the EUNIS classification system to studies of beaches, harbours and bays across northern France. Sokołowski *et al.* (2021) conducted a study on benthic habitat types in Puck Bay, Gdańsk Gulf, Poland. Vasquez *et al.* (2023), within the scope of the EMODnet Seabed Habitats project, identified 40 habitat types for the Mediterranean, from infralittoral to abyssal zone, according to EUNIS.

It is therefore clear that there has been a considerable European effort, often collaboratively, to establish and develop habitat classification as a powerful mapping and monitoring tool. Studies on the determination, distribution and stability of habitat types in Turkish seas have just begun and there are presently very few that have been published in the scientific literature. An additional urgency in this area has been raised by the International Union for the Conservation of Nature (IUCN), which recently employed the EUNIS classification system to establish a Red List of endangered marine habitats (Gubbay *et al.* 2016). This initiative, however, revealed serious data gaps in our knowledge of Eastern Mediterranean marine habitats that require urgent attention.

In conclusion, the similarities and differences of EUNIS and Barcelona Convention marine habitat types have been examined within the scope of a littoral survey in Türkiye. It was determined that the habitat components of the EUNIS habitat classification system represented the studied area better than BC. It is evident, however, that a new or expanded habitat hierarchy is likely to be needed for the Eastern Mediterranean.

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References

1. Aslan-Cihangir, H., Sezgin, M. & Tuncer, S. 2009. New records of two species of Amphipods, *Melphidippella macra* (Norman, 1869) and *Monocorophium sextonae* (Crawford, 1937) for Turkish seas. *Crustaceana*, 82(1): 111-116. https://doi.org/10.1163/156854008X363722

2. Aslan-Cihangir, H. & Pancucci-Papadopoulou, M.A. 2011. Aspects of Decapod Crustacean assemblages from soft bottoms submitted to strong hydrodynamic conditions: an example from Canakkale Strait (Turkish Strait System). *Fresenius Environmental Bulletin*, 20: 2400-2411.

3. Aslan, H., Saunders, G., Kaboğlu, G., Yokeş, M.B., Okudan, E.Ş. & Gönülal, O. 2018. Preliminary spatial study of the shallow marine communities of Gokceada Island using geolocational habitat mapping techniques, 191-196. Paper presented at the 13th International Symposium on Fisheries and Aquatic Sciences, FABA, 21-23 Kasım, Ankara-Türkiye.

4. Aslan, H., Saunders, G., Kaboğlu, G., Yokeş, M.B., Gönülal, O. & Okudan, E.Ş. 2019. EUNIS sınıflandırma şemasının Gökceada kıyı deniz habitatları haritalandırmasına uygulanması, 146-148. Paper presented at the II. Ulusal Denizlerde İzleme ve Değerlendirme Sempozyumu, 11-13 Aralık, Ankara-Türkiye.

5. Aslan, H., Tekeli, Z. & Bacak, Ö. 2021. Effects of mucilage on the benthic crustacean in the North Aegean Sea. *Journal of the Black Sea/Mediterranean Environment*, 27: 214-231.

6. Bakran-Petricioli, T., Antonić, O., Bukovec, D., Petricioli, D., Janeković, I., Križan, J., Kušan, V. & Dujmović, S. 2006. Modelling spatial distribution of the Croatian marine benthic habitats. *Ecological Modelling*, 191(1): 96-105. https://doi.org/10.1016/j.ecolmodel.2005.08.014

7. Barberá, C., Moranta, J., Ordines, F., Ramón, M., de Mesa, A., Díaz-Valdés, M., Grau, A.M. & Massutí, E. 2012. Biodiversity and habitat mapping of Menorca Channel (Western Mediterranean): implications for conservation. *Biodiversity and Conservation*, 21: 701-728. https://doi.org/10.1007/s10531-011-0210-1

8. Clarke K.R. & Gorley R.N. 2015. PRIMER v7: User manual/tutorial. PRIMERE, Plymouth, 296 pp.

9. Connor, D.W., Allen, J.H., Golding, N., Howell, K.L., Lieberknecht, L.M., Northen, K.O. & Reker, J.B. 2004. The Marine Habitat Classification for Britain and Ireland Version 04.05. JNCC, Peterborough ISBN 1 861 07561 8 (internet version) www.jncc.gov.uk/MarineHabitatClassification. (Date accessed: 28.02.2024).

10. Çakmak, M.H. & Aytaç, Z. 2020. Determination and mapping of EUNIS habitat types of Mamak District (Ankara), Turkey. *Acta Biologica Turcica*, 33: 227-236.

11. Çakmak, M.H. & Aytaç, Z. 2021. EUNIS habitat sınıflandırmasının Türkiye durum değerlendirmesi. *Bilge International Journal of Science and Technology Research*, 5(2): 157-163. https://doi.org/10.30516/bilgesci.888297

12. Davies, C.E., Moss, D. & Hill, M.O. 2004. EUNIS habitat classification revised 2004. Report to: European Environment Agency-European topic centre on nature protection and biodiversity, 127-143 pp.

13. Demir, O., Kızılırmaklı, A., Bozdağ, C.M. & Cabi, E. 2022. Determination of terrestrial EUNIS habitat types of Mount Ganos (Işıklar), Tekirdağ, Türkiye. *Frontiers in Life Sciences and Related Technologies*, 3(2): 69-74. https://doi.org/10.51753/flsrt.1109635

14. Dethier, M.N. & Harper, J. 2011. Classes of Nearshore Coasts. pp. 60-61 In: Wolanski, E. & McLusky, D. (eds.). *Treatise on Estuarine and Coastal Science*. Vol. 1. Classification of Estuarine and Nearshore Coastal Ecosystems. Academic Press, London, 327 pp.

15. Devillers, P., Devillers-Terschuren, J. & Ledant, J-P. 1991. *CORINE biotopes manual.* Habitats of the European Community. Office for Official Publications of the European Communities, Luxembourg, 300 pp.

16. European Council, 1992. Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora (Habitats Directive), OJ L206, 22.07.92. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A31992L0043. (Date accessed: 23.07.2024).

17. European Environment Agency, 2022. EUNIS habitats. https://www.eea.europa.eu/en/datahub/datahubitem-view/123d0c9a-a6fa-4f2d-b887-5d8e5468faed?activeAccordion=1069939%2C1069938%2C1085231. (Date accessed: 28.02.2024).

18. European Union, 2008. Directive 2008/56/EC of the European Parliament and of the Council of 17 June 2008 establishing a framework for community action in the field of marine environmental policy (Marine Strategy Framework Directive) [2008] OJ L164/19. https://eur-lex.europa.eu/eli/dir/2008/56/oj. (Date accessed: 23.07.2024).

19. Fraschetti, S., Terlizzi, A. & Boero, F. 2008. How many habitats are there in the sea (and where)? *Journal of Experimental Marine Biology and Ecology*, 366(1-2): 109-115. https://doi.org/10.1016/j.jembe.2008.07.015

20. Galparsoro, I., Rodríguez, J.G., Menchaca, I., Quincoces, I., Garmendia, J.M. & Borja, Á. 2015. Benthic habitat mapping on the Basque continental shelf (SE Bay of Biscay) and its application to the European Marine Strategy Framework Directive. *Journal of Sea Research*, 100: 70-76. https://doi.org/10.1016/j.seares.2014.09.013

21. Gili, J-M., Sardá, R., Madurell, T. & Rossi, S. 2014. Zoobenthos. pp. 213-236. In: Goffredo, S. & Dubinsky, Z. (eds.). *The Mediterranean Sea: its history and present challenges*, Springer Science, Germany, 318 pp.

22. Gubbay, S., Sanders, N., Haynes, T., Janssen, J.A.M., Rodwell, J.R., Nieto, A., García Criado, M., Beal, S., Borg, J., Kennedy, M., Micu, D., Otero, M., Saunders G. & Calix, M. 2016. European Red List of Habitats: Part 1. Marine Habitats. Publications Office of the European Union.

23. Henriques, V., Tuaty Guerra, M., Mendes, B., Gaudêncio, M.J. & Fonseca, P. 2015. Benthic habitat mapping in a Portuguese Marine Protected Area using EUNIS: an integrated approach. *Journal of Sea Research*, 100: 77-90. https://doi.org/10.1016/j.seares.2014.10.007

24. Henseler, C., Nordström, M.C., Törnroos, A., Snickars, M., Pecuchet, L., Lindegren, M. & Bonsdorff, E. 2019. Coastal habitats and their importance for the diversity of benthic communities: a species- and trait-based approach. *Estuarine, Coastal and Shelf Science*, 226: 106272. https://doi.org/10.1016/j.ecss.2019.106272

25. Hiscock, K. 1990. *Marine Nature Conservation Review: methods.* Joint Nature Conservation Committee, Peterborough, Nature Conservancy Council, CSD Report, No. 1072. (Marine Nature Conservation Review Report, No. MNCR/OR/5.).

26. Kaboğlu, G., Akçalı, B., Kızıldağ, N., Tıraşın, E.M., Atgın, O., Özel, Ö., Oğuz Kaboğlu, S., Cihangir, B., Özdaş, A.H., Açık Çınar, Ş., Yılmaz, F., Önen, S., Bitlis, B., Can Yılmaz, E.K., Bizsel, C., Yıldız, İ., Karayalı, O., Özgen, Ö., Güçlüsoy, H., Manap, E., Sghaier, Y.R. & Ouerghi, A. 2022. Benthic habitats and fisheries interactions: MedKeyHabitats II Turkey Project, 17-18. Paper presented at the 5. Ulusal Deniz Bilimleri Konferansı, 01-03 Haziran, Trabzon-Türkiye.

27. Joint Nature Conservation Committee, 2013. SACFOR abundance scale used for both littoral and sublittoral taxa from 1990 onwards. -Joint Nature Conservation Committee. https://mhc.jncc.gov.uk/media/1009/sacfor.pdf (Date accessed: 18.04.2024).

28. McElderry, J.H. 1963. Mediterranean Tides and Currents. *Irish Astronomical Journal*, 6(1): 12.

29. Mergen, O. & Karacaoğlu, C. 2015. Tuz Lake Special Environment Protection Area, Central Anatolia, Turkey: The EUNIS habitat classification and habitat change detection between 1987 and 2007. *Ekoloji*, 24(95): 1-9.

30. Montefalcone, M., Tunesi, L. & Ouerghi, A. 2021. A review of the classification systems for marine benthic habitats and the new updated Barcelona Convention classification for the Mediterranean. *Marine Environmental Research*, 169: 105387. https://doi.org/10.1016/j.marenvres.2021.105387

31. Monteiro, P., Bentes, L., Oliveira, F., Afonso, C., Rangel, M., Alonso, C., Mentxaka, I., Germán Rodríguez, J., Galparsoro, I., Borja, A., Chacón, D., Sanz Alonso, J.L., Guerra, M.T., Gaudêncio, M.J., Mendes, B., Henriques, V., Bajjouk, T., Bernard, M., Hily, C., Vasquez, M., Populus, J. & Gonçalves, J.M.S. 2013. Atlantic Area Eunis Habitats. Adding new habitat types from European Atlantic coast to the EUNIS Habitat Classification. Technical Report No.3/2013 - MeshAtlantic, CCMAR-Universidade do Algarve, Faro, 72 pp.

32. Oğuz, T. & Sur, H.İ. 1989. A two-layer model of water exchange through the Dardanelles Strait. *Oceanologica Acta*, 12: 23-31.

33. Öztürk, B. & Öztürk, A.A. 1996. On the biology of the Turkish straits system. pp. 205-221. In: F. Briand (ed). *Dynamics of Mediterranean Straits and Channels.* Bulletin de l'Institut océanographique, no spécial 17, Monaco, 250 pp.

34. Riedl, R. 1959. Die Hydroiden des Golfes von Neapel und ihr Anteil an der Fauna unterseeischer Höhlen. Ergebnisse der Österreichischen Tyrrhenia-Expedition 1952, Teil XVI. *Pubblicazione della Stazione Zoologica di Napoli*, 30: 591-755.

35. Rolet, C., Spilmont, N., Dewarumez, J.M. & Luczak, C. 2015. Linking macrobenthic communities structure and zonation patterns on sandy shores: Mapping tool toward management and conservation perspectives in Northern France. *Continental Shelf Research*, 99: 12-25. https://doi.org/10.1016/j.csr.2015.03.002

36. Sokołowski, A., Jankowska, E., Balazy, P. & Jędruch, A. 2021. Distribution and extent of benthic habitats in Puck Bay (Gulf of Gdańsk, southern Baltic Sea). *Oceanologia*, 63(3): 301-320. https://doi.org/10.1016/j.oceano.2021.03.001

37. T.C. Çevre, Şehircilik ve İklim Değişikliği Bakanlığı. 2021. Marmara Denizi ve Adalar Özel Çevre Koruma Bölgesi. https://ockb.csb.gov.tr/marmara-denizi-ve-adalar-ozel-cevre-koruma-bolgesi-i-106827 (Date accessed: 25.03.2024).

38. Tekeli, Z. & Aslan, H. 2020. Bivalvia (Mollusca) fauna of the Canakkale Strait (Dardanelles) Coast. *Acta Biologica Turcica*, 33(4): 244-251.

39. Tekeli, Z. & Aslan, H. 2023. Zonation in littoral macrobenthic assemblages in the Çanakkale Strait (Dardanelles). *Marine Biodiversity*, 53. https://doi.org/10.1007/s12526-023-01391-8

40. Topaloğlu, B., Topçu, N.E., Tezcan, D. & Öztürk, B. 2016. EUNIS habitat classification of a potential MPA, Sıle - Turkey (Western Black Sea). Paper presented at the 41st CIESM Congress, 12-16 September, Kiel-Germany.

41. Vasquez, M., Ségeat, B., Cordingley, A., Tilby, E., Wikström, S., Ehrnsten, E., Hamdani, Z.A., Agnesi, S., Skovgaard Andersen, M., Annunziatellis, A., Askew, N., Bekkby, T., Bentes, L., Daniels, E., Doncheva, V., Drakopoulou, V., Brandbyge Ernstsen, V., Gonçalves, J., Karvinen, V., Laamanen-Nicolas, L., Lillis, H., Loukaidi, V., Manca, E., McGrath, F., Mo, G., Monteiro, P., Muresan, M., Nygard, H., O'Keeffe, E., Pelembe, T., Radicioli, M., Sakellariou, D., Teaca, A., Todorova, V., Tunesi, L. & Woods, H. 2023. EUSeaMap 2023, A European broad-scale seabed habitat map, Technical Report.

42. Wijkmark, N., Florén, K., Näslund, J., Ogonowski, M., Aigars, J., Daunys, D., Saskov, A., Fammler, H., Martin, G. & Oganjan, K. 2015. HELCOM, BALSAM Project 2013-2015: Recommendations and Guidelines for Benthic Habitat Monitoring with Method Descriptions for Two Methods for Monitoring of Biotope and Habitat Extent.

43. WoRMS, 2022. World Register of Marine Species. https://www.marinespecies.org/ (Date accessed: 26.07.2024).

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Research Article

Investigation of the putative functional relevance of the *IL-6* 3'UTR genetic variants with athletic phenotype in Turkish triathletes

Dilek Pirim[1,2*], Hasan Faruk Niş[3], Fatih Atilla Bağcı[3]

[1] Bursa Uludag University, Faculty of Arts & Science, Department of Molecular Biology and Genetics, Bursa, TÜRKIYE
[2] Bursa Uludag University, Institute of Health Sciences, Department of Translational Medicine, Bursa, TÜRKIYE
[3] Bursa Uludag University, Institute of Natural and Applied Sciences, Department of Molecular Biology and Genetics, Bursa, TÜRKIYE

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Abstract: Previous research suggests that genetic variants in the interleukin-6 (*IL-6*) gene contribute to sport-related traits and athletic performance. We aimed to identify sequence variants in the *IL-6* gene region comprising the 3' untranslated region (UTR) in the Turkish triathletes and sedentary individuals and assessed their putative roles in tendency to athletic phenotype. Sequence variants were identified in the Turkish triathletes (n = 47) and sedentary individuals (n = 46) by Sanger sequencing. Allele/genotype frequencies and linkage disequilibrium (LD) patterns were calculated by the Haploview program. The functional significance of the detected variants was analyzed using *in silico* prediction tools. Four single nucleotide variants (rs13306435, rs747302620, rs2069849, rs13306436) were detected in saliva samples of the participants by sequencing the target region. Notably, rs13306436-3'UTR/*IL-6* was only seen in the triathletes, while the exonic rs747302620 was observed in only sedentary group. Also, rs13306436G>A causes loss/gain sites for binding multiple miRNAs that may be associated with athletic performance. Our findings indicate that the 3'UTR/*IL-6* may have functional relevance in determining sports talent. Future comprehensive studies focusing on the *IL-6* gene in athletes may pave the way for not only determining the athletic status of the individuals but also have implications for translational medicine.

Özet: Önceki araştırmalar, interlökin-6 (*IL-6*) geni varyantlarının spor ile ilgili özelliklere ve atletik performansa katkı sağladığını ileri sürmektedir. Bu çalışmada, Türk triatletler ve sedanter bireylerde *IL-6* geninin 3' translasyon olmayan bölgelerinde (UTR) dizi varyantlarını tanımlamayı ve bunların atletik fenotipe yatkınlıktaki varsayılan rollerini değerlendirmeyi amaçladık. Türk triatletlerde (n = 47) ve sedanter bireylerde (n = 46) dizi varyantları Sanger dizileme ile tanımlanmıştır. Alel/genotip frekansları ve bağlantı dengesizliği (LD) örüntüleri Haploview programı ile hesaplanmıştır. Tespit edilen varyantların fonksiyonel önemleri *in silico* tahmin araçları kullanılarak analiz edilmiştir. Hedef gen bölgesinin dizilenmesi sonucunda, katılımcıların tükürük örneklerinde dört tek nükleotid varyantı (rs13306435, rs747302620, rs2069849, rs13306436) tespit edilmiştir. rs13306436-3'UTR/*IL-6* sadece triatletlerde görülürken, ekzonik rs747302620 sadece sedanter grupta gözlenmiştir. Ayrıca, rs13306436G>A, miRNA'ların bağlanabileceği kayıp/kazanç bölgeleri yaratarak atletil performans ile ilişkili olabilir. Bulgularımız, 3'UTR/*IL-6*'nın sporcu yeteneğini belirlemede işlevsel bir öneme sahip olabileceğini göstermektedir. Sporcularda *IL-6* genine odaklı yapılacak gelecekteki kapsamlı çalışmalar, yalnızca bireylerin atletik durumlarının belirlenmesine değil, aynı zamanda translasyonel tıp için de çıkarımlara yol açabilir.

Edited by:
Belgin Süsleyici

****Corresponding Author:***
Dilek Pirim
dilekpirim@uludag.edu.tr

ORCID iDs of the authors:
DP. 0000-0002-0522-9432
HFN. 0000-0002-6665-9874
FAB. 0009-0003-5158-1757

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Introduction

Human athletic performance is determined by combinations of intrinsic and extrinsic factors such as strength, endurance, psychology, diets, epigenetic and genetic factors (de la Iglesia *et al.* 2020, Ginevičienė *et al.* 2022). Recently, the contribution of genetic factors to athletic performance has been widely studied and the genetic heritability of exercise-related traits has been estimated to range from 50 to 68% (Konopka *et al.* 2023). Thus, genetic studies related to athletic performance have progressively increased in the last years leading to the emergence of a new field called sporomics, which aims to elucidate the determinants of athletic success using

different omic layers (Bongiovanni *et al.* 2019, Appel *et al.* 2021, Semenova *et al.* 2023). Almost 250 gene regions have so far been found to be associated with a tendency to exercise-related traits and athletic ability (Varillas-Delgado *et al.* 2022, Konopka *et al.* 2023, Semenova *et al.* 2023). However, multiple single nucleotide variants (SNVs) in the *COL61A, IL-6, 5-HTT, MAO-A, BDKRB2, NOS3, PPAR-A, MCT1, HIF1A1,* and *AMPD1* genes have been suggested to be associated with athletic performance in triathletes (Domingo *et al.* 2012, Grealy *et al.* 2015, Saunders *et al.* 2015, Corak *et al.* 2017, Akkoç *et al.* 2020). Yet, more research is needed to elucidate the genetic architecture of the triathletes that may contribute to their talent and well-being for sports performance.

The interleukin-6 (*IL-6)* gene, located in chromosome 7, encodes a pleiotropic cytokine involved in immune regulation, and its regulations have been shown to contribute to distinct pathologies (Ataie-Kachoie *et al.* 2013, Hirano 2021, Kishimoto & Kang 2022). *IL-6* is known to have an essential function in anti-inflammatory processes in skeletal muscle and also plays an active role in muscle repair and hypertrophy after exercise (Rosa Neto *et al.* 2009, Pedersen, 2013). Thus, plasma levels of *IL-6* are observed to be increased during acute exercise, and exercise duration is the primary mediator of the *IL-6* concentrations (Nash *et al.* 2023). Given the idea that *IL-6* production favors the tendency to physical activity and considering its important role in metabolic processes during exercise, studies in sports genetics have focused on single nucleotide polymorphisms (SNPs) in the *IL-6* gene (Akkoç *et al.* 2020, Ben-Zaken, *et al.* 2022, Nash *et al.* 2023). The most studied SNP located in the *IL-6* gene is the rs1800795G>C (c.-174C > G), which is located in the 5' untranslated region (UTR) (Eider *et al.* 2013, Fuku *et al.* 2019, Pickering *et al.* 2019, Moreland *et al.* 2022, Semenova *et al.* 2023). The IL6/rs1800795-G allele has been reported to be associated with high *IL-6* expression and athletic performance in previous studies (Bennermo *et al.* 2004, Kazancı *et al.* 2023). However, future investigations are warranted to fully assess the roles of *IL-6* sequence variants in sports genetics. The 3′UTRs play an important role in regulating of gene expression, mRNA stability, and protein function. Nevertheless, SNPs in the 3'UTRs of the genes may be located in the regulatory sequences that disrupt or enhance miRNA-mRNA interactions. In this regard, our study aimed to resequence a part of the exon 5 of the *IL-6* gene comprising 3′UTR in Turkish triathletes and assess the functional importance of the detected variants using bioinformatic tools.

Materials and Methods

Samples

Saliva samples (2 mL, in saliva collection tubes) collected from 93 volunteers aged 18 or above, including 47 triathletes (38 Males, 9 Females) from the Gelibolu and Balıkesir Avlu Triathlon races organized by the Triathlon Federation in 2022, and 46 sedentary individuals (12 Males, 34 Females) selected from the general population were included in the study. All triathletes who participated in the study were classified as elite status based on their previous performance (1st, 2nd, or 3rd place winners) in international and/or national triathlon races. Collected saliva samples were stored at -20°C until DNA isolation.

DNA Isolation

Genomic DNA was isolated from 500ul of each of the saliva samples using the Saliva DNA Extraction Kit (Hibrigen, Türkiye) by an extra spin-column purification step (Thermo Fisher Scientific, Darmstadt, Germany). Proteinase K treatment (3 hours at 56°C) was applied to all samples before DNA isolation. The NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Darmstadt, Germany) and Qubit 4.0 (Thermo Fisher Scientific, Darmstadt, Germany) were used to assess the DNA concentration and quality.

PCR Amplification and Sequencing

Primers covering the 3'UTR of the *IL-6* gene (NM_000600.5) were designed using the NCBI primer design tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers targeting the fragment in the 5th exon region (600bp) covering 3'UTR (F: AGCATCCCTCCACTGCAAAG, R: TGGTGGCAGTGACAAGAAAC) were used for PCR amplification and Sanger Sequencing. For amplification of the desired fragment 2.5 µl 10X PCR buffer, 2 µl $MgCl_2$, 0.5 µl 20 mM dNTP, 0.6 µl from each primer (10 µM), 2-5 µl DNA template, and 0.15 µl AmpliTaq Gold Taq Polymerase (AmpliTaq Polymerase, ThermoFisher) were used in the final volume of 25 µl. PCR conditions are given in Supplementary Material Table S1. After amplification, PCR products were visualized and confirmed in 1.5% agarose gel electrophoresis. Sanger sequencing was performed in Applied Biosystems 3500 Genetic Analyzer (Thermo Fisher Scientific, Darmstadt, Germany).

Statistical and Bioinformatics Analysis

Haploview software was used to calculate the Hardy-Weinberg Equilibrium (HWE) *p*-value and linkage disequilibrium (LD) patterns of the SNVs. The Chi-square test was conducted using Haploview software (Barrett *et al.* 2005). Sequence chromatograms were analyzed by using the Sequencher (Gene Codes, Ann Harbor, MI) and Uniprogen software. We also used the LDlink online tool to assess the LD pattern and genotype distributions of the variants in the worldwide populations (https://ldlink.nih.gov) (Machiela & Chanock 2015). The regulatory impact of the SNVs was assessed from RegulomeDB (Boyle *et al.* 2012), while SIFT, MutationTaster, and Polyphen databases were used to assess their possible effects on protein function (Ng & Henikoff 2003, Adzhubei *et al.* 2010, Schwarz *et al.* 2014). We used the miRNASNP database (http://bioinfo.life.hust.edu.cn) to predict the potential impacts of the SNVs for miRNA bindings (Liu *et al.* 2021). A *p*-value of less than 0.05 was considered a statistically significant result.

Results

Variant Detection

A total of four variants (rs13306435, rs747302620, rs2069849, rs13306436) with minor allele frequency 0.005-0.022 were identified in the total sample (n = 93) (Table 1). Genotype distributions were found to follow HWE ($p > 0.05$) (Table 1).

We identified only one variant (rs13306436) located in the 3'UTR, and three were located in the coding region of the exon 5. The heterozygote (GA) genotype was observed for rs13306436 (3'UTR variant) in only three triathletes (MAF = 0.016) (Fig. 1) while coding variant rs747302620 was observed in only sedentary individuals (MAF = 0.011) (Tables 1, 2).

The distribution of the alleles and genotypes was not statistically significant, yet a marginal *p*-value (0.08) was observed for rs13306436-A when comparing the two groups (Table 2). The distribution of the detected variants in populations sequenced in 1000 Genome Project was also assessed (the data retrieved from the LDlink online tool is presented in Fig. 2 and Supplementary Material Table S2). The coding rs747302620 was not reported in 1000 Genome Project data, so not included in Fig. 2. Strikingly, 3'UTR variant rs13306436-A was rarely detected in populations of Asian descent (MAF ≤ 0.043) and the A-allele was not reported in remaining worldwide populations. The LD patterns of the variants were analyzed and no significant LD was found in any group (Supplementary Material Fig. S1).

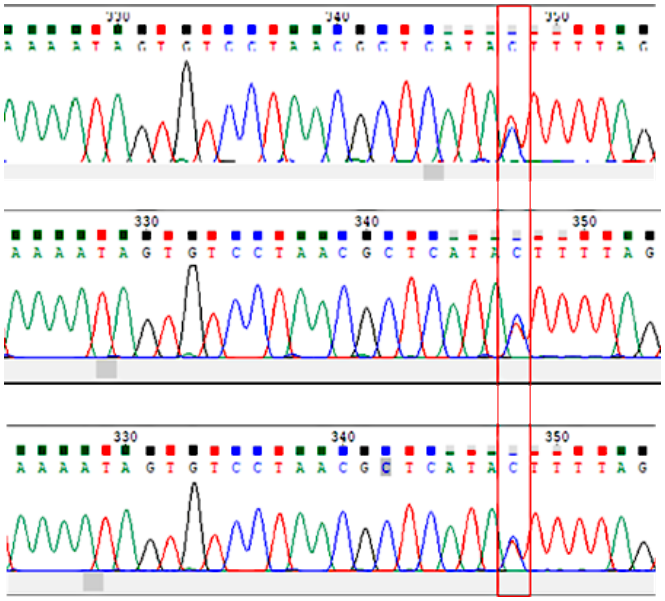

Fig. 1. The chromatograph depicts sequence variant (3'UTR-rs13306436G>A) detected in 3 triathletes.

Table 1. Allele and genotype frequencies of the identified SNVs in the samples (n = 93).

RefSNP ID	Alleles	Chr loc.[a] (GRCh38.p14)	Location	HW-p[b]	Allel Freq[c]	Genotype (n, %)	1000G[d] European MAF	ExAC[e] Global MAF	GnomAD[f] Exomes Global MAF
rs13306435	T:A	22731420	Exon5 (Asp162Glu)	1.0	T: 98.4%, A: 1.6%	TT (n = 89, 89%) TA (n = 3, 3%) AA (n = 0, 0%)	0.017	0.025562	n/a
rs747302620	A:C	22731430	Exon5 (Thr166Pro)	1.0	A: 99.5%, C: 0.5%	AA (n = 92, 92%) AC (n = 1, 1%) CC (n = 0, 0%)	n/a	0.000008	0.000004
rs2069849	C:T	22731537	Exon5 (Phe201Leu)	1.0	C: 97.8%, T: 2.2%	CC (n = 89, 89%) CT (n = 4, 4%) TT (n = 0, 0%)	0.022	0.046119	0.043579
rs13306436	G:A	22731677	3' UTR	1.0	G: 98.4%, A: 1.6%	AA (n = 90, 90%) AG (n = 3, 3%) GG (n = 0, 0%)	0	n/a	0.000699

a: Chromosomal location, b: Hardy-Weinberg *p*-value, c: Allele frequency, d: 1000 Genome project, e: The exome aggregation consortium, f: The genome aggregation database, n/a: not applicable.

Table 2. Allele and genotype frequencies of the identified SNVs in triathletes and sedanter individuals.

RefSNP ID	Associated Allele	Total MAF	Allele Frequency		Genotypes	
			Triathletes (n = 47)	Sedanter (n = 46)	Triathletes (n = 47)	Sedanter (n = 46)
rs13306435 T > A	A	0.016	T: 98.9%, A: 1.1%	T: 97.8%, A: 2.2%	TT (n = 46) TA (n = 1) AA (n = 0) n/a (n = 0) x2/*p*-value: 0.385/0.5351	TT (n = 43) TA (n = 2) AA (n = 0) n/a (n = 1)
rs747302620 A > C	C	0.005	A: 100%, C: 0%	A: 98.9%, C: 1.1%	AA (n = 47) AC (n = 0) CC (n = 0) n/a (n = 0) x2/*p*-value: 1.027/0.3108	AA (n = 45) AC (n = 1) CC (n = 0) n/a (n = 0)
rs2069849 C > T	T	0.022	C: 96.8%, T: 3.2%	C: 98.9%, T: 1.1%	CC (n = 44) CT (n = 3) TT (n = 0) n/a (n = 0) x2/*p*-value: 0.979/0.3225	CC (n = 45) CT (n = 1) TT (n = 0) n/a (n = 0)
rs13306436 G > A	A	0.016	G: 96.8%, A: 3.2%	G: 100%, A: 0%	GG (n = 44) GA (n = 3) AA (n = 0) n/a (n = 0) x2/*p*-value: 2.984/0.0841	GG (n = 46) GA (n = 0) AA (n = 0) n/a (n = 0)

n/a; genotypes not determined

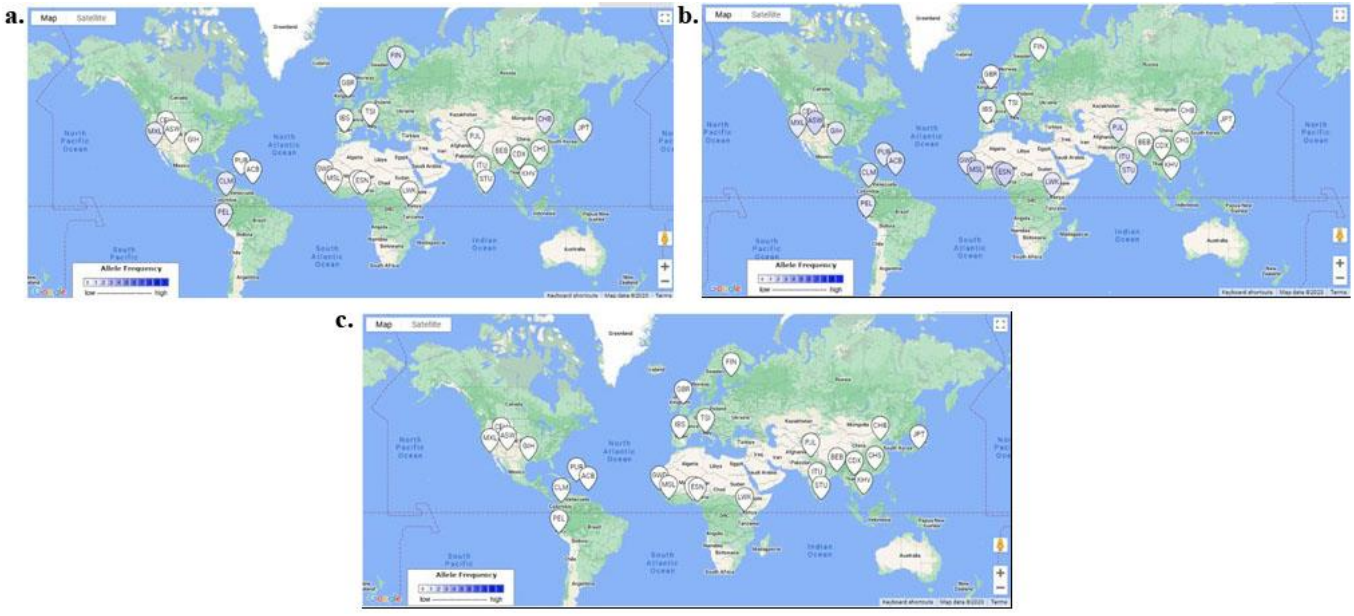

Fig. 2. Allele frequency distribution of **a.** rs13306435, **b.** rs2069849, **c.** rs13306436

In silico Functional Analysis of the Identified Variants

Genetic Changes that Affect the Protein

We identified three variants in the coding region of the exon-5 of which two were missense variants causing amino acid replacement [rs13306435 (p.Asp162Glu) and rs747302620 (p.Thr166Pro)] and one [rs2069849 (p.Phe125=)] was a synonymous variant. The results of the MutationTaster, SIFT, and Polyphen databases indicate that rs13306435 does not have a detrimental effect on protein, yet it is likely to be a regulatory variant by affecting the binding of regulatory proteins (RegulomeDB score=2a) (Table 3). Meanwhile, rs747302620 and rs2069849 had a RegulomeDB score of 4, indicating their possible regulatory role by residing in the transcription factor binding region (Table 3). Distributions of the identified variants in two groups are given in Table 2.

Table 3. *In silico* functional analysis of the detected variants.

RefSNP ID	Genomic Location (NG_011640.1)	Genetic Location	Amino Acid Change	MAF	RegulomeDB Score	SIFT	MT	PP2
rs13306435	g.9274T>A	Exon	D>E	0.016	2a	T	B	B
rs747302620	g.9284A>C	Exon	T>P	0.005	4	T	B	PD
rs2069849	g.9391C>T	Exon	F>F	0.022	4	T	B	PD
rs13306436	g.9531G>A	UTR	-	0.016	5	n/a	B	n/a

MAF; Minor Allele Frequency; RegulomeDB Score; 2a, TF binding + matched TF motif + matched Footprint + chromatin accessibility peak; 4, TF binding + chromatin accessibility peak, 5, TF binding or chromatin accessibility peak; SIFT; T, Tolerated; MT, MutationTaster; B, Benign; PP2, Polyphen2; PD, Probably Damaging.

Genetic Changes that Affect the Binding of Regulatory Molecules

We detected only one 3'UTR variant (rs13306436) with potential as a microRNA-associated single nucleotide polymorphism (mirSNP) and regulatory properties. The rs13306436G>A change was predicted to cause formation of new miRNA binding sites for hsa-miR-5007-3p and hsa-miR-1279 and the loss of existing miRNA binding sites for hsa-miR-539-3p, hsa-miR-5003-3p, hsa-miR-1-5p and hsa-miR-485-3p (Table 4). Meanwhile, all detected variants have been found to have a potential role in *IL-6* gene regulation according to the RegulomeDB scores (<5). The rs13306435 located in exon 5 has a RegulomeDB score of 2a implying the significance of the sequence for binding multiple regulatory proteins. Also, the 3'UTR variant (rs13306436) had a RegulomeDB score of 5, indicating its importance as a transcription factor binding site (Table 3).

Table 4. miRNA binding sites affected by 3'UTR rs13306436G>A.

miRNA	Effect	Target Score	Duplex SNP-miRNA
hsa-miR-5007-3p	Gain	21.58	3'UTR: 5' GUUGUUCUCUAUGGAGAACUAAAAAUAUGAGCGUUAGGACA 3' | | | | | | . miRNA: 3'UAAUCUCAAACCAAGUAUACUA 5'
hsa-miR-1279	Gain	25.09	3'UTR: 5' GUUCUCUAUGGAGAACUAAAAAUAUGAGCGUUAGGAC 3' | | | | | | . miRNA: 3'UCUUUCUUCGUUAUACU 5'
hsa-miR-539-3p	Loss	21.08	3'UTR: 5' GUUGUUCUCUAUGGAGAACUAAAAGUAUGAGCGUUAGGACA 3' X | | | | | . miRNA: 3' UUUCUUUAACAGGAACAUACUA 5'
hsa-miR-5003-3p	Loss	21.56	3'UTR: 5' GUUGUUCUCUAUGGAGAACUAAAAGUAUGAGCGUUAG 3' | | | | X | . miRNA: 3' GGGGUUGUUGGAUCUUUUCAU 5'
hsa-miR-1-5p	Loss	23.63	3'UTR: 5' GUUGUUCUCUAUGGAGAACUAAAAGUAUGAGCGUUAG 3' | | X | | | | . miRNA: 3' UACCCGUAUAUUUCUUCAUACA 5'
hsa-miR-485-3p	Loss	24.08	3'UTR: 5' GUUGUUCUCUAUGGAGAACUAAAAGUAUGAGCGUUAG 3' X | | | | | . miRNA: 3' UCUCUCCUCUCGGCACAUACUG 5'

Discussion

Accumulating efforts have attempted to uncover the genetic determinants causing the interindividual variations in athletic tendencies and sports performance. These studies' findings alluded that the athletes' genetic profile may not only lead them to be successful in sports but also may be associated with advantageous traits for favourable health (Varillas-Delgado *et al.* 2022). However, athletic performance is a highly heterogeneous trait and is influenced by several factors that need to be meticulously investigated. Recent technological advances facilitated the identification of multiple genetic variants associated with exercise-related traits and sporting aptitude. Yet, sports genetics studies are still emerging, and a better understanding of the molecular mechanisms contributing to talent in specific sports disciplines is necessary.

In this study, we seek to determine the regulatory variants located in the 3'UTR region of the *IL-6* gene in triathletes, which may be associated with their athletic status. Thus, 47 triathletes and 46 sedentary individuals participated in the study and the *IL-6* gene region encompassing 3'UTR was sequenced by Sanger sequencing. Three coding SNVs and one 3'UTR SNV (rs13306436) were identified of which rs13306436G>A (MAF = 0.016) was detected in only triathletes. We also compared the MAF distributions of the identified variant to the MAF determined in the large-scale sequencing projects (Table 1 and Supplementary Material Table S2). Our results for two coding SNVs (rs13306435 and rs2069849) were similar to 1000 Genome Project results obtained in populations of European descent. The MAF of the other coding SNV (rs747302620) was not reported in the 1000 Genome project (Supplementary Material Table S2), and it was rarely detected in the exome sequencing projects (ExAC Global MAF = 8E-6 and gnomAD Exomes Global MAF = 4E-6) **(**Table 1). The MAF value of the 3'UTR variant rs13306436 in 1000 Genome Global was 0.0048, and it was not detected in populations of European descent (Supplementary Material Table S2). However, rs13306436G>A change was rarely seen in Asian populations (MAF = 0.0051-0.0433). Although three triathletes were heterozygotes for rs13306436G>A in our study, none of the sedanters in our sample carried the A-allele. A small population size may explain this but still, our results need further consideration as the A allele may lead favourable phenotype for athletic status and thus be observed in only triathletes.

The 3'UTR of the genes is known to comprise functional sequences that are targets for regulatory molecules, including miRNAs (Mayr, 2019). Nevertheless, suggesting evidence implies the substantial role of 3'UTRs for the tendency to physical activity as SNPs located in the 3'UTR region of the multiple genes were reported to be associated with athletic performance (O'Connell *et al.* 2014, Grealy *et al.* 2015, Saunders *et al.* 2015, Heffernan *et al.* 2017, Rivera *et al.* 2020). Recently, miRNAs associated with exercise-related traits have gained attention, and several miRNAs were shown to be differentially expressed during acute or chronic exercise in athletes, which may ease exercise-induced pathologies and lead to their athletic success (de Gonzalo-Calvo *et al.* 2015, Li *et al.* 2018, Massart *et al.* 2021, Zhou *et al.* 2020, Kotewitsch *et al.* 2024). Thus, a better understanding of the roles of the miRSNPs in sport-related genes is important for athlete health and talent identification. Our analyses revealed that the 3'UTR variant (rs13306436G>A) found in only triathlete group is located in miRNA binding sites of hsa-miR-1-5p, hsa-miR-485-3p, hsa-miR-539-3p, hsa-miR-5003-3p, hsa-miR-1279 and hsa-miR-5007-3p (Table 4). The biomarker potentials of hsa-miR-1-5p, hsa-miR-485-3p, hsa-miR-539-3p, hsa-miR-5003-3p, and hsa-miR-1279 were extensively studied in the literature, yet limited evidence exists for the functional relevance of hsa-miR-5007-3p in human diseases and traits (Yang *et al.* 2015, Montalbo *et al.* 2018, Hu *et al.* 2019, Chen *et al.* 2022, Jing *et al.* 2023, Ryu *et al.* 2023, Yue *et al.* 2023). However, our results obtained from the miRNASNP database show that the G>A change disrupts the binding site of hsa-miR-1-5p, hsa-miR-485-3p, hsa-miR-539-3p, and hsa-miR-5003-3p while creating putative binding sites for hsa-miR-1279 and hsa-miR-5007-3p. Previously, hsa-miR-1-5p has been suggested as a muscle-specific/muscle-enriched miRNA (myomiR) due to its crucial role in myogenesis, and its expression has been shown to increase after acute exercise (Meurer *et al.* 2016, Silva *et al.* 2017, Siracusa *et al.* 2018). Meanwhile, the dysregulation of circulating hsa-miR-485-3p was also observed during exercise training, suggesting its potential role in exercise adoption (Silva *et al.* 2017).

IL-6 is a key molecule of the cytokine signaling pathway and is released from active skeletal muscles during exercise while maintaining muscle energy homeostasis (Catoire & Kersten, 2015, Nash *et al.* 2023). *IL-6* acts as a myokine overproduced during muscle contraction and boosts exercise performance by allowing training adaptations (Trinh *et al.* 2021, Leuchtmann *et al.* 2022). The role of *IL-6* in exercise physiology has been widely investigated in previous studies and certain SNVs in the *IL-6* gene were repetitively studied in athletes from different sports disciplines and ethnic populations. The results of a recent study conducted in Turkey demonstrated that *IL-6*/rs1800795G>C was found more frequently (MAF = 0.19) in Ironman triathlon athletes (n =10) (Akkoç *et al.* 2020). The functional IL6/rs1800795-C allele has also been associated with athletic performance in different studies recruited distinct athlete groups and was suggested to have a role in mechanisms related muscle repair (Yamin *et al.* 2008, Ben-Zaken *et al.* 2015, Cenikli *et al.* 2016, Ben-Zaken *et al.* 2017, Akkoç *et al.* 2020, Sofu, 2020, Kazanci *et al.* 2021, Tuna *et al.* 2022). Meanwhile, *IL-6*/rs2228145A>C was also proposed to influence interindividual differences in physical activity levels by fortifying the *IL-6* and soluble fragment of the *IL-6* receptor (s*IL-6*R) complex formation (Nash *et al.* 2023). The underlying mechanisms related to associations of *IL-6* SNVs with athletic talent indeed depend on the functional effects of SNVs in the *IL-6* gene, therefore a deeper understanding of the *IL-6* variations possibly promote

athletic success is highly important (Ben-Zaken *et al.* 2017, Nash *et al.* 2023). To the best of our knowledge, the 3'UTR of the *IL-6* has not been sequenced in triathletes before, and thus, our results yield a novel perspective on the contribution of the *IL-6* in sports genetics. However, our study has some limitiations. First, our sample size can be too small for detecting rare and low frequency variants with possible functional roles so they might have been missed in our analyses. Also, the effects of the variants on gene expression were not evaluated which can be uncovered by further research. Nevertheless, elucidating the miRSNP potential and functional relevance of 3'UTR rs13306436 in athletic predisposition deserves further attention and comprehensive investigations.

In conclusion, our study provides suggestive evidence for the possible functional implications of the 3'UTR region of the *IL-6* in athletic tendency, and future studies are needed to ensure the prominent role of *IL-6* in the tendency to physical activity.

Ethics Committee Approval: Ethics committee approval was received for this study from the Institutional Review Board (IRB) of the Bursa Uludag University by the number 2022-1/18. All participants were informed about the study and participated after signing informed consent forms. Sample collections in the race areas were accomplished with the approval and permission of the Turkish Triathlon Federation.

Data Sharing Statement: All data are available within the study and in the Supplementary Material.

Author Contributions: Concept: D.P., Design: D.P., H.F.N., Execution: D.P., H.F.N., F.A.B., Material supplying: D.P., H.F.N., Data acquisition: D.P., H.F.N., Data analysis/interpretation: D.P., H.F.N., F.A.B., Writing: D.P., H.F.N., F.A.B., Critical review: D.P., F.A.B.

Conflict of Interest: The authors have no conflicts of interest to declare.

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References

1. Adzhubei, I.A., Schmidt, S., Peshkin, L., Ramensky, V.E., Gerasimova, A., Bork, P., Kondrashov, A.S. & Sunyaev, S.R. 2010. A method and server for predicting damaging missense mutations. *Nature Methods*, 7(4): 248-249. https://doi.org/10.1038/nmeth0410-248

2. Akkoç, O., Birlik, A., Doğan, C.S., Ulucan, K. & Kirandi, Ö. 2020. Türk İronman Triatlon Sporcularında *IL-6*, HIF1A, MCT1, PPAR-a Polimorfizm Dağılımının Belirlenmesi. *Spor Eğitim Dergisi*, 4(1): 1-7.

3. Appel, M., Zentgraf, K., Krüger, K. & Alack, K. 2021. Effects of Genetic Variation on Endurance Performance, Muscle Strength, and Injury Susceptibility in Sports: A Systematic Review. *Frontiers in Physiology*, 12: 694411. https://doi.org/10.3389/fphys.2021.694411

4. Ataie-Kachoie, P., Pourgholami, M.H. & Morris, D.L. 2013. Inhibition of the *IL-6* signaling pathway: a strategy to combat chronic inflammatory diseases and cancer. *Cytokine & Growth Factor Reviews*, 24(2): 163-173. https://doi.org/10.1016/j.cytogfr.2012.09.001

5. Barrett, J.C., Fry, B., Maller, J. & Daly, M.J. 2005. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics*, 21(2): 263-265. https://doi.org/10.1093/bioinformatics/bth457

6. Bennermo, M., Held, C., Stemme, S., Ericsson, C.-G., Silveira, A., Green, F. & Tornvall, P. 2004. Genetic Predisposition of the Interleukin-6 Response to Inflammation: Implications for a Variety of Major Diseases? *Clinical Chemistry*, 50(11): 2136-2140. https://doi.org/10.1373/clinchem.2004.037531

7. Ben-Zaken, S., Eliakim, A., Nemet, D., Kaufman, L. & Meckel, Y. 2022. Genetic characteristics of competitive swimmers: A review. *Biology of Sport*, 39(1): 157-170. https://doi.org/10.5114%2Fbiolsport.2022.102868

8. Ben-Zaken, S., Eliakim, A., Nemet, D., Rabinovich, M., Kassem, E. & Meckel, Y. 2015. ACTN3 Polymorphism: Comparison Between Elite Swimmers and Runners. *Sports Medicine - Open*, 1(1): 13. https://doi.org/10.1186/s40798-015-0023-y

9. Ben-Zaken, S., Meckel, Y., Nemet, D., Kassem, E. & Eliakim, A. 2017. Increased Prevalence of the *IL-6*-174C Genetic Polymorphism in Long Distance Swimmers. *Journal of Human Kinetics*, 58: 121-130. https://doi.org/10.1515/hukin-2017-0070

10. Bongiovanni, T., Pintus, R., Dessì, A., Noto, A., Sardo, S., Finco, G., Corsello, G. & Fanos, V. 2019. Sportomics: metabolomics applied to sports. The new revolution? *European Review for Medical and Pharmacological Sciences*, 23(24): 11011-11019. https://doi.org/10.26355/eurrev_201912_19807

11. Boyle, A.P., Hong, E.L., Hariharan, M., Cheng, Y., Schaub, M.A., Kasowski, M., Karczewski, K.J., Park, J., Hitz, B.C., Weng, S., Cherry, J.M. & Snyder, M. 2012. Annotation of functional variation in personal genomes using RegulomeDB. *Genome Research*, 22(9): 1790-1797. https://doi.org/10.1101/gr.137323.112

12. Catoire, M. & Kersten, S. 2015. The search for exercise factors in humans. *The FASEB Journal*, 29(5): 1615-1628. https://doi.org/10.1096/fj.14-263699

13. Cenikli, A., Nursal, A., Tural, E., Polat, Y., Tasmektepligil, M. & Serbulent, Y. 2016. The Correlation between Rs1800795 Variant of IL 6 and Sports Performance among Turkish Elite Athletes. *International Journal of Humanities, Social Sciences and Education*, 3(11): 1-5. http://dx.doi.org/10.20431/2349-0381.0311001

14. Chen, Z., Zhang, L., Ding, C., Ren, K., Wan, D. & Lin, S. 2022. A six-miRNA signature as a novel biomarker for improving prediction of prognosis and patterns of immune infiltration in hepatocellular carcinoma. *American Journal of Translational Research*, 14(6): 3610-3637.

15. Corak, A., Kapici, S., Sercan, C., Akkoç, O. & Ulucan, K. 2017. A pilot study for determination of anxiety related SLC6A4 promoter "S" and "L" alleles in healthy Turkish athletes. *Cellular and Molecular Biology (Noisy-Le-Grand,*

France), 63(5): 29-31. http://dx.doi.org/10.14715/cmb/2017.63.5.6

16. de Gonzalo-Calvo, D., Dávalos, A., Montero, A., García-González, Á., Tyshkovska, I., González-Medina, A., Soares, S.M.A., Martínez-Camblor, P., Casas-Agustench, P., Rabadán, M., Díaz-Martínez, Á.E., Úbeda, N. & Iglesias-Gutiérrez, E. 2015. Circulating inflammatory miRNA signature in response to different doses of aerobic exercise. *Journal of Applied Physiology*, 119(2): 124-134. https://doi.org/10.1152/japplphysiol.00077.2015

17. de la Iglesia, R., Espinosa-Salinas, I., Lopez-Silvarrey, F.J., Ramos-Alvarez, J.J., Segovia, J. C., Colmenarejo, G., Borregon-Rivilla, E., Marcos-Pasero, H., Aguilar-Aguilar, E., Loria-Kohen, V., Reglero, G. & Ramirez-de Molina, A. 2020. A Potential Endurance Algorithm Prediction in the Field of Sports Performance. *Frontiers in Genetics*, 11: 711. https://doi.org/10.3389/FGENE.2020.00711

18. Domingo, R., Sturrock, E. & Collins, M. 2012. ACE Activity and Endurance Performance during the South African Ironman Triathlons. *International journal of sports medicine*, 34(5): 402-408. https://doi.org/10.1055/s-0032-1323820

19. Eider, J., Cieszczyk, P., Leońska-Duniec, A., Maciejewska, A., Sawczuk, M., Ficek, K. & Kotarska, K. 2013. Association of the 174 G/C polymorphism of the IL6 gene in Polish power-orientated athletes. *The Journal of Sports Medicine and Physical Fitness*, 53(1): 88-92.

20. Fuku, N., Kumagai, H. & Ahmetov, I.I. 2019. Chapter Fourteen - Genetics of muscle fiber composition. pp. 295-314. In: Barh, D. & Ahmetov, I.I. (eds). *Sports, Exercise, and Nutritional Genomics*. Academic Press. http://dx.doi.org/10.1016/B978-0-12-816193-7.00014-2

21. Ginevičienė, V., Utkus, A., Pranckevičienė, E., Semenova, E.A., Hall, E.C R. & Ahmetov, I.I. 2022. Perspectives in Sports Genomics. *Biomedicines*, 10(2): 298. https://doi.org/10.3390/biomedicines10020298

22. Grealy, R., Herruer, J., Smith, C.L E., Hiller, D., Haseler, L.J. & Griffiths, L.R. 2015. Evaluation of a 7-Gene Genetic Profile for Athletic Endurance Phenotype in Ironman Championship Triathletes. *PLOS ONE*, 10(12): e0145171. https://doi.org/10.1371/journal.pone.0145171

23. Heffernan, S.M., Kilduff, L.P., Erskine, R.M., Day, S.H., Stebbings, G.K., Cook, C.J., Raleigh, S.M., Bennett, M.A., Wang, G., Collins, M., Pitsiladis, Y.P. & Williams, A.G. 2017. COL5A1 gene variants previously associated with reduced soft tissue injury risk are associated with elite athlete status in rugby. *BMC genomics*, 18(Suppl 8): 820. https://doi.org/10.1186/s12864-017-4187-3

24. Hirano, T. 2021. *IL-6* in inflammation, autoimmunity and cancer. *International Immunology*, 33(3): 127-148. https://doi.org/10.1093/intimm/dxaa078

25. Hu, L., Liu, Y., Wang, B., Wu, Z., Chen, Y., Yu, L., Zhu, J., Shen, W., Chen, C., Chen, D., Li, G., Xu, L. & Luo, Y. 2019. MiR-539-5p negatively regulates migration of rMSCs induced by Bushen Huoxue decoction through targeting Wnt5a. *International Journal of Medical Sciences*, 16(7): 998-1006. https://doi.org/10.7150%2Fijms.33437

26. Jing, J., Chang, M., Jiang, S., Wang, T., Sun, Q., Yang, J., Ma, C. & Li, T. 2023. Clinical value of serum miR-1-3p as a potential circulating biomarker for abdominal aortic aneurysm. *Annals of Medicine*, 55(2): 2260395. https://doi.org/10.1080%2F07853890.2023.2260395

27. Kazanci, D., Polat, T., Doğan, C.S., Aslan, B.T., Oktay, Ş., Bilici, M.F., Kaynar, Ö., Eken, B.F. & Ulucan, K. 2021. The Determination of *IL-6* rs1800795 Polymorphism Distribution in Turkish National Cross-Country Skiing Athletes Sub-groups Created Referring to the 1km CCSTAs. *Clinical and Experimental Health Sciences*, 11(4): 782-786. https://doi.org/10.33808/clinexphealthsci.904524

28. Kazancı, D., Polat, T., Kaynar, Ö., Bilici, M.F., Tacal Aslan, B. & Ulucan, K. 2023. PPARA and IL6: exploring associations with athletic performance and genotype polymorphism. *Cellular and Molecular Biology (Noisy-Le-Grand, France)*, 69(11): 69-75. http://doi.org/10.14715/cmb/2023.69.11.12

29. Kishimoto, T. & Kang, S. 2022. *IL-6* Revisited: From Rheumatoid Arthritis to CAR T Cell Therapy and COVID-19. *Annual Review of Immunology*, 40(1): 323-348. https://doi.org/10.1146/annurev-immunol-101220-023458

30. Konopka, M.J., Sperlich, B., Rietjens, G. & Zeegers, M.P. 2023. Genetics and athletic performance: a systematic SWOT analysis of non-systematic reviews. *Frontiers in Genetics*, 14. https://doi.org/10.3389/fgene.2023.1232987

31. Kotewitsch, M., Heimer, M., Schmitz, B. & Mooren, F.C. 2024. Non-coding RNAs in exercise immunology: A systematic review. *Journal of Sport and Health Science*, 13(3): 311-338. https://doi.org/10.1016/j.jshs.2023.11.001

32. Leuchtmann, A.B., Furrer, R., Steurer, S.A., Schneider-Heieck, K., Karrer-Cardel, B., Sagot, Y. & Handschin, C. 2022. Interleukin-6 potentiates endurance training adaptation and improves functional capacity in old mice. *Journal of Cachexia, Sarcopenia and Muscle*, 13(2): 1164-1176. https://doi.org/10.1002/jcsm.12949

33. Li, Y., Yao, M., Zhou, Q., Cheng, Y., Che, L., Xu, J., Xiao, J., Shen, Z. & Bei, Y. 2018. Dynamic Regulation of Circulating microRNAs During Acute Exercise and Long-Term Exercise Training in Basketball Athletes. *Frontiers in Physiology*, 9: 282. https://doi.org/10.3389/fphys.2018.00282

34. Liu, C.-J., Fu, X., Xia, M., Zhang, Q., Gu, Z. & Guo, A.-Y. 2021. miRNASNP-v3: a comprehensive database for SNPs and disease-related variations in miRNAs and miRNA targets. *Nucleic Acids Research*, 49(D1): D1276-D1281. https://doi.org/10.1093/nar/gkaa783

35. Machiela, M.J., & Chanock, S. J. (2015). LDlink: A web-based application for exploring population-specific haplotype structure and linking correlated alleles of possible functional variants. *Bioinformatics (Oxford, England)*, 31(21): 3555–3557. https://doi.org/10.1093/bioinformatics/btv402

36. Massart, J., Sjögren, R.J.O., Egan, B., Garde, C., Lindgren, M., Gu, W., Ferreira, D.M.S., Katayama, M., Ruas, J.L., Barrès, R., O'Gorman, D.J., Zierath, J.R. & Krook, A. 2021. Endurance exercise training-responsive miR-19b-3p improves skeletal muscle glucose metabolism. *Nature Communications*, 12(1): 5948. https://doi.org/10.1038/s41467-021-26095-0

37. Mayr, C. 2019. What Are 3′ UTRs Doing? *Cold Spring Harbor Perspectives in Biology*, 11(10): a034728. https://doi.org/10.1101/cshperspect.a034728

38. Meurer, S., Krüger, K. & Mooren, F. 2016. MicroRNAs unter Einfluss körperlicher Belastung. *Deutsche Zeitschrift für Sportmedizin*, 2016(02): 27-34.

39. Montalbo, R., Izquierdo, L., Ingelmo-Torres, M., Lozano, J.J., Capitán, D., Alcaraz, A. & Mengual, L. 2018. Prognostic value of circulating microRNAs in upper tract urinary carcinoma. *Oncotarget*, 9(24): 16691-16700. https://doi.org/10.18632/oncotarget.24672

40. Moreland, E., Borisov, O.V., Semenova, E.A., Larin, A.K., Andryushchenko, O.N., Andryushchenko, L.B., Generozov, E.V., Williams, A.G. & Ahmetov, I.I. 2022. Polygenic Profile of Elite Strength Athletes. *Journal of Strength and Conditioning Research*, 36(9): 2509-2514. https://doi.org/10.1519/JSC.0000000000003901

41. Nash, D., Hughes, M.G., Butcher, L., Aicheler, R., Smith, P., Cullen, T. & Webb, R. 2023. *IL-6* signaling in acute exercise and chronic training: Potential consequences for health and athletic performance. *Scandinavian Journal of Medicine & Science in Sports*, 33(1): 4-19. https://doi.org/10.1111/sms.14241

42. Ng, P.C. & Henikoff, S. 2003. SIFT: predicting amino acid changes that affect protein function. *Nucleic Acids Research*, 31(13): 3812-3814. https://doi.org/10.1093/nar/gkg509

43. O'Connell, K., Posthumus, M. & Collins, M. 2014. Collagen gene interactions and endurance running performance. *South African Journal of Sports Medicine*, 26(1): 9-14. http://dx.doi.org/10.7196/SAJSM.523.

44. Pedersen, B.K. 2013. Muscle as a Secretory Organ. *Comprehensive Physiology*, 3(3): 1337-1362. https://doi.org/10.1002/cphy.c120033

45. Pickering, C., Suraci, B., Semenova, E.A., Boulygina, E.A., Kostryukova, E.S., Kulemin, N.A., Borisov, O.V., Khabibova, S.A., Larin, A.K., Pavlenko, A.V., Lyubaeva, E.V., Popov, D.V., Lysenko, E.A., Vepkhvadze, T.F., Lednev, E.M., Leońska-Duniec, A., Pająk, B., Chycki, J., Moska, W., Lulińska-Kuklik, E., Dornowski, M., Maszczyk, A., Bradley, B., Kana-Ah, A., Cięszczyk, P., Generozov, E.V., Ahmetov, I.I 2019. A Genome-Wide Association Study of Sprint Performance in Elite Youth Football Players. *Journal of Strength and Conditioning Research*, 33(9): 2344-2351. https://doi.org/10.1519/JSC.0000000000003259

46. Rivera, M.A., Fahey, T.D., López-Taylor, J.R. & Martínez, J.L. 2020. The Association of Aquaporin-1 Gene with Marathon Running Performance Level: a Confirmatory Study Conducted in Male Hispanic Marathon Runners. *Sports Medicine - Open*, 6: 16. https://doi.org/10.1186/s40798-020-00243-0

47. Rosa Neto, J.C., Lira, F.S., Oyama, L.M., Zanchi, N.E., Yamashita, A.S., Batista, M.L., Oller do Nascimento, C.M. & Seelaender, M. 2009. Exhaustive exercise causes an anti-inflammatory effect in skeletal muscle and a pro-inflammatory effect in adipose tissue in rats. *European Journal of Applied Physiology*, 106(5): 697-704. https://doi.org/10.1007/s00421-009-1070-1

48. Ryu, I.S., Kim, D.H., Cho, H.-J. & Ryu, J.-H. 2023. The role of microRNA-485 in neurodegenerative diseases. *Reviews in the Neurosciences*, 34(1): 49-62. https://doi.org/10.1515/revneuro-2022-0039

49. Saunders, C.J., Posthumus, M., O'Connell, K., September, A.V. & Collins, M. 2015. A variant within the AQP1 3'-untranslated region is associated with running performance, but not weight changes, during an Ironman Triathlon. *Journal of Sports Sciences*, 33(13): 1342-1348. https://doi.org/10.1080/02640414.2014.989535

50. Schwarz, J.M., Cooper, D.N., Schuelke, M. & Seelow, D. 2014. MutationTaster2: mutation prediction for the deep-sequencing age. *Nature Methods*, 11(4): 361-362. https://doi.org/10.1038/nmeth.2890

51. Semenova, E.A., Hall, E.C.R. & Ahmetov, I.I. 2023. Genes and Athletic Performance: The 2023 Update. *Genes*, 14(6): 1235. https://doi.org/10.3390/genes14061235

52. Silva, G.J.J., Bye, A., el Azzouzi, H. & Wisløff, U. 2017. MicroRNAs as Important Regulators of Exercise Adaptation. *Progress in Cardiovascular Diseases, Physical Activity, Exercise and Fitness in Health and Disease*, 60(1): 130-151. https://doi.org/10.1016/j.pcad.2017.06.003

53. Siracusa, J., Koulmann, N. & Banzet, S. 2018. Circulating myomiRs: a new class of biomarkers to monitor skeletal muscle in physiology and medicine. *Journal of Cachexia, Sarcopenia and Muscle*, 9(1): 20-27. https://doi.org/10.1002/jcsm.12227

54. Sofu, M. 2020, January 23. Futbolcularda dayanıklılık ve kas iyileşmesi ile ilişkili interlökin-6 (*IL-6*) rs1800795 polimorfizminin dağılımının belirlenmesi. MasterThesis. Fen Bilimleri Enstitüsü.

55. Trinh, B., Peletier, M., Simonsen, C., Plomgaard, P., Karstoft, K., Pedersen, B.K., van Hall, G. & Ellingsgaard, H. 2021. Blocking endogenous *IL-6* impairs mobilization of free fatty acids during rest and exercise in lean and obese men. *Cell Reports Medicine*, 2(9). https://doi.org/10.1016/j.xcrm.2021.100396

56. Tuna, G., Polat, T., Yilmaz, Ö.Ö., Kapici, S., Doğan, C.S., Sağiroğlu, I., Savaşan, M., Erdil, N.G. & Ulucan, K. 2022. The Relationship between Swimming Styles and *IL-6* Rs1800795 Polymorphism in Professional Swimmers. *Pakistan Journal of Medical & Health Sciences*, 16(07): 444-444. https://doi.org/10.53350/pjmhs22167444

57. Varillas-Delgado, D., Del Coso, J., Gutiérrez-Hellín, J., Aguilar-Navarro, M., Muñoz, A., Maestro, A. & Morencos, E. 2022. Genetics and sports performance: the present and future in the identification of talent for sports based on DNA testing. *European Journal of Applied Physiology*, 122(8): 1811-1830. https://doi.org/10.1007/s00421-022-04945-z

58. Yamin, C., Duarte, J.A.R., Oliveira, J.M.F., Amir, O., Sagiv, M., Eynon, N., Sagiv, M. & Amir, R.E. 2008. IL6 (-174) and TNFA (-308) promoter polymorphisms are associated with systemic creatine kinase response to eccentric exercise. *European Journal of Applied Physiology*, 104(3): 579-586. https://doi.org/10.1007/s00421-008-0728-4

59. Yang, X.-D., Xu, X.-H., Zhang, S.-Y., Wu, Y., Xing, C.-G., Ru, G., Xu, H.-T. & Cao, J.-P. 2015. Role of miR-100 in the radioresistance of colorectal cancer cells. *American Journal of Cancer Research*, 5(2): 545-559.

60. Yue, X., Lan, F. & Liu, W. 2023. CircDDX17 inhibits invasive progression of pituitary adenomas by sponging miR-1279 and regulating CADM2 expression. *Frontiers in Oncology*, 13: 1268644. https://doi.org/10.3389/fonc.2023.1268644

61. Zhou, Q., Shi, C., Lv, Y., Zhao, C., Jiao, Z. & Wang, T. 2020. Circulating microRNAs in Response to Exercise Training in Healthy Adults. *Frontiers in Genetics*, 11: 256 https://doi.org/10.3389/fgene.2020.00256

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Research Article

A new species record for the flora of Türkiye; *Caroxylon vermiculatum* (L.) Akhani & Roalson (*Chenopodiaceae / Amaranthaceae*)

İsa Başköse *, Ahmet Emre Yaprak

Ankara University, Faculty of Science, Department of Biology, 06100 Ankara, TÜRKİYE

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Edited by:
Regina Karousou

***Corresponding Author:**
İsa Başköse
isabaskose@gmail.com

ORCID iDs of the authors:
İB. 0000-0001-7347-3464
AEY. 0000-0001-6464-2641

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Abstract: In this study, *Caroxylon vermiculatum* (L.) Akhani & Roalson, which is distributed in Southwest Europe, Northwest and Central Africa, and the Middle East, is recorded for the first time from Türkiye. The species was collected from the Akçakale district of Şanlıurfa province, close to the Syrian border. The description of the species, synonyms, a distribution map, photographs of the specimens, and its morphological characteristics are given. In addition, the diagnostic characteristics of known perennial species of the genus *Caryxylon* from Türkiye were compared.

Özet: Bu çalışmada, Güneybatı Avrupa, Kuzeybatı ve Orta Afrika ile Orta Doğu ülkelerinde yayılış gösteren *Caroxylon vermiculatum* (L.) Akhani & Roalson türünün kaydı ülkemizden ilk defa verilmektedir. Tür, Şanlıurfa ili Akçakale ilçesinden, Suriye sınırına yakın alanlarda toplanmıştır. Türün betimi, sinominleri, Türkiye ve Dünyadaki dağılım haritası, türün genel ve detaylı morfolojik karakterlerinin fotoğrafları verilmiştir. Ayrıca *Caryxylon* cinsine ait Türkiye'den bilinen çok yıllık türlerin diagnostik özellikleri ile karşılaştırılmıştır.

Introduction

Caroxylon Thunberg was first described in 1782, to place the shrubby *Caroxylon salsola* Thunberg in. It was subsequently recognized as a separate genus with 19 species in a study by Moquin-Tandon (1849). However, in 1851, the genus was transferred to the genus *Salsola* L. by Fenzl and recognized as a section of it (*Salsola* L. sect. *Caroxylon* Fenzl) (Fenzl 1851). This taxonomic status has been accepted in all studies carried out since Fenzl's work in 1851, reaching to early 21st century (Il'in 1936, Brenan 1954, Aellen 1967, Freitag & Rilke 1997, Freitag 2001, Aellen & Akeroyd 2003, Zhu *et al.* 2003).

Based on phylogenetic, morphological, and anatomical studies conducted in the last two decades (Akhani *et al*. 2007, Wen *et al*. 2010, Voznesenskaya *et al*. 2013), many new genera have been separated from the genus *Salsola*, which is considered polyphyletic. One of these is the genus *Caroxylon*. The necessary systematic and taxonomic arrangements for the genus and its representative taxa were established by various authors (Akhani *et al.* 2016, Mucina 2017, Mosyakin 2019, Rudov *et al.* 2020). The genus *Caroxylon* currently includes 128 species naturally distributed in Europe, Africa, and Asia (Powo 2023). In Türkiye, it is represented by eight species, of which *Caroxylon stenopterum* (Wagenitz) Akhani & Roalson is the only endemic taxon (Yaprak 2012). Of these eight taxa, four (*C. dendroides* (Pall.) Tzvelev, *C. ericoides* (M.Bieb.) Akhani & Roalson, *C. laricinum* (Pall.) Tzvelev, and *C. nodulosum* Moq) are perennial woody plants and four (*C. inerme* (Forssk.) Akhani & Roalson, *C. stenopterum* (Wagenitz) Akhani & Roalson, *C. incanescens* (C.A.Mey.), and *C. nitrarium* (Pall.) Akhani & Roalson Akhani & Roalson are annual herbaceous plants. All of them are distributed in saline areas and salty steppes in Central, Eastern, Southeastern Anatolia and the Aegean regions of Türkiye.

In this study, *Caroxylon vermiculatum* was recorded for the first time in Türkiye from Akçakale district of Şanlıurfa province. In addition, habitat information, distribution in Türkiye and the world, morphological characteristics of the species are given and these characteristics are compared with other four perennial *Caraxylon* species in Türkiye.

Materials and Methods

Caroxylon specimens with different morphological characteristics were collected in areas close to the Syrian border of Türkiye during field studies conducted by the authors in 2018, 2022, and 2023 in the Akçakale district of Şanlıurfa province. They were pressed, dried, and preserved for identification in accordance with common herbarium procedures. The first attempt to identify the

specimens using the volumes of "*Flora of Turkey"* (Aellen 1967, Davis *et al*. 1988, Güner *et al*. 2000) showed that the specimens did not belong to any previously recorded *Caroxylon* species naturally distributed in Türkiye. Therefore, the specimens were identified using other relevant literature (Boissier 1875, Il'in 1936, Brenan 1954, Tutin *et al*. 1964, Aellen & Hillcoat 1964, Zohary 1966, Tackholm 1974, Castroviejo 1990, Hedge 1997, Boulos 1999, Freitag 2000). Digital photographs of specimens of this species in international herbaria (BR, DES, L, LISI, LUX, MA, MW, P, W, WAG, WU, TAA, U, and US, with acronyms according to Thiers 2023+) were also examined and compared with the identified specimens of the present study. A list of some of the specimens examined is given in the Supplementary Material by country. *Caroxylon vermiculatum* specimens collected in the present study are included in the collection of the herbarium of the Department of Biology, Faculty of Science, Ankara University (ANK). Detailed information about the collection locality is in the Result section.

Digital measurements of all morphological characters were made in dry specimens using a BAB stereo binocular microscope and Bs200Pro BAB image processing and analysis system (BAB Image Analysing Systems). For each taxonomically valuable character of *C. vermiculatum* used to determine the limits of variation, 100 measurements were made separately. Morphological and morphometric measurements were made on both fruiting and flowering specimens collected in different years and at least ten different individuals were examined (see Supplementary Material). Using the obtained data, a comprehensive description of the population of the species in Türkiye was prepared.

Results

Taxonomy

Based on current data, the species is classified within the tribe *Caroxyleae* Akhani & Roalson of the subfamily *Salsoloideae* Raf. family of *Amaranthaceae* Juss.

Caroxylon vermiculatum (L.) Akhani & Roalson, Int. J. Pl. Sci. 168(6): 948 (2007), (Fig. 1, 2).

≡ *Salsola vermiculata* L. in Sp. Pl.: 223 (1753); *Nitrosalsola vermiculata* (L.) Theodorova in Ukrayins'k. Bot. Zhurn. 72: 444 (2015).

= *Chenopodium flavescens* (Cav.) Schult. in J.J. Roemer & J.A. Schultes, Syst. Veg., ed. 15[bis]. 6: 269 (1820); *Nitrosalsola hispanica* (Botsch.) Theodorova, Ukrayins'k. Bot. Zhurn. 72: 443 (2015); *Nitrosalsola portilloi* (Caball.) Theodorova, Ukrayins'k. Bot. Zhurn. 72: 444 (2015); *Itrosalsola rodinii* (Botsch.) Theodorova, Ukrayins'k. Bot. Zhurn. 72: 444 (2015); *Salsola buxifolia* Dum. Cours., Bot. Cult. 1: 622 (1802); *Salsola ericifolia* Masson ex Link, C.L. von Buch, Phys. Beschr. Canar. Ins.: 141 (1828); *Salsola flavescens* Cav., Icon. 3: 45 (1796); *Salsola frankenioides* (Caball.) Botsch., Novosti Sist. Vyssh. Rast. 11: 281 (1975); *Salsola hispanica* Botsch., Bot. Zhurn. (Moscow & Leningrad) 60: 501 (1975); *Salsola microphylla* Cav., Icon. 3: 45 (1796); *Salsola portilloi* Caball., Bol. Real Soc. Esp. Hist. Nat. 36: 143 (1936); *Salsola rodinii* Botsch., Bot. Zhurn. (Moscow & Leningrad) 60: 504 (1975); *Salsola tamariscifolia* Lag., Gen. Sp. Pl.: 12, nom. illeg. (1816); *Salsola vermiculata* var. *flavescens* (Cav.) Moq., A.P. de Candolle, Prodr. 13(2): 181 (1849); *Salsola vermiculata* var. *frankenioides* (Caball.) Maire, Bull. Soc. Hist. Nat. Afrique N. 28: 378 (1937); *Salsola vermiculata* subsp. *frankenioides* Caball., Bol. Real Soc. Esp. Hist. Nat. 36: 141 (1936); *Salsola vermiculata* var. *glabrescens* Moq., Chenop. Monogr. Enum.: 141 (1840); *Salsola vermiculata* var. *microphylla* (Cav.) Moq., A.P. de Candolle, Prodr. 13(2): 181 (1849); *Salsola vermiculata* var. *portilloi* (Caball.) Maire, Bull. Soc. Hist. Nat. Afrique N. 28: 378 (1937); *Salsola vermiculata* var. *pseudopapillosa* Caball., Bol. Real Soc. Esp. Hist. Nat. 36: 141 (1936); *Salsola vermiculata* var. *pubescens* Moq., Chenop. Monogr. Enum.: 141 (1840).

Woody or dwarf shrub with many stems from the base. **Stems** 40-120 cm long, up to 1 cm in diameter, prostrate to erect, branched at the upper part; branches 5-30 cm long, ascending to spreading, alternate; in addition to the flowering shoots often with numerous shorter vegetative shoots; cream or dirty-white at early stage, brown at maturity, cylindrical, with dense short crisped and sparse long pilose hairs 1-3 mm long; stem epidermis partially peeling on the lower parts of the stem at flowering, whole stem epidermis peeling at maturity. **Leaves** (4-)5-12 × 0.30-0.65 mm, linear, needle-like, triangular, green, greenish-gray, with short crisped and long pilose hairs; base slightly widened, margins entire, apex acute, upper parts semi-terete or terete, alternate, older leaves recurved, younger leaves straight or slightly incurved. **Bract** 1, (1.5-)2.0-7.50 × 1.2-3.2 mm, lower bracts leaf-like, longer than bracteoles, the upper scale-like, equal to bracteoles, sessile, succulent, ovate or ovate-lanceolate to subulate, green, greenish-grey, straw-colored at maturity; margins membranous, apex acute or obtuse, abaxial part with short crisped hairs and prominent single vein. **Bracteoles** 2, 1.9-2.8 × (0.8-)1.0-1.9 mm, ovate, sessile, slightly fleshy, green to greenish-grey, connated only at base, single-veined, apex acute or rarely acuminate, margins membranous (0.5 mm part), with short crisped hairs. **Inflorescence** paniculate-spike or thyrse-spike; flowers alternate, in axils of bracts and bracteoles. **Perianth** with 5 free segments, 1.7-2.5 × 0.6-1.5 mm, 1-veined, glabrous, membranous, ovate to ovate-lanceolate, with green triangular blotch on abaxial part, margins entire, apex acute or rarely acuminate. **Stamens** 5, 1.2-4.2 mm long, glabrous; **anthers** 1.0-2.0 × 0.4-1.0 mm, yellow, ovate-oblong, sagittate, opening longitudinally; **filaments** 0.7-3.7 × 0.10-0.60 mm, gray, flat, short in the early period and then elongated (flowering time), dorsifixed. **Pistil** 1, 2-carpellate, 1-locular, 2.5-4.5 mm long, glabrous; **stigmas** 2, 0.4-1.1 mm long, subulate, papillate, light brown; **style** 1, 0.6-1.2 mm in length, cylindrical ovary 0.6-2.0 × 0.7-1.9 mm, globose-rounded

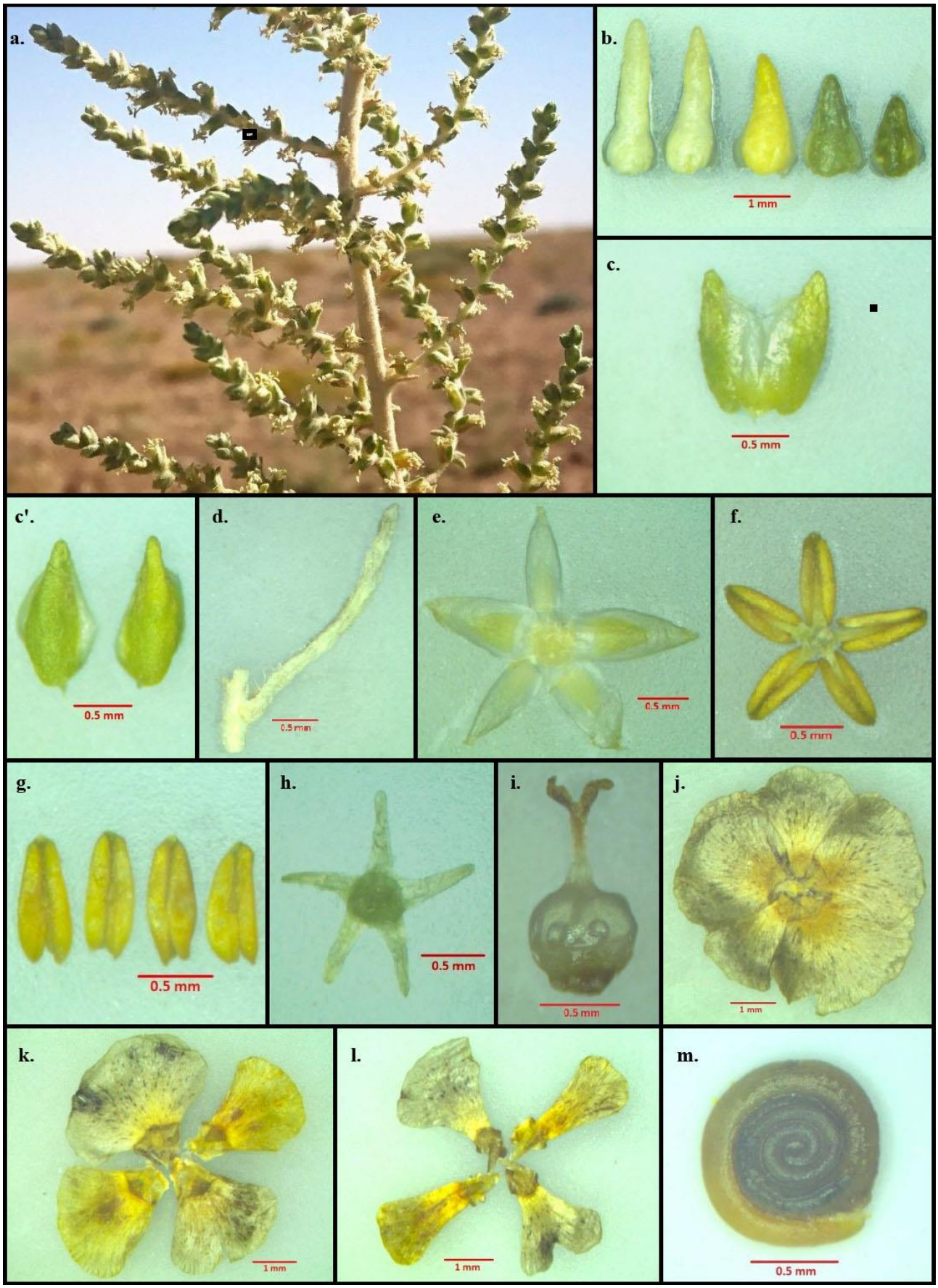

Fig. 1. Morphological characteristics of *Caroxylon vermiculatum*. **a.** Inflorescence, **b.** bracts **c-c'.** bracteoles, **d.** leaf, **e.** perianth segments, **f.** stamens, **g**. anthers, **h.** filaments, **i.** pistil, **j.** fruit, **k.** outer fruit wings, **l.** inner fruit wings, **m.** seed.

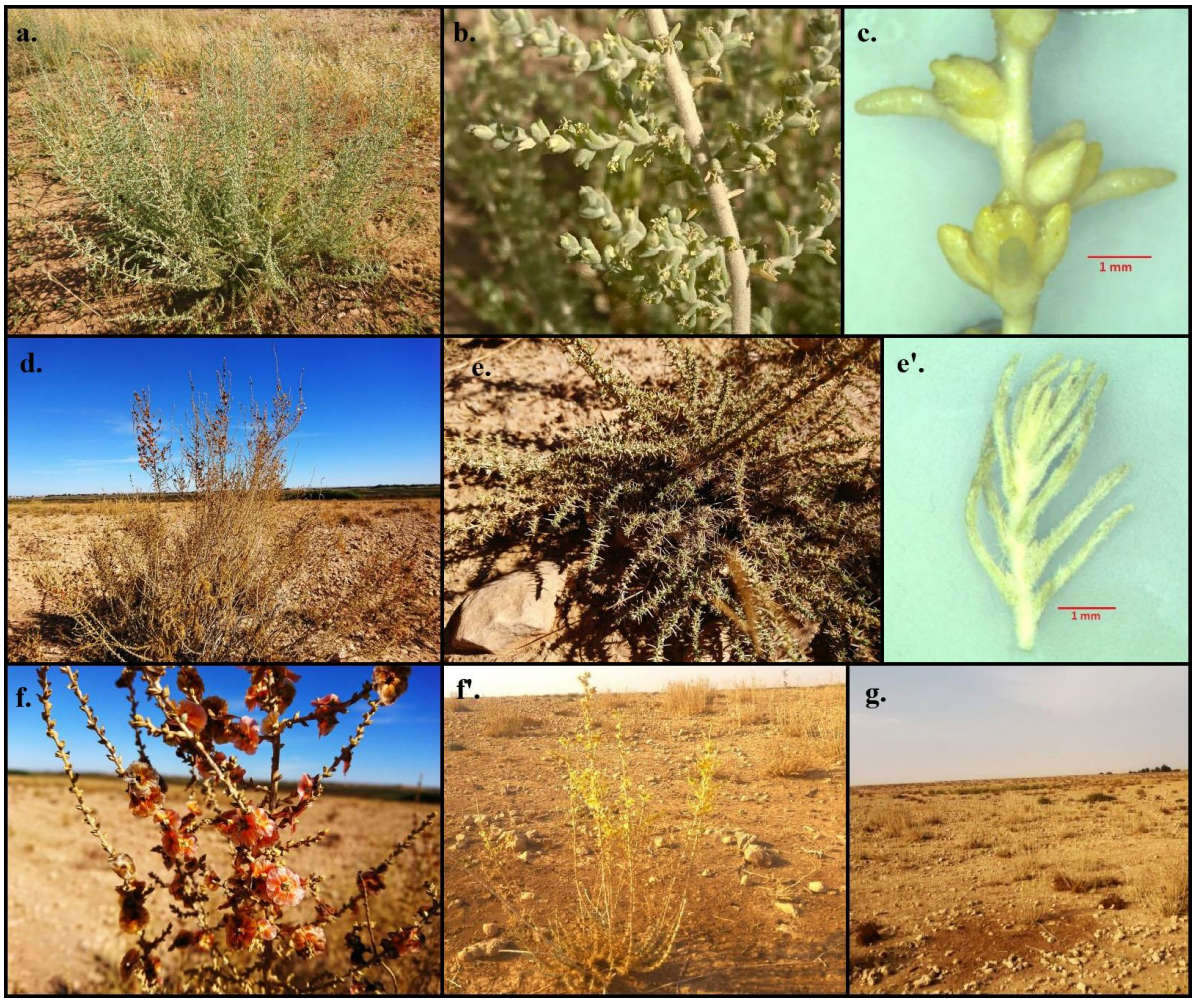

Fig. 2. Habitus, leaf, flower, habitat and fruit structures of *Caroxylon vermiculatum.* **a.** Habitus in flowering time, **b.** inflorescence, **c.** flower structures, **d.** habitus in fruiting time, **e-e'.** leaf structures, **f-f'.** fruit structure (pink, orange and yellow fruit), **g.** habitat.

or rarely pyriform. **Fruit** dry, 8.0-13.0 × 8.0-11.5 mm, rounded or ovoid, glabrous, yellow, orange or pink at early stage, light brown at maturity, with 5 wings, 3 outer and 2 inners; **outer** wings 3.0-5.6 × 3.5-8. 5 mm, obovate, reniform or flabellate, margins entire, tip obtuse or rarely emarginated, sometimes with black spots; **inner** wings 3.2-5.0 × (1.75-)2.0-5.0 mm, obovate or flabellate, margins entire, apex obtuse or rarely emarginated, sometimes with black spots. Seed 1, 1.7-3.0 × 1.7-2.7 mm, horizontally positioned, rounded; testa membranous; embryo spiral, radicle brown, plumula green.

Type: Hispania, L(Oeffling) 200, holotype (LINN. 315.20, photo!)

Locality: C7 Şanlıurfa province, Akçakale district, Öncül village, 2 km east of the village, field or irrigation canal edges, 360 m, 28 October 2018, 36°42'51.84"N, 39° 3'10.81"E, *Başköse-4459* (ANK).

Phenology: The species is flowering in July and August and fruiting in September and October.

Habitat: The species is known from a single locality in Şanlıurfa province, Akçakale district, close to the Syrian border. *Caroxylon vermiculatum* is distributed in slightly saline soils, fallow fields, and field edges or around irrigation canals at an altitude of 360 m a.s.l in Türkiye. The population of the species is represented by about 80 mature individuals and is distributed in an area of 1-2 km². This area is surrounded by agricultural fields and is under great anthropogenic effects. It is also under grazing pressure (cow and sheep) at some periods of the year. In addition, distribution area of the species is rarely used for military activities.

Distribution in Türkiye and the world: *Caroxylon vermiculatum* was first described by Linnaeus in 1753 within the genus *Salsola* as *S. vermiculata.* Today, the species is naturally distributed in Algeria, the Balearic Islands, the Canary Islands, Djibouti, Egypt, Georgia, Iran, Iraq, Israel, Italy (Sicily and Sardinia), Jordan, Lebanon, Mauritania, Morocco, Niger, Portugal, Saudi Arabia, Spain, Sudan, Syria, and Tunisia (Hedge 1997, Gbif 2023, Powo 2023) (Fig. 1). It has also been recorded in the United States, where it is considered an invasive species. With this study, the presence of *C. vermiculata* in Türkiye was confirmed and its distribution in the world was updated (Fig. 3).

Fig. 3. The distribution map of *Caroxylon vermiculatum* in world (**a**, **b**) and Türkiye (**c**).

Discussion

As a result of detailed morphological and morphometric measurements, it was understood that the collected specimens belong to *C. vermiculatum*, which has not been previously recorded from Türkiye. In this study, *C. vermiculatum* is reported from Türkiye for the first time, increasing the number of species of the genus *Caroxylon* in Türkiye to nine. The specimens of *C. vermiculata* were collected from Akçakale district of Şanlıurfa province, close to the Türkiye-Syria border. For identification of new plant records, border regions between countries are particularly important regions in terms of reports of new plant records for the countries in question. For instance, the authors of the present study (Başköse & Yaprak 2021) and other researchers (Kaya *et al.* 2010) have previously reported new plant records from the region where the material of the study was collected.

Caroxylon vermiculatum is the fifth perennial woody species within the genus after *C. dendroides*, *C. ericoides*, *C. laricinum*, and *C. nodulosum* in Türkiye. A comparison of the morphological characteristics of *C. vermiculata* and four other perennial taxa distributed in Türkiye is given in Table 1. The stems of the species are either horizontal or erect and can grow up to 120 cm in length. Considering the stem length, it is the second species with the longest stem structure after *C. dendroides*, which can grow up to 200 cm in length.

Table 1. Diagnostic characteristics of *Caroxylon* species from Türkiye (Boissier 1875, Il'in 1936, Brenan 1954, Aellen & Hillcoat 1964, Tutin *et al*. 1964, Zohary 1966, Tackholm 1974, Castroviejo 1990, Hedge 1997, Boulos 1999, Freitag 2000).

Species► Characteristics▼	*C. vermiculatum*	*C. ericoides*	*C. dendroides*	*C. laricinum*	*C. nodulosum*
Stem length	40-120 cm	Up to 30 cm	75-200 cm	Up to 75 cm	10-30 cm
Vegetative shoots	Present	Absent	Absent	Absent	Absent
Leaves shape and length	Linear or triangular, 5-12 mm	Ericoid, 3-12 mm	Linear, up to 10 mm	Linear, up to 20 mm	Ovate-triangular, up to 3 mm
Leaves hair type	Short crisped and long pilose hairs	Branched hairs	Glabrous	Pubescent or glabrous	Pubescent or glabrous
Perianth length	1.7-2.5 mm	1.0-2.0 mm	1.0-2.0 mm	1.25-2.25 mm	1.5-3.0 mm
Anther appendages shape	Short-obtuse	Rectangular to trapezoid	Short-obtuse	Short-obtuse	Oblong-ovate. elliptic-oblong, acute
Stigma shape	Subulate, papillate	Long papillate	Ribbon-shaped,	Thread-like papillate	Subulate, glabrescent
Style type and length	Long, 0.6-1.2 mm	Short, 0.2-0.4 mm	Long, 0.25-0.65 mm	Sessile or up to 0.25 mm	Short, 0.20-0.60 mm
Fruit diameter	8.0-13.0 mm	5-10 mm	3.75-10.50 mm	4-7.0 mm	4-9 mm
Fruit colors	Variable	Variable	Concolorous	Concolorous	Variable
Seed size (⌀)	1.7-3.0 mm	1.5-1.8 mm	1.10-1.75 mm	1.25-2.20 mm	1.50-2.50 mm

Regarding the habit of the species, it was determined that it has both many-stemmed flowering shoots from the base and many short vegetative shoots. When this condition was compared with other woody *Caroxylon* taxa distributed in Türkiye, it was determined that only *C. vermiculatum* has short vegetative shoots.

Among the perennial taxa of the genus, *Caroxylon ericoides* has the most distinct leaves of 3.0-12.0 mm long, ericoid, succulent and glabrous. In other species, the leaves are variable, linear, ovate, ovate-triangular, not succulent, hairy or glabrous and their length varies between 3.0-20 mm. The smallest leaf belongs to *C. nodulosum* with a length of 3.0 mm. In *C. vermiculatum*, the leaves are 5-12 mm long, linear, needle-like or triangular in shape and covered with both short crisped and long pilose hairs.

Caroxylon vermiculatum has the largest fruits (8.0-13.0 mm in diameter) among other representatives of *Caroxylon*in Türkiye. The fruits are 3.5-10.5 mm in diameter in *C. dendroides*, 5.0-10.0 mm in *C. ericoides*, 4.0-7.0 mm in *C. laricinum* and 4.0-9.0 mm in *C. nodulosum*. The fruits of *C. dendroides* and *C. laricinum* are dirty-white, white or yellowish-gray in the early period (immature). In the remaining perennial taxa, fruit color is variable and is yellow, orange, pink, maroon or red. In all taxa of the genus, ripe fruits are dark or light brown. Fruit color variability (yellow, orange, pink, red, burgundy) is also observed in annual taxa of the genus.

The seeds in all taxa of the genus are horizontal, disc-like, more or less rounded and the embryo is spiral. According to seed size, the largest seed belongs to *C. vermiculatum* with seed diameter varying between 1.7-3.0 mm.

Conclusion

As a result of this study, *Caroxylon vermiculatum* was recorded for the first time from Türkiye. Thus, one more species was added to the flora of Türkiye, contributing to our knowledge of plant biodiversity. The number of species of the genus *Caroxylon* in Türkiye increased to nine.

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References

1. Akhani, H., Edwards, G. & Roalson, E.H. 2007. Diversification of the old world Salsoleae s.l. (Chenopodiaceae): molecular phylogenetic analysis of nuclear and chloroplast data sets and a revised classification. *International Journal of Plant Sciences*, 168: 931-956. http://dx.doi.org/10.1086/518263
2. Akhani, H., Greuter, W. & Roalson, E.H. 2014. Notes on the typification and nomenclature of *Salsola* and *Kali* (Chenopodiaceae). *Taxon*, 63: 647-650. https://doi.org/10.12705/633
3. Akhani, H., Khoshravesh, R. & Malekmohammadi, M. 2016. Taxonomic novelties from Irano-Turanian region and NE Iran: *Oreosalsola*, a new segregate from *Salsola* s.l., two new species in *Anabasis* and *Salvia*, two new combinations in *Caroxylon* and *Seseli*. *Phytotaxa*, 249(1): 159-180. https://doi.org/10.11646/phytotaxa.249.1.7
4. Aellen, P. 1967. Chenopodiaecae. pp. 1-22. In*:* Merxmüller, H. (ed.). *Prodromus einer Flora von Südwestafrika 32.* Cramer, Lehre.
5. Aellen, P. & Akeroyd, J.R. 1993. *Salsola* L. pp. 125-128. In: Tutin, T.G., Burges, N.A., Chater, A.O., Edmondson, J.R., Heywood, V.H., Moore, D.M., Valentine, D.H., Walters, S.M. & Webb, D.A. (eds.). *Flora Europaea 2nd ed., vol. 1.* Cambridge University Press, Cambridge, 629 pp.
6. Aellen, P. & Hillcoat, D. 1964. Chenopodiaceae. pp. 180-212. In: Rechinger, K.H. (ed). *Flora Of Lowland Iraq*, Codicote, Herts. Wheldon&Wesley, LTD., Newyork, N.Y. Hafner Publishing CO, 746 pp.
7. Aellen, P. 1967. Salsola pp. 328-334. In: Davis, P.H. (ed.). *Flora of Turkey and the East Aegean Islands.* Vol. 2, Edinburgh University Press, Edinburgh, 567 pp.
8. Başköse, İ. & Yaprak, A.E. 2021. A new *Suaeda* record for flora of Turkey: *Suaeda aegyptiaca* (Hasselquist) Zohary (Chenopodiaceae/Amaranthaceae). *Trakya University Journal of Natural Science*, 22(2): 179-185. https://doi.org/10.23902/trkjnat.903661
9. Boissier, P.E. 1875. *Salsolaceae* and *Amaranthaceae*, pp. 897- 997. In: Boissier, P.E. (ed.). *Flora Orientalis*, vol. 4, 1033 pp.
10. Boulos, L. 1999. Chenopodiaceae. pp. 92-129. In: Boulos, L. (ed.). *Flora of Egypt-Azollaceae to Oxalidaceae*. Al Hadara Publishing, Cairo, 419 pp.
11. Brenan, J.P.M. 1954. Chenopodiaceae. pp. 1-26. In: Turrill, W.B. & Milne-Readhead, E. (Eds). *Flora of Tropical East Africa.* Crown Agents for Oversea Governments and Administrations, London, 26 pp.
12. Castroviejo, S. 1990. Salola. pp. 541-547. In: Castroviejo, S., Laínz, M., López González, G., Montserrat, P., Muñoz Garmendia, F., Paiva J. & Villar L. (Eds). *Flora Iberica, Vol. II, Platanaceae-Plumbaginaceae (partim)*, Real Jardín Botánico, CSIC, Madrid, 891 pp.
13. Davis, P.H., Tan, K. & Mill, R.R. 1988. *Flora of Turkey and the East Aegean Islands*. Vol. 10 (Suppl. I), Edinburgh University Press, Edinburgh, 590 pp.
14. Fenzl, E. 1851. Ordo Salsolaceae. pp. 689-853. In: Ledebour, C.F. (ed.). *Flora Rossica sive Enumeratio plantarum in totius Imperii Rossici provinciis Europaeis, Asiaticis et Americanis hucusque observatarum*, vol. 3(2). Sumtibus Librariae E. Schweizerbart, Stuttgartiae [Stuttgart]. 866 pp.
15. Freitag, H. 2000. Chenopodiaceae. pp. 161-163. In: Güner, A., Özhatay, N., Ekim, T. & Başer, K.H.C. (eds) *Flora of Turkey and the East Aegean Islands*, Vol. 11, (Suppl. 2), Edinburgh University Press, Edinburgh, 656 pp.
16. Freitag, H. 2001. *Salsola*. pp. 104-126. In: Ali, S.I. & Qaiser, M. (Eds). *Flora of Pakistan, vol. 204.* University of Karachi, Karachi & Missouri Botanical Garden Press, St Louis. 220 pp.
17. Freitag, H. & Rilke, S. 1997. *Salsola*. pp. 154-255. In: Rechinger, K.H. (ed.). *Flora Iranica, vol. 172.* Akademische Druck- und Verlagsanstalt, Graz. 583 pp.
18. Gbif, 2023. *Coroxylon vermiculatum*. https://www.gbif.org (*Global Biodiversity Information Facility*) (Date accessed, 3.10.2023).
19. Güner, A., Özhatay, N., Ekim, T. & Başer, K.H.C. 2000. Flora *of Turkey and the East Aegean Islands, Vol. 11 (Suppl. II)*. Edinburgh University Press, Edinburgh, 656 pp.
20. Hedge, I.C. 1997. *Flora Iranica*. Vol. 172, Akademische Druck-u. Verlagsanst. Graz, 371 pp.
21. Il'in, M.M. 1936. Chenopodiaceae. pp 4-372. In: Shishkin, B.K. (eds). *Flora of The USSR, volume VI, Centrospermae*, Izdatel'stvo Akademii Nauk SSSR Moskva –Leningrad, 731 pp.
22. Kaya, O.F., Cetin, E., Aydogdu, M., Ketenoglu, O., & Atamov, V. 2010. Syntaxonomical analyses of the secondary vegetation of Harran Plain (Sanliurfa/Turkey) ensuing excessive ırrigation by using GIS and remote sensing. *Ekoloji* 19(75): 1-14.
23. Linnaeus, C. 1753. *Species Plantarum, vols. 1.* Laurentius Salvius, Holmiae [Stockholm], 1200 pp.
24. Moquin-Tandon, A. 1849. Salsolaceae. pp. 41-219.In: Candolle, A.P. de (ed.). *Prodromus systematis naturalis regni vegetabilis*, vol. 13(2). Masson, Paris. 468 pp.
25. Mosyakin, S.L. 2019. *Caroxylon camphorosma*, a new combination for *Salsola camphorosma* (*S. camphorosmoides* Iljin, *nom. illeg.*, non Desf.) (Chenopodiaceae). *Phytotaxa*, 409(5): 291-295. https://doi.org/10.11646/phytotaxa.409.5.5
26. Mucina, L. 2017. *Caroxylon* (Chenopodiaceae *s.str.*) in continental southern Africa and Madagascar: a preliminary nomenclatural synopsis and biogeographical considerations. *Phytotaxa*, 312(2): 151-178. https://doi.org/10.11646/phytotaxa.312
27. Powo, 2023. *Coroxylon vermiculatum*. http://www.plantsoftheworldonline.org/taxon (Date accessed, 3.10.2023).
28. Rudov, A., Mashkour, M., Djamali, M. & Akhani, H. 2020. A Review of C4 Plants in Southwest Asia: an ecological, geographical and taxonomical analysis of a region with high diversity of C4 Eudicots. *Frontiers Plant Sciences.* 11: 546518.
29. Tackholm, V. 1974. *Students' Flora of Egypt, Second Edition.* Published by Cairo University, Beirut, 888 pp.

30. Thiers, B. 2023. [contonuously updated]) *Index herbariorum: a global directory of public herbaria and associated staff. New York Botanical Garden*. Available from: http://sweetgum.nybg.org/ih/ (Date accessed, 3.10.2023).

31. Thunberg, C.P. 1782. *Nova genera plantarum, vol. 2*. Joh. Edman, Upsaliae [Uppsala], 114 pp.

32. Tutin, T.G., Heywood, V.H., Burges, N.A., Moore, D.M., Valentine, D.H., Walters, S.M. & Webb, D.A. 1964. *Flora Europaea*, vol. 1, Cambridge University Press, Cambridge.

33. Voznesenskaya, E.V., Koteyeva, N.K., Akhani, H., Roalson, E.H. & Edwards, G.E. 2013. Structural and physiological analyses in Salsoleae (Chenopodiaceae) indicate multiple transitions among C3, intermediate, and C4 photosynthesis. *Journal of Experimental Botany* 64(12): 3583-3604. https://doi.org/10.1093/jxb/ert191

34. Wen, Z.B., Zhang, M.L., Zhu, G.L. & Sanderson, S.C. 2010. Phylogeny of Salsoleae s.l. (Chenopodiaceae) based on DNA sequence data from ITS, *psb*B-*psb*H, and rbcL, with emphasis on taxa of northwestern China. *Plant Systematics and Evolution*, 288: 25-42. http://dx.doi.org/10.1007/s00606-010-0310-5

35. Wikipedia, 2023. *Caroxylon vermiculatum*. https://en.wikipedia.org/wiki/Caroxylonvermiculatum (Date accessed, 7.10.2023).

36. Yaprak, A.E. 2012. Salsola. pp. 28-29. In: Güner, A., Aslan, S., Ekim, T., Vural, M. & Babaç, M.T. (eds). *Türkiye Bitkileri Listesi (Damarlı Bitkiler)*. Nezahat Gökyiğit Botanik Bahçesi ve Flora Araştırmaları Derneği Yayını, İstanbul, 1290 pp.

37. Zhu, G.-L., Mosyakin, S.L. & Clemants, S.E. 2003. Chenopodiaceae. pp. 354-414. In: Wu, Z.-Y., Raven, P.H. & Hong, D.Y. (Eds). *Flora of China, vol. 5*. Science Press, Beijing & Missouri Botanical Garden Press, St. Louis, 506 pp. Available from: www.efloras.org/florataxon.aspx?flora_id=2&taxon_id=10185 (Date accessed, 7.10.2023).

38. Zohary, M. 1966. Chenopodiaceae. pp. 136-180. In: Zohary, M. (ed.). *Flora Palaestina, Equisetaceae to Moringaceae*. The Israel Academy of Sciences and Humanities, Jarusalem, 403 pp.

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Research Article

Molecular characterization and comparative genomic analysis of two triamitovirus isolates hosted by the hypogean fungus *Tuber excavatum* Vittad.

Elifnaz Bora[1], Ilgaz Akata[2], Emre Keskin[3], Ergin Sahin[4,5*]

[1] Ankara University, Graduate School of Natural and Applied Sciences, 06110, Dışkapı, Ankara, TÜRKİYE
[2] Ankara University Faculty of Science, Department of Biology 06100 Tandogan, Ankara, TÜRKİYE
[3] Ankara University, Faculty of Agriculture Department of Fisheries and Aquaculture, Evolutionary Genetics Laboratory (eGL), 06110, Dışkapı, Ankara, TÜRKİYE
[4] Dokuz Eylul University, Faculty of Science, Department of Biology, 35390 Buca, İzmir, TÜRKİYE
[5] Dokuz Eylul University, Fauna and Flora Research and Application Center, 35390 Buca, İzmir, TÜRKİYE

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Boris Assyov

***Corresponding Author:**
Ergin Sahin
ergin.sahin@deu.edu.tr

ORCID iDs of the authors:
EB. 0000-0009-6047-6776
IA. 0000-0002-1731-1302
EK. 0000-0002-7279-313X
ES. 0000-0003-1711-738X

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Truffle
Tuber excavatum

Abstract: The connections between viruses and their hosts are complex and can arise from any combination of different evolutionary events including "codivergence", "switching", and "duplication" of the pathogen. Mycoviruses, a diverse virus group whose members specifically infect fungal hosts, are subject to similar evolutionary processes. In this study, we present the identification and complete genome characterization of the second isolate of a mitovirus, commonly known as *Tuber excavatum* mitovirus, officially named *Triamitovirus tuex1*. This mycovirus infects the hypogean, ectomyrrhizal fungus *Tuber excavatum* Vittad.. Both *Triamitovirus tuex1* isolates, Tekirdağ (identified by us) and Lammspringe, were found in the fruiting bodies of *T. excavatum* isolates collected from Türkiye and Germany, respectively. Comparative genomic analyses revealed that the two virus isolates share 85.33% sequence similarity in their whole genomes, with their protein encompassing RNA-dependent RNA polymerase (RdRp) domain showing an identity rate of 94.60%. The most diverse part of the viral genomes was found to be the 5' untranslated regions (UTRs), with a sequence similarity of 78.53%, while the 3' UTRs were the most conserved, with 91.53% sequence similarity. Considering the shared host species, the emergence of these *Triamitovirus tuex1* isolates appears to reflect a duplication pattern (intra-host divergence) resulting from adaptive radiation.

Özet: Virüsler ile konakları arasındaki bağlantılar karmaşıktır ve "kodivergens (birlikte ıraksama)", "değişim" ve "patojenin çoğaltılması" gibi farklı evrimsel olayların herhangi bir kombinasyonundan kaynaklanabilir. Mikovirüsler, özel olarak mantar konaklarını enfekte eden, çeşitlilik gösteren bir virüs grubudur ve benzer evrimsel süreçlere tabidir. Bu çalışmada, *Tuber excavatum* mitovirüsü olarak bilinen ve resmi olarak *Triamitovirus tuex1* olarak adlandırılan bir mitovirüsün ikinci izolatının tanımlanması ve tüm genom nitelemesi sunulmaktadır. Bu mikovirüs, hipogean, ektomikorizal mantar *Tuber excavatum* Vittad.'ı enfekte eder. Sırasıyla Türkiye ve Almanya'dan toplanan Tekirdağ (bizim tarafımızdan tanımlanan) ve Lammspringe *Triamitovirus tuex1* izolatlarının her ikisi de, *T. excavatum* meyvelerinde tanımlandı. Karşılaştırmalı genom analizleri, her iki virüs izolatının da tüm genomlarında %85,33'lük bir dizi benzerliği paylaştığını ve RNA bağımlı RNA polimeraz (RdRp) alanını (domain) içeren proteinlerinin %94,60'lık bir benzerlik oranına sahip olduğunu ortaya koymuştur. Viral genomların en çok farklılık gösteren kısmının %78,53'lük bir dizi benzerliği gösteren 5' translasyonu yapılmayan bölgeler (UTR'ler) olduğu, 3' UTR'lerin ise %91,53'lük bir dizi benzerliği ile en çok korunmuş kısımlar olduğu bulunmuştur. Konak türlerin ortak olması göz önünde bulundurulduğunda, bu *Triamitovirus tuex1* izolatlarının ortaya çıkışları, adaptif radyasyondan kaynaklanan bir çoğaltma modelini (konak-içi çeşitlenmesi) yansıtıyor gibi görünmektedir.

Introduction

Research on fungal-associated viral communities has experienced a significant increase, propelled by the advancements in high-throughput sequencing (HTS) technologies (Ayllon & Vainio 2023). HTS analyses have unveiled the remarkable diversity of fungal viruses (mycoviruses) and their wide-ranging infectivity across

diverse fungal groups, spanning from basal lineages to highly divergent divisions, each with unique lifestyles (Hough *et al.* 2023). Moreover, HTS methodologies have facilitated the discovery of numerous novel fungal viruses within unassigned virus groups, as well as the identification of various established viral taxa previously undocumented in the fungal kingdom (Ayllon & Vainio 2023) (https://talk.ictvonline.org/ictv-reports/ictv_online_report/n.

Currently, the International Committee on the Taxonomy of Viruses (ICTV) classifies mycoviruses into more than 30 families (https://ictv.global/). The majority of mycoviruses typically possess genomes comprised of double-stranded RNA (dsRNA) or positive-sense single-stranded RNA (+ssRNA) (Ghabrial *et al.* 2015). Additionally, mycoviruses with genomes containing negative-sense single-stranded RNA (−ssRNA) have been identified and are classified within the family *Mymonaviridae* (Wang *et al.* 2018, Lin *et al.* 2019, Walker *et al.* 2020, Guo *et al.* 2021). Recently, several single-stranded DNA (ssDNA) mycovirus species have been discovered, with only two classified within the recognized mycoviral family *Genomoviridae*. These include the monopartite *Gemycircularvirus sclero1* infecting *Sclerotinia sclerotiorum* and the tripartite *Gemytripvirus fugra1* infecting *Fusarium graminearum* (Yu *et al.* 2010, Varsani & Krupovic 2021).

In a single host species, multiple factors can impact the development of diverse genotypes within a virus species. These factors could be mutation rates and genetic recombination (e.g. genome segment reassortment), as well as host factors (e.g. antiviral status and genetic background) and ecological factors (e.g. climate and habitat disruption) that exert selection pressure on viruses (Elena & Sanjuin 2007, Parvez & Parveen 2017, LaTourrette & Garcia-Ruiz 2022). In evolutionary terms, the interactions between viruses and their hosts are complex and can arise from various evolutionary processes. These include "codivergence", where the phylogenies of viruses and hosts show topological congruence, "switching" involving lateral transfer of the virus to a new host that is phylogenetically distant from the previous one, and pathogen "duplication" where the parasite undergoes adaptive radiation within the same host species, resulting in multiple parasite groups with an identical host range (Göker *et al.* 2011).

The *Mitoviridae* family consists of RNA viruses that lack capsids and have a positive-sense, single-stranded RNA (ssRNA) genome, which ranges from 2.1 to 4.9 kb in length (Hillman & Cai 2013, Koonin *et al*. 2020). These viruses feature a single open reading frame (ORF) that employs the mitochondrial genetic code, encoding an RNA-dependent RNA polymerase (RdRp) domain characterized by six conserved protein motifs each denoted with single letters (A-F). The family *Mitoviridae* now encompasses four newly identified genera: *Kvaramitovirus*, *Triamitovirus*, *Duamitovirus*, and *Unuamitovirus* (https://ictv.global/taxonomy). Although these viruses were initially discovered in fungi, more recent findings have identified *Mitoviridae* members in plants and insects (Bruenn *et al.* 2015, Nibert *et al*. 2018, Fonseca *et al*. 2020).

The genus *Tuber* comprises ectomycorrhizal fungi known for their subterranean ascomata (Akata *et al*. 2020). Among these, *Tuber excavatum* Vittad. is distinguished by its underground fruit bodies known with transition from a pale yellowish brown to a reddish brown hue upon maturation. These ascomata are generally spherical or slightly lobed and feature a pronounced cavity. The gleba within begins as white or straw-colored and gradually darkens to reddish brown, with a network of branching yellowish veins. This species typically flourishes in calcareous soils, where it forms symbiotic relationships with both deciduous trees and conifers (Castellano & Türkoğlu 2012, Fan *et al*. 2013).

Despite their ecological importance, virus communities hosted by ectomycorrhizal fungi have received limited attention, with few studies focusing on the ecological roles of these viruses in soil environments (Petrzik *et al.* 2016, Sahin & Akata 2019, Sahin *et al.* 2020, Sutela & Vainio 2020, Sahin & Akata 2021, Sahin *et al.* 2021a, Sahin *et al.* 2021b, Akata *et al*. 2023, Sahin *et al.* 2023). Existing literature reports only two studies on mitoviruses found in hypogeous ectomycorrhizal fungal genus *Tuber* (Stielow *et al.* 2011, Stielow *et al.* 2012). These viruses designated as Tuber excavatum mitovirus (TeV) and Tuber aestivum mitovirus (TaV) were officially classified as member of the virus species *Triamitovirus tuex1* isolate Lammspringe and *Duamitovirus tuae1*, respectively.

In this study, we characterize the full-length genome sequence of the second member (isolate) of *Triamitovirus tuex1* (TeV isolate Tekirdağ) identified in a *Tuber excavatum* isolate. We further make evolutionary inferences about the two *Triamitovirus tuex1* isolates in light of the comparative genomic analyses.

Materials and Methods

Sampling of T. excavatum ascocarp

During a field survey conducted on August 18, 2021, a single ascocarp of *Tuber excavatum* (Fig. 1a) was collected under an oak tree (*Quercus* sp.) in the Tekirdağ province of Türkiye. This specimen was subsequently deposited at the ANK Ankara University Herbarium under the voucher specimen, with identifier Akata & Sahin TT 002.

dsRNA Isolation, in vitro Reverse Transcription, and Polymerase Chain Reaction Amplification

The ascocarp sample was sterilized by submerging it in a 2% sodium hypochlorite solution for one minute, then rinsing it thoroughly with sterile distilled water. After dehydration, the sample was finely milled into a powder. This powdered sample was used to enrich dsRNA with the Viral dsRNA Extraction Mini Kit (iNtRON Biotechnology, South Korea). The dsRNA obtained was

treated with S1 nuclease and DNase I according to the manufacturer's guidelines (Promega). The sample was purified with the GeneJET PCR Purification Kit (Thermo Fisher Scientific) and converted to cDNA using the primer-dN6 (5'-CCTGAATTCGGATCCTCCNNNNNN-3') and the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). This cDNA was then randomly amplified with the rPCR primer (5'-CCTGAATTCGGATCCTCC-3') and DreamTaq DNA Polymerase (Thermo Fisher Scientific), following the method outlined previously (Darissa *et al*. 2010). The rPCR amplicons were then cleaned up using the PureLink™ PCR Purification Kit (Thermo Fisher Scientific). About 1µg of the purified PCR products was sent to the Agrigenomics Hub (Ankara University, Türkiye) for library preparation. Sequencing of 150 bp paired ends, at a minimum depth of 100x, was performed on an Illumina Novaseq 6000 platform.

Sequence Data Analysis and Phylogenetic Study

Raw reads from high-throughput sequencing (HTS) were assembled into contigs *de novo* using CLC Genomic Workbench version 20.0.2 (Qiagen). *De novo* assembly analyses included a word size of 26, a default bubble size of 50, automatic estimation of paired distances, and a minimum contig length of 200 nucleotides (nt). The resulting contigs and their amino acid (aa) sequences, translated using the Swiss Institute of Bioinformatics' (SIB) online tool (https://web.expasy.org/translate/), were analyzed via BLASTx and BLASTp to identify viral sequences with an e-value < 1. Viral protein domains, such as RNA-dependent RNA polymerase, were identified using the Pfam protein family database (https://pfam.xfam.org/). Evolutionary analysis involved aligning the RdRp aa sequences of *Triamitovirus tuex1* isolates Tekirdağ and Lammspringe with other mitovirus species within the *Mitoviridae* family using the ClustalW multiple sequence alignment tool (Madeira *et al*. 2019). Phylogenetic trees were generated using MEGA X software, applying the maximum-likelihood (ML) method and the JTT+G+I substitution model (Kumar *et al*. 2018). The reliableness of the tree branches was tested with 1000 bootstrap replicates.

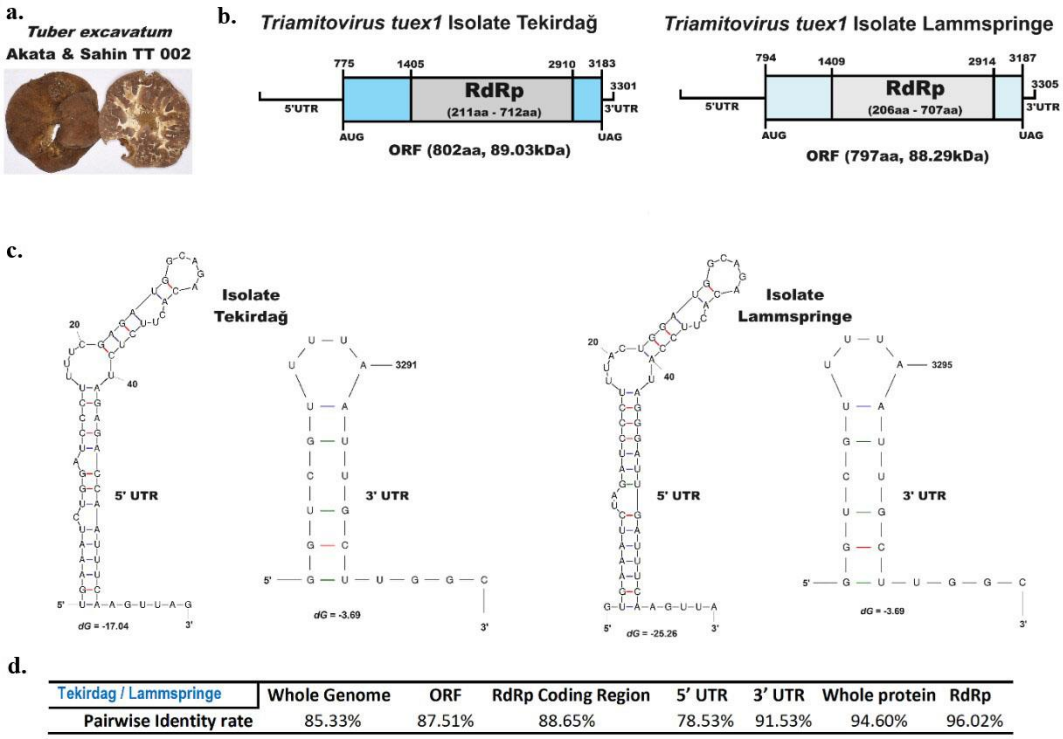
Determining the Sequences of 5' and 3' Termini with RLM-RACE

To sequence the 5'- and 3'-termini, the 3' ends of the extracted dsRNA were tagged with the short DNA oligo RLO (5'p-CATGGTGGCGACCGGTAG-NH2 3') using T4 RNA ligase 1 (New England Biolabs). The tagged dsRNA was cleaned up using the PureLink™ PCR Purification Kit (Thermo Fisher Scientific) and reverse transcribed into cDNA with the primer RTP (5'-CTACCGGTCGCCACCATG-3') and the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The terminal sequences were PCR amplified using the sequence specific reverse and forward oligonucleotide primers, TeV-5RACE1 (5'-ATCCTGTTGCGTCTCACATG-3') and TeV-3RACE1 (5'-TCAGTTGGGTTGGGTAGAGG-3'), respectively, with the RTP primer included in the PCR. The resulting PCR products were inserted into the pGEM-T Easy Vector (Promega) and sequenced using the conventional Sanger seqeuncing with universal M13 oligonucleotides at Agrigenomics Hub (Ankara University, Türkiye).

Results

BLASTx analyses of the assembled contigs derived from *T. excavatum* Akata & Sahin TT 002 (Fig. 1a) revealed a contig exhibiting 94.60% aa sequence similarity to the RdRp of a mitovirus, previously identified in a *T. excavatum* isolate collected from the Lammspringe village located in Lower Saxony, Germany (Stielow *et al.* 2012). This predefined virus was designated as "Tuber excavatum mitovirus (TeV) isolate Lammspringe" at that time. Later, it was officially defined as an exemplar virus of a mitovirus species *Triamitovirus tuex1* by the ICTV. Condsidering the 70% RdRp sequence similarity as the species demarcation threshold for mitovirids (https://ictv.global/ictv/proposals/2021.003F.R.Mitoviridae_100nsp_4ngen.zip), the mitovirus we identified was defined as an isolate of *Triamitovirus tuex1*. We, therefore, used the isolate name "Tekirdağ" to define the mitovirus we identified in the *T. excavatum* specimen Akata & Sahin TT 002. The genetic makeup of TeV isolate Tekirdağ and isolate Lammspringe consist of 3,301 and 3,305 nucleotides (nt) and have G+C contents of 38.17% and 37.70%, respectively (Supplementary Material). The genome sequences of TeV isolate Tekirdağ was kept in the NCBI GenBank database with the accession number OR157964.1. Using the fungal mitochondrial genetic code, where the opal stop codon UGA codes for tryptophan, the analysis showed that TeV isolate Tekirdağ genome harbors a single open reading frame (ORF) as similar to the genome of TeV isolate Lammspringe (Fig. 1b). The predicted polypeptides encoded by the ORFs of isolates Tekirdağ and Lammspringe composed of 802 and 797 aa with the molecular weights of 89.03 kDa and 88.29 kDa as calculated using the online Protein Molecular Weight tool (https://www.aatbio.com/tools/calculate-peptide-and-protein-molecular-weight-mw). The lengths of the 5′ and 3′ untranslated regions (UTRs) of isolate Tekirdağ are 774 and 118 nt, and the sizes of the corresponding regions in isolate Lammspringe are 793 and 118 nt (Fig. 1b). The 5′- and 3′- terminals of both isolates were analyzed by RNA Folding Form V2.3 of the RNA mfold server (http://www.unafold.org/mfold/applications/rna-folding-form-v2.php) and shown to have similar stem-loop secondary structures (Fig. 1c). No potential cyclization motif forming a panhandle structure which is often present in genomes of mitovirids was predicted in TeV isolates Tekirdağ and Lammspringe.

Searches in the Conserved Domains Database (CDD) of NCBI showed that the polypeptides encoded by TeV isolates Tekirdağ and Lammspringe contain RdRp domains located between aa positions 211 and 712, and 206 and 707, respectively (Fig. 1b).



d.

Tekirdag / Lammspringe	Whole Genome	ORF	RdRp Coding Region	5' UTR	3' UTR	Whole protein	RdRp
Pairwise Identity rate	85.33%	87.51%	88.65%	78.53%	91.53%	94.60%	96.02%

Fig. 1. a. Ascocarp of the *Tuber excavatum* Akata & Sahin TT 002, **b.** schematic representation of the genome organizations of *Triamitovirus tuex1* isolates Tekirdağ and Lammspringe. The UTRs and the ORFs encoding for RdRp were shown for each genome. The portion of the ORFs encoding for the RdRp domains are also specified, **c.** Predicted secondary structures of the 5' and 3' UTRs of both *Triamitovirus tuex1* isolates. The initial free energy values calculated for the secondary structures were stated, **d.** the percentage of sequence similarity rates observed between the *Triamitovirus tuex1* isolates Tekirdağ and Lammspringe at both the nucleotide and protein levels.

Further analysis in CDD showed that these conserved RdRp domains of both isolates are part of the mitovirus RdRp family (Accession: cl05469, with E-values of 3.09586e-67 and 9.95093e-67, respectively). Additionally, BLASTp analyses indicated that the RdRps of TeV isolates Tekirdağ and Lammspringe exhibit 94.60% sequence similarity to each other and the RdRp of Tuber mitovirus 3 (NCBI GenBank accession: WZN15221.1) was found to be their second best hit with 68.09% and 68.34% identities, respectively.

After comparing the genome sequences of both isolates, we found that the 3' UTRs displayed the highest similarity, with 91.53% nt sequence identity. Following this, the RdRp domain encoding regions exhibited 88.65% nt identity, while the ORFs showed 87.51% nt identity. The overall similarity across the entire genomes was 85.33%. Conversely, the most diverse regions were the 5' UTRs, with a nt sequence similarity of 78.53% (Fig. 1c). At the protein level, the isolates share 94.60% aa sequence similarity in their entire protein encoded by their single ORFs. Additionally, the RdRp domains of both isolates have a 96.02% aa sequence overlap (Fig. 1c).

To elucidate the relationship between TeV isolates Tekirdağ and Lammspringe and other mycoviruses, a phylogenetic tree was generated using the RdRp sequences from both isolates along with those from various mitoviruses. The resulting tree revealed that both isolates are grouped within the genus *Triamitovirus*, clustering with several members such as Tuber mitovirus 3, Geopora sumneriana mitovirus 1, Rhizictonia solani mitovirus 39, and Ceratobasidium mitovirus A (Fig. 2a). A multiple sequence alignment analysis of the RdRp domains from ten different *Triamitovirus* genus members showed that the RdRp domains of TeV isolates Tekirdağ and Lammspringe encompass all six conserved motifs (F, A, B, C, D, and E, arranged from the N-terminal to the C-terminal). Especially, this includes the most conserved motifs A, B, and C, which are located in the catalytic palm subdomain (Fig. 2b).

Discussion

Taking into account the species demarcation criteria for mitoviruses, which sets a 70% RdRp sequence identity threshold, along with the high sequence similarity rates exceeding 85% and 94% at the genome and protein levels respectively, the two TeV isolates, infecting the same host species *Tuber excavatum*, are considered as two representative members (strains, genotypes, or variants) of the mitovirus species *Triamitovirus tuex1*.

Within a single host species, various factors can influence the emergence of different genotypes of a virus species. In the context of the emergence of various mycovirus strains, these factors include, but are not limited to, 1) the viral mutation rate, 2) genetic recombination events, 3) specific host factors, and 4) ecological differences.

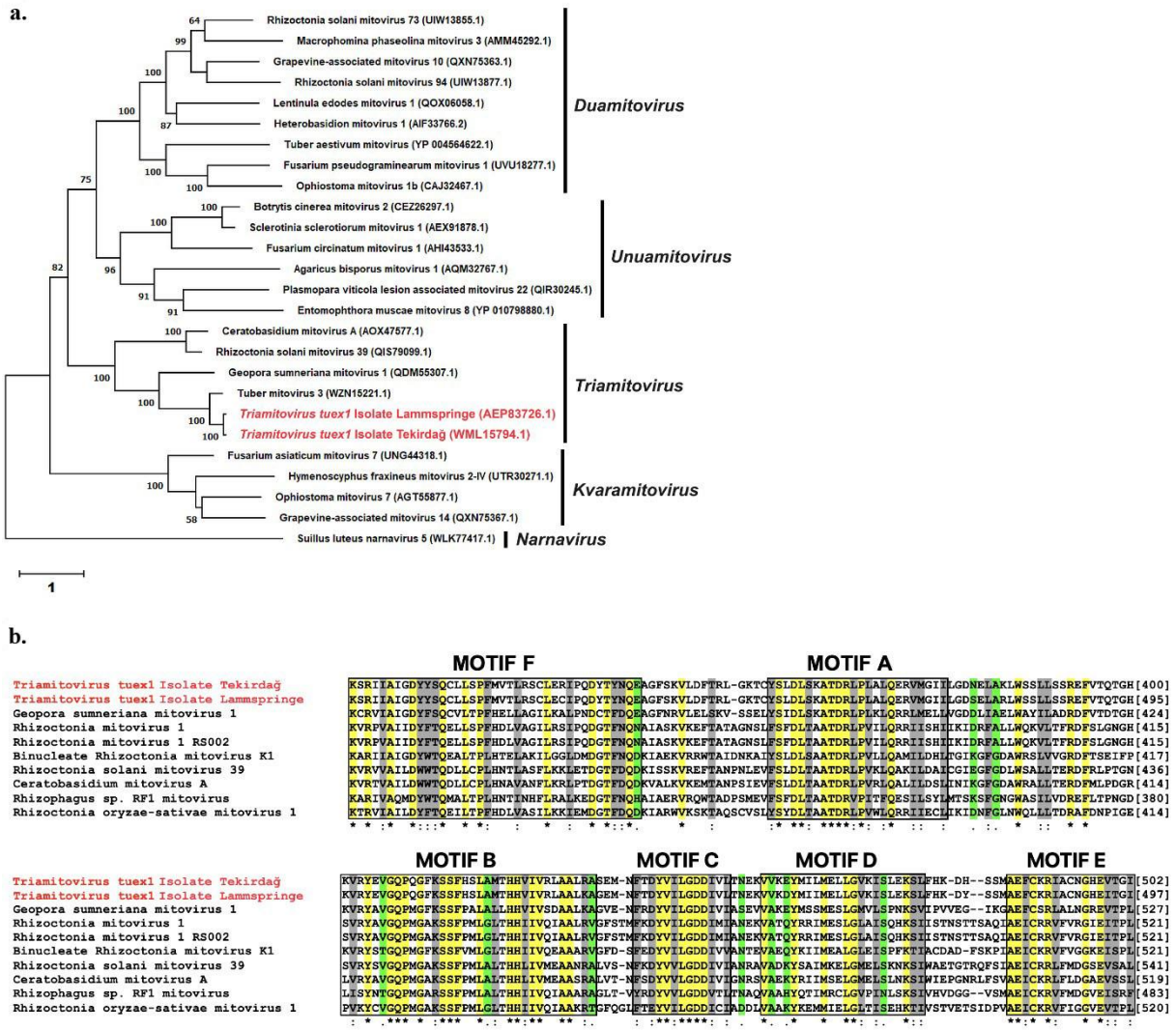


Fig. 2. a. Unrooted maximum-likelihood tree of the RdRps of both *Triamitovirus tuex1* isolates and related mitoviruses. Bootstrap values (>50%) are shown for each branch. GenBank accession numbers are also stated for each virus. Suillus luteus narnavirus 5 was selected as the outgroup sequence in the phylogenetic tree. The bars (lower left) show a genetic distance of 1 for the phylogenetic tree, **b.** the comparison of the RdRp conserved motifs (F, A, B, C, D, and E in the order from N-terminal to C-terminal) among both *Triamitovirus tuex1* isolates and closely related viruses within the genus *Triamitovirus* is shown. Matching amino acid residues are highlighted in yellow boxes, while amino acid residues sharing similar chemical properties are shown in grey and green boxes.

If we briefly mention each of these factors; 1) Viruses mutate rapidly due to their high replication rates and lack of proofreading mechanisms in their replication machinery. Thus, mutations can occur randomly during viral genome replication and lead to the emergence of new virus strains. This phenomenon can generally be observed in mitoviruses as well. 2) Mitoviruses have RNA genomes that can undergo genetic recombination (for instance via intermolecular template switch or via non-replicative recombination involving strand break and ligation) when multiple mitovirus strains infect the same host cell. In fact, multiple mycovirus infections are commonly observed in fungal hosts of diverse origin. This infection state of the host can result in the creation of novel viral genotypes with combinations of genetic material from different viral strains. 3) Variability in host factors such as antiviral status and genetic background, can influence which viral genotypes are more successful in establishing infection and spreading within a host population. It is plausible that at least some of the genetic variations in TeV isolates might be the result of the accumulation of host adaptive mutations 4) Environmental factors such as climate, habitat disruption, and interspecies interactions can impact the distribution and prevalence of viruses, potentially creating selection pressure on viruses. As a result, certain genotypes/variants may have advantages in specific environments, leading to their propagation and survival. Overall, the interplay of these factors can result in the diversification of viral genotypes even within a single host species, contributing to the ongoing evolution and adaptation of viruses.

A fundamental question in evolutionary biology concerns the extent to which the evolution of parasites is tied to the evolution of their hosts (Klassen 1992, Johnson

et al. 2003). If viruses are specific to their hosts and transmitted solely vertically (for instance, without natural vectors), their phylogeny should be congruent with that of their hosts, adhering to Fahrenholz' rule of strict codivergence (de Vienne *et al.* 2013). However, a combination of events such as host switching, duplication (intra-host divergence), and parasite extinction can result in incongruence between the phylogenies of viruses and their hosts (Göker *et al.* 2011). In this context, "switching" denotes the lateral transfer of the parasite and its successful establishment in a new host that is phylogenetically distant from the previous one. If this transfer leads to parasite speciation, it is termed a "complete switch"; otherwise, it is an "incomplete switch." "Duplication" refers to the parasite's adaptive radiation within the same host species, producing multiple parasite groups with an identical host range.

In a prior investigation, advanced statistical methods were employed to evaluate the hypothesis that mycoviruses from different lineages codiverge/coevolve with their hosts (Göker *et al.* 2011). With a dataset limited to 25 mitovirus-related sequences, the researchers observed that the evolutionary patterns of mitoviruses closely resembled, though not precisely mirrored, the duplication-switching pattern rather than codivergence. In this sense, the emergence of *Triamitovirus tuex1* isolates within the same host species appears to reflect a duplication (intra-host divergence) event resulting from adaptive radiation.

It is important to highlight that various genomic regions of *Triamitovirus tuex1* members evolve at different rates. For example, comparative analyses revealed that the 3' UTRs of both isolates are highly conserved (91.53% similarity), whereas the 5' UTRs show the most diversity (78.53%). The relatively high sequence similarity observed in the 3' UTRs of the virus isolates could be attributed to their widely acknowledged crucial roles in synthesizing the minus (-) strand during the genome replication of positive (+) ssRNA viruses.

The untranslated regions (UTRs) of mitoviruses show considerable variability in length and sequence diversity, even within the same species. Although it is believed that translation factors interact with these sites, the exact host factors (non-coding RNAs and/or proteins) that participate in these interactions have yet to be identified. Additionally, the functions of these non-coding regions, and whether they undergo any epitranscriptomic modifications affecting the host's physiology, have yet to be experimentally explored. It is currently theorized that the terminal sequences of each UTR region function as cis-elements, aiding in their interaction with the viral RdRps during the replication of the mitovirus genome. To enhance the understanding of these regulatory cis elements, comparative genomic analyses using advanced deep learning models could be advantageous, provided that a substantial amount of sequence data is available.

In conclusion, our objective was to enhance the expanding mycovirus sequence database by sequencing and characterizing the complete genome of a mitovirus isolate. Additionally, through comparative genomic analyses with closely related mitovirus isolates, we aimed to provide a deeper understanding of the evolutionary processes influencing mitovirus genome and protein

Ethics Committee Approval: Since the article does not contain any studies with human or animal subject, its approval to the ethics committee was not required.

Data Sharing Statement: All data are available within the study and in the Supplementary Material.

Author Contributions: Concept: E.Ş., Design: E.Ş., Execution: E.B., Material supplying: I.A, Data acquisition: E.B., E.Ş., Data analysis/interpretation: E.B., I.A., E.K., E.Ş., Writing: I.A., E.Ş., Critical review: E.B., I.A., E.Ş.

Conflict of Interest: The authors have no conflicts of interest to declare.

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References

1. Akata, I., Sevindik, M. & Şahin, E. 2020. *Tuber fulgens* Quél., a new record for Turkish truffles. *Turkish Journal of Agriculture-Food Science and Technology*, 8(11): 2472-2475. https://doi.org/10.24925/turjaf.v8i11.2472-2475.3884

2. 2. Akata, I., Edis, G., Keskin, E. & Sahin, E. 2023. Diverse partitiviruses hosted by the ectomycorrhizal agaric *Hebeloma mesophaeum* and the natural transmission of a partitivirus between phylogenetically distant, sympatric fungi. *Virology*, 581: 63-70. https://doi.org/10.1016/j.virol.2023.03.002

3. 3. Ayllon, M.A. & Vainio, EJ. 2023. Mycoviruses as a part of the global virome: Diversity, evolutionary links and lifestyle. *Advances in Virus Research*, 115: 1-86. https://doi.org/10.1016/bs.aivir.2023.02.002

4. 4. Bruenn, J.A., Warner, B.E. & Yerramsetty, P. 2015. Widespread mitovirus sequences in plant genomes. *PeerJ*, 3: e876. https://doi.org/10.7717/peerj.876

5. 5. Castellano, M.A. & Türkoğlu, A. 2012. New Records of Truffle Taxa in *Tuber* and *Terfezia* from Turkey. *Turkish Journal of Botany*, 36: 295-298. https://doi.org/10.3906/bot-1106-10

6. 6. Darissa, O., Willingmann, P. & Adam, G. 2010. Optimized approaches for the sequence determination of double-stranded RNA templates. *Journal of Virological Methods*, 169(2): 397-403. https://doi.org/10.1016/j.jviromet.2010.08.013

7. 7. De Vienne, D.M., Refrégier, G., López-Villavicencio, M., Tellier, A., Hood, M.E. & Giraud, T.J.N.P. 2013. Cospeciation vs host-shift speciation: methods for testing, evidence from natural associations and relation to coevolution. *New Phytologist*, 198(2): 347-385. https://doi.org/10.1111/nph.12150

8. 8. Elena, S.F. & Sanjuán, R. 2007. Virus evolution: insights from an experimental approach. *Annual Review of Ecology*,

Evolution, and Systematics, 38: 27-52. https://doi.org/10.1146/annurev.ecolsys.38.091206.095637

9. 9. Fan, L., Cao, J.Z. & Li, Y. 2013. A reassessment of excavated *Tuber* species from China based on morphology and ITS rDNA sequence data. *Mycotaxon*, 124(1): 155-163. https://doi.org/10.5248/124.155

10. 10. Fonseca, P., Ferreira, F., da Silva, F., Oliveira, L.S., Marques, J.T., Goes-Neto, A., Aguiar, E. & Gruber, A. 2020. Characterization of a novel mitovirus of the sand fly *Lutzomyia longipalpis* using genomic and virus-host interaction signatures. *Viruses*, 13(1): 9. https://doi.org/10.3390/v13010009

11. 11. Ghabrial, S.A., Castón, J.R., Jiang, D., Nibert, M.L. & Suzuki, N. 2015. 50-plus years of fungal viruses. *Virology*, 479: 356-368. https://doi.org/10.1016/j.virol.2015.02.034

12. 12. Göker, M., Scheuner, C., Klenk, H.P., Stielow, J.B. & Menzel, W. 2011. Codivergence of mycoviruses with their hosts. *PLoS One* 6(7): e22252. https://doi.org/10.1371/journal.pone.0022252

13. 13. Guo, M., Shen, G., Wang, J., Liu, M., Bian, Y. & Xu, Z. 2021. Mycoviral diversity and characteristics of a negative-stranded RNA virus LeNSRV1 in the edible mushroom *Lentinula edodes*. *Virology*, 555: 89-101. https://doi.org/10.1016/j.virol.2020.11.008

14. 14. Hillman, B.I. & Cai, G. 2013. The family *narnaviridae*: simplest of RNA viruses. *Advances in Virus Research*, 86: 149-176. https://doi.org/10.1016/B978-0-12-394315-6.00006-4

15. 15. Hough, B., Steenkamp, E., Wingfield, B. & Read, D. 2023. Fungal viruses unveiled: a comprehensive review of mycoviruses. *Viruses*, 15(5): 1202. https://doi.org/10.3390/v15051202

16. 16. Johnson, K.P., Adams, R.J., Page, R.D. & Clayton, D.H. 2003. When do parasites fail to speciate in response to host speciation? *Systematic Biology*, 52(1): 37-47. https://doi.org/10.1080/10635150390132704

17. 17. Klassen, G.J. 1992. Coevolution: a history of the macroevolutionary approach to studying host-parasite associations. *The Journal of Parasitology*, 78: 573-587. https://doi.org/10.2307/3283532

18. 18. Koonin, E.V., Dolja, V.V., Krupovic, M., Varsani, A., Wolf, Y. I., Yutin, N., Zerbini, F.M. & Kuhn, J.H. 2020. Global organization and proposed megataxonomy of the virus world. *Microbiology and Molecular Biology Reviews*, 84(2): 10-1128. https://doi.org/10.1128/mmbr.00061-19

19. 19. Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, 35(6): 1547-1549. https://doi.org/10.1093/molbev/msy096

20. 20. LaTourrette, K. & Garcia-Ruiz, H. 2022. Determinants of virus variation, evolution, and host adaptation. *Pathogens*, 11(9): 1039. https://doi.org/10.3390/pathogens11091039

21. 21. Lin, Y.H., Fujita, M., Chiba, S., Hyodo, K., Andika, I.B., Suzuki, N. & Kondo, H. 2019. Two novel fungal negative-strand RNA viruses related to mymonaviruses and phenuiviruses in the shiitake mushroom (*Lentinula edodes*). *Virology*, 533: 125-136. https://doi.org/10.1016/j.virol.2019.05.008

22. 22. Madeira, F., Park, Y.M., Lee, J., Buso, N., Gur, T., Madhusoodanan, N., Basutkar, P., Tivey, A.R.N., Potter, S.C., Finn, R.D. & Lopez, R. 2019. The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Research*, 47(W1): W636-W641. https://doi.org/10.1093/nar/gkz268

23. 23. Nibert, M.L., Vong, M., Fugate, K.K. & Debat, H.J. 2018. Evidence for contemporary plant mitoviruses. *Virology*, 518: 14-24. https://doi.org/10.1016/j.virol.2018.02.005

24. 24. Parvez, M.K. & Parveen, S. 2017. Evolution and emergence of pathogenic viruses: past, present, and future. *Intervirology*, 60(1-2): 1-7. https://doi.org/10.1159/000478729

25. 25. Petrzik, K., Sarkisova, T., Starý, J., Koloniuk, I., Hrabáková, L. & Kubešová, O. 2016. Molecular characterization of a new monopartite dsRNA mycovirus from mycorrhizal *Thelephora terrestris* (Ehrh.) and its detection in soil oribatid mites (Acari: Oribatida). *Virology*, 489: 12-19. https://doi.org/10.1016/j.virol.2015.11.009

26. 26. Sahin, E. & Akata, I. 2019. Complete genome sequence of a novel mitovirus from the ectomycorrhizal fungus *Geopora sumneriana*. *Archives of Virology*, 164: 2853-2857. https://doi.org/10.1007/s00705-019-04367-x

27. 27. Sahin, E. & Akata, I. 2021. Full-length genome characterization of a novel alphapartitivirus detected in the ectomycorrhizal fungus *Hygrophorus penarioides*. *Virus Genes*, 57(1): 94-99. https://doi.org/10.1007/s11262-020-01814-9

28. 28. Sahin, E., Akata, I. & Keskin, E. 2020. Novel and divergent bipartite mycoviruses associated with the ectomycorrhizal fungus *Sarcosphaera coronaria*. *Virus Research*, 286: 198071. https://doi.org/10.1016/j.virusres.2020.198071

29. 29. Sahin, E., Akata, I. & Keskin, E. 2021a. Molecular characterization of a new endornavirus inhabiting the ectomycorrhizal fungus *Hygrophorus penarioides*. *Brazilian Journal of Microbiology*, 52(3): 1167-1172. https://doi.org/10.1007/s42770-021-00500-8

30. 30. Sahin, E., Keskin, E. & Akata, I. 2021b. Novel and diverse mycoviruses co-inhabiting the hypogeous ectomycorrhizal fungus *Picoa juniperi*. *Virology*, 552: 10-19. https://doi.org/10.1016/j.virol.2020.09.009

31. 31. Sahin, E., Ozbey Saridogan, B.G., Keskin, E. & Akata, I. 2023. Identification and complete genome sequencing of a novel betapartitivirus naturally infecting the mycorrhizal desert truffle *Terfezia claveryi*. *Virus Genes*, 59(2): 254-259. https://doi.org/10.1007/s11262-023-01972-6

32. 32. Stielow, B., Klenk, H.P., Winter, S. & Menzel, W. 2011. A novel *Tuber aestivum* (Vittad.) mitovirus. *Archives of Virology*, 156: 1107-1110. https://doi.org/10.1007/s00705-011-0998-8

33. 33. Stielow, J.B., Bratek, Z., Klenk, H.P., Winter, S. & Menzel, W. 2012. A novel mitovirus from the hypogeous ectomycorrhizal fungus *Tuber excavatum*. *Archives of Virology*, 157: 787-790. https://doi.org/10.1007/s00705-012-1228-8

34. 34. Sutela, S. & Vainio, E.J. 2020. Virus population structure in the ectomycorrhizal fungi *Lactarius rufus* and *L. tabidus* at two forest sites in Southern Finland. *Virus Research*, 285: 197993. https://doi.org/10.1016/j.virusres.2020.197993

35. 35. Varsani, A. & Krupovic, M. 2021. Family *Genomoviridae*: 2021 taxonomy update. *Archives of Virology*, 166: 2911-2926. https://doi.org/10.1007/s00705-021-05183-y

36. 36. Walker, P.J., Siddell, S.G., Lefkowitz, E.J., Mushegian, A.R., Adriaenssens, E.M., Dempsey, D.M., Dutilh, B.E., Harrach, B., Harrison, R.L., Hendrickson, R.C., Junglen, S., Knowles, N.J., Kropinski, A.M., Krupovic, M., Kuhn, J.H., Nibert, M., Orton, R.J., Rubino, L., Sabanadzovic, S., Simmonds, P., Smith, D.B., Varsani, A., Zerbini, F.M. & Davison, A.J. 2020. Changes to virus taxonomy and the statutes ratified by the International Committee on Taxonomy of Viruses (2020). *Archives of Virology*, 165: 2737-2748. https://doi.org/10.1007/s00705-020-04752-x

37. 37. Wang L., He, H., Wang, S., Chen, X., Qui, D., Kondo, H. & Guo, L. 2018. Evidence for a novel negative-stranded RNA mycovirus isolated from the plant pathogenic fungus *Fusarium graminearum. Virology*, 518: 232-240. https://doi.org/10.1016/j.virol.2018.03.008

38. 38. Yu, X., Li, B., Fu, Y., Jiang, D., Ghabrial, S.A., Li, G., Peng, Y., Xie, J., Cheng, J., Huang, J. & Yi, X. 2010. A geminivirus-related DNA mycovirus that confers hypovirulence to a plant pathogenic fungus. *Proceedings of the National Academy of Sciences of the U.S.A."*, 107(18): 8387-8392. https://doi.org/10.1073/pnas.0913535107

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Research Article

ATM kinase phosphorylates Ser15 of p53 in a pH-dependent manner

Serap Pektaş

Recep Tayyip Erdogan University, Faculty of Arts and Sciences, Department of Chemistry, 53020, Rize, TÜRKİYE
e-mail: serap.pektas@erdogan.edu.tr, ORCID: 0000-0003-0497-6257

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Abstract: The phosphorylation of Ser15 in the transactivation domain (TAD) of the tumor suppressor protein 53 (p53) by ataxia-telangiectasia mutated (ATM) kinase is a crucial step in the tumor suppressor function of p53. An understanding of the factors that affect the rate of Ser15 phosphorylation may provide new strategies for the manipulation of the ATM-p53 pathway in cancer therapy. In this study, the effect of electrostatic interactions between ATM and p53 was investigated by measuring the phosphorylation of Ser15 at varying pH ranges from 5 to 9. To achieve this, two different kinase assay methods were utilized: the ELISA technique, which directly quantifies the phosphorylated Ser15, and the Universal Kinase Assay, which assesses the formation of ADP. The results revealed that Ser15 phosphorylation was pH-dependent, with higher phosphorylation rates observed in the alkaline range. To ascertain whether the lower phosphorylation rates observed at acidic pH were due to protein denaturation, a pH-dependent solubility profile was generated using the CamSol server. The obtained results demonstrated comparable solubility rates within the pH range of the kinase assays performed. Furthermore, the significance of negatively charged residues in TAD_{1-39} was evaluated by substituting Asp and Glu residues with hydrophobic and uncharged hydrophilic residues in TAD_{1-39} using ChimeraX and subsequently comparing their interactions with the ATM using the protein-protein docking server HADDOCK2.4. The results of the docking simulations indicated that the alteration of negatively charged residues with uncharged ones resulted in a reduction in the efficiency of the interaction between the ATM and TAD_{1-39}. In conclusion, it can be stated that electrostatic interactions between the ATM and TAD are important for optimal Ser15 phosphorylation.

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Özet: Tümör baskılayıcı protein 53'ün (p53) transaktivasyon domainindeki (TAD) Ser15'in ataksi-telenjiektazi mutasyonlu (ATM) kinaz tarafından fosforilasyonu, p53'ün tümör baskılayıcı işlevinde çok önemli bir adımdır. Ser15 fosforilasyon oranını etkileyen faktörlerin anlaşılması, kanser tedavisinde ATM-p53 yolağının manipülasyonu için yeni stratejiler sağlayabilir. Bu çalışmada, ATM ve p53 arasındaki elektrostatik etkileşimlerin etkisi, Ser15'in 5 ila 9 arasında değişen pH aralıklarında fosforilasyonu ölçülerek araştırılmıştır. Bunu başarmak için iki farklı kinaz tahlil yöntemi kullanılmıştır: fosforile Ser15'i doğrudan ölçen ELISA tekniği ve ADP oluşumunu değerlendiren Universal Kinase Assay. Sonuçlar, Ser15 fosforilasyonunun pH'a bağlı olduğunu ve alkali aralıkta daha yüksek fosforilasyon oranlarının gözlendiğini ortaya koymuştur. Asidik pH'da gözlenen daha düşük fosforilasyon oranlarının protein denatürasyonundan kaynaklanıp kaynaklanmadığını tespit etmek için CamSol sunucusu kullanılarak pH'ya bağlı bir çözünürlük profili oluşturulmuştur. Elde edilen sonuçlar, gerçekleştirilen kinaz deneylerinin pH aralığı içinde karşılaştırılabilir çözünürlük oranları göstermiştir. Ayrıca, TAD_{1-39}'daki negatif yüklü kalıntıların önemi, ChimeraX kullanılarak TAD_{1-39}'daki Asp ve Glu kalıntılarının hidrofobik ve yüksüz hidrofilik kalıntılarla değiştirilmesi ve ardından protein-protein yerleştirme sunucusu HADDOCK2.4 kullanılarak ATM ile etkileşimlerinin karşılaştırılmasıyla değerlendirilmiştir. Yerleştirme simülasyonlarının sonuçları, negatif yüklü kalıntıların yüksüz olanlarla değiştirilmesinin ATM ve TAD_{1-39} arasındaki etkileşimin etkinliğinde bir azalmaya yol açtığını göstermiştir. Sonuç olarak, ATM ve TAD arasındaki elektrostatik etkileşimlerin optimal Ser15 fosforilasyonu için önemli olduğu söylenebilir.

Introduction

Ataxia-telangiectasia mutated (ATM) kinase is a member of the phosphatidylinositol 3-kinase-related kinases (PIKKs) family. The ATM protein, which is approximately 346 kDa in size, is encoded by the *ATM*

gene, and is located on chromosome 11q22-23 (Banin *et al*. 1998 Canman & Lim 1998, Li *et al*. 2020). ATM kinase phosphorylates its substrates at a serine or threonine residue preceding a glutamine residue (SQ/TQ motif) (Traven & Heierhorst 2005). The kinase domain (KD) of ATM is located in the C-terminal region and exhibits a high degree of homology to the phosphatidylinositol 3-kinases (PI3Ks) family of lipid kinases. ATM kinase has been shown to phosphorylate over one hundred substrates involved in DNA damage repair, cell cycle checkpoints, and apoptosis (Kastan & Lim 2000). The tumor suppressor protein 53 (p53) is one of the well-known substrates of ATM. In the absence of DNA double-strand breaks (DSBs), p53 is continuously expressed and degraded in cells (Ozaki & Nakagawara 2011). However, upon the occurrence of DSBs, the ATM kinase is activated and phosphorylates the p53 protein at the Ser15 residue within the transactivation domain (TAD) (Dumaz & Meek 1999). The p53 protein is encoded by the *TP53* gene, which is one of the most frequently mutated genes in human cancers (Goh *et al*. 2011, Bouaoun *et al*. 2016, Marei *et al*. 2021). The Ser15 phosphorylation event stabilizes p53 and facilitates its transactivation function. Stabilized p53 transactivates genes involved in apoptosis, cell cycle arrest, DNA repair, and other processes. Given the role of ATM and p53 in cancer biology, elucidating the factors that regulate the Ser15 phosphorylation event is of significant importance for controlling its function and may ultimately contribute to the cancer therapy targeting ATM and p53-dependent pathways (Yogosawa & Yoshida 2018, Cheng *et al*. 2018, Marei *et al*. 2021). The factors influencing enzyme-substrate recognition and substrate specificity include electrostatic interactions, hydrogen bonding, hydrophobic interactions, and Van der Waals forces. By modifying or blocking these interactions through a molecule, residue modification, or by changing the charge of interacting residues, it is possible to manipulate enzymatic activity (Hansen *et al*. 2005, Fadeyi *et al*. 2017, Lin 2023.

Amino acid residues with ionizable side chains such as Asp, Glu, His, Lys, and Arg can play a crucial role in enzyme-substrate recognition. These residues facilitate electrostatic interactions, which may influence the specificity of protein interactions. Modulating the charges of these residues by pH or post-translational modifications could disrupt protein-protein interactions (Schreiber *et al*. 2009, Zhou & Pang 2018). The three-dimensional (3D) structure of the ATM kinase domain, resolved by cryo-electron microscopy, reveals an active site opening lined predominantly with positively charged and hydrophobic residues. In contrast, the TAD sequence of p53 contains 16 negatively charged residues (Table 1). Given that electrostatic interactions can occur even at relatively large distances (5-10 Å) and are critical for the specificity of protein-protein recognition (Schreiber *et al*. 2009), the charge distribution in the active site region of ATM and the TAD of p53 suggests that electrostatic interactions may play a role in ATM-TAD recognition (Fig. 1). Previous mutational studies have demonstrated that prolines and hydrophobic residues within the TAD are essential for its transactivation function and interaction with its negative regulator mouse double minute 2 homolog (MDM2), as well as the Taz1 and Taz2 domains of coactivator p300 (Grossman 2001, Feng *et al*. 2009, Teufel *et al*. 2009, Miller *et al*. 2015, Li *et al*. 2022). Furthermore, the double TAD^{E2E3} or TAD^{D41D42} mutations have also been reported to affect the transactivation function of p53, although to a lesser extent than prolines and hydrophobic residues (Chang *et al*. 1995). However, the contribution of these residues to the Ser15 phosphorylation event remains to be elucidated.

In this study, to determine the effect of electrostatic interactions on the ATM-TAD interaction, the phosphorylation of Ser15 of p53 was monitored at a varying pH range from 5 to 9. In addition to kinase assays to elucidate the contribution of electrostatic interactions, two TAD variants were generated by replacing the negatively charged residues in TAD_{1-39} with either Asn and Gln or with Leu, and their interactions with the ATM kinase, were evaluated through protein-protein docking using HADDOCK2.4 (Van Zundert *et al*. 2016).

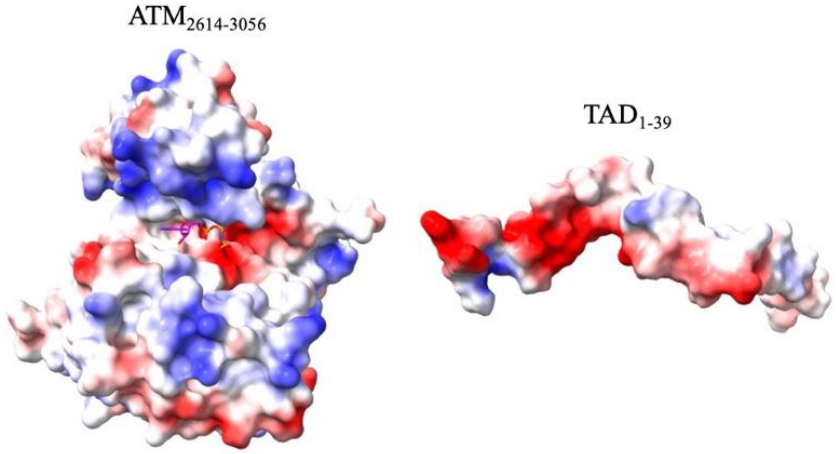

Fig. 1. Surface charge of ATM-$KD_{2614-3056}$ and TAD_{1-39}. Red colors represent negative charge, white regions are neutral, and blue regions are positively charged (Image generated by UCSF ChimeraX) (Meng *et al*. 2023).

Materials and Methods

All chemicals were purchased from commercial vendors and used as received, except for the human p53 and human ATM expression plasmids. Recombinant human p53 plasmid (corresponding to residues 1-393) was a gift from Cheryl Arrowsmith (Addgene plasmids # 24859; https://www.addgene.org/24859/; RRID: Addgene_24859) (Ayed *et al*. 2001). Recombinant human full-length ATM plasmid pcDNA3.1(+)Flag-His-ATM wt was a gift from Michael Kastan (Addgene plasmid # 31985; https://www.addgene.org/31985/; RRID:Addgene_31985) (Canman *et al*. 1998). *E. coli* BL21(DE3)plysS cells and 10 kDa dialysis tubing were purchased from Thermo Fisher Scientific. QIAprep Spin Miniprep Kit (#27106, QIAGEN) and PureLink HiPure Plasmid Maxiprep Kit (#K210007, Invitrogen) were used for plasmid DNA isolation. LB broth miller, ATP, $MgCl_2$, $MnCl_2$, ampicillin, chloramphenicol, Ni-NTA affinity resin, and imidazole were purchased from Merck. Isopropyl β-D-1-thiogalactopyranoside (IPTG) purchased from Biogen. Universal Kinase Assay Kit (Fluorometric) (ab138879) and Human p53 ELISA Kit (pSer15) were purchased from Abcam (ab156027).

p53 Transformation and Expression

DH5α cells containing recombinant human p53 plasmid were grown overnight at 37°C in a shaker incubator. Plasmid DNA was then isolated using the QIAprep Spin Miniprep Kit (QIAGEN, USA) according to the kit instructions. The isolated plasmid was transformed into the *E. coli* BL21(DE3)pLysS cells by the heat shock method (Froger & Hall 2007). In summary, the *E. coli* BL21(DE3)pLysS competent cells were incubated with the isolated plasmid at 42°C for 45 seconds, and then placed on ice. Subsequently, 500 µL of SOC media was added to the cell-plasmid mixture, which was then incubated at 37°C for 1 hour. The plasmid-transformed cells were identified based on their capacity to form bacterial colonies on an ampicillin-containing plate. Subsequently, p53 was expressed as previously described with some modifications. Briefly, *E. coli* BL21(DE3)plysS cells transformed with the human p53 gene were grown overnight in a 220-rpm shaking incubator at 37°C in 25 mL of LB broth (Miller) medium containing 100 µg/mL ampicillin and 37 µg/mL chloramphenicol. The next morning, 4 mL of the overnight culture was inoculated into 400 mL of LB broth (Miller) medium containing ampicillin and chloramphenicol. When the OD_{600nm} reached 0.5-0.6, IPTG was added to a final concentration of 1 mM to induce the cells. After IPTG induction, the cells were incubated at 20°C for 6 hours. Finally, the cells were harvested by centrifugation at 8,000 rpm and stored at -86°C for later purification.

p53 Purification

Recombinant human p53 was purified as previously described with some modifications (Ayed *et al*. 2001). Cells were lysed in a lysis buffer (50 mM NaH_2PO_4, pH 8.00, 0.3 M NaCl, 10 mM imidazole, 1% glycerol, 0.1% tween, 1 mM DTT, 0.5 mM PMSF) and sonication was used to lyse the cells (5 seconds on, 10 seconds off, and 30% amplitude for 3-5 minutes). After sonication, the pellet was removed by centrifugation (at 15,000 rpm for 40 minutes). The obtained supernatant was applied to the Ni-NTA column at a flow rate of 0.25 min/mL. The column was then washed with the five-column volume of wash buffer (lysis buffer minus DTT, plus 25 mM imidazole, 5 mM ATP, 1 mM $MgCl_2$, and 1 M NaCl). The purified protein was eluted with a buffer containing 250 mM imidazole. The eluted protein was then buffer exchanged to 50 mM Tris-HCl pH 8.00 by dialysis and stored at -20°C. The purity of the protein was confirmed by polyacrylamide gel electrophoresis (SDS-PAGE) (Shapiro *et al*. 1967) (Fig. 3a).

Transient Transfection of ATM in Expi293F Cells

Stbl2 cells containing the recombinant human ATM plasmid were grown in a shaker incubator for 24 hours at 30°C. Plasmid DNA was then isolated using a PureLink HiPure Plasmid Maxiprep Kit (Thermo Fisher Scientific, USA). Expi293F cells were grown in an Expi293 expression medium to a cell density of 2×10^6 cells/mL. PEI 40K Max was used as the transfection reagent (Baretić *et al*. 2017). PEI was dissolved in Expi293 expression medium and then added dropwise to the isolated DNA in a final ratio of 1:3 (PEI: DNA). After 15 min of incubation at room temperature, the PEI-DNA mixture was added dropwise to Expi293F cells. 24 hours after transfection, a fresh Expi293F expression medium was added to the transfected cells. At 72 hours post-transfection, the cells were harvested at 4,500 rpm and stored at -86°C for later purification.

ATM Purification

Recombinant human ATM was purified as described previously (Baretić *et al*. 2017). Briefly, Expi293F cells transiently transfected with human ATM plasmid were lysed in lysis buffer (50 mM Tris-HCl, pH 8.00, 0.3 M NaCl, 1.0 mM DTT, 0.5 mM PMSF, 10% glycerol). The cells were then homogenized with 50 strokes using a Dounce homogenizer. The homogenate was centrifuged at 14,500 rpm for 45 min at 4°C. The resulting cell lysate was mixed with anti-Flag M2 affinity gel and incubated for 1 hour at 4°C on a benchtop rotator. The cell lysate gel mixture was then loaded onto an empty chromatography column. The column was washed with five times the column volume of lysis buffer. Finally, ATM was eluted from the column using Flag peptide (2 µg/mL). The purity of the eluted protein was checked using SDS-PAGE (Shapiro *et al*. 1967) (Fig. 3b). Flag peptide was removed by dialysis using 10k snakeskin dialysis tubing in 50 mM Tris-HCl pH 8.00, 10% glycerol.

Kinase Activity Assays

A fluorometric-based universal kinase assay measuring ADP formation and ELISA measuring phosphorylated Ser15 were applied to monitor kinase activity. All activity assays were performed with saturating concentrations of ATP (800 µM), $MgCl_2$ (20 mM), $MnCl_2.H_2O$ (20 mM), ATM (25 nM), and p53 (25

μM). To study the effect of pH on Ser15 phosphorylation, a buffer cocktail containing 25 mM MES and 25 mM Tris-HCl was prepared to obtain acidic, neutral, and basic pH ranges. ATM and p53 were added to the buffer cocktails, and each kinase reaction was initiated by adding the ATP-Mg^{2+}-Mn^{2+} complex to the reaction mixture. The reactions were then quenched by the addition of a stop solution (12.5 mM HEPES, pH 8.00, 1% glycerol, 250 mM EDTA) after 15 minutes. For each reaction, phosphorylated Ser15 ($Ser15^P$) was measured using a p53 (pS15) ELISA kit, and ADP formation was determined using a fluorometric-based Universal Kinase Assay Kit according to the kit instructions (Abcam, #ab138879).

Stability Prediction of ATM and p53 at Different pH Values

The stabilities of ATM kinase and p53 were predicted at different pH ranges using the CamSol server (Sormanni *et al*. 2015). For this purpose, the amino acid sequences of human ATM_{1-3056} and human $p53_{1-393}$ were obtained from the NCBI website (the accession number for ATM kinase is AAB65827.1, and for p53 it is BAC16799.1) (Sayers *et al*. 2022). The pH range was set from 1 to 14 for the predictions. Since proteins are unstable at their isoelectric point (p*I*), in addition to stability at different pH values, the p*I* of ATM_{1-3056} and $p53_{1-393}$ was determined based on their amino acid sequences using the ExPASy ProtParam tool (Wilkins *et al*. 1999) and avoided in kinase assays (p*I* of ATM is 6.39 and of p53 is 6.33).

ATM and TAD_{1-39} Docking

In docking experiments, the 3D structures of TAD and ATM, which are available in the Protein Data Bank (PDB), were used. For the TAD, the 3D structure with PDB ID: 2k8f, which contains the TAD_{1-39} chain, was utilized as a template, and the remaining chains were removed (Feng *et al*. 2009). For the ATM-$KD_{2614-3056}$ structure, the PDB structure with the PDB ID 8oxo was used as a template (Howes *et al*. 2023). The effect of negatively charged residues on the ATM-TAD interaction was investigated by generating two variants, using ChimeraX (Meng *et al*. 2023). One variant was generated by changing two Asp residues to Asn and five Glu residues to Gln in TAD_{1-39} (TAD^{NQ}), maintaining similar steric effects while eliminating the charge effect. The other variant was generated by replacing Asp and Glu residues with Leu, thus eliminating polar character (TAD^{L}). The HADDOCK2.4 server was utilized for ATM-TAD_{1-39} docking predictions (Van Zundert *et al*. 2016, Honorato *et al*. 2021). The docking employed to the C-terminal region of $ATM_{2614-3056}$, with the remaining residues in the 8oxo PBD structure removed. Actively interacting residues were defined based on the $ATM_{1462-3056}$-TAD_{12-18} structure complex (PDB ID: 8oxo) (Fig. 2). In the TAD_{1-39} structure, the residues Leu14, Ser15, and Gln16 were identified as actively interacting. Similarly, in the $ATM_{2614-3056}$ structure, residues His2872, Thr2902, and Phe3049 were identified as actively interacting (Fig. 2). The docking was performed for the TAD^{WT}, TAD^{NQ} and TAD^{L} variants (Table 1).

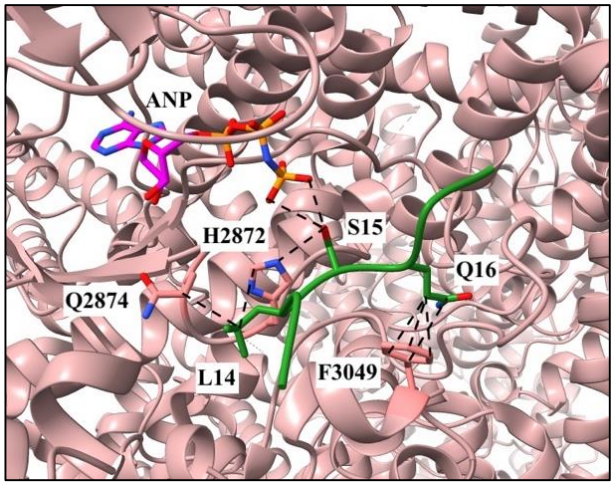

Fig. 2. The active site of the ATM interacting with TAD_{12-18} (shown in green) (PDB ID: 8oxo), ANP is a structural analogue of ATP (image generated by UCSF ChimeraX) (Meng *et al*. 2023).

Table 1. The amino acid sequences of the wild-type TAD_{1-39} (TAD^{WT}) and its TAD^{NQ} and TAD^{L} mutant variants.

TAD_{1-39}	Sequence
TAD^{WT}	MEEPQSDPSVEPPLSQETFSDLWKLLPENNVLSPLPSQA
TAD^{NQ}	MQQPQSNPSVQPPLSQQTFSNLWKLLPQNNVLSPLPSQA
TAD^{L}	MLLPQSLPSVLPPLSQLTFSLLWKLLPLNNVLSPLPSQA

Results

Effect of pH on Ser15 Phosphorylation

Kinase assays were conducted using a saturating concentration of ATP, Mg^{2+}, and $p53_{1-393}$ to ensure maximal kinase activity in pH-adjusted buffers. The formation of precipitates in kinase reactions was observed at the physiological temperature of 37°C. To prevent protein denaturation, kinase assays were performed at room temperature (approximately 20°C). The pH dependency of Ser15 phosphorylation was observed over the pH range of 5 to 9. Phosphorylation occurred at a faster rate at the basic pH values than at the acidic pH values (Figs 4a, b). The phosphorylation rate was found to be lowest at pH 5, with an increase observed as the pH increased, reaching a maximum in the pH range of 7 to 9. Due to the tendency of the ATM denaturation to occur at pH values below 5 and above 9, these pH values were avoided. Both methods gave comparable pH profiles.

The pH Stability Profile of ATM and p53

The full-length ATM_{1-3056} exhibits a negative solubility score between pH 1 and 14 (-3.80639 to -3.07764), indicating poor solubility (Fig. 5a). However, within the pH range of the kinase assay (pH 5 to 9), the solubility scores are relatively close, -3.71628 to -3.79001, differing by only about 0.07373 unit of solubility. In comparison to ATM, p53 showed generally higher solubility scores, ranging from 1.78721 to 2.0416, over the pH range of 1 to 14 (Fig. 5b).

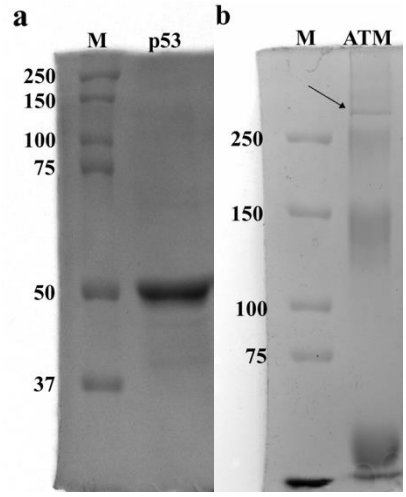

Fig. 3. The SDS-PAGE and Coomassie blue staining of the purified p53 and ATM. **a.** Purified p53 was loaded onto 5% stacking gel and 10% separating gel, M: protein marker (10-250 kDa), **b.** purified ATM was loaded onto 4% stacking gel and 6% separating gel. The arrow points to the ATM band at around 346 kDa, M: protein marker (10-250 kDa).

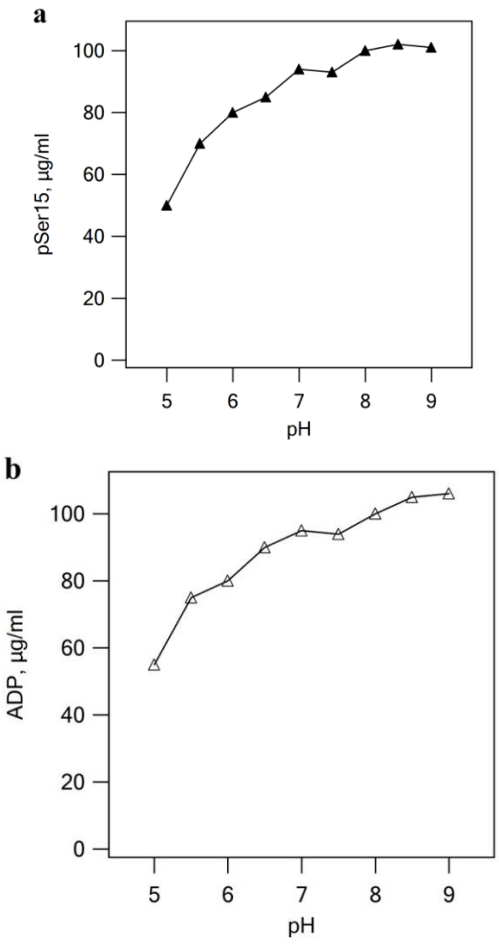

Fig. 4. The pH profile of Ser15 phosphorylation. **a.** pH profile using the Human p53 (pSer15) ELISA Kit, **b.** pH profile using the ADP-based Universal Kinase Assay Kit (Fluorometric).

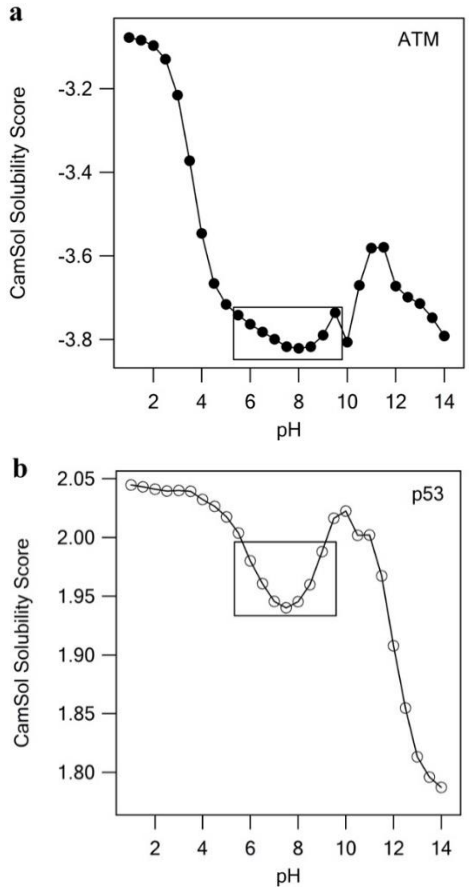

Fig. 5. pH solubility profile generated by the CamSol server (Sormanni *et al*. 2015). **a.** ATM_{1-3056}, pH solubility profile, **b.** $p53_{1-393}$, pH solubility profile. The pH range of the kinase assay is indicated by a rectangle.

In the kinase assay pH range (5 to 9), the solubility scores of p53 exhibited a range of 2.01747 to 1.98791, with a difference of 0.02956 units. Overall, p53 appears to be more stable than ATM.

The Importance of Charged Residues of TAD_{1-39} on ATM-TAD Recognition

To evaluate the importance of the negatively charged residues in TAD_{1-39}, two mutant TAD_{1-39} variants were generated by replacing two Asp residues with Asn or Leu and five Glu residues with Gln or Leu within the TAD_{1-39} using ChimeraX (Meng *et al*. 2023). The interaction of these variants with the kinase domain of ATM was then analyzed using the HADDOCK2.4 server (Honorato *et al*. 2021; Van Zundert *et al*. 2016). The generated docking complexes were evaluated based on their HADDOCK2.4 scores, with more negative scores indicating stronger interactions. The most reliable docking complex in HADDOCK2.4 is identified based on the more negative z-scored complexes among the generated structures. The interaction energies of both the TAD^{WT}, TAD^{NQ}, and TAD^{L} domains with the $ATM_{2614-3056}$ are presented in Table 2. The HADDOCK2.4 scores indicate that the ATM-TAD^{WT} complex exhibits a higher interaction

efficiency compared to the ATM-TAD^{NQ} and ATM-TAD^{L} variants (Table 2). Conversely, the ATM-TAD^{NQ} and ATM-TAD^{L} complexes showed relatively similar and lower interaction efficiencies than the ATM-TAD^{WT} complex. Significant reductions were observed in the electrostatic energies, which decreased from -341.6 to -192.7 for the ATM-TAD^{NQ} complex and to -221.8 for the ATM-TAD^{L} complex in comparison to the ATM-TAD^{WT} complex. Another notable difference was observed in the desolvation energies. The ATM-TAD^{WT} complex had a desolvation energy of -8.2, which increased to -28.2 for the ATM-TAD^{NQ} complex and to -24.8 for the ATM-TAD^{L} complex. The hydrophobic interactions were found to be comparable between TAD^{WT}, TAD^{NQ}, and TAD^{L}, with -36.3, -33.5, and -34.4, respectively. Overall, replacement of acidic residues on TAD$_{1-39}$ with uncharged or hydrophobic residues resulted in alterations to the residues involved in the ATM-TAD interaction. The list of residues involved in this interaction is provided in Table 3.

The structural alignment of the ATM-TAD$_{1-39}$ complexes demonstrates the change in binding orientation (Figs 6a-d). In the ATM-TAD^{WT} complex, TAD$_{1-39}$ exhibited a predominant interaction with residues between 2 and 21, whereas TAD^{NQ} demonstrated a more pronounced interaction with residues between 9 and 24, with Gln5 also involved in the interaction. In contrast, the TAD^{L} variant, which has fewer residues supporting electrostatic interactions, exhibited a predominant interaction with residues between 15 and 31. A more detailed examination of the ATM-TAD complexes, focusing on the residues involved in the interaction within a 3.5 Å distance, is presented in Figs 6e to 6f. The Phe19 of TAD$_{1-39}$ has a conserved interaction with ATM residues (Pro2901, Thr2902, Phe3049) in both TAD^{WT} and its mutant variants (Figs 6e-f). In addition to Phe19 residue, Leu14, Ser15, Gln16, and Glu17 residues of TAD$_{1-39}$ are also involved in the interaction with ATM, albeit with different residue contacts. Furthermore, Gln2802, Asp2870, and Lys3053 within ATM are also involved in stabilizing the ATM-TAD$_{1-39}$ interaction.

Table 2. HADDOCK2.4 docking energy scores for ATM-TAD^{WT}, ATM-TAD^{NQ}, and ATM-TAD^{L} complexes.

TAD$_{1-39}$	HADDOCK Score	Electrostatic energy	Van der Waals energy	Desolvation energy	Z-Score
TAD^{WT}	-111.7 ± 7.1	-341.6 ± 51.0	-36.3 ± 2.9	-8.2 ± 2.6	-1.6
TAD^{NQ}	-99.5 ± 5.7	-192.7 ± 19.4	-33.5 ± 3.1	-28.2 ± 1.2	-1.3
TAD^{L}	-100.3 ± 12.5	-221.8 ± 34.5	-34.4 ± 1.9	-24.8 ± 1.5	-2.0

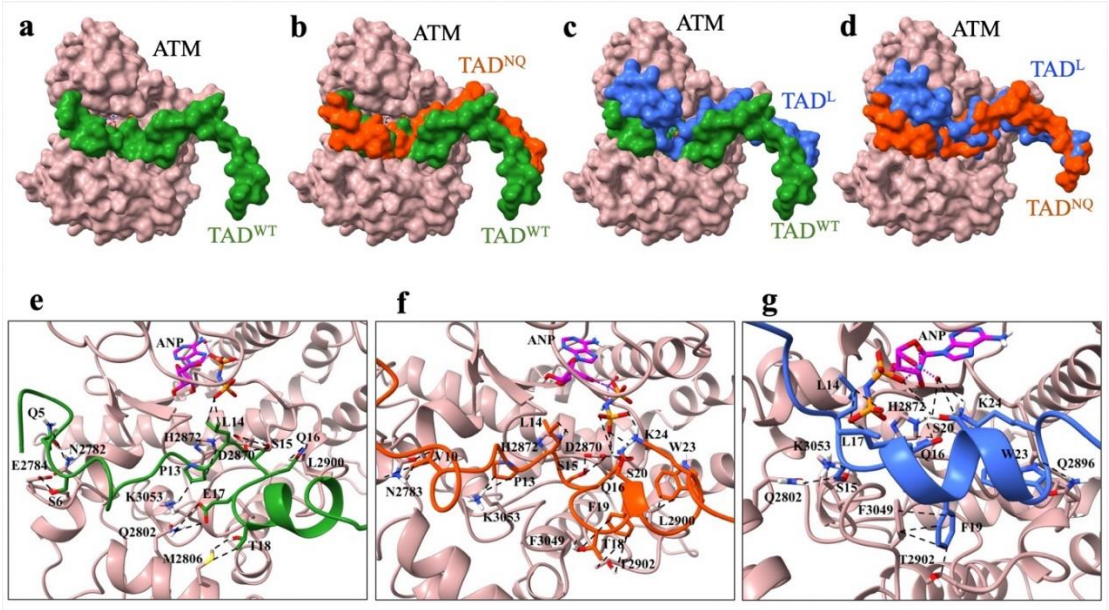

Fig. 6. ATM$_{2614-3056}$-TAD$_{1-39}$ interactions. **a.** ATM$_{2614-3056}$-TAD^{WT} complex surface representation, **b.** ATM$_{2614-3056}$-TAD^{WT} and ATM$_{2614-3056}$-TAD^{NQ} complexes alignment. **c.** ATM$_{2614-3056}$-TAD^{WT} and ATM$_{2614-3056}$-TAD^{L} complexes alignment surface depictions, **d.** ATM$_{2614-3056}$-TAD^{NQ} and ATM$_{2614-3056}$-TAD^{L} complexes alignment surface depictions, **e.** ATM$_{2614-3056}$-TAD^{WT} complex interacting residues around the active site, **f.** ATM$_{2614-3056}$-TAD^{NQ} complex interacting residues around the active site, **g.** ATM$_{2614-3056}$-TAD^{L} complex interacting residues around the active site (ANP is a structural analogue of ATP) (Images were generated by UCSF ChimeraX) (Meng *et al*. 2023).

Table 3. Residues involved in the $ATM_{2614-3056}$-TAD_{1-39} interaction within up to 4 Å of each other.

TAD^{WT}/TAD^{NQ}/ TAD^{L}	ATM-(TAD^{WT})	ATM-(TAD^{NQ})	ATM-(TAD^{L})
E2/Q2/L2	R2642, Q2641, K2643	-	-
E3/Q3/L3	R2691, K2643	-	-
P4	R2642	-	T2640
Q5	N2782, E2783	E2784	-
S6	N2783, E2784	-	-
D7/N7/L7	R2642	-	K2636
P8	-	-	-
S9	N2783	F2799	-
V10	N2783	N2783	-
E11/Q11/L11	K3053	K3053	ANP
P12	-	-	ANP
P13	K3053	K3053	-
L14	H2872, ANP	H2872	ANP
S15	D2870, H2872	D2870, H2872	Q2802, K3053
Q16	L2900, P2901	D2870	D2870, H2872, F3049
E17/Q17/L17	Q2802, M2806	Q2802	ANP
T18	M2806	F3049	-
F19	P2901, T2902, F3049	P2901, T2902, F3049	P2901, T2902, F3049
S20	-	V2696, ANP	ANP
D21/N21/L21	V2696	-	-
L22	-	P2901	P2901
W23	-	L2900	V2891, Q2896
K24	-	V2696, ANP	ANP
L28	-	-	N2963
N29	-	-	T2961, M2962
V31	-	-	N2963

Discussion

The Ser15 phosphorylation of the highly dynamic and acidic TAD of p53 is known to be important for its transactivation function and cellular stability (Kubbutat *et al*. 1997, Dumaz & Meek 1999, Jenkins *et al*. 2012). However, the factors that are affecting this phosphorylation event need further investigation. The present results indicate that electrostatic interactions between ATM and TAD play a role in efficient phosphorylation of Ser15. Modulation of charged amino acid side chains by pH led to a change in the Ser15 phosphorylation rate. The higher phosphorylation rates were observed at basic pH ranges compared to acidic pH ranges (Figs 3a, b). This alteration in the phosphorylation rate with pH indicates that electrostatic interactions between relatively positively charged residues in the active site opening of ATM and negatively charged residues in TAD may be significant for optimal ATM-TAD recognition (Fig. 1). The results also suggest that electrostatic interactions may be used to regulate Ser15 phosphorylation, a process that is of critical importance in the ATM and p53-mediated pathway in cancer biology.

The modulation of ionizable amino acid side chains by pH may affect protein structural stability through alterations to side-chain interactions that form folded protein, potentially may lead to denaturation (Schaefer *et al*. 1997, Tollinger *et al*. 2003). The structural stability of ATM_{1-3056} and $p53_{1-393}$ at varying pH ranges was evaluated using the CamSol server (Sormanni *et al*. 2015). According to the CamSol sever result, similar solubility scores in the kinase assay pH range suggest a low likelihood of pH-dependent denaturation (Fig. 5b). The pH-dependent solubility profiles of ATM_{1-3056} and $p53_{1-393}$ provide evidence that changes in electrostatic interactions, rather than denaturation, may be responsible for the observed decrease in phosphorylation rate. Nevertheless, this prediction requires experimental validation to substantiate the conclusions drawn.

A reduction in electrostatic interactions is observed for both TAD^{NQ} and TAD^{L} variants in comparison to TAD^{WT}, indicating that the negatively charged residues in TAD^{WT} play a crucial role in stabilizing the interaction through electrostatic forces. In the mutants, the remaining polar residues (6, Ser; 3, Gln; 2, Asn; 1, Thr) can provide hydrogen bonding and other polar interactions, but they are unable to fully compensate for the loss of the strong electrostatic interactions provided by the acidic residues, thereby compromising binding efficiency. In addition to the observed changes in electrostatic interactions, alterations in desolvation energies were also observed in the TAD^{NQ} and TAD^{L} variants. The TAD^{NQ} and TAD^{L} variants exhibited a considerable increase in desolvation energy compared to the TAD^{WT}. These indicate that the replaced residues in TAD^{NQ} and TAD^{L} are less favorable for interaction in an aqueous environment, potentially due to their uncharged and hydrophobic nature. In contrast to the electrostatic and desolvation energies, the hydrophobic interactions were relatively similar across all variants, suggesting that replacing the charged residues with uncharged or hydrophobic ones does not significantly affect the hydrophobic components of the interaction (Table 2). In addition to the alteration of interaction energies, the orientation of the ATM-TAD interface also changed for TAD^{NQ} and TAD^{L} compared to

TAD^{WT}. In the TAD^{WT}, the interaction was predominantly observed in the more polar N-terminal region. In contrast, in TAD^{NQ}, the interaction shifted to the central region due to the loss of electrostatic characteristics at the N-terminal region. In the TAD^{L} variant, the interaction interface shifted to the C-terminal region, which contains a greater proportion of polar amino acids than the N-terminal region. These results further illustrate the significance of the electrostatic interactions in the ATM-TAD interaction. These findings contribute to a more comprehensive understanding of the molecular mechanisms underlying ATM-TAD recognition.

Conclusion

The results of this study highlight the critical role of electrostatic interactions between the kinase domain of ATM and the TAD of p53 in facilitating Ser15 phosphorylation. The observed variations in phosphorylation rate across different pH ranges indicate that these electrostatic interactions play an important role in regulating the optimal interaction between ATM and TAD. Moreover, the CamSol solubility predictions support the notion that the observed changes in phosphorylation rate are not primarily due to pH-dependent stability issues. Furthermore, exploration of negatively charged residues within the TAD by residue substitution and subsequent protein-protein docking revealed that these residues are indeed essential for optimal interaction between ATM and TAD. Taken together, these results demonstrate the importance of electrostatic interactions between ATM and TAD for optimal Ser15 phosphorylation and further our understanding of the molecular mechanisms underlying ATM-p53 signaling.

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Data Sharing Statement: All data are available within the study.

Conflict of Interest: The author has no conflicts of interest to declare.

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References

1. Ayed, A., Mulder, F.A., Yi, G.S., Lu, Y., Kay, L.E. & Arrowsmith, C.H. 2001. Latent and active p53 are identical in conformation. *Nature structural biology*, 8(9): 756-760. https://doi.org/10.1038/nsb0901-756
2. Banin, S., Moyal, L., Shieh, L., Taya, Y., Anderson, C.W., Chessa, L., Prives, C., Reiss, Y., Shiloh, Y. & Ziv, Y. 1998. Enhanced Phosphorylation of P53 by ATM in Response to DNA Damage. *Science*, 281(5383): 1674-77. https://doi.org/10.1126/science.281.5383.1674
3. Baretić, D., Pollard, H.K., Fisher, D.I., Johnson, C.M., Santhanam, B., Truman, C.M. & Williams, R.L. 2017. Structures of closed and open conformations of dimeric human ATM. *Science advances*, 3(5): e1700933. https://doi.org/10.1126/sciadv.1700933
4. Bouaoun, L., Sonkin, D., Ardin, M., Hollstein, M., Byrnes, G., Zavadil, J. & Olivier, M. 2016. TP53 variations in human cancers: new lessons from the IARC TP53 database and genomics data. *Human mutation*, 37(9): 865-876. https://doi.org/10.1002/humu.23035
5. Canman, C.E., Lim, D.S., Cimprich, K.A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M.B. & Siliciano, J.D. 1998. Activation of the ATM Kinase by Ionizing Radiation and Phosphorylation of P53. *Science*, 281(5383): 1677-79. https://doi.org/10.1126/science.281.5383.1677
6. Canman, C.E. & Lim, D.S. 1998. The role of ATM in DNA damage responses and cancer. *Oncogene*, *17*(25), 3301-3308. https://doi.org/10.1038/sj.onc.1202577
7. Chang, J., Kim, D.H., Lee, S.W., Choi K.Y. & Sung, Y.C. 1995. Transactivation Ability of P53 Transcriptional Activation Domain Is Directly Related to the Binding Affinity to TATA-Binding Protein. *Journal of Biological Chemistry*, 270(42): 25014-25019. https://doi.org/10.1074/jbc.270.42.25014
8. Cheng, J., Dwyer, M., Okolotowicz, K.J., Mercola, M. & Cashman, J.R. 2018. A novel inhibitor targets both Wnt signaling and ATM/p53 in colorectal cancer. *Cancer Research*, 78(17): 5072-5083. https://doi.org/10.1158/0008-5472.CAN-17-2642
9. Dumaz, N. & Meek, D.W. (1999). Serine 15 phosphorylation stimulates p53 transactivation but does not directly influence interaction with HDM2. *The EMBO journal*, 18(24): 7002-7010. https://doi.org/10.1093/emboj/18.24.7002
10. Fadeyi, O.O., Hoth, L.R., Choi, C., Feng, X., Gopalsamy, A., Hett, E.C. & Jones, L.H. 2017. Covalent enzyme inhibition through fluorosulfate modification of a noncatalytic serine residue. *ACS Chemical Biology*, 12(8): 2015-2020. https://doi.org/10.1021/acschembio.7b00403
11. Feng, H., Lisa M. Miller Jenkins, L.M.M., Durell, S.R., Hayashi, R., Mazur, S.J., Cherry, S., Tropea, J.E., Miller, M., Wlodawer, A., Appella, E. & Bai., Y. 2009. Structural Basis for P300 Taz2-P53 TAD1 Binding and Modulation by Phosphorylation. *Structure*, 17(2): 202-210. https://doi.org/10.1016/j.str.2008.12.009
12. Froger, A. & Hall, J.E. 2007. Transformation of plasmid DNA into E. coli using the heat shock method. *JoVE*

(Journal of visualized experiments), (6): e253. https://doi.org/10.3791/253

13. Goh, A.M., Coffill, C.R. & Lane, D.P. 2011. The role of mutant p53 in human cancer. The Journal of pathology, 223(2): 116-126. https://doi.org/10.1002/path.2784
14. Grossman, S.R. 2001. p300/CBP/p53 interaction and regulation of the p53 response. *European journal of biochemistry*, 268(10): 2773-2778. https://doi.org/10.1046/j.1432-1327.2001.02226.x
15. Hansen, S.K., Cancilla, M.T., Shiau, T.P., Kung, J., Chen, T. & Erlanson, D.A. 2005. Allosteric inhibition of PTP1B activity by selective modification of a non-active site cysteine residue. *Biochemistry*, 44(21), 7704-7712. https://doi.org/10.1021/bi047417s
16. Honorato, R.V., Koukos, P.I., Jiménez-García, B., Tsaregorodtsev, A., Verlato, M., Giachetti, A., Rosato, A. & Bonvin, A.M. 2021. Structural biology in the clouds: the WeNMR-EOSC ecosystem. *Frontiers in molecular biosciences*, 8: 729513. https://doi.org/10.3389/fmolb.2021.729513
17. Howes, A.C., Perisic, O. & Williams, R.L. 2023. Structural insights into the activation of ataxia-telangiectasia mutated by oxidative stress. *Science Advances*, 9(39): eadi8291. https://doi.org/10.1126/sciadv.adi8291
18. Jenkins, L.M. M., Durell, S.R., Mazur, S.J. & Appella, E. 2012. p53 N-terminal phosphorylation: a defining layer of complex regulation. *Carcinogenesis*, 33(8): 1441-1449. https://doi.org/10.1093/carcin/bgs145
19. Kastan, M.B. & Lim, D.S. 2000. The many substrates and functions of ATM. *Nature reviews Molecular cell biology*, 1(3): 179-186. https://doi.org/10.1038/35043058
20. Kubbutat, M.H., Jones, S.N. & Vousden, K.H. 1997. Regulation of p53 stability by Mdm2. *Nature*, 387(6630): 299-303. https://doi.org/10.1038/387299a0
21. Li, T., Motta, S., Stevens, A.O., Song, S., Hendrix, E., Pandini, A. & He, Y. 2022. Recognizing the binding pattern and dissociation pathways of the p300 Taz2-p53 TAD2 complex. *JACS Au*, 2(8): 1935-1945. https://doi.org/10.1021/jacsau.2c00358
22. Li, W., Peng, X., Lang, J. & Xu, C. 2020. Targeting mouse double minute 2: current concepts in DNA damage repair and therapeutic approaches in cancer. *Frontiers in Pharmacology*, 11: 537486. https://doi.org/10.3389/fphar.2020.00631
23. Lin, H. 2023. Substrate-selective small-molecule modulators of enzymes: mechanisms and opportunities. *Current opinion in chemical biology*, 72: 102231. https://doi.org/10.1016/j.cbpa.2022.102231
24. Marei, H.E., Althani, A., Afifi, N., Hasan, A., Caceci, T., Pozzoli, G. & Cenciarelli, C. 2021. p53 signaling in cancer progression and therapy. *Cancer cell international*, 21(1): 703. https://doi.org/10.1186/s12935-021-02396-8
25. Meng, E C., Goddard, T.D., Pettersen, E.F., Couch, G.S., Pearson, Z.J., Morris, J.H. & Ferrin, T.E. 2023. UCSF ChimeraX: Tools for structure building and analysis. *Protein Science*, 32(11): e4792. https://doi.org/10.1002/pro.4792
26. Miller Jenkins, L.M., Feng, H., Durell, S.R., Tagad, H.D., Mazur, S.J., Tropea, J.E., Bai., T.Y. & Appella, E. 2015. Characterization of the p300 Taz2–p53 TAD2 complex and comparison with the p300 Taz2–p53 TAD1 complex. *Biochemistry*, 54(11): 2001-2010. https://doi.org/10.1021/acs.biochem.5b00044
27. Ozaki, T. & Nakagawara, A. 2011. Role of p53 in cell death and human cancers. *Cancers*, 3(1): 994-1013. https://doi.org/10.3390/cancers3010994
28. Sayers, E.W., Bolton, E.E., Brister, J.R., Canese, K., Chan, J., Comeau, D.C., Connor, R., Funk, K., Kelly, C., Kim, S., Madei, T., Marchler-Bauer, A., Lanczycki, C., Lathrop, S., Lu, Z., Thibaud-Nissen, F. & Sherry, S.T. 2022. Database resources of the national center for biotechnology information. *Nucleic acids research*, 50(D1): 20-26. https://doi.org/10.1093/nar/gkab1112
29. Schaefer, M., Sommer, M. & Karplus, M. 1997. pH-dependence of protein stability: absolute electrostatic free energy differences between conformations. *The Journal of Physical Chemistry B*, 101(9): 1663-1683. https://doi.org/10.1021/jp962972s
30. Schreiber, G., Haran, G. & Zhou, H.X. 2009. Fundamental aspects of protein− protein association kinetics. *Chemical reviews*, 109(3): 839-860. https://doi.org/10.1021/cr800373w
31. Shapiro, A.L., Viñuela, E. & Maizel Jr, J.V. 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. *Biochemical and biophysical research communications*, 28(5): 815-820. https://doi.org/10.1016/0006-291X(67)90391-9
32. Sormanni, P., Aprile, F.A. & Vendruscolo, M. 2015. The CamSol method of rational design of protein mutants with enhanced solubility. *Journal of molecular biology*, 427(2): 478-490. https://doi.org/10.1016/j.jmb.2014.09.026
33. Teufel, D.P., Bycroft, M. & Fersht, A.R. 2009. Regulation by phosphorylation of the relative affinities of the N-terminal transactivation domains of p53 for p300 domains and Mdm2. *Oncogene*, 28(20): 2112-2118. https://doi.org/10.1038/onc.2009.71
34. Tollinger, M., Crowhurst, K.A., Kay, L.E. & Forman-Kay, J.D. 2003. Site-specific contributions to the pH dependence of protein stability. *Proceedings of the National Academy of Sciences*, 100(8), 4545-4550. https://doi.org/10.1073/pnas.0736600100
35. Traven, A. & Heierhorst, J. 2005. SQ/TQ cluster domains: concentrated ATM/ATR kinase phosphorylation site regions in DNA-damage-response proteins. *Bioessays*, 27(4), 397-407. https://doi.org/10.1002/bies.20204
36. Wilkins, M.R., Gasteiger, E., Bairoch, A., Sanchez, J.C., Williams, K.L., Appel, R.D. & Hochstrasser, D. F. 1999. Protein Identification and Analysis Tools in the ExPASy Server. *Methods in Molecular Biology*, 112: 531-532. https://doi.org/10.1385/1-59259-584-7:531
37. Van Zundert, G.C.P., Rodrigues, J.P.G.L.M., Trellet, M., Schmitz, C., Kastritis, P.L., Karaca, E., Melquiond, A.S.J. & Bonvin, A.M.J.J. 2016. The HADDOCK2. 2 web server: user-friendly integrative modeling of biomolecular complexes. *Journal of molecular biology*, 428(4): 720-725. https://doi.org/10.1016/j.jmb.2015.09.014

38. Yogosawa, S. & Yoshida, K. 2018. Tumor suppressive role for kinases phosphorylating p53 in DNA damage-induced apoptosis. *Cancer science*, 109(11): 3376-3382. https://doi.org/10.1111/cas.13792

39. Zhou, H.X. & Pang, X. 2018. Electrostatic interactions in protein structure, folding, binding, and condensation. *Chemical reviews*, 118(4): 1691-1741. https://doi.org/10.1021/acs.chemrev.7b00305

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Research Article

Rhizogenesis in Shrub rose cultivated *in vitro*

Larysa Koldar, Iryna Denysko, Alla Konopelko*, Yevhen Mazur

Department of Ornamental and Fruit Plants of the Sofiyivka National Dendrological Park of the National Academy of Sciences of Ukraine, UKRAINE

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Abstract: The study of the reproduction characteristics of roses of the garden class Shrub, the definition of the dependence of the hormonal determination of explant rhizogenesis on the concentrations of phytohormones that are part of the nutrient medium, are relevant and has both scientific and practical interest. This study presents the results of studies of hormonal determination of rhizogenesis in explants of cultivars of roses of the garden class Shrub: Gärtnerfreude, Lavender Dream, Pomponella, Red Cascade, Sommerabend cultivated *in vitro* on nutrient medium containing growth regulators. It has been established that of the nutrient medium modified by the addition of 0.2-1.0 mg/l α-naphthylacetic acid (α-NAA), the most effective was the medium with the content of α-NAA 0.5 mg/l, the content of macro- and microelements half of the Murashige and Skoog prescriptions, and a decrease in the sucrose content to 2.0%. On this medium, the frequency of rhizogenesis averaged 61.2% for the studied cultivars. Hormonal determination of rhizogenesis and efficiency of root formation *in vitro* in the Shrub rose regenerants depended on the genotype of the plant: cv. Lavender Dream (66.0%) and cv. Sommerabend (67.0%) had the highest rhizogenesis ability. The use of the universal growth regulator Humifield in combination with 0.5 mg/l α-NAA contributed to an increase in the rooting rate of the studied rose cultivars up to 70.0-86.0%.

Özet: Çalı güllerinin üreme özelliklerinin incelenmesi ve eksplant kök oluşumunun hormonal kontrolünün kullanılan besleyici ortamın fitohormon konsantrasyonuna bağlı olduğunun tanımlanması önemlidir ve hem bilimsel hem de pratik açıdan üzerinde durulan konulardır. Bu çalışmada, büyüme düzenleyicileri içeren besin ortamı üzerinde *in vitro* olarak yetiştirilen çalı gülü kültivarlarının (Gärtnerfreude, Lavender Dream, Pomponella, Red Cascade, Sommerabend) eksplantlarında kök oluşumunun hormonal belirlenmesine yönelik denemelerin sonuçları sunulmuştur. 0,2-1,0 mg/l a-naftilasetik asit (a-NAA) ilavesiyle modifiye edilen besin ortamlarından en etkilisinin, 0,5 mg/l a-NAA içerikli, sukroz oranı %2'ye kadar düşürülmüş ortam olduğu tespit edilmiştir. Bu ortamda, çalışılan kültivarlar için kök oluşumu sıklığı ortalama %61,2 olarak belirlenmiştir. Kullanılan kültivarlardaki kök oluşumunun *in vitro* etkinliğindeki hormonal katkının kültivarların genotiplerine bağlı olduğu belirlenmiştir: Lavender Dream kültivarı (%66,0) ve Sommerabend kültivarı (%67,0) en yüksekkök oluşumu yeteneği sergilemişlerdir. Bir büyüme düzenleyicisi olan Humifield'in 0,5 mg/l α-NAA ile kombinasyon halinde kullanılması, incelenen gül kültvarlarında köklenme oranının %70,0-86,0'a kadar artmasına katkıda bulunmuştur.

Edited by:
Yıldız Aydın

****Corresponding Author:***
Alla Konopelko
konopelko_alla@ukr.net

ORCID iDs of the authors:
LK. 0000-0002-6756-4172
ID. 0000-0002-2385-9331
AK. 0000-0002-5214-6170
YM. 0009-0009-8045-0768

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Introduction

The modern technology of plant propagation (use of plant tissue culture methods) effectively complements traditional methods, when propagation of plants by seed or vegetative means is not effective. Traditionally, self-rooted seedlings of garden roses are obtained by rooting of stem cuttings, layering, dividing the plants (Kroin 2016). An alternative to traditional methods of plant propagation is the introduction of modern technologies, among which the leading place belongs to micropropagation (Chawla 2011, Kumar & Reddy 2011, Hasnain *et al* 2022).

The method of tissue culture for micropropagation of roses using isolated meristems began to be developed in the 70-80s of the 20th century (Horn 1992). However, the issue of the induction and course of the stages of organogenesis of each plant genotype has not yet been practically studied, approaches to the selection of effective inducers of various stages of organogenesis *in vitro* have not been determined, and the mechanisms that are responsible for the effective organogenesis of roses, the cultivation of which is of great interest for green building have not been sufficiently studied.

The expediency of using ornamental plants for architectural and artistic design of residential areas depends, first of all, on the ecological and biological properties of these plants including drought resistance, winter hardiness, dust and gas resistance, resistance to pests and pathogens etc. In the process of creating decorative plantings for outdoor gardening, special attention is paid to flowering shrubs that do not need systematic care and have a long bloom period. These requirements are met by roses of the garden class Shrub, which include, in particular, roses of the selection by D. Austin (English Roses), the so-called ground cover roses, etc. Observations have shown that the roses of this group are well adapted to the soil and climatic conditions of the Right-Bank Forest-Steppe Zone of Ukraine, showing high decorative qualities (Moroz *et al*. 2010, Moroz *et al*. 2012, Denysko 2022).

In vitro propagation makes it possible to juvenile a culture, heal plants from fungal and bacterial infections, preserve their species and varietal characteristics, and significantly increase the multiplication factor, which plays an important role in the development of cost-effective propagation technologies and accelerated plant cloning (Kalinin *et al*. 1992, Jain & Ishii 2003, Nikbakht *et al.* 2005, Hameed *et al.* 2006, Tytarenko & Tesliuk 2020).

The final stage of plant propagation *in vitro* is the rhizogenesis of the resulting explants, which combines many vital biochemical, physiological and histological processes, the efficiency of which further affects the viability of regenerated plants obtained *in vitro* (De Klerk 2002). The process of adventitious root formation occurs in several stages: induction, initiation, appearance and growth of roots in explants (Podwyszynska 2003).

The success of the stage of rhizogenesis depends on many factors including the genotype of the culture itself, the hormonal composition of the nutrient medium, the number of passages, the conditions of conducting the experiment (Bidabadi & Jain 2020). To achieve a high percentage of root formation, the addition of growth regulators of auxin nature to nutrient medium is used, a decrease by 1/2, and sometimes by 1/4 of the content of macro- and microelements and sucrose can be used (Arnold *et al*. 1995, Carelli & Echeverrigaray 2002).

The key role in the induction of the formation and development of roots is played by plant growth and development regulators of the auxin type of action (Kalinin *et al*. 1992, Vedmid *et al*. 2002, Rugini & Pesce 2006). They contribute to the stimulation of morphogenetic and physiological processes in explants, affect the division and growth of plant cells and provide a regular sequence of phases of individual development (Koldar *et al*. 2021). Recently, as evidenced by the results of the analysis of scientific literature, the role of plant growth and development regulators of low-toxic environmentally friendly substances that are effective when used in small quantities is increasing (Koldar 2008, Molnar *et al*. 2011, Chauhan *et al*. 2018).

A high number of studies addresses the characteristic features of reproduction, growth and development *in vitro* of different classes of roses (Dubois *et al*. 1988, Datta *et al.* 2002, Hameed *et al*. 2006, Attia *et al.* 2012). But in most cases, technologies developed for a certain genotype are not effective for other plants, and each of the next genotype under study requires the development of its own *in vitro* propagation technology.

Therefore, the study of the characteristics of regeneration, morphogenesis of Shrub class roses under *in vitro* condition, in particular, the achievement of rhizogenesis by explants is relevant and needs appropriate research. Stimulation of shoot and root formation is achieved by selecting the necessary ratios of cytokinins and auxins and their concentrations in the nutrient medium. They are external factors that contribute to the activation of competent cells capable of perceiving inducing factors, thereby excluding determination only in a certain direction (Koldar 2012, Podgajeckyj *et al*. 2018, Kosenko *et al*. 2021). Hence, the preparation of nutrient medium is carried out individually for definite plant genotypes, taking into account their species and cultivar characteristics (Nebykov *et al*. 2016, Khudolieieva *et al*. 2017, Mishchenko & Krivosheeva 2018).

The aim of the study is to define the dependence of hormonal determination of rhizogenesis in explants of roses of the Shrub class on the concentrations of α-naphthylacetic acid (α-NAA) and the universal growth regulator Humifield in nutrient medium under *in vitro* propagation.

Materials and Methods

Description of the study site

The hormonal determination of rhizogenesis by rose regenerants was carried out in the laboratory of microclonal propagation of the Department of Ornamental and Fruit Plants of the Sofiyivka National Dendrological Park of the National Academy of Sciences of Ukraine (hereinafter NDP "Sofiyivka"). NDP "Sofiyivka" is located in the northern part of the city of Uman, Cherkasy region (Ukraine).

Plant material for in vitro studies

Plant material of Shrub roses (Gärtnerfreude, Lavender Dream, Pomponella, Red Cascade, Sommerabend) from the NDP "Sofiyivka" collection fund was used for the study (Fig. 1).

Cultivar (cv.) Gärtnerfreude (W. Kordes Söhne, 1991). The flowers are carmine-red, diameter 3-4 cm, cup-shaped, double, no fragrance, in inflorescences. The leaves are dark green, glossy. The plant is spreading, branched, 0.5-0.7 m high. The bloom is abundant, repeated (June, August).

cv. Lavender Dream (G. P. Ilsink, 1984). The flowers are pinkish-lilac, diameter 3-4 cm, cup-shaped, semi-double, mild fragrance, in large inflorescences. The

Fig. 1. The Shrub roses from the NDP "Sofiyivka" collection fund. **a.** Gärtnerfreude, **b.** Lavender Dream, **c.** Pomponella, **d.** Red Cascade, **e.** Sommerabend.

leaves are light green, matte. The plant is spreading, 1-1.5 m high. The bloom is abundant, repeated (June, August).

cv. Pomponella (W. Kordes Söhne, 2005). The flowers are pink, diameter 4-6 cm, spherical, double, mild fragrance, collected in inflorescences. The leaves are dark green, semi-glossy. The plant is upright, branched, 0.8-1.5 m high. The bloom is abundant, repeated (June, August).

cv. Red Cascade (R. S. Moore, 1976). The flowers are dark red, cup-shaped, diameter 2-3 cm, double, no fragrance, in large inflorescences. The leaves are green, semi-glossy. The plant is creeping, up to 0.5 m high with prickly stems 0.8-1.2 m long. The bloom is abundant, almost ceaseless throughout the season.

cv. Sommerabend (W. Kordes Söhne, 1995). The flowers are dark red, cup-shaped, diameter 4-6 cm, double, no fragrance, in large inflorescences. The leaves are green and glossy. The plant is creeping, up to 0.4 m high with prickly stems up to 4 m long. The bloom is abundant, ceaseless throughout the season.

Cultivation conditions and determinants of stimulating root formation in explants

The single nodes (5.0-15.0 mm) taken from anually shoots from 6-14-years-old paternal rose plants was the source of explants for an introduction into *in vitro* culture. Pretreatment of roses explants was carried out using a disinfectant BTC 885 containing ammonium chloride salts in a concentration of 21.7% (IPAX CLEANOGEL, USA), and the main treatment was carried out with the treatment of mercury dichloride ($HgCl_2$) with the addition of the emulsifier Tween-80 (Scharlau Chemie, Spain) with an exposure of 1.5 min. After two-stage sterilization, aseptic and viable explants were cultivated on nutrient medium supplemented with phytohormones of the auxin (0.01 mg/l β-IBA) and cytokin (2.0 mg/l 6-BAP) groups (Kosenko *et al*. 2021).

The plant material was cultivated at a temperature of 24 ± 1°C, a 16-hour photoperiod, an illumination intensity of 3000 lx, and a relative humidity of 70.0%. Nutrient medium, materials and instruments were prepared according to the methodological recommendations by Pierik (1997), Kunakh (2005) and Chawla (2011).

The objects of the study were explants of the second and further passages, which reached a height of 2.0-4.0 cm with 2-3 pairs of leaves (Fig. 2). The authors used the method of induction of rhizogenesis in explants by adding α-NAA of various concentrations to the nutrient medium (0.2, 0.5, 0.8, 1.0 mg/l). The cultivation of explants was carried out on a universal basic agar nutrient medium according to the prescription of Murashige and Skoog (MS) with the addition of a half dose of macro- and microelements (Murashige & Skoog 1962). To increase the percentage of rooting of explants, the universal plant growth regulator Humifield (potassium humate) was used — a natural product manufactured by Humintech GmbH (Germany) from leonardite, a special type of brown coal with a high content of humic acids. The stimulant (coal humate) contains about 80.0% humic acids and a full range of microelements with 100% solubility. In order to optimize the rhizogenesis of explants of the studied rose cultivars, the study was made of the complex effect of α-NAA at a concentration of 0.5 mg/l and Humifield — 5.0; 10.0; 15.0 and 20.0 mg/l.

Fig. 2. Explants of roses were selected for cultivation in rooting medium.

Experimental design, data collection and statistical analysis

Percentage of rooted explants and quantitative parameters of their development were defined within 30-40 days. For quantitative analysis, at least 25 explants were taken per experimental variant. Data on the parameters of the development of rooted explants, in particular the number of roots, the root length, and the shoot height were determined as the average of the measurements of 5 separate plants. All experiments were performed in triplicate. Results were presented as mean value (x) ± standard deviation (SD). Data were subjected to ANOVA and the means were compared by Duncans multiple range test ($p \leq 0.05$).

Results

When cultivating explants of different cultivars of roses on nutrient medium for 10-18 days in the basal part of the explant, the initial stage of rhizogenesis determination was observed — the appearance of callus in the explants. Depending on the cultivar and the concentration of auxin in nutrient medium, root primordia appeared with different growth rates, from which roots formed within 28-36 days. In the course of the study, the dependence of the efficiency of rhizogenesis on the plant genotype and auxin concentration was established. Root formation was not observed on a hormone-free medium (control) (Fig. 3).

Of the nutrient medium modified by the addition of 0.2–1.0 mg/l α-NAA, the most effective was the medium containing 0.5 mg/l α-NAA, during cultivation in which the frequency of rhizogenesis for the studied cultivars averaged 61.2% of the number of explants planted for rooting. The susceptibility of each cultivar to the studied concentrations of α-NAA showed that regenerated plants of the cv. Lavender Dream (66.0%) and cv. Sommerabend (67.0%) had the highest percentages for rhizogenesis (Fig. 4). The number of rooted explants decreased by an average of 8% with an increase in concentration of α-NAA up to 0.8 mg/l. An increase in the concentration of α-NAA to 1.0 mg/l did not activate rhizogenesis more; instead, it inhibited it, whereupon the number of rooted plants by variety was in the range of 23.0-43.0%. The proportion of rooted plants was relatively low with a decrease in the concentration of α-NAA to 0.2 mg/l, which resulted in the decrease of the rhizogenesis of regenerated plants to 4.0-18.0%.

Fig. 4. Regenerated plant cv. Sommerabend.

The cultivars differed in the number of roots, the root length, and the shoot height (Table 1).

The number of formed roots per explant on average ranged from 1.9 (at a concentration of 1.0 mg/l α-NAA) to 3.2 (0.5 mg/l α-NAA) pcs., while the highest number

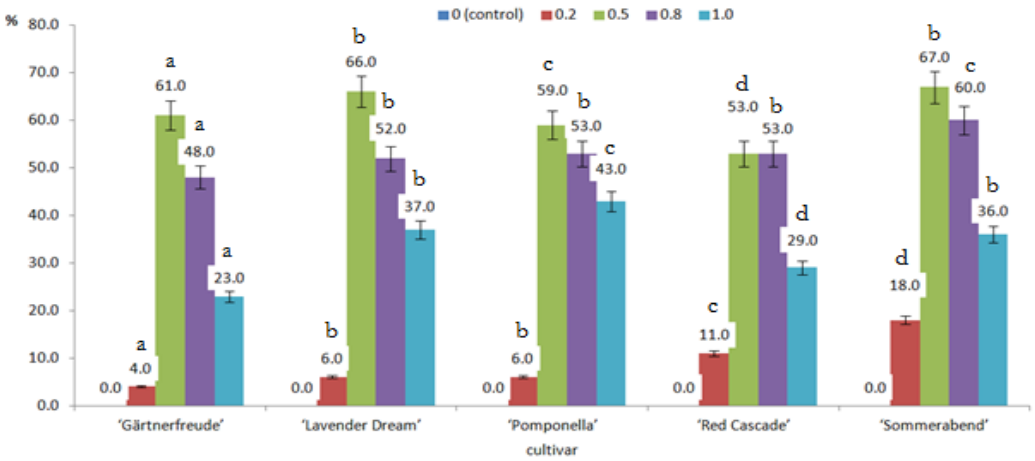

Fig. 3. Rooted rose plants on MS nutrient medium containing α-NAA (mg/l).
Note: The letters (**a-d**) define homogeneity groups (Duncan test, $p < 0.05$).

of roots in explants of Gärtnerfreude (3.1 pcs.) were formed on nutrient medium with a concentration of 0.8 mg/l α-NAA, and in explants of Lavender Dream, Pomponella, Red Cascade, Sommerabend (respectively 2.8; 3.9; 3.7; 3.5 pcs.) they were formed by concentration of 0.5 mg/l α-NAA. The shortest roots in all cultivars of roses were noted on the nutrient medium with the addition of 0.2 mg/l and 1.0 mg/l of α-NAA (with an average of 2.6 and 2.2 cm). When 0.5 mg/l of α-NAA was added to the nutrient medium, the roots reached a length from 3.2 cm (Gärtnerfreude) to 5.3 cm (Lavender Dream).

The main shoots continued to grow in the explants, and within 25-38 days, they reached 2.5-4.8 cm in height and formed three to four pairs of well-developed leaves. With a change in the concentration of the hormonal component of the nutrient medium, simultaneously with root formation, the growth of the vegetative part of plants was different (Fig. 5). At 0.5 mg/l concentration of α-NAA, vegetative shoots reached a maximum height in cv. Pomponella which became 4.8 cm, in cv. Red Cascade — 4.6 cm, and in cv. Lavender Dream, cv. Sommerabendand cv. Gärtnerfreude —4.4, 4.3 and 3.6 cm, respectively.

Fig. 5. Rooted rose plants

In order to increase the rhizogenes potential of regenerated plants of the studied culrivars, the MS nutrient medium containing 0.5 mg/l α-NAA was optimized by adding Humifield growth regulator. The composition of the regulator includes humic acids, which have a versatile effect on plant growth processes and carry out their regulation throughout the entire period of vegetation, promote the enzymatic activity of cells, and stimulate vital processes (Rugini & Pesce 2006, Kashyap *et al.* 2017).

The complex use of the growth regulators resulted in the activation of the initiation of root primordia and the growth of roots. The highest frequency of rhizogenesis was observed in explants cultivated on a medium containing α-NAA 0.5 mg/l and Humifield 10 mg/l (Fig. 6).

The highest proportion of rooted plants was obtained for the cv. Lavender Dream (86.0%), which is 20.0% higher than when using α-NAA only. A high percentage of rooting was also noted for the cv. Red Cascade (80.0%). Rooting percentages for cultivars Pomponella and Sommerabend with the addition of 10 mg/l Humifield were slightly lower, and were 78% and 70%, respectively (Fig. 7). With the content of 5 mg/l of Humifield the number of rooted roses explants was 9.0-27.0% higher than the control.

With the content of 5 mg/l of Humifield in the medium, the rooting rates were similar to the control and amounted to 65.0-73.0%, and an increase in its amount to 15 mg/l contributed to raising the percentage of rooting increased by 3.0-5.0% for the cv. Gärtnerfreude, Lavender Dream, Red Cascade, Sommerabend. Slightly lower rates of rhizogenesis were obtained by adding 20 mg/l of Humifield to the nutrient medium, on which the rooting rates for all the cultivars became 46.0-59.0%.

Fig. 7. Rhizogenesis in cv. Red Cascade explants

Discussion

The rooting of explants *in vitro* of most taxa has always been problematic, especially after long-term cultivation in isolated culture. Many scientists noted that for the formation of roots, it was necessary to completely remove cytokinins from the composition of the nutrient medium and add increased concentrations of auxins. Preparations of this group of phytohormones are the main ones for inducing rhizogenesis (Kalinin *et al.* 1992, Arnold *et al.* 1995, Carelli & Echeverrigaray 2002, Kunakh 2005, Figas *et al.* 2016, Zapolsky 2021).

The successful use of different auxins for root induction in rose *in vitro* has been reported, in particular, indole-3-butyric acid (IBA) for *Rosa ×centifolia* L. and cv. Gruss an Teplitz (Baig *et al*. 2011) *R. hybrida* Vill. Al-Taif (Attia *et al*. 2012); *R. × damascena* Herrm. Isfahan and Kashan (Saremi-Rad & Mohammadi 2020), for *R. pisiformis* (Christ.) D. Sosn. (Özel *et al*. 2023); α-NAA for *R. canina* L. (Davoudi Pahnekolayi *et al*. 2016), *R. hybrida* (Oo *et al*. 2021); or a combination thereof for *R. canina* та *R. beggeriana* Schrenk ex Fisch. & C.A.Mey. (Moradian & Bagheri, 2019), hybrid tea rose

Table 1. Morphometric indicators of the development of rose during rooting *in vitro.*

Cultivar	α-NAA concentrations to the nutrient medium (mg/l)			
	0.2	0.5	0.8	1.0
Number of roots				
Gärtnerfreude	1.8 ± 0.2 a	2.1 ± 0.1 a	3.1 ± 0.1 a	1.7 ± 0.1 a
Lavender Dream	2.3 ± 0.2 b	2.8 ± 0.2 b	2.7 ± 0.1 b	1.6 ± 0.1 b
Pomponella	1.8 ± 0.1 a	3.9 ± 0.1 c	3.1 ± 0.1 a	2.4 ± 0.1 c
Red Cascade	2.7 ± 0.1 c	3.7 ± 0.1 d	3.4 ± 0.1 c	2.6 ± 0.1 d
Sommerabend	2.1 ± 0.1 d	3.5 ± 0.2 e	2.8 ± 0.2 b	1.4 ± 0.1 e
x ± SD	2.1 ± 0.4	3.2 ± 0.7	3.0 ± 0.3	1.9 ± 0.5
Root length (cm)				
Gärtnerfreude	2.4 ± 0.2 a	3.2 ± 0.2 a	2.6 ± 0.2 a	2.3 ± 0.3 a
Lavender Dream	2.7 ± 0.2 b	5.3 ± 0.2 b	4.8 ± 0.2 b	2.8 ± 0.2 b
Pomponella	2.3 ± 0.2 a	4.2 ± 0.2 c	3.8 ± 0.2 c	2.5 ± 0.3 ab
Red Cascade	2.8 ± 0.2 b	4.1 ± 0.2 c	3.2 ± 0.2 d	2.2 ± 0.2 a
Sommerabend	2.9 ± 0.2 b	3.6 ± 0.2 d	2.7 ± 0.2 a	1.4 ± 0.2 c
x ± SD	2.6 ± 0.3	4.1 ± 0.8	3.4 ± 0.9	2.2 ± 0.9
Shoot height (cm)				
Gärtnerfreude	2.9 ± 0.17 a	3.6 ± 0.21 a	4.1 ± 0.13 cd	2.5 ± 0.18 a
Lavender Dream	3.4 ± 0.16 b	4.4 ± 0.09 b	4.6 ± 0.15 ac	3.7 ± 0.14 bc
Pomponella	3.4 ± 0.11 bc	4.8 ± 0.7 b	4.1 ± 0.15c	2.7 ± 0.12 ad
Red Cascade	2.9 ± 0.9 ac	4.6 ± 0.16 b	3.9 ± 0.12 c	3.1 ± 0.15 cd
Sommerabend	2.6 ± 0.09 a	4.3 ± 0.15 b	3.8 ± 0.17 db	2.7 ± 0.16 a
x ± SD	3.0 ± 0.4	4.3 ± 0.5	4.1 ± 0.3	2.9 ± 0.5

Note: The letters (a-e) define homogeneity groups (Duncan test, $p < 0.05$)

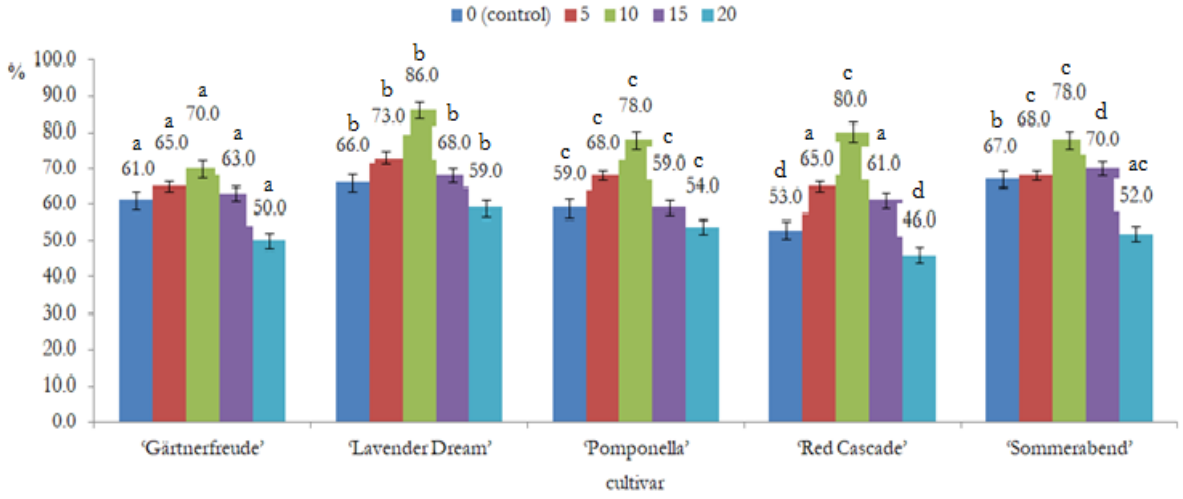

Fig. 6. Rooting of the Shrub rose depending on the content of growth regulators α- NAA (0.5 mg/l) and α-NAA + Humifield (mg/l) in the nutrient medium.
Note: The letters (a-d) define homogeneity groups (Duncan test, $p < 0.05$).

cv. Raktagandha (Kumari *et al*. 2017) and *R. hybrida* (Afrin *et al*. 2022); indole-3-acetic acid (IAA) for *R. mini* L. (Tsygankova *et al*. 2022). The result of *in vitro* rooting depended primarily on the concentration of the stimulator and, of course, it was a species-specific reaction. In earlier reports, it was noted that the ability to in vitro root formation was lower in the old world rose species *R. canina* and *R. ×damascena* compared to *R. hybrida* cultivars (Khosh-Khui & Sink 1982, Rezanejad *et al*. 2023). Rezanejad *et al.* (2023) demonstrated the efficacy of pretreatment on solid MS medium for a duration of two weeks, containing 3 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), for the *in vitro* rooting of miniature rose Modern Hybrid and *R. × damascena*. Then, explants were transferred to a half strength MS liquid medium containing 0.05 mg/l IAA and without IAA, respectively (Rezanejad *et al*. 2023). Reports of successful rooting of roses without auxins are rare (Ozel & Arslan 2006, Šiško 2011).

In the course of our research, rooting of the researched cultivars of roses was not observed on a hormone-free MS medium. The determinants of stimulating root formation in explants were different ratios of α-NAA in the nutrient medium. The use of this external factor contributed to the activation of rhizogenesis processes and the formation of up to 61.2% of rooted explants.

Putrescine (Musavi Ahmadabadi *et al.* 2023), chitosan (Yegorova *et al*. 2023) and phloroglucinol (Deltalab *et al*. 2023) in conjunction with phytohormones were used to enhance the root formation of certain rose cultivars under *in vitro* conditions. Numerous studies proved the effectiveness of using humic substances to stimulate root growth, which was depended on the growth regulator's source, the rate of application and, to a lesser extent, on the plant's type and growing conditions (Chen & Aviad 1990, Rose *et al*. 2014, Nardi *et al*. 2021). According to the results of research by scientists from different countries of the world, the use of humic acid was effective for shoot and root growths of pea plants (Gawlik *et al*. 2014), rooting azaleas (Elmongy *et al*. 2018), enhancing Sorbonne lily bulb and root growth under *in vitro* conditions (Wu *et al*. 2016), rooting and acclimatization of pears (Marino *et al*. 2009), *ex vitro* acclimatization of strawberry (Neri *et al*. 2022). During the Date palm (*Phoenix dactylifera* L.) micropropagation, the addition of humic acid and zinc oxide nanoparticles to the medium increased the level of macronutrients Nitrogen (N), Phosphorus (P), Potassium (K), Sulfur (S), and the micronutrient Zinc (Zn) in the shoots, and showed high effectiveness at the stages of callus formation, shoot reproduction and rooting *in vitro*, compared to other treatments (Al-Mayahi 2021).

For this reason, we investigated the effect of plant growth regulator Humifield with a high content of humic acid on rhizogenesis in Shrub rose regenerants cultivated *in vitro*. Its effectiveness was previously proven under *ex vitro* conditions. The incorporation of Humifield in a mixture with fertilizers was instrumental in augmenting the grain yield of blue lupine (Kotelnytska *et al*. 2021). In wheat, the combination of herbicides and Humifield provided the best performance in weed control, including perennial ones (Korotkova *et al*. 2021).

The use of 0.5 mg/l α-NOC in combination with 10 mg/l Humifield increased the frequency of rhizogenesis up to 70-86%, depending on the rose genotype. *In vitro* hormonal determination in Shrub class rose regenerants contributed to a significant increase in the frequency of rhizogenesis and accelerated plant cloning, what is the basis for obtaining mass planting material necessary for green building.

Conclusions

Hormonal determination of rhizogenesis and efficiency of *in vitro* root formation in regenerants of the Shrub class roses depended on the plant genotype (cultivar) and concentrations of α-NAA in nutrient medium. The addition of 0.5 mg/l of α-NAA to the nutrient medium contributed to the production of 61.2% of rooted explants. The cultivars with the highest rhizogenesis ability were Lavender Dream (66.0%) and Sommerabend (67.0%)

The complex use of growth regulators containing 0.5 mg/l α-NAA and 10 mg/l Humifield contributed to the rooting of 70.0 to 86.0% of roses of the studied cultivars. The highest rhizogenic activity was shown by the cv. Lavender Dream, in which 86% of rooted regenerated plants were obtained.

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Data Sharing Statement: All data are available within the study.

Author Contributions: Concept: L.K., Y.M., Design: L.K., I.D., Execution: I.D., Y.M., Material supplying: I.D., Y.M., Data acquisition: LK., A.K., Data analysis/interpretation: LK., A.K., Writing: L.K., Y.M., Critical review: I.D., A.K.

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References

1. Afrin, S., Rahman, M., Khalekuzzaman, M., Hasan, M., Fahim, A. & Alam, M. 2022. Study on *in vitro* micropropagation of *Rosa* sp. *Bangladesh Journal of Agriculture*, 47(1): 66-74. https://doi.org/10.3329/bjagri.v47i1.60593
2. Al-Mayahi, A. 2021. The effect of humic acid (HA) and zinc oxide nanoparticles (ZnO-NPS) on *in vitro* regeneration of date palm (*Phoenix dactylifera* L.) cv. Quntar. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 145: 445-456.
3. Arnold, N., Binns, M., Cloutier, C., Barthakur, N. & Pellerin, R. 1995. Auxins, salt concentrations and their interactions during *in vitro* rooting of winter-hardy and hybrid tea roses. *Horticultural Science*, 30(7): 1436-1440.
4. Attia, A., Dessoky, E. & El-Tarras, A. 2012. *In vitro* propagation of *Rosa hybrida* L. cv. Al-Taif Rose plant. *African Journal of Biotechnology*, 11(48): 10888-10893. https://doi.org/10.5897/AJB12.781
5. Baig, M., Hafiz, I., Hussain, A., Ahmad, T. & Abbasi, N. 2011. An efficient protocol for in vitro propagation of *Rosa* gruss an teplitz and *Rosa centifolia*. *African Journal of Biotechnology*, 10(22): 4564-4573. https://doi.org/10.5897/AJB10.2051
6. Bidabadi, S. & Jain, S. 2020. Cellular, molecular, and physiological aspects of *in vitro* plant regeneration. *Plants*, 9(6): 702.
7. Carelli, B. & Echeverrigaray, S. 2002. An improved system for the *in vitro* propagation of rose cultivars. *Scientia Horticulturae*, 92: 69-74. https://doi.org/10.1016/S0304-4238(01)00280-1
8. Chauhan, U., Singh, A., Godani, D., Handa, S., Gupta, P., Patel, S. & Joshi, P. 2018. Some natural extracts from plants as lowcost alternatives for synthetic PGRs in rose

micropropagation. *Journal of Applied Horticulturae*, 20: 103-111. https://doi.org/10.37855/jah.2018.v20i02.19

9. Chawla, H. 2011. *Introduction to plant biotechnology*. CRC Press, 760 pp.

10. Chen, Y. & Aviad, T. 1990. Effects of humic substances on plant growth. pp. 161-186. In: Maccarthy, P., Clapp, C.E., Malcolm, R.L. & Bloom, P.R. (eds). *Humic substances in soil and crop sciences: Selected readings. Madison: Soil Science Society of America*. https://doi.org/10.2136/1990.humicsubstances.c7

11. Datta, S., Chakraborty, D., Deepti D., Mandal, A. & Saxena, M. 2002. *In vitro* petal culture and callus formation in *Rosa* species. *Indian Journal of Agricultural Sciences*, 72(5): 271-276.

12. Davoudi Pahnekolayi, M., Tehranifar, A., Samiei, L. & Shoor, M. 2016. Optimization of the micro-propagation protocol of two native rose species of Iran (*Rosa canina* and *Rosa beggeriana*). *Acta Hortic*, 1131: 87-96. https://doi.org/10.17660/ActaHortic.2016.1131.12

13. De Klerk, G. 2002. Rooting of microcuttings: theory and practice. *In Vitro Cellular & Developmental Biology-Plant*, 38(5): 415-422. https://doi.org/10.1079/IVP2002335

14. Deltalab, B., Kaviani, B. & Kulus, D. 2023. *In vitro* propagation of oil-bearing *Rosa damascena* using phloroglucinol: A protocol for rapid and high-quality shoot multiplication and rooting. *Industrial Crops and Products*, 203: 117139. https://doi.org/10.1016/j.indcrop.2023.117139

15. Denysko, I. 2022. Assessment of introduction prospects of David Austin's roses to the conditions of the Right Bank Forest-Steppe Zone of Ukraine. *Journal of Native and Alien Plant Studies*, 18: 16-28. [In Ukrainian]. https://doi.org/10.37555/2707-3114.18.2022.269920

16. Dubois, L., Roggemans, J., Soyeurt, G. & De Vries, D. 1988. Comparison of the growth and development of dwarf rose cultivars propagated *in vitro* and *in vivo* by softwood cuttings. *Scientia Horticulturae*, 35: 293-299. https://doi.org/10.1016/0304-4238(88)90123-9

17. Elmongy, M., Zhou, H., Cao, Y., Liu, B. & Xia, Y. 2018. The effect of humic acid on endogenous hormone levels and antioxidant enzyme activity during in vitro rooting of evergreen azalea. *Scientia Horticulturae*, 227: 234-243. https://doi.org/10.1016/j.scienta.2017.09.027

18. Figas, A., Tomaszewska-Sowa, M., Sawilska, A. & Keutgen, A. 2016. Improvement of *in vitro* propagation and acclimation of *Helichrysum arenarium* L. Moench. *Acta scientiarum Polonorum. Hortorum cultus*, 15(4): 17-26.

19. Gawlik, A., Kulpa, D., Gołębiowska, D. & Bejger, R. 2014. Influence of the auxin-like activity of humic acid on bio and microbiometric parameters of *Pisum sativum* L. by *in vitro* cultures of pea plants. *Journal of Food, Agriculture & Environment*, 12(3-4): 209-212.

20. Hameed, N., Shabbir, A., Ali, A. & Bajwa, R. 2006. *In vitro* micropropagation of disease-free rose (*Rosa indica* L.). *Mycopathology*, 4(2): 35-38.

21. Hasnain, A., Naqvi, S., Ayesha, S., Khalid, F., Ellahi, M., Iqbal, S., Hassan, M. Z., Abbas, A., Adamski, R., Markowska, D., Baazeem, A., Mustafa, G., Moustafa, M, Hasan, M. & Abdelhamid, M. 2022. Plants *in vitro* propagation with its applications in food, pharmaceuticals and cosmetic industries; current scenario and future approaches. *Frontiers in Plant Science*, 13: 1009395. https://doi.org/10.3389/fpls.2022.1009395

22. Horn, W. 1992. Micropropagation of rose (*Rosa* sp. L.). pp. 320-342. In: Bajaj YPS (ed.) *Biotechnology in Agriculture and Forestry*. Springer, Berlin.

23. Jain, S. & Ishii, K. 2003. Micropropagation of Woody Trees and Fruits. Forestry sciences. Dordrecht Kluwer Academic Publishers, 840 pp. https://doi.org/10.1007/978-94-010-0125-0

24. Kalinin, F., Kushnir, G., Sarnatskaya, V., Lobov, V. 1992. Technology of microclonal propagation of plants. Kiev, Scientific opinion, 488 pp. (In Russian)

25. Kashyap, S., Kapoor, N., Kale, R. 2017. Micropropagation of *B. monnieri* using humin media inplant tissue culture. *Annals of Plant Sciences*, 6(5): 1625-1629. https://doi.org/10.21746/aps.2017.05.004

26. Khosh-Khui, M. & Sink, K. 1982. Micropropagation of new and old world rose species. *Journal of Horticultural Science*, 57(3): 315-319. https://doi.org/10.1080/00221589.1982.11515058

27. Khudolieieva, L., Kutsokon, N., Nesterenko, O., Rashydov, N. & Dugan, O. 2017. *In vitro* establishing of poplar and willow clones perspective for renewable energetics. *Biological sytems*, 9(1): 18-22. https://doi.org/10.31861/biosystems2017.01.018

28. Koldar, L. 2008. Features of ontogeny of *Cercis siliquastrum* L. plants *in vitro* culture. *Autochthous and Alien Plants*, 3-4: 53-57. [In Ukrainian].

29. Koldar, L. 2012. The role of phytohormones in the determination of *Cerasus serratula* Lindl. explants cultivated *in vitro*. *Biosphere Reserve "Askania Nova" Reports*, 14: 152-155. [In Ukrainian].

30. Koldar, L., Dzhus, L. & Nebykov, M. 2021. Regeneration capacity of narrow-localized endemic species *Dianthus hypanicus* Andrz. *in vitro*. *Biotechnologia Acta*, 14(3): 39-45. [In Ukrainian]. https://doi.org/10.15407/biotech14.03.039

31. Korotkova, I., Marenych, M., Hanhur, V., Laslo, O., Chetveryk, O. & Liashenko, V. 2021. Weed control and winter wheat crop yield with the application of herbicides, nitrogen fertilizers, and their mixtures with humic growth regulators. *Acta Agrobotanica*, 74(1): 748. https://doi.org/10.5586/aa.748

32. Kosenko, I., Koldar, L., Denysko, I., Balabak, O., Nebykov, M., Balabak, A. & Balabak, A. 2021. Morphogen development of *in vitro* cultivated Shrub roses. *Ukrainian Journal of Ecology*, 11(2): 229-235. https://doi.org/10.15421/2021_104

33. Kotelnytska, A., Tymoshchuk, T., Kravchuk, M., Sayuk, O. & Nevmerzhytska, O. 2021. Mineral nutrition optimization as a factor affecting blue lupine crop productivity under conditions of global climate warming. Romanian Agricultural Research, 38: 223-230.

34. Kroin, J. 2016. Effective methods to propagate plants from cuttings, by adventitious root formation, grafting & stenting, layering, and improved transplanting using Hortus and

Rhizopon plant rooting hormones. Includes a case study of rose propagation. Hortus USA Corp.

35. Kumar, N. & Reddy, M. 2011. *In vitro* plant propagation: a review. *Journal of forest and environmental science*, 27(2): 61-72.

36. Kumari, S., Singh, K., Singh, S., Kumar, S. & Sarkhel. S. 2017. Establishment of in vitro propagation protocol for Hybrid Tea rose cv. Raktagandha. Indian Journal of Horticulture, 74(2): 245-250. https://doi.org/10.5958/0974-0112.2017.00050.0

37. Kunakh, V. 2005. *Biotechnology of medicinal plants. Genetic, Physiological and biochemical basis.* Logos, Kyiv, 730 pp. [In Ukrainian].

38. Marino, G., Cellini, A., Masia, A., Simoni, A., Francioso, O. & Gessa, C. 2009. *In vitro* treatment with a low molecular weight humic acid can improve growth and mineral uptake of pear plantlets during acclimatization, 565-572 pp. Paper presented at the XI International Symposium on Plant Bioregulators in Fruit Production, 20 September, Bologna-Italy. https://doi.org/10.17660/ActaHortic.2010.884.73

39. Mishchenko, S. & Krivosheeva, L. 2018. *In vitro* callusogenesis and organogenesis of different *Linum usitatissimum* L. accessions. *Plant Genetic Resources*, 23: 49-58. https://doi.org/10.36814/pgr.2018.23.04

40. Molnar, Z., Virag, E. & Ordog, V. 2011. Natural substances in tissue culture media of higher plants. *Acta Biologica Szegediensis*, 55: 123-127.

41. Moradian, M. & Bagheri, A. 2019. Effect of media composition and plant growth regulators on in vitro regeneration of *Rosa canina* and *Rosa beggeriana*. *Journal of Plant Research (Iranian Journal of Biology*, 32(1): 218-230. https://doi.org/20.1001.1.23832592.1398.32.1.14.7

42. Moroz, O., Denysko, I., Bank, V. 2010. The collection of ground-cover roses in the National Dendrological Park "Sofiyivka" of the National Academy of Sciences of Ukraine. *Autochthous and Alien Plants*, 6: 106-109. [In Ukrainian].

43. Moroz, O., Denysko, I. & Bank, V. 2012. Collection of Shrub roses in the National Dendrological Park "Sofievka" NAS of Ukraine. *Biosphere Reserve "Askania Nova" Reports*, 14: 181-185. [In Ukrainian].

44. Murashige, T. & Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. Ph*ysiologia Plantarum*, 15: 473-497.

45. Musavi Ahmadabadi, M., Ahmadi, N. & Dehestani-Ardakani, M. 2023. Putrescine and IBA enhanced the adventitious root formation in Damask rose (*Rosa*× *damascena* Mill.) under *in vivo* and *in vitro* conditions. *Journal of Horticulture and Postharvest Research*, 6(4): 383-396. https://doi.org/10.22077/jhpr.2023.6862.1339

46. Nardi, S., Schiavon, M. & Francioso, O. 2021. Chemical structure and biological activity of humic substances define their role as plant growth promoters. *Molecules*, 26(8): 2256. https://doi.org/10.3390/molecules26082256

47. Nebykov, M., Koldar, L., Bonyk, Z., Trofimenko, N. & Belemets, N. 2016. Microclonal breeding is whitish-grey meadowsweet (*Spiraea cana* Waldst. et Kit.). *Autochthous and Alien Plants*, 12: 160-165. [In Ukrainian]. https://doi.org/10.37555/.12.2016.173394

48. Neri, J., Meléndez-Mori, J., Tejada-Alvarado, J., Vilca-Valqui. N., Huaman-Huaman, E., Oliva, M &, Goñas, M. 2022. An optimized protocol for micropropagation and acclimatization of strawberry (*Fragaria*× *ananassa* Duch.) variety 'Aroma'. *Agronomy*, 12(4): 968. https://doi.org/10.3390/agronomy12040968

49. Nikbakht, A., Kafi, M., Mirmasoudi, M. & Babalar, M. 2005. Micropropagation of Damask rose (*Rosa damascena* Mill.) cvs Azaran and Ghamsar. *International Journal of Agriculture and Biology*, 7: 535-538.

50. Oo, K., Lwin, K. & Khai, A. 2021. *In vitro* micropropagation of rose (Hybrid Rosa spp.) through plant tissue culture technique. *Journal of Scientific and Innovative Research*, 10(1): 1-4.

51. Ozel, C. & Arslan, O. 2006. Efficient micropropagation of English shrub rose 'Heritage' under *in vitro* conditions. *International Journal of Agriculture and Biology*, 8(5): 626-629.

52. Özel, O., Demiray, H., Eroglu, V. & Ecer, C. 2023. Axillary shoot proliferation and regeneration of red rose [Rosa Pisiformis (Christ.) D. Sosn. - an under threat extinction species endemic to Turkey. *Bangladesh journal of botany*, 52(4): 949-958. https://doi.org/10.3329/bjb.v52i4.70576

53. Podgajeckyj, A., Matskevych, V. & Podhaiets'kyj, A. 2018. *Peculiarities of microclonal propagation of plant species*. BNAU, Bila Tserkva, 209 pp. [In Ukrainian].

54. Pierik, R. 1997. *In vitro culture of higher plants*. Springer science & business media, 348 pp.

55. Rezanejad, F., Abdirad, S. & Abarian, M. 2023. Comparison of shoot and root regeneration of miniature potted rose (*Rosa* x *hybrida* L.) and Damask rose (*R. damascena* Mill.) in microculture system. *Acta agriculturae Slovenica*, 119(1): 1-10. https://doi.org/10.14720/aas.2023.119.1.2380

56. Rose, M., Patti, A., Little, K., Brown, A., Jackson, W. & Cavagnaro, T. 2014. A meta-analysis and review of plant-growth response to humic substances: practical implications for agriculture. *Advances in agronomy*, 124: 37-89. https://doi.org/10.1016/B978-0-12-800138-7.00002-4

57. Podwyszynska, M. 2003. Rooting of Micropropagated Shoots. 66-76 pp. In: Roberts, A., Debener, T. & Gudin, S. (eds.) *Encyclopedia of Rose Science*. Academic press.

58. Rugini, E. & Pesce, P. 2006. Genetic improvement of olive. *Pomologia Croatica*, 12: 43-74. https://doi.org/10.1016/j.biotechadv.2016.03.004

59. Saremi-Rad, A. & Mohammadi, A. 2020. Optimizing the propagation of Damask rose (*Rosa damascena* Mill.) rootstocks under *in vitro* conditions. *Iranian Journal of Medicinal and Aromatic Plants*, 36(3): 509-521. https://doi.org/10.22092/ijmapr.2020.126120.2673

60. Šiško, M. 2011. Micropropagation of roses (*Rosa* spp.): the effects of different media on *in vitro* rooting. *Agricultura (Slovenia)*, 8(2): 19-22.

61. Tsygankova, V., Oliynyk, O., Kvasko, O.Y., Pilyo, S. & Klyuchko, S. 2022. Effect of plant growth regulators ivin,

methyur and kamethur on the organogenesis of miniature rose (*Rosa mini* L.) *in vitro*. *International Journal of Medical Biotechnology & Genetics*, 1: 1-8.

62. Tytarenko, N. & Tesliuk, N. 2020. Improvement of the processes of microclonal reproduction of blackberry Rubus Caesius L.var. Thornfree. *Microbiology&Biotechnology***,** 2: 72-84. https://doi.org/10.18524/2307-4663.2020.2(49).209806

63. Vedmid, M., Yatsenko, S. & Popov, O. 2002. Application of plant growth regulators in the process of seedlings growing and in the creation of planted forest. *Scientific bulletin of UNFU*, 12(4): 240-245.

64. Wu, Y., Xia, Y., Zhang, J., Du, F., Zhang, L. & Zhou, H. 2016. Low humic acids promote in vitro lily bulblet enlargement by enhancing roots growth and carbohydrate metabolism. *Journal of Zhejiang University. Science*. 17(11): 892. https://doi.org/10.1631/jzus.B1600231

65. Yegorova, N., Stavtzeva, I., Tevfik, A., Tikhonov, V. & Sushinskaya, N. 2023. Effect of chitosan on rose and lavender micropropagation *in vitro*. Paper presented at the *AIP Conference Proceedings* 2931(1). AIP Publishing. https://doi.org/10.1063/5.0180661

66. Zapolsky, Y. 2021. Efficiency of reproduction of honeysuckle (*Lonicera caerulea* var. Edulis Turcz. ex Herder). *Taurida Scientific Herald. Series: Rural Sciences*, 117: 44-53. https://doi.org/10.32851/2226-0099.2021.117.7

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Research Article

Co-expression of P53 and P60-katanin shapes transcriptome dynamics

Şirin Korulu[1,2]

[1] Tallinn University, Institute of Natural and Health Sciences, 10120, Tallinn, ESTONIA
[2] İstanbul Arel University, Department of Molecular Biology and Genetics, 34537, Istanbul, TÜRKİYE
e-mail: sirinkorulu@gmail.com, ORCID: 0000-0001-6762-0659

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Abstract: Microtubules (MT), essential elements of the cytoskeleton have important roles in the cell such as intracellular cargo transport, cell motility and cell division. They provide support, growth and maintenance of the axonal and dendritic processes in neurons. Microtubule severing proteins such as katanin and spastin have roles in microtubule reconfiguration. Katanin is one of the best characterized severing proteins and is composed of catalytic subunit p60-katanin and regulatory subunit p80-katanin. The microtubule severing mechanism of p60-katanin has been depicted in detail, but how p60-katanin itself is regulated is still little-known. p53 is an important protein between proliferation and differentiation. It regulates different cellular mechanisms such as cell cycle arrest, senescence, differentiation, and apoptosis. p53 controls proliferation in dividing cells and is related to differentiation by means of affecting neuronal process length in non-dividing neurons. Both p53 and p60-katanin have critical roles in proliferation and differentiation separately. Moreover, these proteins were shown to physically interact, but their combined effect remains unclear. To this aim, the current study reveals the effects of p53 – p60-katanin co-expression on transcriptome of the fibroblast cells. Data indicated that the transcriptome of many different pathways such as actin regulation, neuroactive ligand-receptor interaction, and serotonergic synapses pathways were altered under p53 – p60-katanin co-expression conditions. Exploring combined effect of p53 and p60-katanin will help in design of new studies to better understand not only microtubule regulation but also neurodegenerative diseases that are linked to the reactivation of cell cycle and neuronal damage where two of these players take place.

Özet: Mikrotübüller (MT), hücre iskeletinin temel elemanları olup hücre içi kargo taşınması, hücre hareketliliği ve hücre bölünmesi gibi hücrede önemli rollere sahiptir. Ayrıca sinir hücreleri olan nöronlarda, aksonal ve dendritik yapıların desteklenmesi ve uzaması için önemli görevlere sahiptirler. Katanin ve spastin gibi mikrotübül kesici proteinler, mikrotübüllerin yeniden yapılandırılmasında rol oynar. Katanin, en iyi karakterize edilmiş MT kesici proteinlerden olup, katalitik alt birim p60-katanin ve düzenleyici alt birim p80-katanin'den oluşur. p60-katanin'in mikrotübül kesme mekanizması oldukça iyi bilinmektedir, ancak p60-katanin'in kendisinin nasıl düzenlendiği halen az bilinen bir konudur. p53, proliferasyon ve farklılaşma arasında kritik bir proteindir. Hücre döngüsünü, yaşlanma, farklılaşma ve apoptoz gibi farklı hücresel mekanizmaları düzenler. p53'ün bölünen hücrelerde proliferasyonu kontrol ettiği, bölünmeyen nöronlarda ise farklılaşma ile ilişkili olduğu ortaya konmuştur. Hem p60-katanin hem de p53, ayrı ayrı proliferasyon ve farklılaşmada kritik rollere sahiptir. Ayrıca, bu proteinlerin fiziksel olarak etkileşimde bulunduğu da gösterilmiştir, ancak bu proteinlerin birleşik etkisi belirsizliğini korumaktadır. Bu amaçla, mevcut çalışma, p53 ve p60-katanin'in birlikte eksprese edilmesinin fibroblast hücrelerinin transkriptomu üzerindeki etkilerini ortaya koymaktadır. Veriler, aktin düzenlenmesi, nöroaktif ligand-reseptör etkileşimi, serotonerjik sinaps yolları gibi birçok farklı yolakların transkriptomlarının p53 – p60-katanin'in birlikte ekprese edildiğinde değiştiğini göstermiştir. p53 ve p60-katanin'in birleşik etkisinin araştırılması, sadece mikrotübül düzenlemesini daha iyi anlamak için değil, aynı zamanda bu iki proteinin rol oynadığı hücre bölünmesinin yeniden aktifleşmesi ve nöronal hasarla ilişkili nörodejeneratif hastalıkları daha iyi anlamak için yeni çalışmaların tasarlanmasına da öncülük edecektir.

Introduction

The tumor suppressor protein p53 and the microtubule-severing enzyme p60-katanin play essential roles in various cellular processes such as cell cycle control, DNA damage response, neuronal differentiation

and cytoskeletal organization (Lane 1992, McNally & Vale 1993, Vousden & Prives 2009). p53 acts as a key guardian of genomic integrity by managing the cells' reactions to stress, guiding processes like cell cycle arrest, DNA repair, or apoptosis to ensure genomic stability (Lane 1992, Vousden & Prives 2009). On the other hand, p60-katanin influences microtubule dynamics, which affects cell division, intracellular transport, and overall cell shape (McNally & Vale 1993, McNally 2013). p53 and p60-katanin have roles both in dividing and in non-dividing cells, specifically in the differentiation process of neurons. The role of p60-katanin in neuronal processes is extensively studied. Similarly, p53 was shown to affect neuronal process lengths in non-dividing neurons (Ferreira & Kosik 1996, Hudson *et al.* 2005, Kim *et al.* 2011, Di Giovanni *et al.* 2006).

Although each of these proteins has been studied extensively on its own, their interactions and combined effects on cells' transcriptome are not yet fully understood (Baas 1997, Hayashi & Karl Seder 2013). Available findings suggest that they might affect shared signaling pathways or transcriptional networks, thereby altering how cells respond to different stimuli (Hayashi & Karl Seder 2013). A recent study showed for the first time that p53 and p60-katanin interact physically at protein level via p53's DNA binding domain and p60-katanin's C-terminal (Korulu & Yildiz 2020). However, the exact mechanisms behind this interaction and the full extent of their impact on gene expression are still not clear and need more comprehensive investigation.

This novel finding prompted us to explore the molecular changes that occur when p60-katanin and p53 are co-expressed in the cell. Investigating how p53 and p60-katanin co-expression affects transcriptome of the cell is a crucial step for understanding their complex roles in cellular regulation and disease development (Dai & Lu 2004, Duan *et al.* 2006). These preliminary findings also offer a reliable starting point for upcoming research in the field. Abnormalities in p53 and p60-katanin are linked to various human diseases, including cancer, neurodegenerative conditions, and developmental disorders (Vousden & Prives 2009, McNally 2013). Unraveling the details of their combined effect could provide new insights into disease mechanisms and potentially lead to innovative therapeutic approaches.

Materials and Methods

Construction of the plasmids

Constructs were obtained by cloning p53 (AB082923) and p60-katanin (NM_007044) into 3XFLAG-CMV™-10 and pcDNA3.1/myc-His vectors respectively.

Cell transfection

Rat RFL-6 cells were gifted by Prof. Dr. Arzu Karabay Korkmaz (Istanbul Technical University) and were cultivated in F12K (Lonza, Switzerland) medium containing 20% FBS (Thermo Fisher, USA), NEAA (Lonza) and L-Glutamine (Thermo Fisher). One day prior to transfection, cells were seeded in 6 well-plates, as 500,000/well. The following day, cells were transfected by using Lipofectamine 3000 (Thermo Fisher Scientific, USA). Cells were transfected with either p60-katanin-pcDNA3.1/myc-His and p53-3XFLAG-CMV™-10 (p60-katanin and p53 co-overexpressed) or pcDNA3.1/myc-His and 3XFLAG-CMV™-10 vectors (control cells). RNA extraction was performed 48 hours post transfection by using High Pure RNA Isolation Kit (Roche, Switzerland).

Sample labeling and purification

The Agilent One-Color Microarray-Based Gene Expression Analysis protocol was used for RNA labeling/hybridization. Briefly, total RNA was labeled with Cy3-dCTP during amplification. The labeled cRNAs were purified with the help of RNAeasy Mini Kit (Qiagen, Switzerland) and quantified with the NanoDrop ND-1000 spectrophotometer.

Hybridization and Scan

Labeled cRNA were fragmented to an average size of approximately 50±100 nucleotides by heating with the help of blocking agent and fragmentation buffer. Fragmented cRNA was hybridized and analyzed with the Agilent SurePrint G3 Human GE 8X60K, V3 Microarrays (Agilent®).

Statistical analysis

Gene-enrichment and functional annotation analyses were performed using Gene Ontology (www.geneontology.org) and KEGG (http://kegg.jp).

Results

The transcriptome analysis of RFL-6 cells where p60-katanin and p53 genes were overexpressed revealed significant changes in the mRNA levels of several genes. These changes were relative to control cells where cells were transfected with mock plasmids only and did not contain excessive expression of the proteins. For the current study, briefly, the genes that have over 3-fold up- or down-regulation have been summarized (Fig. 1). FOXO4, PLK5, and LPIN3 showed increased expression, suggesting activation of cellular pathways involved in stress response, cell cycle regulation, and lipid metabolism, respectively. Conversely, EMC10, BTBD8, and HOXC-AS1 exhibited decreased expression, indicating potential disruptions in ER function, transcriptional regulation, and chromatin remodeling (Table 1).

KEGG enrichment pathway analysis was also performed to elucidate the biological significance of the differentially expressed genes/proteins identified. This analysis revealed several enriched pathways associated with various cellular processes summarized in Fig. 2. These findings provide important insights into the possible functional effects of the observed changes in gene expression and shed light on the molecular mechanisms involved in the co-overexpression of p60-katanin and p53.

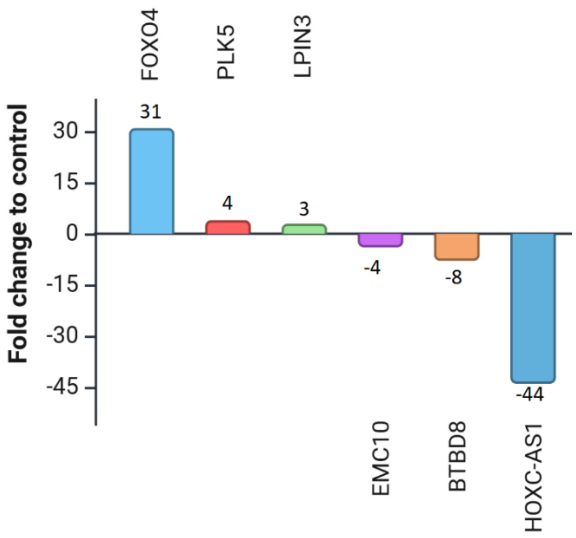

Fig. 1. Genes found to have increased and decreased expression change over 3-fold.

Table 1. Putative biological functions of the differentially expressed genes.

Putative Biological Function	Gene
Transcription factor promoting cell cycle arrest and apoptosis.	FOXO4
Kinase involved in cell cycle progression.	PLK5
Protein involved in lipid phosphate signaling and cytoskeletal remodeling.	LPIN3
Component of the ER exit sites complex, potentially regulating protein trafficking.	EMC10
Protein involved in clathrin-mediated endocytosis and neuronal development.	BTBD8
Antisense RNA potentially regulating HOXC cluster genes important for development.	HOXC-AS1

For instance, transcriptional misregulation in cancer, proteoglycans in cancer, and pathways in cancer were significantly enriched, suggesting a strong link between p60-katanin and p53 co-overexpression and tumor-related processes

Pathways like MAPK signaling, PI3K-Akt signaling, Wnt signaling, and p53 signaling showed notable enrichment. These are crucial pathways regulating cell survival, apoptosis, and growth, further indicating the potential role of p60-katanin and p53 in cell fate decisions. Additionally, pathways like regulation of actin cytoskeleton and cell cycle were also enriched, highlighting changes in cytoskeletal dynamics and cell division, consistent with p60-katanin's role in microtubule severing. Moreover, neuroactive ligand-receptor interaction, serotonergic synapse, and synaptic vesicle cycle pathways were implicated, suggesting that the co-overexpression might also affect neuronal function and differentiation.

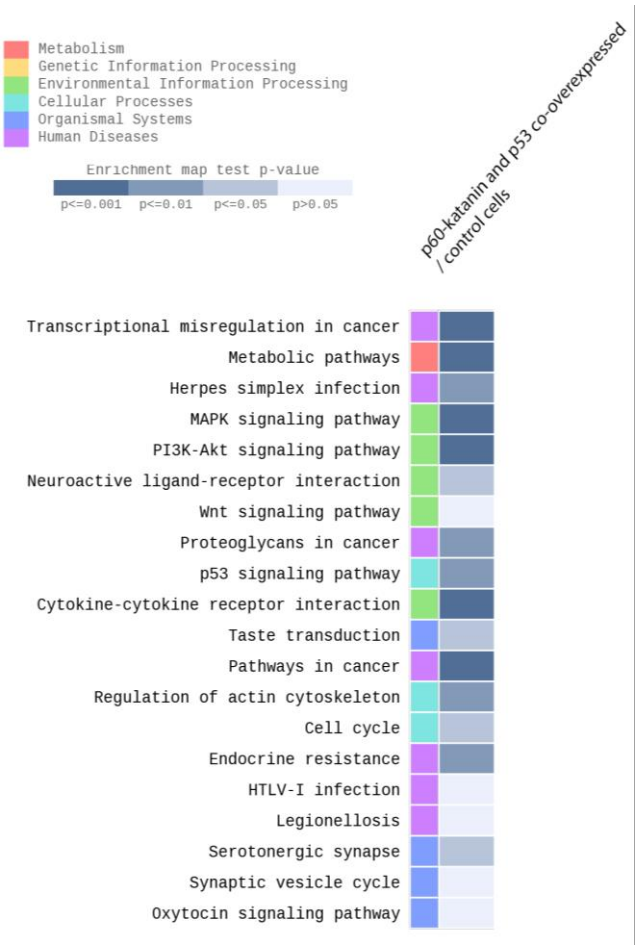

Fig. 2. KEGG enrichment pathway analysis.

Discussion

When comparing transcription changes in cells co-overexpressing p60-katanin and p53, the gene expression profile reveals significant alterations in the expression of key genes involved in various cellular processes. For instance, FOXO4, known to interact with p53 in regulating senescence and apoptosis, may play a critical role in how the co-overexpression of p60-katanin and p53 affects the transcriptional misregulation observed in cancer-related pathways. (Zhang *et al.* 2023). FOXO4's role in inhibiting p53-mediated apoptosis aligns with the observed enrichment in pathways like the p53 signaling pathway and transcriptional misregulation in cancer (Fig. 1). This indicates that FOXO4 could modulate p53's effects under co-overexpression, either by enhancing or shadowing p53-driven pathways (Zhang *et al.* 2023). On the other hand, PLK5 and LPIN3 suggest a coordinated cellular response to environmental stimuli or metabolic needs (de Cárcer *et al.* 2011). The modest rise in PLK5, a cell cycle regulator, may signal an active cell division phase or a reaction to DNA damage, aiding in cellular proliferation and repair. The enrichment in pathways like MAPK signaling and PI3K-Akt signaling, both of which are crucial for cellular growth and survival (de Cárcer *et al.* 2011) points towards a potential regulatory role for PLK5 in these pathways. PLK5 may interact with these

signaling cascades, particularly in neuronal cells, where p60-katanin is involved in cytoskeletal dynamics. Since PLK5 is also involved in the DNA damage response (de Cárcer *et al.* 2011), it could synergize with p53 to enhance cellular responses to stress, including regulation of actin cytoskeleton and neuronal differentiation processes. On the other hand, the human PLK5 gene is significantly silenced in astrocytoma and glioblastoma by promoter hypermethylation, suggesting a tumor suppressor function for this gene (de Cárcer *et al.* 2011). Additionally, the upregulation of LPIN3, a lipid metabolism regulator (Su *et al.* 2023), points to increased lipid biosynthesis or metabolic adjustments to fulfill cellular energy requirements. PLK5, the least studied member of the PLK family, is involved in neurodevelopment and tumor suppression (Su *et al.* 2023). Finally, LPIN3's role in maintaining lipid homeostasis could influence cellular membrane dynamics, which may affect processes like synaptic vesicle cycling, an enriched pathway in this analysis. This suggests that LPIN3 could be influencing neuronal structure and signaling indirectly through its regulation of lipid metabolism, which may further connect with p60-katanin's known role in regulating microtubule dynamics.

In contrast, the decreased expression of EMC10, BTBD8, and HOXC-AS1 highlights potential disruptions in cellular balance or regulatory pathways.

In response to cellular stress, p53 triggers apoptosis. However, when overexpressed, it can disrupt protein synthesis and cause ER stress, affecting the levels of essential proteins like EMC10. On the other hand, since p53 regulates promoter of p60-katanin (Kırımtay *et al.* 2020), overexpression of both proteins could probably result with excessive activity of p60-katanin, hence severing and disruption of microtubules, thus disruption of railways required for protein delivery. Reduced levels of EMC10, which is involved in ER protein translocation and quality control, may impair protein folding or ER function, leading to cellular stress as well. Moreover, EMC10 was shown to be a strong candidate that plays a key role in developmental milestones, with the potential to cause neurodevelopmental disorders in humans (Umair *et al.* 2020).

BTBD8, also known as AP2-Interacting Protein, silencing in neurons was associated with severe impairment of maturation at early developmental stages, reduced synaptic vesicle density, enlarged endosome-like structures, and defects in synaptic transmission, consistent with an impaired clathrin/AP2-mediated synaptic vesicle recycling (Piccini *et al.* 2017). Since BTBD8 is involved in clathrin-mediated endocytosis and synaptic vesicle recycling, the disruption of microtubule dynamics caused by p60 overexpression can impair these processes, leading to reduced synaptic vesicle density and defects in synaptic transmission. This suggests that BTBD8's function is closely tied to the stability and organization of the microtubule network.

The significant downregulation of HOXC-AS1 (A long non-coding RNA HOXC cluster antisense RNA 1), suggests significant changes in chromatin remodeling and developmental processes. p53 can epigenetically suppress the expression of non-coding RNAs like HOXC-AS1 (Parfenyev *et al.* 2021). On the other hand, the effects of p60-katanin on the cytoskeleton can lead to disruptions in chromatin structure and the organization of genetic material (Lombino *et al.* 2019). Both p53 and p60-katanin can directly or indirectly suppress HOXC-AS1 transcription. In addition, HOXC-AS1 was shown to have cancer-promoting effect (Yang *et al.* 2023). Overexpression of p53 enhances its ability to induce cell cycle arrest and apoptosis, preventing the proliferation of damaged cells. Simultaneously, downregulation of HOXC-AS1 reduces oncogenic signals, supporting p53's tumor-suppressing functions. Together, these mechanisms maintain cellular homeostasis.

Overall, these gene expression changes reflect the dynamic responses of cells to internal and external signals, underscoring the complex interplay of cellular pathways and gene regulation in maintaining homeostasis and adapting to environmental challenges.

As a result, it is thought that p53 and p60-katanin proteins may play a role in the regulation of molecules such as Foxo4, PLK5, LPIN3, EMC10, BTBD8 and HOXC-AS1 by working together. This suggests that critical cellular processes such as cell cycle, apoptosis, intracellular protein traffic, and endocytosis can be coordinated under the joint influence of these two proteins and a wide range of cellular functions can be managed. This cooperation of p53 and p60-katanin may play an important role in maintaining cellular homeostasis by affecting many vital processes from cell cycle control to gene expression regulation.

Additional experimental validation, by means of i.e. qRT-PCR, functional assays, and network analysis, may provide further depth to the findings, and these additional experiments may be pursued in future work when resources allow. Despite these limitations, the current data offer valuable insights for the research in this area.

Ethics Committee Approval: Since the article does not contain any studies with human or animal subject, its approval to the ethics committee was not required.

Data Sharing Statement: All data are available within the study.

Conflict of Interest: The author has no conflicts of interest to declare.

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References

1. Baas, P.W. 1997. Microtubules and axonal growth. *Current Opinion in Cell Biology*, 9(1): 29-36. https://doi.org/10.1016/s0955-0674(97)80148-2
2. Chen, Y. Rui, B.B., Tang, L.Y. & Hu, C.M. 2015. Lipin family proteins--key regulators in lipid metabolism. *Annals of Nutrition and Metabolism*, 66(1): 10-8. https://doi.org/10.1159/000368661
3. Dai, C. & Lu, Y. 2004. Tumor suppressor p53 and its gain-of-function mutants in cancer. *Acta Biochimica et Biophysica Sinica*, 36(5): 283-293. https://doi.org/10.1093/abbs/gmt144
4. de Cárcer, G., Escobar, B., Higuero, A. M., García, L., Ansón, A., Pérez, G. & Malumbres, M. 2011. PLK5, a Polo Box Domain-Only Protein with Specific Roles in Neuron Differentiation and Glioblastoma Suppression. *Molecular and Cellular Biology*, 31(6): 1225-1239. https://doi.org/10.1128/MCB.00607-10
5. Di Giovanni, S., Knights, C.D., Rao, M., Yakovlev, A., Beers, J., Catania, J., Avantaggiati, M.L.& Faden, A.I. 2006. The tumor suppressor protein p53 is required for neurite outgrowth and axon regeneration. *European Molecular Biology Organization Journal*, 25: 4084-4096. https://doi.org/10.1038/sj.emboj.7601292
6. Duan, S., Cermak, L., Pagan, J.K., Rossi, M., Martinengo, C., di Celle, P.F. & Soucek, L. 2006. FBXO11 targets BCL6 for degradation and is inactivated in diffuse large B-cell lymphomas. *Nature*, 443(7111): 235-239.
7. Ferreira, A. & Kosik, K.S. 1996. Accelerated neuronal differentiation induced by p53 suppression. *Journal of Cell Science*, 109: 1509-1516. https://doi.org/10.1002/stem.641
8. Hayashi, T. & Karl Seder, J. 2013. DNA damage associated with mitosis and cytokinesis failure. *Oncogene*, 32(39): 4593-4601. https://doi.org/10.1038/onc.2012.615
9. Hudson, C.D., Morris, P.J., Latchman, D.S. & Budhram-Mahadeo, V.S. 2005. Brn-3a transcription factor blocks p53-mediated activation of proapoptotic target genes Noxa and Bax in vitro and in vivo to determine cell fate. *Journal of Biological Chemistry*, 280: 11851-11858. https://doi.org/10.1074/jbc.M408679200
10. Kim, J., Lengner, C.J., Kirak, O., Hanna, J., Cassady, J.P., Lodato, M.A., Wu, S., Faddah, D.A., Steine, E.J., Gao, Q., Fu, D., Dawlaty, M. & Jaenisch, R. 2011. Reprogramming of postnatal neurons into induced pluripotent stem cells by defined factors. *Stem Cells*, 29(6): 992-1000. https://doi.org/10.1002/stem.641
11. Kırımtay, K., Selçuk, E., Kelle, D., Erman, B. & Karabay, A. 2020. p53 regulates katanin-p60 promoter in HCT 116 cells. *Gene*, 727: 144241. https://doi.org/10.1016/j.gene.2019.144241
12. Korulu, S. & Yildiz, A. 2020. p60-katanin: a novel interacting partner for p53. *Molecular Biology Reports*, 47: 4295-4301. https://doi.org/10.1007/s11033-020-05557-6
13. Lane, D.P. 1992. Cancer. p53, guardian of the genome. *Nature*, 358(6381): 15-16. https://doi.org/10.1038/358015a0
14. Lombino, F. L., Muhia, M., Lopez-Rojas, J., Brill, M. S., Thies, E., Ruschkies, L., Lutz, D., Richter, M., Hausrat, T. J., Lopes, A. T., McNally, F. J., Hermans-Borgmeyer, I., Dunleavy, J. E. M., Hoffmeister-Ullerich, S., Frotscher, M., Misgeld, T., Kreutz, M. R., de Anda, F. C. & Kneussel, M. 2019. The Microtubule Severing Protein Katanin Regulates Proliferation of Neuronal Progenitors in Embryonic and Adult Neurogenesis. *Scietific Reports*, 9: 15940. https://doi.org/10.1038/s41598-019-52367-3
15. McNally, F.J. & Vale, R.D. 1993 Identification of katanin, an ATPase that severs and disassembles stable microtubules. *Cell*, 75(3): 419-429. https://doi.org/10.1016/0092-8674(93)90377-3
16. McNally, F.J. 2013. Mechanisms of spindle positioning. *Journal of Cell Biology*, 200(2): 131-140. https://doi.org/10.1083/jcb.201210007
17. Parfenyev, S., Singh, A., Fedorova, O. Daks, A., Kulshreshtha, R., Barlev, N. A. 2021. Interplay between p53 and non-coding RNAs in the regulation of EMT in breast cancer. *Cell Death and Disease*, 12: 17. https://doi.org/10.1038/s41419-020-03327-7
18. Piccini, A., Castroflorio, E., Valente, P., Guarnieri, F. C., Aprile, D., Michetti, C., Bramini, M., Giansante, G., Pinto, B., Savardi, A., Cesca, F., Bachi, A., Cattaneo, A., Wren, J. D., Fassio, A., Valtorta, F., Benfenati, F. & Giovedì, S. 2017. APache Is an AP2-Interacting Protein Involved in Synaptic Vesicle Trafficking and Neuronal Development. *Cell reports*, 21(12): 3596-3611. https://doi.org/10.1016/j.celrep.2017.11.073
19. Su, S., Ndiaye, M.A., Guzmán-Pérez, G., Baus, R.M., Huang, W., Patankar, M.S. & Ahmad, N. 2023. Potential Tumor Suppressor Role of Polo-like Kinase 5 in Cancer. *Cancers*, 15(22): 5457. https://doi.org/10.3390/cancers15225457
20. Umair, M., Ballow, M., Asiri, A., Alyafee. Y., Al Tuwaijri, A., Alhamoudi, K.M., Aloraini, T., Abdelhakim, M., Althagafi, A.T., Kafkas, S., Alsubaie, L., Alrifai, M.T., Hoehndorf, R., Alfares, A. & Alfadhel, M. 2020. EMC10 homozygous variant identified in a family with global developmental delay, mild intellectual disability, and speech delay. *Clinical Genetics*. 98(6): 555-561. https://doi.org/10.1111%2Fcge.13842
21. Vousden, K.H. & Prives, C. 2009. Blinded by the light: The growing complexity of p53. *Cell*, 137(3): 413-431. https://doi.org/10.1016/j.cell.2009.04.037
22. Yang, Z., Wan, J., Ma, L., Li, Z., Yang, R., Yang, H., Li, J., Zhou, F. & Ming, L. 2023. Long non-coding RNA HOXC-AS1 exerts its oncogenic effects in esophageal squamous cell carcinoma by interaction with IGF2BP2 to stabilize SIRT1 expression. *Journal of clinical laboratory analysis*, 37(1): e24801. https://doi.org/10.1002/jcla.24801
23. Zhang, R., Gao, K., Sadremomtaz, A., Ruiz-Moreno, A.J., Monti, A., Al-Dahmani, Z.M, Gyau, B.B., Doti, N. & Groves, M.R. 2023. Identification of hotspots in synthetic peptide inhibitors of the FOXO4:p53 interaction. *Gene & Protein in Disease*, 2(3): 1491. https://doi.org/10.36922/gpd.1491

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Research Article

Tuber magnatum Picco: a new record for the Turkish mycobiota

Hasan Hüseyin Doğan[1*], İsmail Şen[2], Hakan Allı[3]

[1] Selçuk University, Faculty of Science, Department of Biology, 42130, Konya, TÜRKİYE
[2] Demirsoy Agriculture Company, R & D Department, Seydikemer, Muğla, TÜRKIYE
[3] Muğla Sıtkı Koçman University, Faculty of Science, Department of Biology, 48000, Kötekli/Muğla, TÜRKİYE

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Corresponding Author:
Hasan Hüseyin Doğan
hhdogan@selcuk.edu.tr

ORCID iDs of the authors:
HHD. 0000-0001-8859-0188
İŞ. 0000-0001-5760-5535
HA. 0000-0001-8781-7029

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Hypogeous fungi
Taxonomy
White truffle
Türkiye

Abstract: *Tuber magnatum* Picco, the Italian white truffle mushroom recorded for the first time in Türkiye, is a rare and economically valuable mushroom. Samples were collected from Sakarya province during field studies in 2024. The samples were identified as *T. magnatum* according to their macro- and microscopic features. To make molecular confirmation of the species, the samples' ITS1 and ITS4 gene regions were analysed and registered in Genbank with the number PP239641. The sample showed 100% compatibility with other *T. magnatum* accessions in Genbank. The distributions of *Tuber* species in Türkiye were given, and their taxonomic features were compared.

Özet: Türkiye'den ilk kez kaydedilen *Tuber magnatum* Picco, Italyan beyaz trüf mantarı, nadir bulunan ve ekonomik açıdan oldukça değerli bir mantardır. 2024 yılında Türkiye'nin Sakarya bölgesinde yapılan arazi çalışmalarında örnekler toplanmıştır. Bu örnekler makro ve mikroskobik özelliklerine göre *T. magnatum* olarak teşhis edilmiştir. Türlerin moleküler teyidini yapmak için örneklerin ITS1 ve ITS4 gen bölgeleri çalışılmış ve Genbank'a PP239641 numarası ile kayıt edilmiştir. Örnek Genbank'taki diğer *T. magnatum* kayıtlarıyla %100 uyum göstermektedir. Ayrıca Türkiye'de yayılış gösteren *Tuber* türlerinin dağılımları verilmiş ve taksonomik özellikleri karşılaştırılmıştır.

Introduction

Truffles form a symbiotic relationship with various plants, particularly with coniferous or broad-leaved trees). They develop their ascocarps in soil, resembling tubers, and may have different colours ranging from white to black. Unlike other fungal species that form fructification above soil, truffles complete their entire developmental stages in soil. As the fruiting bodies of truffle species begin to mature in soil, they begin to spread aromatic scents with different chemical structures they create in their bodies from the soil to the environment (Jeandroz *et al.* 2008). As the mushroom matures, the odour emitted gradually increases and this attractive odour affects animals. These gorgeous scents are also the main reason why truffles are valued for human consumption. Truffles' unique aroma gives them high economic value, totalling a market of nearly 3 billion euros (Lovrić *et al.* 2020).

According to Bonito *et al.* (2010) and Leonardi *et al.* (2021), approximately 200 *Tuber* species have been identified worldwide. In the Index Fungorum database (2024), 406 names related to *Tuber* genus (including synonyms) appear of which 198 are listed as accepted *Tuber* species. The addition of newly described *Tuber* species to the list in recent years is particularly noteworthy. For instance, 41 new *Tuber* species have been described worldwide within the last five years, including four species in 2019, six in 2020, nine in 2021, 15 in 2022, and seven in 2023.

The collection and consumption of truffle species dates back to ancient times (Hall *et al.* 2008). *Tuber* species grow naturally in Spain, Portugal, Italy, southern Germany, the European part of Russia, North Africa and America (California), where warm and temperate climate prevails (Alsheikh & Trappe 1983, Castellano *et al.* 2004, Wedén *et al.* 2009). Among the truffle species, those with economic value are *Tuber aestivum* (Wulfen) Spreng., *T. brumale* Vittad., *T. borchii* Vittad., *T. magnatum* Picco and *T. melanosporum* Vittad. *Tuber magnatum* and *T. melanosporum* grow in a relatively small geographical area compared to *T. aestivum, T. brumale* and *T. borchii*. While *T. magnatum* grows naturally only in Italy, France, Sweden, and the Balkan Peninsula, *T. melanosporum* grows in Spain, France, and Italy. In contrast throughout Europe (Castellano *et al.* 2004, Jeandroz *et al.* 2008).

The studies carried out on truffle species in Türkiye led to identification of 11 new species so far (Table 1). Öztürk *et al.* (1997) gave the first truffle record, *T. brumale*, in the country from Niğde. The same species

was also recorded from Denizli, Niğde, Osmaniye, and Samsun at different times. The second record, *T. aestivum*, was reported by Gezer *et al.* (2014) from Denizli. Akata *et al.* (2022) provided the last truffle record as *T. oligospermum*.. A general consideration of the regions where truffles distribute in Türkiye, it appears that they were recorded more frequently in the Aegean and Marmara regions, most probably due to the optimal climate and vegetation these regions provide for a better development. On the other hand, when the forest presence and some suitable geographical features of other regions of Türkiye are considered, it is also possible to find more truffle species. With the special attention of targeted studies in the country, we believe that revealing the existence of truffles is very important in terms of biodiversity and in determining species with commercial value. In field studies carried out in this context, *T. magnatum* was collected for the first time in Türkiye and included in the list of Turkish mushrooms.

Materials and Methods

Macro- and microscopic investigation

Within the scope of the *Tuber* inventory project organised by the General Directorate of Forestry, some regions of Türkiye were investigated to identify truffle species. Truffle hunters Şen Kalyoncu and Onur Özmet participated in the Sakarya region with their Lagotto dogs. *Tuber* samples were checked from areas indicated by specially trained dogs digging the soil. In identifying the collected samples, the specific odours of the fresh samples and habitat characteristics were noted, and colour photographs were taken. Morphological and microscopic characteristics were studied in the laboratory using a microscope (Leica DM3000) microscope and a software measurement module (Leica) were used for microscopic examinations. Melzer's reagent, 10% KOH, methylene blue, Congo red, and distilled water were used as examination media. In determining the spore measurements, width and length of 25 different spores (n) were measured in Melzer's reagent, and the length-to-width ratio (*Q*) was determined. Spores are yellowish to yellow-brown in water, and there is no specific colour in other chemicals (Melzer's reagent or Congo red).

For the identification of the samples, the relevant literature was used (Trappe & Castellano 1991, Pegler *et al.* 1993, Montecchi & Sarasini 2000, Breitenbach & Kränzlin 1983, Trappe *et al.* 2007, Riousset *et al.* 2012). The samples were then dried at +45°C for 3-5 days. The dried samples were placed in ziplock polyethene bags, the collection number and location information was written on them, and stored at the Fungarium of Selcuk University Mushroom Application and Research Centre. The collected samples were given consecutive numbers starting with "HHD", which is the personal collection ID.

Molecular analysis

Total DNA was extracted from dried ascocarp tissue by using the DNeasy Plant Mini Kit (Qiagen, USA) following the manufacturer's protocol. The quality of the DNA was checked based on an electropherogram in 1% TBE-agarose gel. Polymerase chain reaction (PCR) amplification and sequencing amplification of the ITS region of the template DNA was performed using the primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) (White *et al.* 1990). The PCR product was purified using A & A Biotechnology (Gdynia). A Clean-up kit was used following the manufacturer's protocol before the sequencing. The sequences of *T. magnatum* obtained in this work were deposited at GenBank (National Center for Biotechnology Information, NCBI).

Table 1. Distribution of *Tuber* species in Türkiye.

***Tuber* species**	**Collected regions**	**References**
T. aestivum	Denizli, Muğla, Konya	Gezer *et al.* 2014, Türkoğlu *et al.* 2015, Şen *et al.* 2016, Alkan *et al.* 2018, Özderin *et al.* 2018
T. borchii Vittad.	Kahramanmaraş, Aydın, Denizli, Muğla, Samsun, Tekirdağ, Konya, Gaziantep	Gezer *et al.* 2014, Elliott *et al.* 2016, Kaya *et al.* 2019, Çelik *et al.* 2020, Uzun & Kaya 2020, Çevik *et al.* 2021
T. brumale Vittad.	Niğde, Denizli, Osmaniye, Samsun	Öztürk *et al.* 1997, Gezer *et al.* 2014, Türkoğlu & Castellano 2014, Şen *et al.* 2016
T. ferrugineum Vittad.	Aydın, Muğla, Denizli, Antalya, Konya	Elliott *et al.* 2016, Şen *et al.* 2016, Çelik *et al.* 2020
T. fulgens Quél.	Kırklareli	Akata *et al.* 2020
T. mesentericum Vittad.	Denizli	Castellano & Türkoğlu 2012, Şen *et al.* 2016
T. nitidum Vittad.	Denizli, Uşak, Burdur, Burdur, Kastamonu, Osmaniye, Karaman, Konya	Türkoğlu & Castellano 2014, İleri *et al.* 2020, Çelik *et al.* 2020
T. oligospermum (Tul. & C. Tul.) Trappe	Şanlı Urfa	Akata *et al.* 2022
T. macrosporum Vittad.	Tekirdağ	Doğan 2021
T. puberulum Berk. & Broome	Denizli, Muğla, Aydın, Osmaniye, Artvin and Trabzon	Elliott *et al.* 2016, Şen *et al.* 2016, Uzun & Yakar 2018
T. rufum Pico.	Burdur, Aydın, Antalya, Bolu, Denizli, Kastamonu, Konya, Muğla and Osmaniye	Türkoğlu & Castellano 2014, Türkoğlu *et al.* 2015, Şen *et al.* 2016

For the molecular phylogeny, the Sanger reads obtained from ITS1/ITS4 were assembled using Bioedit version 7.2, and BLAST analyses were performed with the assembled sequences for the identity rate search. The assembled sequences and the nucleotide sequences of the retrieved in-group and out-group members were aligned using the ClustalW algorithm of MEGAX software (Kumar *et al.* 2018). The phylogenetic trees demonstrating the evolutionary history of HHD19491 (Genbank accession no: PP239641) were constructed using the Maximum Likelihood method and K2 nucleotide substitution model with a gamma distribution (Kimura 1980). The bootstrap method was implemented for the accuracy estimation using 1000 bootstrap replicates (Felsenstein 1985).

Results

Phylum ASCOMYCOTA (Berk.) Caval.-Sm.

Class Pezizomycetes O.E.Eriksson & Winka

Order Pezizales J.Schröt.

Family Tuberaceae Dumort.

Tuber magnatum Picco, 1788 (Figs 1, 2)

Ascocarp: Hypogeous, tuberiform, irregular in shape, lobed, gibbous, sometimes flattened, deformed, striated, angular, turbinate, usually 2–4(6) cm in diameter (Fig. 1a). Ascocarp can reach 20 cm or larger (REF). In addition, some specimens can reach to a mass value of 500 grams, representing notable dimensions for the species (Montecchi & Sarasini 2000).

Peridium: Surface smooth, white to pale yellow, yellow, light yellowish when fresh, sometimes with greyish spots, pale yellowish ochraceous to brown when dry, with small greenish papillae (Fig. 1 a); under a magnifying glass, the surface is finely grained. The rounded outline of a ribbed conical base can be observed even in young specimens, unlike other species of *Tuber*. Whitish in section, pseudo-parenchymatic (Fig. 1b).

Gleba: Solid, white when young, then pinkish yellowish, pinkish ochraceous to grey pinkish when ripe, turning brown afterwards, crossed by anastomose whitish veins. The flesh is initially compact and hard and has a somewhat soapy feel.

Smell: *Tuber magnatum* has an intense odour, fragrant with notes of methane and cheese, an intense garlic-like odour or flour-exquisite flavour, smell also like a mixture of lighting gas, fermented cheese, garlic, and shallot; it is characteristic and exquisite.

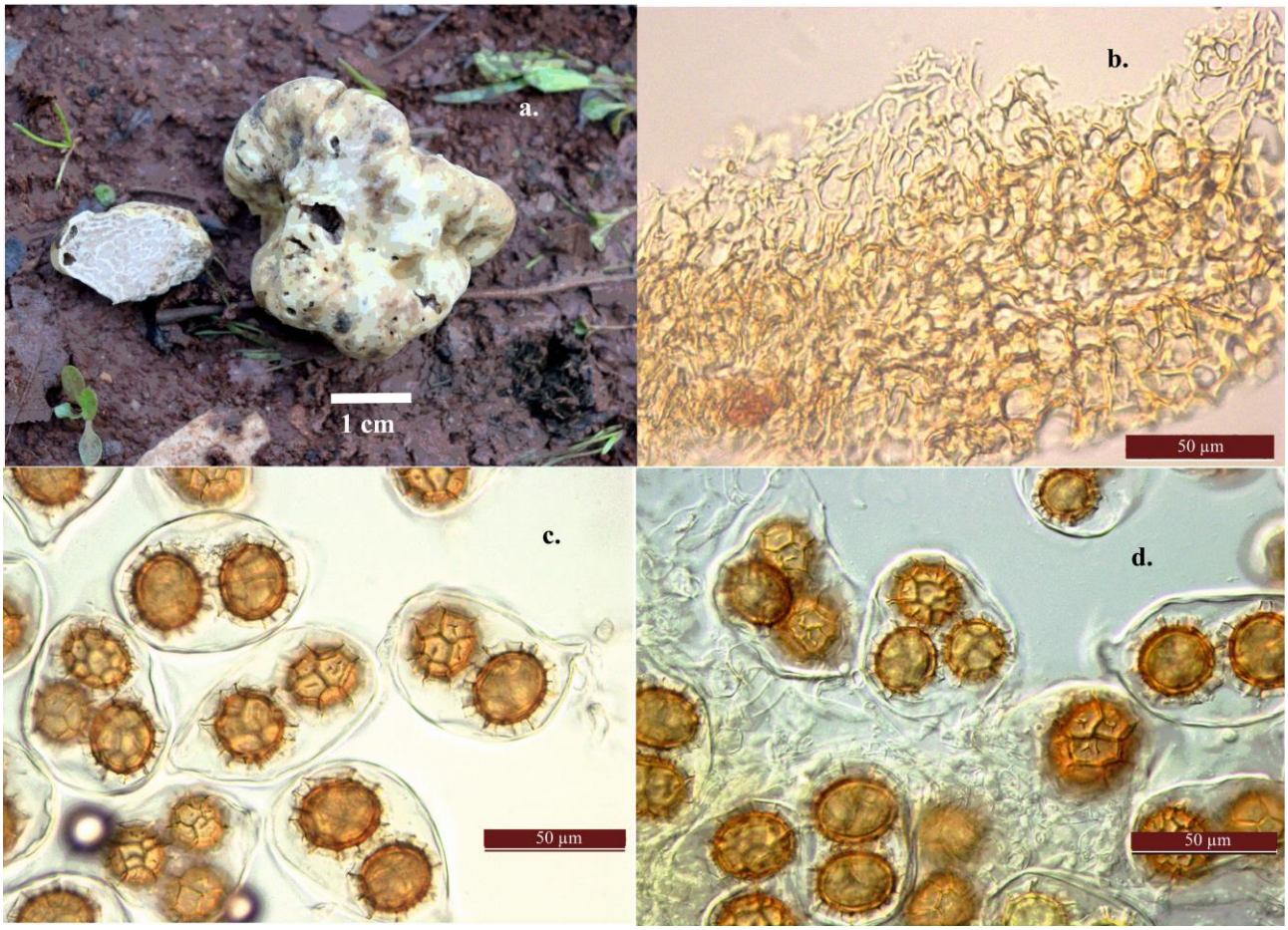

Fig. 1. a. Macroscopic view of *T. magnatum*, **b.** cross section of peridium, **c-d.** asci and ascospores.

Asci: Globose to subglobose or ellipsoid, hyaline, with thin or slightly thickened walls, stalked or non-pedunculate up to 1–2 µm, (60.81–) 65.72 × 77.83 (–80.27) µm in diameter and contain 1 to 3 spores, but could be sometimes 4-spored, (Fig 1. c-d).

Ascospores: Subglobose to broadly ellipsoid, hyaline when young, becoming light yellow to yellowish brown at maturity, with reticulo-alveolate ornamentation, with large meshes, constituting mostly of hexagonal meshes with 4–7 µm across, ornamentation 2–5 µm high. Including their alveolate-reticulate ornamentation, the ascospores measure (27.42–) 31.29–38.66(–41.27) × 30.33–30.47(–36.28) µm, *Q* = 1.031–1.27 (Fig. 1c, d).

Species examined: Sakarya-Hendek, in Poplar plantation, 40°55'03.99"N, 30°55'49.24"E, 122 m, 03.01.2024, HHD19491 (GenBank No: PP239641)

Discussion

Due to cultivation of *Tuber magnatum* is extremely difficult, it is the most expensive of the truffles and a good deal of research has been done to understand its ecology and biology. However, we still know little about its life cycle which remains currently unclear. *Tuber magnatum* is the most valued truffle because of its characteristic, exquisite, very strong smell, and particular, pleasant taste for Italians (Gori 2005, Flammer *et al.* 2013). It might be considered that a white truffle is worth two black one (*Tuber aestivum* and *T. brumale*) as it sells for twice as much and more than the Périgord truffle (*Tuber melanosporum*). It is used in thin strips and should not be heated (Gori 2005, Flammer *et al.* 2013). Its price can reach 7000 €/kg at auctions (Graziosi *et al.* 2022). It is commonly known as the "Italian white truffle," "Piedmont truffle," or "Tartufo Bianco di Alba," as it grows in a very restricted area (Riccioni *et al.* 2016). It is more common in Balkan Peninsula and European countries (Spain, France, Switzerland, Italy, Croatia, Hungary, Romania, Serbia, Bulgaria and Greece) (Hall *et al.* 2008, Marjanović *et al.* 2010, Büntgen *et al.* 2019, Graziosi *et al.* 2022). Although the distribution of *T. magnatum* is restricted in Eastern Europe, more interestingly, it was reported from Thailand based on molecular and morphological investigations (GenBank No: KY427074, KY427075 and KY427076) (Suwannarach *et al.* 2017), suggesting that its distribution is not yet fully determined, or that its natural habitat is continuously expanding due to climate change scenarios (Büntgen *et al.* 2019). *Tuber magnatum* is a whitish truffle characterized with a smooth to suede-like surface, and pale-coloured ascomata (Hall *et al.* 2008, Graziosi *et al.* 2022). It has a yellowish-clay surface and is finely papillose, the spore pattern has few large links, and the colour of the gleba is generally paler than that of other similar species. *Tuber excavatum* Vittad., *T. fulgens* Quél., and *T. dryophilum* Tul. & C. Tul. also have spores with few and large meshes partially similar to those of this species, but the spore size and number of meshes are very different. *Tuber excavatum* has a distinct cavity on the ascocarp, *T. fulgens* has more meshes (up to ten) on the spores, and *T. dryophilum* has dark gleba, globose spores and more meshes than *T. magnatum* (Mello *et al*. 2000, Bonito *et al.* 2011, Alvarado *et al.* 2012, Suwannarach *et al.* 2017).

Table 2. Accession numbers and % identities of sequences used in phylogenetic tree.

Genbank accession number	Species name	Total Score	Max Score	% Identity	Query Cover in %	Reference
PP239641	*Tuber magnatum*	1242	1242	100	-	Current **study**
JQ925646	*Tuber magnatum*	1216	1216	100	92	Bonito *et al.* (2013)
AJ002509	*Tuber magnatum*	1118	1118	100	100	Mello *et al.* (1997)
MZ423175	*Tuber magnatum*	948	948	100	100	Leonardi *et al.* (2021)
KJ524534	*Tuber excavatum*	289	289	93.37	31	Hilszczanska *et al.* (2014)
KC330228	*Tuber excavatum*	289	289	93.37	33	N/A
FM205687	*Tuber excavatum*	289	289	93.37	31	Marjanović *et al.* (2010)
JN392211	*Tuber gennadii*	384	285	95.05	37	N/A
KJ524540	*Tuber maculatum*	356	278	94.94	35	Hilszczanska *et al.* (2014)
FM205644	*Tuber maculatum*	356	278	94.94	36	Marjanović *et al.* (2010)
HM485361	*Tuber gennadii* Castellano	278	278	94.48	28	Bonito *et al.* (2013)
FM205642	*Tuber puberulum*	349	270	94.83	35	Marjanović *et al.* (2010)
FJ809882	*Tuber oregonense*	459	270	90.87	21	Bonito *et al*. (2010)
FJ809881	*Tuber oregonense*	404	270	90.87	21	Bonito *et al.* (2010)
FJ809868	*Tuber gibbosum*	402	270	91.50	19	Bonito *et al.* (2010)
AJ557541	*Tuber borchii*	270	270	94.83	30	Halász *et al*. (2005)
KP276184	*Tuber pseudomagnatum*	340	267	93.37	36	Fan *et al.* (2016)
JN392266	*Tuber oligospermum*	380	267	94.25	40	N/A
JN392259	*Tuber oligospermum*	380	267	94.25	40	N/A
FJ809870	*Tuber gibbosum*	398	267	93.85	17	Bonito *et al.* (2010)
OQ398590	*Choiromyces meandriformis*	360	261	93.71	35	Assyov & Slavova (2023)
OQ779652	*Choiromyces meandriformis*	360	261	93.71	35	N/A
JQ771192	*Tuber pseudomaganatum*	343	265	94.74	34	Fan & Cao (2013)
JF261384	*Tuber borchii*	265	265	94.25	30	Stielow *et al.* (2011)

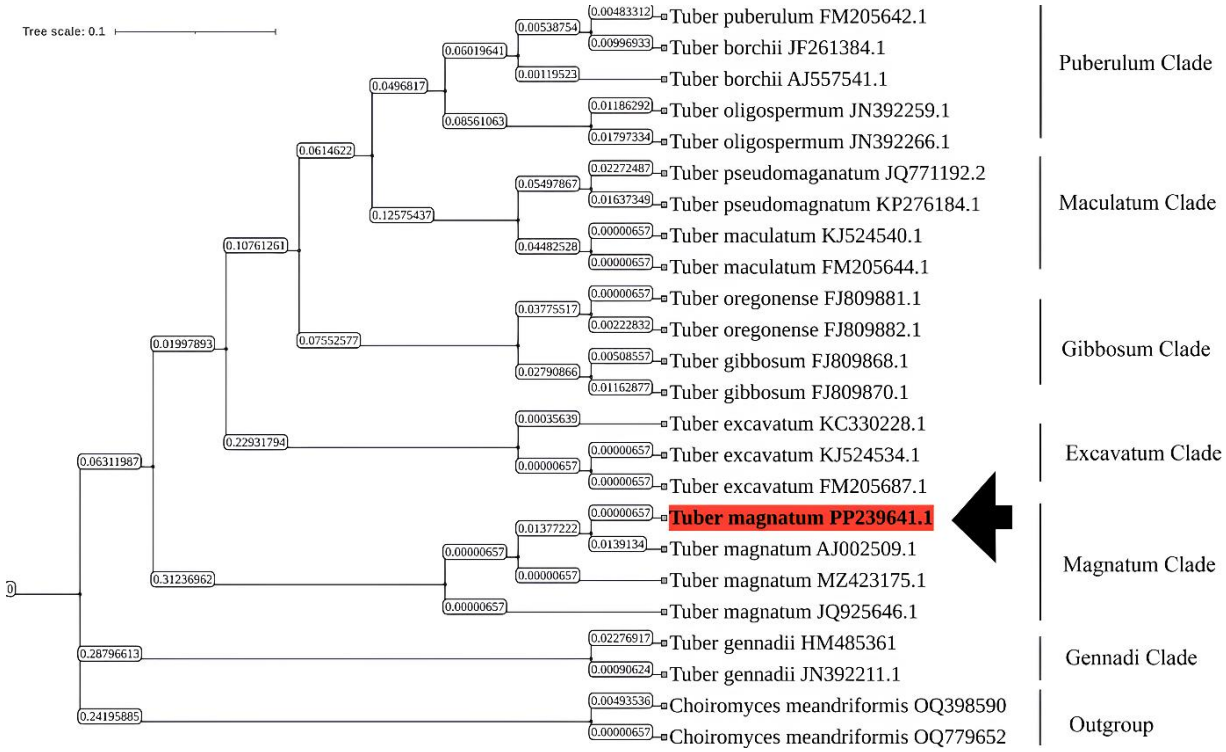

Fig. 2. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model (Tamura, 1992). The tree with the highest log likelihood (-6135.83) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the Maximum Parsimony method. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 4.2756)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 37.29% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendent clade is shown next to each internal node in the tree. This analysis involved 24 nucleotide sequences. There were a total of 1691 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura *et al.* 2021).

Tuber magnatum resembles *T. borchii, T. gennadii* (Chatin) Pat., *T. gibbosum* Harkn., *T. maculatum* Vittad., *T. oligospermum*, and *T. oregonense* Trappe, Bonito & P. Rawl. *Tuber magnatum* can be easily separated from others by the cells of peridium. *Tuber gennadii, T. maculatum, T. oligospermum*, and *T. oregonense* have prosenchymatous cells while *T. magnatum* has pseudoparenchymatous (Mello *et al*. 2000, Bonito *et al.* 2011, Alvarado *et al.* 2012, Suwannarach *et al.* 2017). Sometimes *T. magnatum* is macroscopically confused with *Choiromyces meandriformis* Vittad., the false meander truffle. This, however, has an intense unpleasant odour and presents round spores with digitiform warts (Montecchi & Sarasini 2000).

Tuber magnatum (PP239641) was compared phylogenetically with close species on the NCBI database. All the isolates have query covers above 90% and accession lengths are around 600 base pairs for each specimen. White *Tuber* species were divided into six main clades (Excavatum, Gennadii, Gibbosum, Maculatum, Magnatum, and Puberulum) by Bonito *et al.* (2011). An ITS-based phylogram of PP239641 (Fig. 2) places it within the monophyletic Magnatum clade with high bootstrap (100%) support and Bayesian posterior probabilities (1.0). The analysis findings demonstrated that PP239641 shared a great deal of similarity with other sequences of *T. magnatum* (Table 2 and Fig. 2). The Max scores of *T. magnatum* species are significantly higher than other close species as well as query cover of the other species is very low (Table 2). Compared to other sequences, PP239641 is undoubtedly closest to *T. magnatum* according to the BLAST algorithm and phylogenetic analysis. BLAST algorithm results are given in Table 2. Clades of *Tuber* species are distinctly separated with maximum likelihood analysis with 1000 bootstrap replicates (Fig 2).

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Data Sharing Statement: All data are available within the study.

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References

1. Akata, I., Sevindik, M. & Şahin, E. 2020. *Tuber fulgens* Quél. A new record for Turkish truffles. *Turkish Journal of Agriculture - Food Science and Technology*, 8(11): 2472-2475. https://doi.org/10.24925/turjaf.v8i11.2472-2475.3884
2. Akata, I., Şen, İ., Sevindik, M. & Kabaktepe Ş. 2022. Truffle checklist of Türkiye II with a new record. *Turkish Journal of Agriculture - Food Science and Technology*, 10(10): 1913-1920. https://doi.org/10.24925/turjaf.v10i10.1913-1920.5482
3. Alkan, S., Aktaş, S. & Kaşık G. 2018. Türkiye'deki *Tuber* türleri ve *Tuber aestivum* için yeni bir lokalite. *Selçuk Üniversitesi Fen Fakültesi Dergisi,* 44(1): 25-29.
4. Alsheikh, A.M. & Trappe, J.M. 1983. Taxonomy of *Phaeangium lefebvrei*, a desert truffle eaten by birds. *Canadian Journal of Botany*, 61: 1919-1925.
5. Alvarado, P., Moreno, G. & Manjón, J.L. 2012. Comparison between *Tuber gennadii* and *T. oligospermum* lineages reveals the existence of the new species *T. cistophilum* (Tuberaceae, Pezizales). *Mycologia*, 104: 894-910. https://doi.org/10.3852/11-254
6. Assyov, B. & Slavova, M. 2023. Macrofungi in stands of the endemic pine *Pinus peuce* as inferred from morphological and molecular data. *Proceedings of the Bulgarian Academy of Sciences*, 76(5): 707-715. https://doi.org/10.7546/CRABS.2023.05.06
7. Bonito, G. M., Gryganskyi, A. P., Trappe, J. M. & Vilgalys, R. 2010. A global meta-analysis of *Tuber* ITS rDNA sequences: species diversity, host associations and long-distance dispersal. *Molecular Ecology*, 19(22): 4994-5008. https://doi.org/10.1111/j.1365-294X.2010.04855.x
8. Bonito, G., Smith, M. E., Nowak, M., Healy, R. A., Guevara, G., Cazares, E., Kinoshita, A., Nouhra, E. R., Dominguez, L. S., Tedersoo, L., Murat, C., Wang, Y., Moreno, B. A., Pfister, D. H., Nara, K., Zambonelli, A., Trappe, J. M. & Vilgalys, R. 2013. Historical biogeography and diversification of truffles in the *Tuberaceae* and their newly identified southern hemisphere sister lineage. *PLoS One*, 8(1): e52765. https://doi.org/10.1371/journal.pone.0052765
9. Bonito, G., Trappe, J.M., Donovan, S. & Vilgalys, R. 2011. Improved resolution of major clade within *Tuber* and taxonomy of species within the *Tuber gibbosum* complex. *Mycologia*, 102: 1042-1057. https://doi.org/10.3852/09-213
10. Breitenbach, J. & Kränzlin, F. 1983. *Fungi of Switzerland: Vol. 1. Ascomycetes*. Verlag Mykologia, Luzern, 310 pp.
11. Büntgen, U., Lendorff, H., Lendorff, A., Leuchtmann, A., Peter, M., Bagi, I. & Egli, S. 2019. Truffles on the move. *Frontiers in Ecology and the Environment*, 17: 200-202. https://doi.org/10.1002/fee.2033
12. Castellano, M.A. & Türkoğlu, A. 2012. New records of truffle taxa in *Tuber* and *Terfezia* from Türkiye. *Turkish Journal of Botany*, 36: 295-218. https://doi.org/10.3906/bot-1106-10
13. Castellano, M.A., Trappe, J.M. & Luoma, D.L. 2004. Sequestrate fungi. In: G.M. Mueller, G.F. Bills and M.S. Foster (eds.). *Biodiversity of Fungi: inventory and monitoring methods*, Elsevier, Burlington, 197-213.
14. Çelik, A., Uzun, Y. & Kaya, A. 2020. Macrofungal biodiversity of Güneysınır district (Konya-Türkiye). *Mantar Dergisi*, 11(1): 75-83. https://doi.org/10.30708/mantar.662015
15. Çevik, F.T., Uzun, Y. & Kaya A. 2021. Ereğli (Konya) yöresinde belirlenen makromantarlar. *Mantar Dergisi*, 12(2): 138-178. https://doi.org/10.30708/mantar.960666
16. Doğan, H.H. 2021. A new truffle species addition, *Tuber macrosporum* Vittad., to Turkish mycota. *Trakya University Journal of Natural Sciences*, 22(2): 139-146. https://doi.org/10.23902/trkjnat.873651
17. Elliott T.F., Türkoğlu A., Trappe J.M. & Yaratanakul Güngör M. 2016. Turkish truffles 2: eight new records from Anatolia. *Mycotaxon*, 131: 439-453. https://doi.org/10.5248/131.439
18. Fan, L., & Cao, J.Z. 2013. Two new species of white truffle from China. *Mycotaxon*, 121(1): 297-304.
19. Fan, L., Han, L., Zhang, P.R. & Yan, X.Y. 2016. Molecular analysis of Chinese truffles resembling *Tuber californicum* in morphology reveals a rich pattern of species diversity with emphasis on four new species. *Mycologia*, 108(2): 344-353.
20. Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, 39: 783-791. https://doi.org/10.2307/2408678
21. Flammer, R., Flammer, T. & Reil P. 2013. *Les truffes*, IHW Verlag. 80 pp.
22. Gezer, K., Kaygusuz, O., Çelik, A. & Işıloğlu M. 2014. Ecological characteristics of truffles growing in Denizli province, Türkiye. *Journal of Food Agriculture & Environment*, 12(2): 1105-1109.
23. Gori, L. 2005. *Funghi Ipogei Della Lucchesia di Aitre Province Italiane e dall' Estero*. Pacini Fazzi, 316 pp.
24. Graziosi, S., Hall, I.R. & Zambonelli, A. 2022. The mysteries of the white truffle: Its biology, ecology and cultivation. *Encyclopedia*, 2: 1959-1971. https://doi.org/10.3390/encyclopedia2040135
25. Halász, K., Bratek, Z., Szegő, D., Rudnóy, S., Rácz, I., Lásztity, D. & Trappe, J.M. 2005. Tests of species concepts of the small, white, European group of *Tuber spp.* based on morphology and rDNA ITS sequences with special reference to *Tuber rapaeodorum*. *Mycological Progress*, 4: 281-290.

26. Hall, I., Brown, G. & Zambonelli, A. 2008. *Taming The Truffle*: *The History, Lore and Science of The Ultimate Mushroom*. Timber Press, Portland, 304 pp.

27. Hilszczanska, D., Rosa-Gruszecka, A., Sikora, K. & Szmidla, H. 2014. Soil factors determining the production of *Tuber* spp. ascocarps in natural stands. Database: http://www.ncbi.nlm.nih.gov/nuccore/KJ524536

28. İleri, R., Uzun, Y. & Kaya, A. 2020. Macromycetes of Karadağ (Karaman) and its environs. *Mantar Dergisi*, 11(1): 57-63. https://doi.org/10.30708/mantar.654111

29. Index Fungorum, (2024). https://www.indexfungorum.org/names/Names.asp

30. Jeandroz, S., Murat, C., Wang, Y., Bonfante, P & Le Tacon, F. 2008. Molecular phylogeny and historical biogeography of the genus *Tuber*, the 'True Truffles'. *Journal of Biogeopraphy*, 35(5): 815-829. https://doi.org/10.1111/j.1365-2699.2007.01851.x

31. Kaya, A., Uzun, Y., Karacan, H.İ. & Yakar, S. 2019. Contributions to the macromycota of Gaziantep province. *Kastamonu Universitesi Orman Fakültesi Dergisi,* 19(3): 329-341. https://doi.org/10.30616/ajb.749820

32. Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, 16(2): 111-1120. https://doi.org/10.1007/BF01731581

33. Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, 35(6): 1547-1549. https://doi.org/10.1093/molbev/msy096

34. Leonardi, M., Iotti, M., Mello, A., Vizzini, A., Paz-Conde, A., Trappe, J. & Pacioni, G. 2021. Typification of the four most investigated and valuable truffles: *Tuber aestivum* Vittad., *T. borchii* Vittad., *T. magnatum* Picco and *T. melanosporum* Vittad. *Cryptogamie Mycologie*, 42(9): 149-170. https://doi.org/10.5252/cryptogamie-mycologie2021v42a9

35. Lovrić, M., Da Re, D., Vidale, E., Prokofieva, I., Wong, J., Pettenella, D., Verkerk, P.J. & Mavsar, R. 2020. Non-wood forest products in Europe – a quantitative overview. *Forest Policy and Economics*, 116: 102175. https://doi.org/10.1016/j.forpol.2020.102175

36. Marjanović, Ž., Grebenc T., Marković, M., Glišić, A. & Milenković, M. 2010. Ecological specificity and molecular diversity of truffles (Genus *Tuber*) originating from mid-west of the Balkan Peninsula. *Sydowia*, 62: 67-87.

37. Mello, A., Garnero, L., Meotto, F. & Bonfante, P. 1997. Specific primers for rapid typing of *Tuber borchii* mycorrhizal roots. *Symposium on Plant Biotechnology as a tool for the Exploitation of Mountain Lands*, ISHS Acta Horticulturae 457. https://doi.org/10.17660/ActaHortic.1998.457.28

38. Mello, A., Vizzini, A., Rollo, F., Bonfante, P. & Trappe, J.M. 2000. *Tuber borchii* versus *Tuber maculatum*: neotype studies and DNA analysis. *Mycologia*, 92: 326-331. https://doi.org/10.2307/3761569

39. Montecchi, A. & Sarasini, M. 2000. *Funghi Ipogei d'Europa*. Associazione Micologica Bresadola, Trento, 714 pp.

40. Özderin, S., Yılmaz, F. & Allı, H. 2018. Determining mycorrhiza rate in some oak species inoculated with *Tuber aestivum* Vittad. (Summer Truffle), *Turkish Journal of Forestry*, 19(3): 226-232. http://dx.doi.org/10.18182/tjf.435372

41. Öztürk, C., Kaşık., G. & Toprak, E. 1997. Ascomycetes makrofunguslarından Türkiye için iki yeni kayıt. *Ot Sistematik Botanik Dergisi*, 4(1): 53-56.

42. Pegler, D.N., Spooner, B.M. & Young, T.W.K. 1993. *British Truffles*: *A revision of British Hypogeous fungi*. The Royal Botanic Gardens, Kew, 216 pp.

43. Riccioni, C., Rubini, A., Belfiori, B., Gregori, G. & Paolocci, F. 2016. *Tuber magnatum:* the special one. What makes it so different from other *Tuber spp.* In: Zambonelli, A., Iotti, M., & Murat, C. (eds.). *True truffle (Tuber spp.) in the world. Soil ecology, systematics and biochemistry*. Springer, Switzerland. 87-103.

44. Riousset, G., Riousset, L., Chevalier, G. & Bardet, M.C. 2012. *Truffe d'Europe et de Chine*. INRA, Paris.

45. Şen, İ., Allı, H. & Civelek, H.S. 2016. Checklist of Turkish truffles. *Türk Yaşam Bilimleri Dergisi,* 1(2): 103-109.

46. Stielow, B., Bratek, Z., Orczán, A.K.I, Rudnóy, S., Hensel, G., Hoffmann, P., Klenk, H.P. & Göker, M. 2011. Species delimitation in taxonomically difficult fungi: the case of *Hymenogaster*. *Plos One*, 6(1): e15614. https://doi.org/10.1371/journal.pone.0015614

47. Suwannarach, N., Kumla, J., Meerak, J. & Lumyong, S. 2017. *Tuber magnatum* in Thailand, a first report from Asia. *Mycotaxon*, 132: 635-642. https://doi.org/10.5248/132.635

48. Tamura, K. 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. *Molecular Biology and Evolution*, 9: 678-687.

49. Tamura, K., Stecher, G. & Kumar, S. 2021. MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution*, 38(7): 3022-3027. https://doi.org/10.1093/molbev/msab120

50. Trappe, J.M. & Castellano M.A. 1991. Keys to the genera of truffles *(Ascomycetes). McIlvanea*, 10: 47-65.

51. Trappe, M., Evans, F. & Trappe, J. 2007. *Field Guide to North American Truffles*: *Hunting, Identifying, and Enjoying the World's Most Prized Fungi*. Ten Speed Press, Berkeley, Toronto, 136 pp.

52. Türkoğlu, A. & Castellano, M.A. 2014. New records of some Ascomycete truffle fungi from Türkiye. *Turkish Journal of Botany*, 38: 406-416. https://doi.org/10.3906/bot-1303-24

53. Türkoğlu, A., Castellano, A.M., Trappe, M.J. & Güngör-Yaratanakul, M. 2015. Turkish truffles I: 18 new records for Türkiye. *Turkish Journal of Botany*, 39: 359-376. https://doi.org/10.3906/bot-1406-42

54. Uzun, Y. & Kaya, A. 2020. The Checklist of the macromycetes determined in Gaziantep province. *Anatolian Journal of Botany*, 4(2): 106-115. https://doi.org/10.18016/ksutarimdoga.vi.856014

55. Uzun, Y. & Yakar, S. 2018. New locality record for two *Tuber* species in Türkiye. *Anatolian Journal of Botany*, 2(2): 88-92. https://doi.org/10.18016/ksutarimdoga.vi.857201

56. Wedén, C., Pettersson, L. & Danell, E. 2009. Truffle cultivation in Sweden: Results from *Quercus robur* and *Corylus avellana* field trials on the island of Gotland. *Scandinavian Journal of Forest Research*, 24(1): 37-53. https://doi.org/10.1080/02827580802562056

57. White, T.J., Bruns, T., Lee, S. & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pp. 315-322. In: Innis M.A., Gefland D.H., Sninsky J.J. & White T.J. (eds). *PCR Protocols: A Guide to Methods and Applications.* Academic Press Inc., New York, 482 pp.

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Review

Salmonellae in the air environment: A review

Abdel Hameed A. Awad

Air Pollution Research Department, Environment and Climate Change Research Institute, National Research Centre, Dokki, Giza, EGYPT
e-mail: abed196498@yahoo.com, ORCID: *orcid.org/0000-0003-0784-9835*

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Bio-indicator

Abstract: *Salmonella* bacteria, a zoonotic pathogen, are frequently transmitted through food and water, causing foodborne outbreaks and illnesses. Bioaerosols are a growing concern as pathogenic microorganisms could be transmitted to the indoor and ambient air environments. The airborne transmission of pathogenic microorganisms is considered a risk of contamination or a route of infection. *Salmonella* have been found in rare numbers in the air, but their detection indicate their ability to survive in the air environment. Physical, biological and environmental stressors affect the survival of airborne microorganisms. The infectivity of airborne *Salmonella* is determined by its pathogenicity, infective dose and individual health conditions. The accurate assessment of *Salmonella* in aerosols is a problem due to the synergistic influence of many uncontrollable environmental conditions and a lack of standardized analysis and sampling protocols. Knowledge of the airborne transmission of *Salmonella* and factors influencing their viability is critical to understanding their potential health risk and the related control measures. This review provides evidence for the transmission of *Salmonella* in different air environments, focusing on the presence of *Salmonella* in the air as a risk of biocontamination. The sampling, detection and enumeration methodologies of *Salmonella* in the air are discussed with recommended mitigation and control strategies.

Özet: Zoonotik bir patojen olan *Salmonella* cinsi bakteriler sıklıkla gıda ve su yoluyla bulaşarak gıda kaynaklı salgınlara ve hastalıklara neden olmaktadırlar. Patojenik bakterilerin hava ortamına bulaşabilmesine aracılık ettikleri için biyo-aerosoller giderek artan bir sorun olarak ele alınmaktadırlar. Patojenik mikroorganizmaların hava yoluyla bulaşması, kontaminasyon veya enfeksiyon riski olarak kabul edilir. *Salmonella*'nın havada az sayılarda bulunması, hava ortamında hayatta kalma yeteneklerini göstermektedir. Fiziksel, biyolojik ve çevresel stres etkenleri havadaki mikroorganizmaların hayatta kalmasını etkileyen faktörlerdir. Hava ortamında bulunan *Salmonella* üyelerinin bulaşıcılığı patojeniteleri, enfektif doz ve bireylerin sağlık koşullarınca belirlenir. Aerosollerle taşınan *Salmonella* üyelerinin doğru bir şekilde değerlendirilmesi, kontrol edilemeyen birçok çevresel koşulun sinerjik etkisine ve standartlaştırılmış analiz ve numune alma protokollerinin eksikliğine bağlı bir sorun olarak görülmektedir. *Salmonella* üyelerinin hava yoluyla bulaşması ve canlılıklarını etkileyen faktörlerin bilinmesi, potansiyel sağlık risklerinin ve ilgili kontrol önlemlerinin anlaşılması açısından kritik öneme sahiptir. Bu derleme, biyolojik kontaminasyon riski olarak havadaki *Salmonella* varlığına odaklanarak *Salmonella* üyelerinin farklı hava ortamlarında bulaştığına dair kanıtlar sunmaktadır. Hava ortamında bulunan *Salmonella* üyelerinin örnekleme, tespit ve sayımı metodolojileri, önerilen azaltma ve kontrol stratejileriyle birlikte tartışılmıştır.

Introduction

Aerosols are ubiquitous in the earth's atmosphere and they are central to many environmental issues and public health (Colbeck & Lazaridis 2010, Zhang 2020). Atmospheric aerosols are suspensions of liquid, solid or mixed particles with highly variable chemical composition and size distribution (Putaud *et al.* 2010). Bioaerosols are particles of biological origin (e.g. bacteria, viruses, fungi, algae, biological fragments and pollen) suspended in the air and are an important part of aerosols (Wéry 2014, Smets *et al.* 2016). Bioaerosols, which considerably vary in composition and size (0.2-100 µm) (Stetzenbach 2009), are produced in the environment from a variety of natural and anthropogenic sources (Kim *et al.* 2018, Xie *et al.* 2021), affecting living organisms

through infectivity, allergenicity and toxicity (Cox & Wathes 1995) (Fig. 1). Moreover, bioaerosols could be a source of pollution for plants, animals and surface water (Michalkiewicz, 2019). Biological particles are transported up in the air as free (single cells, spores or aggregates) or attached to non-biological particles (Jones & Harrison 2004), thus leading to considerable differences in their stability, survivability, composition and dispersal mechanisms (Cambra-López *et al.* 2010). Sewage treatment plants, biosolid landfills, spray irrigation (untreated / or insufficiently treated), wastewater (Brooks *et al.* 2004), composting, livestock facilities and herb processing have been considered as potential sources of bioaerosols and pathogenic microorganisms (Hickey & Reist 1975, Skórska *et al.* 2005, Zhang *et al.* 2019, Dai *et al.* 2020). Transmission of pathogenic microorganisms is of a great concern due to their ability to affect worker's and the nearby residents' health.

The transmission of pathogenic microorganisms in the atmosphere has to be paid attention, as their transmission is attributed to the initial health symptoms resembling enteric diseases among workers and population living near sewage treatment plants, biosolid landfills, composting and livestock facilities (Chinivasagam *et al.* 2009). The detection of pathogenic microorganisms in the air environment indicates their ability to persist harsh atmospheric conditions. Nowadays, the potential of aerosolization of pathogenic microorganisms has become a debated issue. The available information on emission, source apportionment and transmission of pathogenic microorganisms into the air environment is scarce (Xie *et al*. 2021).This review aims to highlight the transmission of *Salmonella* in the air environment, factors affecting their survivability, sampling and analysis methods and control strategies.

Salmonella bacteria

Salmonellae belong to *Enterobacteriaceae*, a family of Gram-negative bacteria represented with facultative anaerobic bacilli with 2-5 µm long and 0.5-1.5 µm wide and are motile by peritrichous flagella (Andino & Hanning 2015). *Salmonella* grow at temperatures in the range of 5-45°C, with ideal temperatures between 35-37ºC, but some species can grow at temperatures as high as 54ºC and as low as 2°C (Gray & Fedorka-Cray 2002) and at optimum pH range of 6.5 and 7.5 (Shaji *et al.* 2023). *Salmonella* can be distinguished from other bacterial species by their biochemical and antigenic features. Salmonellae are a complex group containing ≥2600 serovars based on somatic (O), flagellar (H) and surface capsule (Vi) antigens (Mumy 2014).

Salmonellae are ubiquitous human and animal pathogens and can be divided into 2 groups, typhoidal *Salmonella* (TS) and Non-typhodial *Salmonella* (NTS) (Wang *et al.* 2023a). *Salmonella enterica* ser. Enteritidis (*S*. Enteritidis) and *Salmonella enterica* ser. Typhimurium (*S*. Typhimurium), belonging to NTS group, are responsible for the majority of human salmonellosis (Ashurst, *et al.* 2022). NTS group is responsible for ~ 93 million cases of gastroenteritis and 155,000 fatalities annually and is frequently zoonotic (Gordon 2011, Cosby *et al.* 2015). The natural habitat of *Salmonella* is the gastrointestinal tract of humans and animals. Historically, transmission of *Salmonella* and enteric zoonotic infections (e.g. Q-fever, brucellosis, and avian and swine influenza) via aerosols has been neglected (Kallapura *et al.* 2014).

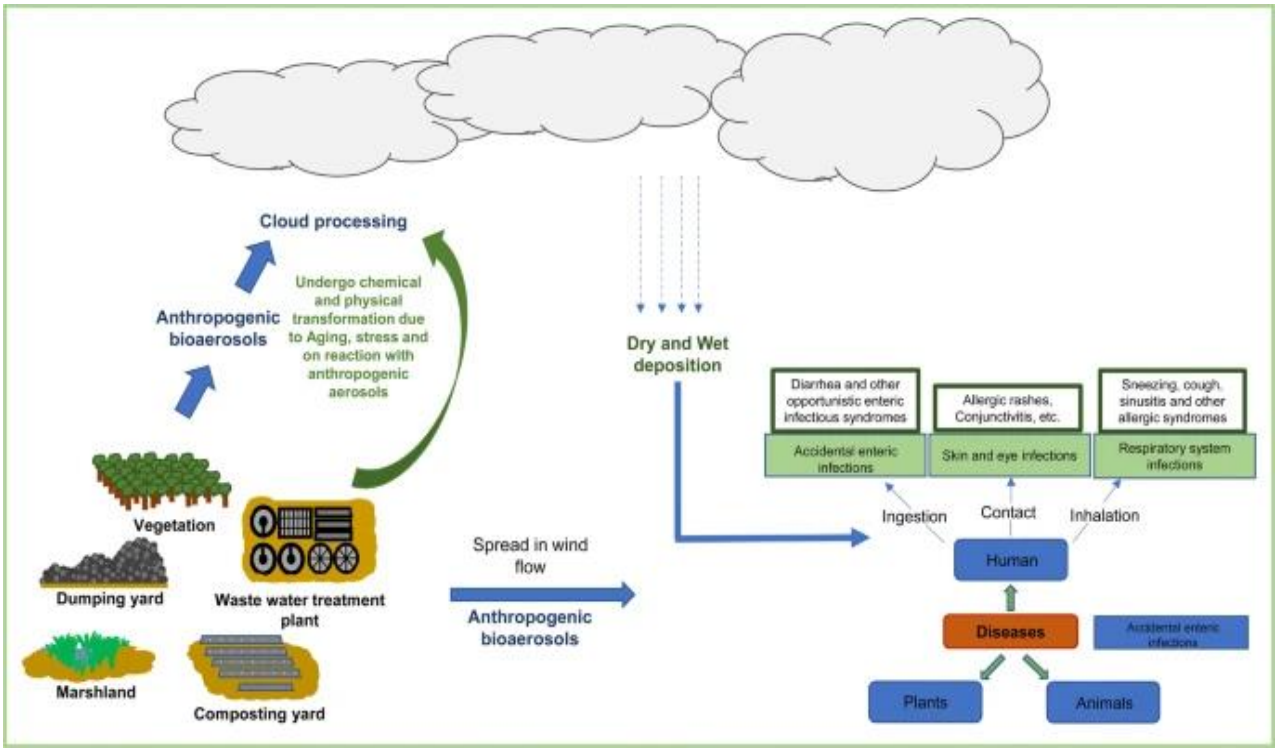

Fig. 1. Diagram of bioaerosols emission sources and fate (Krishnamoorthy *et al.* 2020).

Transmission of *Salmonella* aerosols is less pathogenic and rarely occurs (Shuval *et al.* 1986). The possibility of transmission and survival of *Salmonella* (Oliveira *et al.* 2006, López *et al.* 2012) in aerosols should be considered.

Salmonella aerosol is a concern route for vegetables contamination and foodborne outbreaks. *Salmonella* directly enter water and agricultural environments via waste and sewage irrigation (Heaton & Jones 2008) or indirectly via *Salmonella* aerosols. *Salmonella*, pathogenic *Escherichia coli* and *Listeria monocytogenes* have been linked to bacterial outbreaks of foodborne diseases associated with ready-to-eat fruit and vegetables (Thomas *et al.* 2024).

A low number of *Salmonella* cells may be sufficient to cause disease in a large number of people (Werber *et al.* 2005). For instance, ~ 13 CFU/g is enough to cause salmonellosis outbreaks. Infectious dose of outbreaks of salmonellosis is found between 10 and 1,000 cells (Blaser & Lee 1982, Vought & Tatini 1998). The infectious dose of *Salmonella* via respiratory pathway is lower than the oral route (Darlow *et al.* 1961). Inhalation of *S.* Typhimurium by mice was reported to cause disease in animals in a dose dependent manner, where the lowest dose was reported as ~150 CFU that could produce a disease (Wathes *et al.* 1988).

Sources of Salmonella aerosols

Wastewater and sludge applications

The primary concern of wastewater treatment plants (WWTPs) is to remove contaminants and inactivate pathogenic organisms to protect environment and human health. The enteric bacteria, viruses, protozoa and helminths are the common groups of microorganisms present in municipal wastewater (Akin *et al.* 1978). The enteric bacteria are commonly found in wastewater, with *Escherichia coli* and *Enterococcus feacalis* frequently at concentrations of ~10^9/l and ~10^8/l, respectively. However, concentrations of *E. coli* and total coliform are significantly 1 to 3 folds higher in the influent than the effluent (Ajonina *et al.* 2015). Salmonellae are the most prevalent pathogenic bacterial species in raw wastewater with a concentration ~ 5000 bacteria/l (Foster and Engelbrecht 1973). *Salmonella* concentrations averaged 130 bacteria/100 ml in the raw sewage water and 3 bacteria/100 ml in the treated sewage water (Langeland 1982). The presence and concentration of pathogenic microorganisms in sewage are determined by their prevalence among other populations and their ability to persist treatment processes. Biological wastewater treatment plants leave ~1-10% of *Salmonella*, *Mycobacterium* and viruses in the treated wastewater (Sorber & Sagik 1979). The aerosols containing pathogenic and non-pathogenic microorganisms are generated during wastewater treatment processes, as wastewater undergoes turbulent mixing or mechanical agitation (Sorber & Guter 1975, Sánchez-Monedero *et al.* 2008, Liu *et al.* 2020).

The transportation of bioaerosols is a function of time and distance (Pepper & Gerba 2015). Concentrations of total aerobic bacteria, total and fecal coliforms, fecal enterococci and coliphage were reported to significantly increase in the air within the perimeter of a WWTP in USA after operation (Fannin *et al.* 1985). Aeration tanks and trickling filters at WWTPs are the main sources emitting microorganisms into the ambient air (Han *et al.* 2020). High airborne microbial concentrations were found near aeration tanks and mechanical agitation, ranging within few to more than $8x10^4$ CFU/m^3 (Korzeniewska *et al.* 2008). Concentrations of mesophilic bacteria, bacteria-associated certain waterborne virulence factors, mesophilic fungi and thermophilic fungi were 1.7×10^4 CFU/m^3, 2×10^3 CFU/ m^3, 1.7×10^3 CFU/m^3 and 4.5×10^1 CFU/m^3, respectively in aerosols emitted by aeration tanks of an activated sludge plant (Bauer *et al.* 2002).

Airborne Gram negative bacteria, fecal indicators (*E. coli* & *Clostridia*), *Salmonella* and *P. aeruginosa* were detected at WWIP but in lower counts than Gram positive bacteria. *Escherichia coli* and *Salmonella* were found up to a distance of 300m and 10 m downwind of the aeration tanks, respectively, and a higher number of positive findings were observed during higher wind velocity and low sunshine (Müller 1980). Coliforms were found up in the air to a distance of 0.8 mile downwind of trickling-filter sewage treatment plant (Adams & Spendlove 1970). The dissemination of *Salmonella* by the air was low in relation to *Salmonella* content of the sewage itself (Müller 1980).

A microorganism can be released into the air from aerated sewage only when its concentration exceeds 10^3 cells/cm^3 in the sewage (Teltsch *et al.* 1980), a higher number of a given microorganism in sewage has a higher aerosol emission rate (Sawyer *et al.* 1993). Composition of airborne microflora is closely related with the type and number of microorganisms present in sewage waste (Ossowska-Cypryk 1991). The majority of the released aerosols do not travel very far distances. However, smaller particles tend to travel a considerable distance away from the source point (Mckinney 2004). The composition and size of microbial aerosols are influenced by type of treated wastewater, treatment technology, ambient conditions and shear stress force (Heinonen-Tanski *et al.* 2009). The highest emission of *P. fluorescens*, *E. coli*, *Enterococcus* sp. and *Salmonella* was detected in the air at the first stage of the purification in a municipal wastewater plant, Toruń, Poland (Paluszak *et al.* 2003). *Salmonella* and *Shigella* were not isolated from the air samples despite their presence in sewage water (Sekla *et al.* 1980).

Airborne microbial contamination greatly differed in the vicinity of aeration tank, maturing composting plant and 100 m downwind of municipal treatment plant in Poland, where the concentrations of *E. coli*, *Enterobacter* and *Salmonella* were ~10^1CFU/m^3 in average (Breza-Boruta & Paluszak 2007). The highest microbial air contamination was found in the pretreatment of wastewater (screening, aerated grit removal and pumping) in a WWTP in Finland, where somatic coliphage and

enterococci were found in higher numbers and no *Salmonella* bacteria were detected (Heinonen-Tanski *et al.* 2009). The ratio between *Salmonella* to coliphage densities in sewage aerosols was 1:100,000 (Grunnet & Tramsen 1974) and *Salmonella* bacteria were not recovered in any of the air samples collected at a WWTP in Egypt (Abdel Hameed 1992). Airborne pathogenic enteric bacteria (*S.* Enteritidis and *S.* Boydii), reovirus and enterovirus were isolated in 2%, 46% and 9%, respectively of the total samples collected at different sites in sewage sludge treatment plants in Italy (Carducci *et al.* 2000).

A given quantity of pathogens present in sewage aerosols could represent a source of a threat to workers who are daily exposed to aerosols associated with a variety of infectious microorganisms (Grisoli *et al.* 2009). Wastewater treatment processes bring the workers in contact with multiple pathogens and infectious agents such as viruses (*Hepatitis*-A, *Polio, Coxsackie, Echo, Rota* and *Adeno*), bacteria (*Salmonella* spp., *Shigella* spp., *Campylobacter jejuni, Yersinia enerocolitica, Legionella pneumophila, Helicobacter pylori, Listeria monocytogenes* and *Mycobacterium xenopi*) and protozoa (*Giardia lamblia, Entamoeba histolytica* and *Helminthes)* (Mulloy 2001).

Municipal sewage sludge is utilized worldwide on agricultural lands to solve the problem of sewage disposal, water scarcity and environmental contamination. However, the increase of wastewater in land application has magnified problems such as production of aerosols containing pathogens and contamination of crop and ground and surface waters (Bitton 1980). In the United States, ~33% of the produced municipal sludge is applied onto agricultural lands (Mclamarra & Pruitt 1995), increasing accumulation of pathogens and toxic substances that may be released into the air environment.

Climatic and environmental factors differently affect the survival of airborne pathogenic bacteria. Temperature, relative humidity, oxygen content, UV radiation and reactive chemical radicles are the main factors affecting viability of airborne microorganisms (Ruiz-Gil *et al.* 2020). Salmonellae in sewage sludge spread on grass and may survive up to 72 weeks, and neither aerobic stabilization nor anaerobic digestion significantly reduces the contamination with Salmonellae (Hess & Breer 1975). Raw sludge from municipal sewage may release more airborne pathogens than aerobic/or anaerobic digestion, lime stabilization and thermal drying sludge (Straub *et al.* 1993). The application of raw sludge on agricultural lands has been prohibited in many countries due to its hazardous effects that may be presented by direct contact/or inhalation of infectious aerosols (Cole *et al.* 1999).

Low concentrations of *Salmonella*, coliforms and enteroviruses were detected in air samples collected downwind wastewater spray -irrigated fields. *Salmonella* was detected in 78% and 18% of wastewater and air samples ~40m downwind, respectively, and enteroviruses in 71% and 44% in wastewater and aerosols, respectively, as an indication of the prevalence of enteroviruses than *Salmonella* in aerosols (Teltsch *et al.* 1980). This is attributed to viral contamination may be more resistance to inactivation processes than enteric bacteria and may be concentrated in aerosols than suspending fluid (Baylor *et al.* 1977). Table 1 shows the concentrations of *Salmonella* bacteria in wastewater and aerosols at WWTPs.

Biosolids and composts

Composting is used to stabilize biosolids, as organic substrates are subjected to microbial degradation. Composting produces substrates suitable for cultivation or aids in the disposal of wastes (Fig. 2). Application of composted sludge improves soil quality, but the microbiological safety should be considered (Brooks *et al.* 2005). The risk of infection posed to biosolid handlers reached 34% and 2% annually from exposure to Coxsackievirus A2I and *Salmonella*, respectively (Tanner 2004).

Growth and death rate of pathogens in biosolids, including *Salmonella*, depend on several factors such as moisture content, temperature, available nutrient, associated flora and indigenous microorganisms (Sidhu *et al.* 2001). Most of enteric pathogenic bacteria are non-spore formers and relatively sensitive to environmental factors (Vilanova & Blanch 2005). *Salmonella*, *E. coli* and fecal coliforms can regrow in moist conditions after treatment (Lang *et al.* 2007).

Table 1. Concentrations of *Salmonella* in wastewater and aerosols at WWTPs.

Environment	Concentration	Reference
Wastewater	2-60 MPN/100 ml	Katzenelson & Teltsch (1976)
Wastewater	*Salmonella*: coliforms 2:60 MPN/100 ml	Teltsch et al. (1980)
Aerosols	*Salmonella*: coliforms 3.2×10^{-2} : 5.410^{-2} MPN/m^3 43:1076 CFU/m^3	
Dry sewage sludge Raw wastewater Treated wastewater	140-14000 CFU/100gm 130 bacteria/100 ml 3 bacteria/100 ml	Langeland (1982)
Bulk sludge	0.3-17000 CFU/gm	Hussong *et al.* (1985)
Raw sewage	5000 CFU/ml	Prazmo (1980)
Aerosols/aeration tank	$\leq 10^1$ $CFUm^{-3}$	Breza-Boruta & Paluszak (2007)
Aerosols	*Salmonella*: Coliphage 1:100,000	Grunnet & Tramsen (1974)
Aerosols	≤1 CFU/m^3	Heinonen-Tanski *et al.* (2009), Abdel Hameed (1992), Pillai *et al.* (1996)

The bacterial concentrations were reported to range between 10^4-10^6 CFU/g in a well-managed compost, decreased over time to 150 CFU/g and increased over 6 weeks in poorly managed composts (Ogden *et al.* 2001). Wastewater biosolids generally contain *Salmonella* at a range of 10^2-10^3 CFU/g dry weight (Epstein 1997) and ~10^5 CFU/g in dewatered anaerobically digested sludge (Russ & Yanko 1981). *Salmonella* bacteria are known to survive composting process in low concentration (Gibbs *et al.* 1997) and can form filaments under moderately low-water conditions and upon rehydration can achieve high bacterial loads within a short period of time (Stackhouse *et al.* 2012). The active indigenous flora of compost establishes a homeostatic barrier against *Salmonella* which is considered an invader. However, in the absence of indigenous compost flora, the inoculated *Salmonella* may grow to potentially hazardous levels (Sidhu *et al.* 2001).

Microorganisms are released into the air when compost piles are formed or dismantled. The potential of aerosolization of pathogenic microorganisms from biosolids has become an important debated issue worldwide. The nature of the airborne microflora depends on the existing contamination of the starting materials and microbial development between disposal and composting (Lacey *et al.* 1996). The elevated temperature in composting kills-off coliforms and pathogens, however inadequate compost turning leads to temperature stratification and survival of pathogens (*Salmonella*) in cooler layers which may be emitted into the air during mechanical agitation/ or by wind action (Millner *et al.* 1980).

The biosolid land application generates bioaerosols through soil agitation and weathering of biosolid. Biosolids left on the soil surface are subjected to drying; rendering it friable, becoming airborne with the associated pathogens (Pillai 2007). At a municipal solid waste recycling and composting plant stations in Quebec, Canada, the concentrations of airborne total culturable bacteria and Gram-negative bacteria were above 10^4 CFU/m^3 and 10^3 CFU/m^3, respectively, at six of the nine work stations (Marchand *et al.* 1995). *Salmonella* and *Enterobacter* bacteria were found in the air samples only in the vicinity of the compost piles in Poland (Breza-Boruta & Paluszak 2007). The generation and disposal of bio-wastes potentially increase aerosolization of a wide variety of microbial pathogens.

Fig. 2. Photograph of a drying sewage sludge used as fertilizer.

Livestock houses

Livestock houses have significant hazards to biocontamination of food (Hutchison *et al.* 2004), water (Devane *et al.* 2018) and soil (Nolan *et al.* 2020). Pathogenic microorganisms are shed in animals' excretions, secretions or exhaled in breath, litter (e.g. straw, sawdust or wood chippings) and feed (Chien *et al.* 2011). Poultry litter and manure can pose a serious threat to environmental and human health and need to be managed properly (Gržinić *et al.* 2023). *Salmonella* bacteria are ubiquitous in farm environment, and bioaerosols may be released into the air environment as free/ or associated dust particles (Zhao *et al.* 2014). In agricultural livestock farming, bioaerosols account for well over 90% of airborne dust (Aengst 1984), reaching ~10^7 CFU/m^3 (Dungan 2010). The concentration of airborne total bacteria was 6.43 log CFU/m^3 in broiler houses, 5.1 log CFU/m^3 in pig buildings and 4.3 log CFU/m^3 in cattle buildings, and the overall concentrations of *Enterobacteriaceae* ranged between 3 and 4 log CFU/m^3 (Seedorf *et al.* 1998). In animal houses, the majority of airborne microbial composition is non-pathogenic and Gram-negative bacteria constituted 0.02 and 5.2% of the total amount of aerobic bacteria (Zucker *et al.* 2000).

There is evidence that enteric pathogens are important in airborne transmission of diseases among animals (Pepper & Gebra 2015). *Salmonella* Typhimurium aerosols are transmitted among calve houses (Hinton *et al.* 1983). *Salmonella* Typhimurium could survive for long periods in the air, and calves and mice exposed to *Salmonella* developed gastrointestinal symptoms, proving that pathogens could be spread by aerosolization (Wathes *et al.* 1988). *Bordetella bonchiseptica*, *Brucella suis*, *Haemophilus* spp., *Corynebacterium equi*, *Listeria moncytogenes*, *Mycobacterium* spp., *Mycoplasma* spp., *Pasteurella* spp., *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus*, *Streptococcus suis* and *Leptospira* Pomona are some pathogenic bacteria in pigs and poultry houses that can be airborne/or aerosol transmitted (Wathes 1995).

The aerosolized *Salmonella* Enteritidis could infect laying hens (Baskerville *et al.* 1992). *Salmonella* colonized and persisted in different tissues in broilers following exposure to aerosolized *Salmonella* (Pal *et al.* 2021). Chickens and animals are direct or indirect sources of *Salmonella* through feces and dust (Venter *et al.* 2004, Gale & Velazquez 2020). *Salmonella* infection has been experimentally proven to occur by oral, intracloacal, intratracheal, intraocular, navel and aerosol administration (Cox *et al.* 1990). The hatchery is the most significant contributor of *Salmonella* with a prevalence of 48.5%. Litter, feces, and indoor environment of poultry house are the other 3 major contributing factors with prevalences of 25.4, 16.3, and 7.9%, respectively (Wang *et al.* 2023b).

Cross-contamination of *Salmonella* from contaminated to uncontaminated eggs could be spread by fan-driven air (Berrang *et al.* 1995). *Salmonella* Typhimurium DT104 strain could be efficiently

transmitted to eggs up to 15 times more when laying hens were inoculated via aerosol route than crop route (Leach *et al.* 1999). Airborne transmission of *Salmonella enterica* serovar Typhimurium was demonstrated in chicks hatching in a cabinet containing infected hatchmates (Cason *et al.* 1994). *Salmonella* Enterifidis from infected chicks in an isolation cabinet rapidly transmitted to chicks physically separated from the infected seeder chicks but sharing the same air (Gast *et al.* 1998). However, the transmission mechanism of airborne *S*. Enteritidis has not been fully defined in chick's cabinets. *Salmonella* move through the air by itself or via water droplets, dust, dander or feathers need further studies (Cox *et al.* 1990, Davies & Wray 1996, Holt *et al.* 1999). These types of media may have important role in the transmission process of Salmonellae. Higher *Salmonella* and *E. coli* concentrations were detected in litter samples with water activity ≥ 0.90 and moisture ≥ 35% in a broiler litter (de Rezende *et al.* 2001). Airborne bacterial levels were linked to their densities in litter/or dust, *Salmonella* and *E. coli* averaged 10^4 MPN/g and ~10^8 CFU/g of litter, respectively (Davies & Wray 1994). *Salmonella* concentrations ranged between 10^3-10^5 MPN/g in litter and 2.2×10^{-1}- 44×10^{-1} MPN/m^3 inside the air environment of a poultry house (Chinivasagam *et al.* 2009). The prevalence of Salmonellae isolated from both poultry farm and processing plant environments were 5.4% and 4.7%, respectively with no *Salmonella* bacteria detected in the air samples (Alzenki *et al.* 2007). *Salmonella* Typhimurium was detected in the air at dehairing and evisceration locations in an Irish pig slaughtering plant (Pearce *et al.* 2006).

The number of total airborne aerobic bacteria and Gram negative bacteria varied between 780 and 20100 CFU/m^3 and 39 and 1030 CFU/m^3, respectively, in Chinese rabbit houses (Duan *et al.* 2006). The median of airborne mesophilic bacteria at the processing area of the moving rail was 1.7×10^6 CFU/m^3 with no *Salmonella* species detected from the air samples at a poultry house in Styria, Australia (Haas *et al.* 2005). On the other hand *Salmonella* bacteria only represented ~ 0.56% of the total airborne bacterial colonies in a small poultry house in Egypt (Abdel Hameed *et al.* 2010). *Salmonella* were identified in 10% of total airborne bacterial colonies, with *S.* Choleraesuis, *S.* Typhi and *S.* Typhimurium constituting 5.5% of the total bacterial counts in three pig and three beef plants in USA (Cosenza-Sutton 2004). The concentration of airborne *Salmonella* in a poultry production unit constituted up 3.3% of total bacterial cell counts measured by 4, 6-diamidino 2-phenylindole, ranging from 2.2×10^1 to 3×10^6 *Salmonella* targets/m^3 using *Salmonella*-specific *invA* genes of DNA (Fallschissel *et al.* 2009).

Salmonella bacteria have been isolated from the settled dust within unoccupied poultry shed as a result of the residual effect (Chinivasagam *et al.* 2009). *Salmonella* can survive for ~ 53 weeks in dust (Davies & Wray 1994) and 26 months in thin layers of litter of dried feces and feed (Davies & Breslin 2003). Pathogenic microorganisms were found in low counts, suggesting that air environment is not a significant source of enteric biocontamination. However, the transmission of airborne *Salmonella* within the livestock environment may impact the bird's and worker's health. Table 2 shows levels of *Salmonella* in raw and aerosols at livestock facilities.

Factors influencing the survival of Salmonella aerosols

The air environment is not an optimal medium for the survival of microorganisms. Aerosolized pathogenic bacteria are subject to considerable stressors leading to cell injury and/or death in both Gram positive and negative bacteria (Heidelberg *et al.* 1997). The persistence of airborne microorganisms depends on their tenacity. The tenacity (the ability to survive the airborne) of different microbial species depends on meteorological factors (temperature and humidity, UV radiation, solar radiation), air pollution, free radicals and ozone-olefin reaction products (Open Air Factor, OAF) (Stärk 1999, Clauss *et al.* 2016). These factors lethally affect microbial viability and infectivity through chemical, physical and biological modifications to phospholipid, protein and nucleic acid moieties (Karra & Katsivella, 2007).

Gram negative bacteria, including Salmonellae, are rapidly die-off in the airborne state (Cox 1995). Some bacteria (anaerobic species) are highly sensitive and cannot grow in the presence of oxygen (Tang 2009). Desiccation is experienced by Gram-negative bacteria, ~ 90% immediately loss their viability after aerosolization, due to denaturation of outer phospholipid bilayer membranes (Cox 1989).

Table 2. Levels of *Salmonella* in raw and aerosols at livestock facilities

Environment	Level	Reference
Swine house units	No *Salmonella*	Elliott *et al.* (1976)
Chick dust	10^4 CFU/g	Davies & Wray (1994)
Chick dust Poultry house- air	10^3 - 10^5 MPN/g 0.22 - 4.4 MPN/m^3	Chinivasagam *et al.* (2009)
Poultry house-air (DAPI) Poultry house-air (culture method) Duck stalls (molecular method)	$2.8 \times 10^5 \pm 1.9 \times 10^5$ cell/ m^3 $3.3 \times 10^2 \pm 1.2 \times 10^2$ CFU/m^3 2.5×10^1 - 3×10^6 genes/m^3	Fallschissel *et al.* (2009)
Small poultry house	~0.56% of total bacterial isolates	Abdel Hameed *et al.* (2010)
Poultry house- picking area	2 - 598 CFU/m^3	Heber *et al.* (2006)

Microorganisms generated from liquid suspension undergo desiccation (loss of water) and those generated as dust particles partially rehydrated (Cox 1995, Cox & Wathes 1995).

Long distance transport of microorganisms in the air depends on atmospheric dispersion, dilution, deposition, particle size and meteorological conditions (Gregory 1973). The immission concentrations of bioaerosols decreased exponentially with increasing distance from the source of emission. In the air environment, bioaerosols are exposed to wind and weather and their extent dependens on the tenacity, size and composition of bioaerosol particles (Clauß 2020). The behavior of *Salmonella* in the air environment remains unpredictable (Carrique-Mas & Davis 2008). Temperature affects the molecular structure of the microorganism and consequently its inherent thermodynamic instability (Maillard reaction), involving the elimination of water molecules (Stärk 1999). At warmer temperatures, phospholipid membranes of Gram negative bacteria undergo many complex transition, separation and aggregation phases, leading to changes in biological functions. However, at cooler temperature, exothermic crystallization of lipid moieties together with protein subunit formation leads to loss of viability (Cox 1989). The effect of relative humidity on airborne microorganisms is difficult to determine, however surface damage (inactivation at high RH) and rehydration (inactivation at low RH) are the most influential factors (de Jong *et al.* 1973).

Airborne *Salmonella* are affected by sunlight and other environmental factors, because *Salmonella* bacteria are enteric microbes, adapting to live in a protected environment (Müller 1980). Aerosol particles play a crucial role in the transmission of airborne bacteria, as particles may protect microorganisms from harsh environmental conditions. A significant positive relationship was found between concentrations of aerosol sizes of 0.5-1.0µm and *Salmonella* species in a dairy house (Aminul Islam *et al.* 2020).

Climate change and global warming have contributed to the spread of pathogens. Several studies have recognized the importance of increased ambient temperature and precipitation in the spread and persistence of *Salmonella* in soil and food. The impact of extreme weather events on *Salmonella* infection rates among the most prevalent serovars has not been evaluated worldwide (Jiang *et al.* 2015; Morgado *et al.* 2021). Dust storms have positive (e.g. fertilization of aquatic and terrestrial ecosystems) and negative (e.g. transport of toxins and pathogenic microorganisms) effects. S*almonella* proliferate rapidly at higher temperature, increasing their spread through different environmental media (Akil *et al.* 2014). Emergence or resurgence of numerous infectious diseases is influenced by environmental factors such as climate or land use change (Mills *et al.* 2010). However, the impact of extreme weather events on *Salmonella* growth and persistence in the air environment should be fully evaluated.

Airborne Salmonella: Sampling and analysis techniques

The detection of airborne pathogenic bacteria is of great concern. The efficiency of collection depends on the sampling strategy, analysis technique and media used. Different air sampler types exist and not all are suitable for collecting a specific microorganism. The ideal air sampler is efficiently able to recover all microorganisms from the air and allow all the required analysis to be performed. Currently, there is a lack of standardized techniques to quantify airborne microorganisms. The advantages and drawbacks of different sampling methods (filtration, impingement, impaction, and sedimentation) have been previously reviewed (Buttner *et al.* 1997, Griffin *et al.* 2011, Adell *et al.* 2014).

The collection and analysis methods may represent a stress factor on microbial viability. Sampling technique, type of medium, cut-off diameter of sampling device and its detection limits play important stress factors on the survivability of microbial aerosols. Non-detection of *Salmonella* bacteria from the air environment could be attributed to their low concentrations at point sources (Kocwa-Haluch 1996). Moreover, the presence of many competing bacteria limits isolation of *Salmonella* in air samples (Carrique-Mas & Davies 2008). Several official organizations for standardization have developed reference methods for the isolation of *Salmonella.* Conventional detection methods for *Salmonella* bacteria are based on culturing techniques, using pre-enrichment broths, and selective enrichment media, followed by biochemical and serological reactions. Liquid impinge sampler using pre-enrichment broths (buffered peptone, selenite, tetrathionate brilliant green, Muller-Kauffmann tetrathionate and Rappaport-Vassiliadis soya) have been preferred to collect airborne *Salmonella* (ISO 2002). The efficiency of the enrichment broths depends on type of sample, addition of antibiotics, portion of the inoculum used and incubation temperature (35-37^{o}C). Isolation of Salmonellae is enhanced by incubation of pre-enrichment broth into selective enrichment media (Carrique-Mas & Davis 2008) to detect low levels of pathogens; enabling reproduction of the injured cells and subsequently overestimate pathogens density (Sidhu & Toze, 2009).

Salmonellae can be isolated using numerous low-selective media (MacConkey agar, deoxycholate agar), intermediate-selective media (*Salmonella-Shigella* [SS] agar, Hektoen [HE] agar) and highly selective media (selenite agar with brilliant green), (Cooke *et al.* 1999). Most of the conventional plating media (e.g. brilliant green agar) are non-specific, developing a large number of false positive Salmonellae (*Citrobacter* and *Proteus*). XLD and HE agar are the most popular media for isolating *Salmonella* and their differentiation abilities rely on the characteristics of *Salmonella* (Rambach 1990).

Salmonella colonies are isolated and screened using different biochemical reactions. The main biochemical reactions are Triple sugar iron (TSI) agar (alkaline slant,

with acid, gas and H_2S in the butt), lysine iron agar (Alkaline slant with alkaline, rare gas and H_2S in the butt), oxidase reaction (–ve), predominantly lactose-negative and urease reaction (–ve) and confirm with polyvalent anti-sera (Table 3).

Airborne microbial concentrations cannot be accurately determined using only culture-dependent method; because microorganisms could be viable but non cultivable (Alvarez *et al.* 1995). The selective enrichment media may not restrict the growth of undesirable microorganisms (Albrecht & Kämpher 2006). The majority of naturally occurring pathogenic microorganisms cannot be cultivated using the traditional cultivation techniques (Amann *et al.* 1995). A range of chromogenic media has been developed for the detection of *Salmonella*, based on combination of chromogenic substrate and conventional biochemical reactions. These media produce distinctive colonies; making *Salmonella* identification easier and faster. Rambach agar and *Salmonella* detection media (O'Neill *et al.* 2003) and BBLTH CHROM agar are the common chromogenic media used (Eigner *et al.* 2001). Chromogenic media offer a much higher degree of specificity than conventional media which are based on absence of lactose fermentation within *Salmonella* and/or their ability to generate hydrogen sulphide.

The culture independent technique, based-on DNA amplification by polymerase chain reaction (PCR) is used to complement /or replace culture based technique (Gugliandolo *et al.* 2011). The qualitative ISO 6579:2002 technique is the most sensitive and specific method among presence /absence PCR/ or ELISA for detecting *Salmonella* in the environmental samples (Eriksson & Aspan 2007). Molecular base methods offer advantages of a more rapid, sensitive and specific detection of pathogenic microorganisms (Kolb *et al.* 2005).

qPCR is a potential method for specific/ or genus specific quantification of aerosol samples (Dutil *et al.* 2007, Oppliger *et al.* 2008, Fallschissel *et al.* 2009). The qPCR analysis of airborne microorganisms gives higher counts than conventional cultivation methods as molecular method determines cultivable and non-cultivable cells. The accuracy and detection limit of qPCR are influenced by DNA extraction and analytical phases (Hospodsky *et al.* 2010). The drawback of the PCR is related to its inability to provide information on pathogen viability which is necessary to investigate microbial infectivity (Zeng *et al.* 2016). The most frequently target species-specific and virulence associated genes used in the PCR of *Salmonella* are shown in Table 4.

Salmonella as air bioindicator

The criteria considered in selecting a microbial indicator include 1) the ability of a microorganism to survive in the environment of concern, 2) the correlation between the presence of the indicator and pathogens, 3) ease and speed of detection, and 4) non-pathogenicity of the indicator. The presence of fecal coliform is a good indicator of the possible presence of associated pathogenic bacteria, particularly *Salmonella*. However, pathogens are difficult to assay and seldom occur at readily detectable concentrations but high levels of coliforms and total bacterial counts may indicate the existence of enteric pathogens (Sorber & Sagik 1979). In contrast to Gram positive bacteria, Gram negative bacteria have a thinner cell wall; therefore they are more sensitive to dehydration and not viable in the air state for a long time. Gram negative bacteria represent ~1 - 10% of the airborne total bacteria (Matkovi̧ć *et al.* 2007), however Gram-negative bacteria may include pathogens such as Salmonellae. As a result of their thicker cell wall and the accompanying greater "robustness" towards the airborne state, most of the bacteria from the air detected via cultivation methods are Gram-positive bacteria (Zhao 2011).

Table 3. Appearance of *Salmonella* bacteria on different selective media.

Selective medium	Appearance
Bismuth sulfite agar	Fully developed colonies, convex, 1-3 mm in diameter, black with lustrous surface, form a shallow, soft, black pit with light edge
Brilliant green agar	Transparent pink colonies surrounded by a brilliant color
MacConkey and SS agar	Colonies usually colorless, transparent with light tan, light pinkish or yellow appearance tan centers, 1-5 mm
XLD medium	Pink to red with black center colonies
Hektoen enteric agar	Green or blue green colonies

Table 4. The target species specific and virulence associated genes used in the PCR of *Salmonella*

Target gene	Primer	Sequence (5^{-}-3^{-})	Amplicon size (bP)	Reference
Invasion plasmid Antigen-B (*ipa*B)	*ipa*B-F *ipa*B-R	GGACTTTTTAAAAGCGGCGG GCCTCTCCCAGAGCCGTCTGG	314 429	Kaniga *et al.* (1995)
-	ST11 ST15	AGCCAACCATTGCTAAATTGGCGCA GGTAGAAATTCCAGCGGGTACTG	-	Adell *et al.* (2014)
-	Sef.B127L	5'-AGATTGGGCACTACACGTGT-3'	535	Wang *et al.* (2009)
-	SefB661R	(5'-TGTACTCCACCAGGTAATTG-3'	535	Santos *et al.* (2021)

Enterobacteriaceae are sensitive towards the airborne state, as they already die before/or during sampling and thus are barely detectable. The survival of coliforms in the air environment is still controversial. Coliforms have lower survivability in the air environment than *Salmonella* (Teltsch *et al.* 1980) and do not fulfill the main requirement of microbial indicator "its ability to survive in the environment is equal to/or more than the tested pathogenic microorganism". The stability of coliform in the air environment appears to be lower than certain viruses (Scarpino 1975). *Salmonella*, *Citrobacter*, *Clostridium*, *Proteus*, *Edwardsiella* and *Klebsiella* species have been associated with the presence of fecal contamination (Kromoredjo and Fujioka 1991) and Clostridia are better indicator of airborne pathogens (Hill *et al.* 1993).

Control measures

The practical control measures are crucial in livestock and waste applications to prevent release and spread of pathogenic microorganisms into the air environment (Hendriksen *et al.* 2004). Biosecurity management includes a set of practical measures to prevent and limit the spread of infections to humans and animals (Amass 2005). Biosecurity includes replacement of animal and husbandry (Andres & Davies 2015), dust reduction, air filtration and proper air disinfectants (Stärk 1999), electrostatic filtration, fogging and oil based spray, negative air ionization, vacuum cleaning, ventilation and wet scrubbers (Holt *et al.* 1999, Ritz *et al.* 2006). Rodent and insect control and disinfection between flocks are recommended to reduce *Salmonella* in farms (Gosling *et al.* 2014). Pressurized steam followed by forced hot air reduces levels of *Salmonella* and *Campylobacter* in transport cage flooring and reduce cross-contamination of broilers (Reina *et al.* 2024). Assessing biosecurity includes measuring the potential routes for disease transmission. Air is yet another vector by which pathogens can contaminate the final products. The adjacent nearby residential areas require higher standards of amenity. The width of a buffer zone > 400m between waste and livestock applications and residential areas should be taken in consideration during city planning.

The International Life Sciences Institute (ILSI) outlined a number of measures that should be considered with regard to air entering production floors (Beuchat *et al.* 2011), including a positive pressure air system to prevent the contaminated air infiltrating controlled production areas and eliminating residual moisture (Podolak *et al.* 2010). Filtering air entering production zones may also be effective as well as continuous monitoring of *Salmonella* in the air is important to maintain the appropriate state of the environment.

Conclusion

Salmonella bacteria are potentially generated into the air from livestock farms and waste application facilities. These facilities are hotspots associated with high infection risks of aerosols- containing *Salmonella*. Salmonellae are found in aerosols in detectable counts. Transmission of *Salmonella* via the air pathway is less pathogenic and rarely occurs, however airborne *Salmonella* may represent a threat to public health, but no greater than that of the same count of pathogens ingested. The low count of *Salmonella* in the air is attributed to their enteric adapted to living in the protected environment, short time survives and occurrence is sporadic related to the incidence of disease infection. The efficiency of sampler, analytical technique and nutrient medium in use are important factors in detecting airborne *Salmonella*. The qPCR is fast, rapid and accurate for quantification of *Salmonella* in air samples. More sensitive laboratory methodological techniques should be created. The absence of correlation between the presence of Salmonellae and fecal coliforms make them fail to fulfill one of the main requirements of microbial indicator for air biocontamination. There is an urgent to identify more reliable alternative indicators which could be used for potential public health risk assessment. The development of new diagnostic tools (less labour and more rapid and sensitive) and vaccines targeting specific pathogenesis factors could be used in comparative investigations and control *Salmonella* transmission and infection. Finally, the presence of *Salmonella* in the air may have a hypothetical potential to cause infection.

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References

1. Abdel Hameed, A. 1992. Comparison of salmonellae and coliform bacteria in aerosols emitted from wastewater treatment plants and related irrigation systems. MSc. Thesis, Botany Dept., Faculty of Science, Mansoura Univ., Egypt.

2. Abdel Hameed, A., Elmorsy, T., Tarwarter, P., Green, C. & Gibbs, S. 2010. Air biocontamination in a variety of agricultural industry environments in Egypt: a pilot study. *Aerobiologia*, 26(3): 223-232. https://doi.org/10.1007/s10453-010-9158-y

3. Adams, A. & Spendlove, J. 1970. Coliform aerosols emitted by sewage treatment plants. *Science*, 169(3951): 1218-1220. https://doi.org/10.1126/science.169.3951.1218

4. Adell, E., Moset V., Zhao, Y, Jiménez Belenguer, A., Cerisuelo, A. & Cambra-López, M. (2014). Comparative performance of three sampling techniques to detect airborne Salmonella species in poultry farms. *Annals of Agricultural and Environmental Medicine*, 21(1): 15-24.

5. Aengst, C. 1984. The composition of dust in a pig fattening house [Zur Zusammensetzung des Staubes in einem Schweinemaststall]. Dissertation, Tierärztliche Hochschule Hannover [In German].

6. Akil, L., Ahmad, H. & Reddy, R. 2014. Effects of climate change on Salmonella infections. *Foodborne Pathogens and Disease*, 11(12): 974-80. https://doi.org/10.1089/fpd.2014.1802

7. Akin, E.; Jakubowski, J.; Lucas, B. & Pahren, H. 1978. Health hazards associated with wastewater effluents and sludge: Microbiological considerations, pp. 9-25. In: Sagik, B. & Sorber, C. (eds), *Proc. Conf. Risk Assessment and Health Effects of Land Application of Municipal Wastewater and Sludge.* Center for Applied Research and Technology, University of Texas at San Antonio, San Antonio.

8. Ajonina, C., Buzie, C., Rubiandini, R. & Otterpohl, R. 2015. Microbial pathogens in wastewater treatment plants (WWTP) in Hamburg. *Journal of Toxicology and Environmental Health, Part A,* 78(6): 381-387. https://doi.org/10.1080/15287394.2014.989626

9. Albrecht, A. & Kämpfer, P. 2006. Potential overestimation of the actinomycetes concentrations by growth of thermophilic species of Bacillus and Geobacillus on selective media for thermophilic actinomycetes. *Gefahrstoffe Reinhaltung der Luft*, 66(9): 365-367

10. Alvarez, A., Buttner, M. & Stetzenbach, L. 1995. PCR for bioaerosol monitoring: sensitivity and environmental interference. *Applied and Environmental Microbiology*, 61(10): 3639-3644. https://doi.org/10.1128/aem.61.10.3639-3644.1995

11. Alzenki, S., Al-Nasser, A., Al-Safar, A. Alomirah, H., Al-Haddad, A., Hendriksen, R. & Aarestrup, F. 2007. Prevalence and antibiotic resistance of *Salmonella* isolated from a poultry farm and processing plant environment in the state of Kuwait. *Foodborne Pathogens and Disease*, 4(3): 363-373. https://doi.org/10.1089/fpd.2007.0017

12. Amann, R., Ludwig, W. & Schleifer, K. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews*, 59 (1): 143-169. https://doi.org/10.1128/mr.59.1.143-169.1995

13. Amass, S. 2005. Biosecurity: stopping the bugs from getting in. *The Pig Journal*, 55: 104-114.

14. Aminul Islam, M., Ikeguchi, A. & Naide, T. 2020. Influence of temperature and humidity on the dynamics of aerosol numbers and airborne bacteria in a dairy calf house. *Biosystems Engineering*, 194(7): 213-226. https://doi.org/10.1016/j.biosystemseng.2020.04.003

15. Andres, V. & Davies, R. 2015. Biosecurity measures to control Salmonella and other infectious agents in Pig farms: a Review. *Comprehensive Reviews in Food Science and Food Safety*, 14(4): 317-335. https://doi.org/10.1111/1541-4337.12137

16. Andino, A. & Hanning, I.2015. *Salmonella* enterica: survival, colonization, and virulence differences among serovars. *Scientific World Journal*, 2015: 520179. https://doi.org/10.1155/2015/520179

17. Ashurst, J., Truong, J. & Woodbury, B. 2022. *Salmonella* Typhi. In: *StatPearls*; StatPearls Publishing: Treasure Island, FL, USA, 2022.

18. Baskerville, A., Humphrey, T., Fitzgeorge, R., Cook, R., Chart, H., Rowe B. & Whitehead, A. 1992. Airborne infection of laying hens with Salmonella enteritidis phage type 4. *Veterinary Record*, 130(18): 395-398. https://doi.org/10.1136/vr.130.18.395

19. Bauer, H., Fuerhacker, M., Zibuschka, F., Schmid, H. & Puxbaum, H. 2002. Bacteria and fungi in aerosols generated by two different types of wastewater treatment plants. *Water Research*, 36(16): 3965-70. https://doi.org/10.1016/s0043-1354(02)00121-5

20. Baylor, E., Peters, V. & Baylor, M. 1977. Water to air transfer of virus. *Science*, 197(4305): 763-764. https://doi.org/10.1126/science.329413

21. Berrang, M., Cox, N. & Bailey, J. 1995. Measuring airborne microbial contamination of broiler hatching cabinets. *Journal of Applied Poultry Research*, 4(1): 83-87. https://doi.org/10.1093/japr/4.1.83

22. Beuchat, L., Komitopoulou, E., Beckers, H., Betts, R., Bourdichon, F. & Joosten, H. 2011. Persistence and survival of pathogens in dry food processing environments. Available at: http://ilsi.org/Europe/documents/Persistence%20and%20survival%20report.pdf (Date accessed: January 10, 2024).

23. Blaser, M. and Lee, S. 1982. A review of human salmonellosis: I. Infective dose. *Reviews of Infectious Diseases* 4(6): 1096-1106.

24. Bitton, G. 1980. Introduction to environmental virology, pp. 200-242. JohnWiley & Sons, Inc., NewYork.

25. Breza-Boruta, B., Paluszak, Z. 2007. Influence of water treatment on microbiological composition of air bioaerosols. *Polish Journal of Environmental Studies*, 16(5): 663-670.

26. Brooks, J., Tanner, B., Josephson, K., Haas, C., Gebra, C. & Pepper, I. 2005. A national study on the residential impacts of biological aerosols from the land application of biosolids. *Journal of Applied Microbiology*, 99(2): 310-322. https://doi.org/10.1111/j.1365-2672.2005.02604.x

27. Brooks, J., Tanner, B., Josephson, K., Gerba, C. & Pepper, I. 2004. Bioaerosols from land application of biosoilds in the desert south west USA. *Water Science and Technology*, 50(1): 7-12. PMID: 15318479

28. Buttner, M., Willeke, K. & Grinshpun, S. 1997. Sampling and analysis of airborne microorganisms, pp. 629-640. In: Hurst C., Knudsen G., McInerney M., Stetzenbach L. &

Walter M. (eds), *Manual of environmental microbiology*, Washington D.C., American Society for Microbiology Press.

29. Cambra-López, M., Aarnink, A., Zhao, Y., Calvet, S. & Torres, A. 2010. Airborne particulate matter from livestock production systems: A review of an air pollution problem. *Environmental Pollution*, 158(1): 1-17. https://doi.org/10.1016/j.envpol.2009.07.011

30. Carducci, A., Tozzi, E., Rubulotta, E., Casini, B., Cantiani, L., Rovini, E., Muscillo, M. & Pacinim R. 2000. Assessing airborne biological hazard from urban wastewater treatment. *Water Research*, 34(4), 1173-1178. https://doi.org/10.1016/S0043-1354(99)00264-X

31. Carrique-Mas, J. & Davies, R. 2008. Sampling and bacteriological detection of Salmonella in poultry and poultry premises: a review.*Revue scientifique et technique*, 27(3): 665-677. https://doi.org/10.20506/rst.27.3.1829

32. Cason, J., Cox, N. & Bailey, J., 1994. Transmission of salmonella typhimurium during hatching of broiler chicks. *Avian Diseases*, 38 (3): 583-58. PMID: 7832712

33. Chien, Y., Chen, C., Lin, T., Chen, S. & Chien, Y. 2011. Characteristics of microbial aerosols released from chicken and swine feces. *Journal of the Air & Waste Management Association*, 61(8): 882-889. https://doi.org/10.3155/1047-3289.61.8.882

34. Chinivasagam, H., Tran, T., Maddock, L., Gale, A. & Blackall, P. 2009. Mechanically ventilated broiler sheds: a possible source of aerosolized *Salmonella*, *Camplylobacter* and *Escherichia coli*. *Applied and Environmental Microbiology*, 75(23): 7417-7415. https://doi.org/10.1128/AEM.01380-09

35. Clauß, M. 2020. *Emission of bioaerosols from livestock facilities: methods and results from available bioaerosol investigations in and around agricultural livestock farming*. Thünen Institute of Agricultural Technology Bundesallee 47, Braunschweig/Germany, January.

36. Clauss, M., Huf, A. & Clauß, A. 2016. An unconventional bioaerosol chamber for tenacity studies of airborne microorganisms under almost ambient air conditions, Conference: Bioaerosol Chamber Expert Meeting, 14-15 April, Vienna, Austria.

37. Colbeck, I. & Lazaridis, M. 2010. Aerosols and environmental pollution. *Naturwissenschaften*, 97(2): 117-131. https://doi.org/10.1007/s00114-009-0594-x

38. Cole, D., Hill, V., Humenick, F. & Sobsey, M. 1999. Halth, safety and environmental concerns of farm animal waste. *Occupational Medicine*, 14(2): 423-448. PMID:10329913

39. Cooke, V., Miles, R., Price, R. & Richardson, A. 1999. A novel chromogenic ester agar medium for detection of salmonellae. *Applied and Environmental Microbiology*, 65(2): 807-812. https://doi.org/10.1128/AEM.65.2.807-812.1999

40. Cosby, D., Cox, Ne., Harrison, M., Wilson, J., Jeff Buhr, R. & Fedorka-Cray, P. 2015. *Salmonella* and antimicrobial resistance in broilers: A review. *Journal of Applied Poultry Research*, 24(I3): 408-426. https://doi.org/10.3382/japr/pfv038

41. Cosenza-Sutton, G. 2004. Enumeration of total airborne bacteria, yeast and mold contaminants and identification of *Escherichia coli* 0157:H7, *Listeria spp*, *Salmonella spp* and *Staphylococcus spp* in beef and pork slaughter facility. A dissertation presented to the graduate school of the University of Florida in partial fulfillment of the requirement for degree of doctor of philosophy, University of Florida.

42. Cox, C. 1989. Airborne bacteria and viruses. *Science Progress*, 73(4): 469-499. https://www.jstor.org/stable/43421049

43. Cox, C. 1995. Stability of airborne microbes and allergens, pp. 77-99. In: Cox C. & Wathes C. (eds), *Bioaerosols Handbook*. Lewis Publisher, NY.

44. Cox, C. & Wathes, C. 1995. Bioaerosols Handbook. Lewis Puplishers, Boca Raton, FL., USA.

45. Cox, N., Bailey J., Mauldin, J. & Blankenship L. 1990. Presence and impact of *Salmonella* contamination in commercial broiler hatcheries. *Poultry Science*, 69(9): 1606-1609. https://doi.org/10.3382/ps.0691606

46. Dai, P., Shen, D., Tang, Q., Huang, K. & Li, C. 2020. PM2.5 from a broiler breeding production system: The characteristics and microbial community analysis. *Environmental Pollution*, 256: 113368. https://doi.org/10.1016/j.envpol.2019.113368

47. Darlow, H., Bale, W. & Carter, G. 1961. Infection of mice by the respiratory route with *Salmonella typhimurium*. *Journal of Hygiene* (Camb.), 59(3): 303-308. https://doi.org/10.1017/s0022172400038961

48. Davies, R. & Breslin, M. 2003. Investigation of *Salmonella* contamination and disinfection in farm egg-packing plants. *Journal of Applied Microbiology.*, 94(2): 191-196. https://doi.org/10.1046/j.1365-2672.2003.01817.x

49. Davies, R. & Wray, C. 1994. An approach to reduction of Salmonella infection in broiler chicken flocks through intensive sampling and identification of cross- contamination hazards in commercial hatcheries. *International Journal of Food Microbiology* 24(1-2): 147-160. https://doi.org/10.1016/0168-1605(94)90114-7

50. Davies, R.H: & Wray, C. 1996. Persistence of *Salmonella* in poultry units and poultry food. *British Poultry Science*, 37(3): 589-596. https://doi.org/10.1080/00071669608417889

51. de Jong, J., Trouwborst, T. & Winkler, K. 1973. *The mechanism of virus decay in aerosols*, pp. 124-130. In: Hers, J. & Winkler, K. (eds), *Airborne transmission and airborne infection*. John Wiley & Sons, New York, NY.

52. de Rezende, C., Mallinson, E., Tablante, N., Morales, R. & Park, A. 2001. Effect of dry litter and airflow in reducing *Salmonella* and *Escherichia coli* populations in the broiler production environment. *Journal of Applied Poultry Research*, 10(3): 245-251. https://doi.org/10.1093/japr/10.3.245

53. Devane, M., Weaver, L., Singh, S. & Gilpin, B. 2018. Fecal source tracking methods to elucidate critical sources of pathogens and contaminant microbial transport through New Zealand agricultural watersheds – a review. *Journal of Environmental Management*, 222: 293-303. https://doi.org/10.1016/j.jenvman.2018.05.033

54. Duan, H., Chai, T., Müller, W. & Zucker, B. 2006. Concentration of airborne endotoxins and airborne bacteria in Chinese rabbit houses. *Berliner und Münchener*

Tierärztliche Wochenschrift, 119(1-2): 40-44, PMID:16450707

55. Dungan, R. 2010. Board-invited review: fate and transport of bioaerosols associated with livestock operations and manures. *Journal of Animal Science*, 88(11): 3693-3706. https://doi.org/10.2527/jas.2010-3094

56. Dutil, S., Veillette, M., Meriaux, A., Lazure, L., Barbeau, J. & Duchaine, C 2007. Aerosolization of *Mycobacteria* and *Legionella* during dental treatment: low exposure despite dental unit contamination. *Environmental. Microbiology*, 9(11): 2836-2843. https://doi.org/10.1111/j.1462-2920.2007.01395.x

57. Eigner, U., Reissbrodt, R., Hammann, R. & Fahr, A. 2001. Evaluation of a new chromogenic medium for the isolation and presumptive identification of *Salmonella* species from stool specimens. *European Journal of Clinical Microbiology & Infectious Diseases*, 20(8): 558-565. https://doi.org/10.1007/s100960100546

58. Elliott, L., McCalla, T. & Deshazer, J. 1976. Bacteria in the air of housed swine units. *Applied and Environmental Microbiology*, 32(2): 270-273. https://doi.org/10.1128/aem.32.2.270-273.1976

59. Epstein, E. 1997. The science of composting. CRC Press LLC, Florida, 504 p.

60. Eriksson, E. & Aspan, A. 2007. Comparison of culture, ELISA and PCR techniques for *Salmonella* detection in faecal samples for cattle, pig and poultry. *BMC Veterinary Research*, 3: 21. https://doi.org/10.1186/1746-6148-3-21

61. Fallschissel, K., Kämpker, P. & Jäckel, M. 2009. Direct detection of *Salmonella* cells in the air of livestock stables by real time PCR. *The Annals of Occupational Hygiene*, 53(8): 859-868. https://doi.org/10.1093/annhyg/mep060

62. Fannin, K, Vana, S & Jakubowski, W. 1985. Effect of an activated sludge wastewater treatment plant on ambient air densities of aerosols containing bacteria and viruses. *Applied and Environmental Microbiology*, 49(5): 1191-1196. https://doi.org/10.1128/aem.49.5.1191-1196.1985

63. Foster, D & Engelbrecht, R. 1973. Microbial hazard of disposing of wastewater on soil, pp. 247-270. In: Sopper, W. & Kardos, L. (eds), *Recycling treated municipal wastewater and sludge through forest and cropland*, Pennsylvania State University Press.

64. Forgie, D., Sasser, L. & Neger, M. 2004. Compost facility requirements guideline: How to comply with part 5 of the organic matter recycling regulation, http://www.env.gov.bc.ca/epd/codes/omr/pdf/compost.pdf

65. Gast, R., Mitchell B. & Holt, P. 1998. Airborne transmission of *Salmonella enteritidis* infection between groups of chicks in controlled environment isolation cabinets. *Avian Diseases*, 42(2): 315-320. PMID: 9645323

66. Gibbs, R., Hu, C., Ho, G. & Unkovich, I. 1997. Regrowth of faecal coliforms and salmonellae in stored biosolids and soil amended with biosolids. *Water Science and Technology*, 35(11-12): 269-275. https://doi.org/10.2166/wst.1997.0745

67. Gordon, M. 2011. Invasive nontyphoidal *Salmonella* disease: epidemiology, pathogenesis and diagnosis. *Current Opinion in Infectious Diseases*, 24(5): 484-489. https://doi.org/10.1097/QCO.0b013e32834a9980

68. Gray, J. & Fedorka-Cray, P. 2002. *Salmonella*, pp. 55-68, In: Cliver, D. & Riemann H. (eds.), *Foodborne diseases* (2nd ed), Academic Press, ISBN 0121765598

69. Gregory, P. 1973. *The microbiology of the atmosphere*, 2nd edition. Leonard Hill Books, Aylesbury, Bucks, England.

70. Griffin, D., Gonzalez, C., Teigell, N., Petrosky, T., Northup, D. & Lyles, M. 2011. Observations on the use of membrane filtration and liquid impingement to collect airborne microorganisms in various atmospheric environments. *Aerobiologia*, 27(1): 25-35. https://doi.org/10.1007/s10453-010-9173-z

71. Grisoli, P., Rodolfi, M., Villani, S., Grignan, E., Cottica, D., Beiri, A., Picco, A. & Dacarro, C. 2009. Assessment of airborne microorganism contamination in an industrial area characterized by an open composting facility and wastewater treatment plant. *Environmental Research*, 109(2): 135-142. https://doi.org/10.1016/j.envres.2008.11.001

72. Grunnet, K. & Tramsen, C. 1974. Emission of airborne bacteria from a sewage treatment plant. *Revue Internationale d'Océanographie Médicale*, 34: 117-126.

73. Gugliandolo, C., Lentini, V., Spanò, A. & Maugeri, T. 2011. Conventional and molecular methods to detect bacterial pathogens in mussels. *Letters in Applied Microbiology*, 52(1): 15-21. https://doi.org/10.1111/j.1472-765X.2010.02959.x

74. Gale C. & Velazquez E., 2020. *Salmonella* spp in pigs: an update on diagnostics and control. *Livestock*, 25(1): 38-43. https://doi.org/10.12968/live.2020.25.1.38

75. Gržinić, G, Piotrowicz-Cieślak, A., Klimkowicz-Pawlas, A., Górny, R., Ławniczek-Wałczyk, A, Piechowicz L, Olkowska E, Potrykus M, Tankiewicz M, Krupka M, Siebielec G & Wolska, L. 2023. Intensive poultry farming: A review of the impact on the environment and human health. *Science of The Total Environment*, 858, Part 3: 160014. https://doi.org/10.1016/j.scitotenv.2022.160014

76. Gosling, R., Martelli, F., Wintrip, A., Sayers, A., Wheeler, K. & Davies, R. 2014. Assessment of producers response to *Salmonella* biosecurity issues and uptake of advice on laying hen farms in England and Wales. *British Poultry Science*, 55(5): 559-568. https://doi.org/10.1080/00071668.2014.949620

77. Haas, D., Posch, J., Schmidt, S., Wüst, G., Sixl, W., Feierl, G., Marth, E. & Reinthaler, F. 2005. A case study of airborne culturable microorganisms in a poultry slaughter house in Styria, Austria. *Aerobiologia*, 21: 193 -201. https://doi.org/10.1007/s10453-005-9003-x

78. Han, Y., Li, L., Wang, Y., Ma, J., Li, P., Han, C. & Liu, J. 2020. Composition, dispersion, and health risks of bioaerosols in wastewater treatment plants: A review. *Frontiers of Environmental Science and Engineering*, 15(3): 38. https://doi.org/10.1007/s11783-020-1330-1

79. Heaton, J. C. & Jones, K. 2008. Microbial contamination of fruit and vegetables and the behaviour of enteropathogens in the phyllosphere: a review. *Journal of Applied Microbiology*, 104 (3): 613-626. https://doi.org/10.1111/j.1365-2672.2007.03587.x

80. Heber, A., Peugh M., Lutgring K., Zimmerman N. & Linton R. 2006. Poultry slaughtering plants: concentrations of

microbial aerosols in poultry slaughtering and processing plants. *ASHRAE Transactions*, 112: 644-655.

81. Heidelberg, J., Shahamat, M., Levin, M., Rahman, I., Stelma, G., Grim, C. & Colwell R.R. 1997. Effect of aerosolization on culturability and viability of gram-negative bacteria. *Applied and Environmental Microbiology*, 63(9): 3585-3588. https://doi.org/10.1128/aem.63.9.3585-3588.1997

82. Heinonen-Tanski, H., Reponen, T. & Koivunen, J. 2009. Airborne enteric coliphages and bacteria in sewage treatment plants. *Water Research*, 43(9): 2558-2566. https://doi.org/10.1016/j.watres.2009.03.006

83. Hendriksen, S., Orsel, K., Wagenaar, J., Miko, A. & Van Duijkeren, E. 2004. Animal-to-human transmission of *Salmonella Typhimurium* DT104A variant. *Emerging Infectious Diseases*, 10(12): 2225-27. https://doi.org/10.3201/eid1012.040286

84. Hess, E. & Breer, C. 1975. Epidemiology of salmonellae and fertilizing of grassland with sewage sludge. *Zentralbl Bakteriol Orig B*, 161(1): 54-60. PMID:1189797

85. Hickey, J. & Reist, P. 1975. Health significance of airborne microorganisms from wastewater treatment processes. Part II: Health significance and alternatives for action. *Journal of the Water Pollution Control Federation*, 47(12): 2758-2773. PMID: 1107606

86. Hill, R., Knight, I., Anikis, M. & Colwell, R. 1993. Benthic distribution of sewage sludge indicated by *Clostridium perfringens* at a deep ocean dump site. *Applied and Environmental Microbiology*, 59(1): 47-51. https://doi.org/10.1128/aem.59.1.47-51.1993

87. Hinton, M., Ali, E., Allen, V. & Linton, A. 1983. The excretion of *Salmonella typhimurium* in the feces of cows fed milk substitute. *Journal of Hygiene* (Camb), 91(1): 33-45. https://doi.org/10.1017/s0022172400060009

88. Holt, P., Mitchell, B., Seo, K-H. & Gast, R. 1999. Use of negative air ionization for reducing airborne levels of *Salmonella enterica* serovar Enteritidis in a room containing infected caged layer. *Journal of Applied Poultry Research*, 8: 440-446.

89. Hospodsky, D., Yamamoto, N. & Peccia, J. 2010. Accuracy, precision, and method detection limits of quantitative PCR for airborne bacteria and fungi. *Applied and Environmental Microbiology*, 76(21): 7004-7012. https://doi.org/10.1128/AEM.01240-10

90. Hussong, D., Burge, W. & Enkiri, N. 1985. Ocurrence, growth and suppression of Salmonellae in composted sewage sludge. *Applied and Environmental Microbiology*, 50(4): 887-893. https://doi.org/10.1128/aem.50.4.887-893.1985

91. Hutchison, M.L., Gittins, J., Walker, A., Sparks, N., Humphrey, T.J., Burton, C. & Moore, A. 2004. An assessment of the microbiological risks involved with egg washing under commercial conditions. *Journal of food protection*, 67(1): 4-11.

92. ISO: 6579 2002. International Organization for Standardization, Microbiology of food and animal feeding stuffs — horizontal method for the detection of *Salmonella* spp., ISO 6579:2002.

93. Jiang, C., Shaw, K., Upperman, C., Blythe, D., Mitchell, C., Murtugudde, R., Sapkota A . & Sapkota, A. 2015. Climate change, extreme events and increased risk of salmonellosis in Maryland, USA: Evidence for coastal vulnerability. *Environment International*, 83: 58-62. https://doi.org/10.1016/j.envint.2015.06.006

94. Jones, A. & Harrison, R. 2004. The effects of meteorological factors on atmospheric bioaerosol concentrations- a review. *Science of The Total Environment*, 326(1-3): 151-180. https://doi.org/10.1016/j.scitotenv.2003.11.021

95. Kallapura, G., Morgan, M., Pumford, N., Bielke, L., Wolfenden, A., Faulkner, O., Latorre, J. , Menconi, A., Hernandez-Velasco, X., Kuttappan, V., Hargis, B. & Tellez, G. 2014. Evaluation of the respiratory route as a viable portal of entry for *Salmonella* in poultry via intratracheal challenge of *Salmonella Enteritidis* and *Salmonella Typhimurium*. *Poultry Science*, 93(2): 340-346. https://doi.org/10.3382/ps.2013-03602

96. Kaniga, K., Tucker, S., Trollinger, D. & Galán, J. 1995. Homologs of the Shigella IpaB and IpaC invasins are required for *Salmonella typhimurium* entry into cultured epithelial cells. *Journal of Bacteriology*, 177(14): 3965-3971. https://doi.org/10.1128/jb.177.14.3965-3971.1995

97. Karra, S. & Katsivella, E. 2007. Microorganisms in bioaerosol emission from wastewater treatment plants during summer at Mediterranean site. *Water Research*, 41(6): 1355-1365. https://doi.org/10.1016/j.watres.2006.12.014

98. Katzenelson, E. & Teltsch, B. 1976. Dispersion of enteric bacteria by spray irrigation. *Journal Water Pollution Control Federation*, 48(4): 710-716. https://www.jstor.org/stable/25038569

99. Kim, K., Kabir, E. & Jahan, S. 2018. Airborne bioaerosols and their impact on human health. *Journal of Environmental Sciences*, 67: 23-35. https://doi.org/10.1016/j.jes.2017.08.027

100. Kocwa-Haluch, R. 1996. Comparison of the airborne spread of coliform and hemolytic bacteria around a sewage treatment plant. *Annals of Agricultural and Environmental Medicine*, 3(1): 13-17.

101. Kolb, S., Carbrera, A., Kammann, C., Kämpfer, P. & Conrad, R. 2005. Quantitative impact of CO_2 enriched atmosphere on abundance of methanotrophic bacteria in a meadow soil. *Biology and Fertility of Soils*, 41(5): 337-342. https://doi.org/10.1007/s00374-005-0842-y

102. Korzeniewska, E., Filipkowska, Z., Gotkowska-Ptachta, A., Janczukowicz,W. & Rutkowski, B. 2008. Bacteriological pollution of the atmospheric air at the municipal and dairy wastewater treatment plant area and its surroundings. *Archives of Environmental Protection*, 34(4): 13-23.

103. .Krishnamoorthy, S., Muthalagu, A., Priyamvada, H., Akkal, S, Valsan, A., Raghunathan, R., Kanawade, P. & Gunthe, S. 2020. On distinguishing the natural and human-induced sources of airborne pathogenic viable bioaerosols: characteristic assessment using advanced molecular analysis. *SN Applied Sciences*, 2: 1162. https://doi.org/10.1007/s42452-020-2965-z

104. Kromoredjo, P. & Fujioka, R. 1991. Evaluating three simple methods to assess the microbial quality of drinking water in Indonesia. *Environmental Toxicology & Water Quality*, 6(2): 259-270. https://doi.org/10.1002/tox.2530060214

105. Lacey, J., Williamson, P. & Crook, B. 1996. Microbial emission from composts and associated risks- trials and

tribulations of occupational aerobiologist, Chapter- 1, In: Muilenberg, M. & Burge, H. (eds), Aerobiology, Lewis Publishers, NY.

106. Langeland, G. 1982. *Salmonella* spp. in the working environment of sewage treatment plants in Oslo, Norway. *Applied and Environmental Microbiology*, 43(5): 1111-1115. https://doi.org/10.1128/aem.43.5.1111-1115.1982

107. Lang, N., Bellett-Travers M. & Smith S. 2007. Field investigations on the survival of *Escherichia coli* and presence of other enteric microorganisms in biosolids amended agricultural soil. *Journal of Applied Microbiology*, 103(5): 1868-1882. https://doi.org/10.1111/j.1365-2672.2007.03489.x

108. Leach, S., Williams, A., Davies, A., Wilson, J., Marsh, P. & Humphery, T. 1999. Aerosol route enhances the contamination of intact eggs and muscle of experimentally infected laying hens by *Salmonella typhimurium* DT104. *FEMS Microbiology Letters*, 171(2): 203-207. https://doi.org/10.1111/j.1574-6968.1999.tb13433.x

109. Liu, M., Nobu, M., Ren, J., Jin, X., Hong, G. & Yao, H. 2020. Bacterial compositions in inhalable particulate matters from indoor and outdoor wastewater treatment processes. *Journal of Hazardous Materials*, 385: 121515. https://doi.org/10.1016/j.jhazmat.2019.121515

110. López, F., de las Mercedes Pescaretti, M., Morero, R. & Delgado M. 2012. *Salmonella Typhimurium* general virulence factors: A battle of David against Goliath? *Food Research International*, 45(2): 842-851. https://doi.org/10.1016/j.foodres.2011.08.009

111. Marchand, G., Lavoi, J. & Lazure, L. 1995. Evaluation of bioaerosols in a municipal solid waste recyciling and compositing plant. *Journal of the Air & Waste Management Association*, 45(10): 778-781. https://doi.org/10.1080/10473289.1995.10467406

112. Matković K., Vucemilo, M., Vinkovic, B., Seol, B., Pavicic, Z., Matkovic, S. 2007. Qualitative structure of airborne bacteria and fungi in dairy barn and nearby environment. *Czech Journal of Animal Science*, 52(8): 249-254. https://doi.org/10.17221/2280-CJAS

113. Mckinney, R. 2004. *Environmental Pollution Control Microbiology* (Ed), Chapter- 12, Air microbiology, Marcel Dekker Inc, New York, USA.

114. Mclamarra, J. & Pruitt, J. 1995. Beneficial reuse in the southest. *Industrial. Wastewater*, 3(2): 22-24.

115. Michalkiewicz, M. 2019. Wastewater treatment plants as a source of bioaerosols. *Polish Journal of Environmental Studies*, 28(4): 2261-2271. https://doi.org/10.15244/pjoes/90183

116. Millner, P., Bassett, D. & Marsh, P. 1980. Dispersal of Aspergillus fumigatus from Sewage Sludge Compost Piles Subjected to Mechanical Agitation in Open Air. *Applied and Environmental Microbiology*, 39(5): 1000-1009. https://doi.org/10.1128/aem.39.5.1000-1009.1980

117. Mills, J., Gage, K. & Khan A. 2010. Potential influence of climate change on vector-borne and zoonotic diseases: A review and proposed research plan. *Environ Health Perspect*, 118 (11): 1507-1514. https://doi.org/10.1289/ehp.0901389

118. Morgado, M., Jiang, C., Zambrana, J., Upperman, C., Mitchell, C. Boyle, M., Sapkota, A. & Sapkota, A. 2021. Climate change, extreme events, and increased risk of salmonellosis: foodborne diseases active surveillance network (FoodNet), 2004-2014. *Environmental Health*, 20: 105. https://doi.org/10.1186/s12940-021-00787-y

119. Müller, G. 1980. Airborne dissemination of bacteria from sewage treatment plants. *Environmental International*, 3(4): 283-291. https://doi.org/10.1016/0160-4120(80)90139-7

120. Mulloy, K. 2001. Sewage workers: toxic hazards and health effects. *Occupational Medicine*, 16(1): 23-38. PMID: 11107222

121. Mumy, L. K. 2014. *Salmonella*, Editor (s), Philip Wexler, Encyclopedia of Toxicology (Third Edition), Academic Press, pp. 211-212. https://doi.org/10.1016/B978-0-12-386454-3.00537-6

122. Nolan, S., Thorn, C., Ashekuzzaman, S., Kavanagh, I., Nag, R., Bolton, D., Cummins, E., O'Flaherty, V., Abram, F., Richards, K. & Fenton, O. 2020. Land spreading with co-digested cattle slurry, with or without pasteurization, as a mitigation strategy against pathogen, nutrient and metal contamination associated with untreated slurry. *Science of The Total Environment*, 744(140841), 1-15. https://doi.org/10.1016/j.scitotenv.2020.140841

123. Ogden, L., Fenlon, D., Vinten, A. & Lewis, D. 2001. The fate of *Escherichia coli* O157 in soil and its potential to contaminate drinking water. *International Journal of Food Microbiology*, 66(1-2): 111-7. https://doi.org/10.1016/S0168-1605(00)00508-0

124. Oliveira, C., Carvalho, L. & Garcia, T. 2006. Experimental airborne transmission of *Salmonella* Agona and *Salmonella* Typhimurium in weaned pigs. *Epidemiology and Infection*, 134(1): 199-209. https://doi.org/10.1017/S0950268805004668

125. O'Neill, W., Cooke, R.P., Plumb, H. & Kennedy, P. 2003. ABC chromogenic agar: a cost-effective alternative to standard enteric media for *Salmonella* spp. isolation from routine stool samples. *British Journal of Biomedical Science*, 60(4): 187-190. https://doi.org/10.1080/09674845.2003.11783697

126. Oppliger, A., Charriere, N., Droz, P. & Rinsoz, T. 2008. Exposure to bioaerosols in poultry houses at different stages of fattening use of real time PCR for airborne bacterial quantification. *The Annals of Occupational Hygiene*, 52 (5): 405-412. https://doi.org/10.1093/annhyg/men021

127. Ossowska-Cypryk, K. 1991. Application of indicator microorganisms for the assessment of air pollution level in the vicinity of the industrial waste water treatment plant. *Gaz, Woda i Technika Sanitarna*, 5:105 [in Polish].

128. Pal, A., Riggs, M., Urrutia, A., Osborne, R., Jackson, A., Bailey, M., Macklin, K., Price, S., Buhret, R. & Bourassa, D. 2021. Investigation of the potential of aerosolized *Salmonella* Enteritidis on colonization and persistence in broilers from day 3 to 21. *Poultry Science*, 100(12): 101504. https://doi.org/10.1016/j.psj.2021.101504

129. Paluszak, Z., Ligocka, A. & Breza-Boruta B. 2003. Effectiveness of sewage treatment based on selected fecal bacteria elimination in municipal wastewater treatment plant in Toruń. *Polish Journal of Environmental Studies*, 12(3): 345-349.

130. Pearce, R., Sheridan, J. & Bolton, D. 2006. Distribution of airborne microorganisms in commercial pork slaughter process. *International Journal of food Microbiology*, 107(2): 186-19. https://doi.org/10.1016/j.ijfoodmicro.2005.08.029

131. Pepper, I. & Gebra, C. 2015. Aeromicrobiology, Chapter 5, pp. 89-110, In: Pepper I., Gerba C. & Gentry T. Environmental Microbiology, 3rd edition, Academic Press Publishers, Waltham, MA. https://doi.org/10.1016/B978-0-12-394626-3.00005-3

132. Pillai, S. 2007. Bioaerosols from land-applied biosolids: issues and needs. *Water Environment Research*, 79(3): 270-278. https://doi.org/10.2175/106143007x156763

133. Pillai, S., Widmer, K., Dowd, S. & Ricke, S. 1996. Occurrence of airborne bacteria and pathogen indicators during land application of sewage sludge. *Applied and Environmental Microbiology*, 62(1): 296-299. https://doi.org/10.1128/aem.62.1.296-299.1996

134. Podolak, R., Enache, E., Stone, W., Black, D. & Elliott, P. 2010. Sources and risk factors for contamination, survival, persistence, and heat resistance of Salmonella in low-moisture foods. *Journal of Food Protection*, 73(10): 1919-1936. https://doi.org/10.4315/0362-028X-73.10.1919

135. Prazmo, Z. 1980. *Salmonella* in municipal sewage for agricultural use. *Przegląd Epidemiologiczny*, 34: 147-153 [in Polish].

136. Putaud, J., Van Dingenen, R., Alastuey, A., Bauer, H., Birmili, W., Cyrys, J., Flentje, H., Fuzzi, S., Gehrig, R.H,. Hansson, C., Harrison, R.M., Herrmann, H., Hitzenberger, R., Hüglin, C., Jones, A.M., Kasper-Giebl, A., Kiss, G., Kousa, A., Kuhlbusch, T.A.J., Löschau, G., Maenhaut, W., Molnar, A., Moreno, T., Pekkanen, J., Perrino, C., Pitz, M., Puxbaum, H., Querol, X., Rodriguez, S., Salma, I., Schwarz, J., Smolik, J., Schneider, J., Spindler, G., ten Brink, H., Tursic, J., Viana, M., Wiedensohler, A. & Raes, F. 2010. A European aerosol phenomenology – 3: Physical and chemical characteristics of particulate matter from 60 rural, urban, and kerbside sites across Europe. *Atmospheric Environment*, 44(10): 1308-1320. https://doi.org/10.1016/j.atmosenv.2009.12.011

137. Rambach, A. 1990. New plate medium for facilitated differentiation of *Salmonella* spp. from Proteus spp. and other enteric bacteria. *Applied and Environmental Microbiology*, 56(1): 301-303. https://doi.org/10.1128/aem.56.1.301-303.1990

138. Reina, M., Urrutia, A., Figueroa J., Riggs M, Macklin K., Buhr R., Price S. & Bourassa D. 2024. Application of pressurized steam and forced hot air for cleaning broiler transport container flooring. *Poultry Science*, 103(2): 103276. https://doi.org/10.1016/j.psj.2023.103276

139. Ritz, C., Mitchell, B., Fairchild, B., Czarick, M. & Worley, J. 2006. Improving in-house air quality in broiler production facilities using an electrostatic space charge system. *Journal of Applied Poultry Research*, 15(2): 333-340. https://doi.org/10.1093/japr/15.2.333

140. Ruiz-Gil, T., Acuña, J., Fujiyoshi, S., Tanaka, D., Noda, J., Maruyama, F. & Jorquera, M. 2020.Airborne bacterial communities of outdoor environments and their associated influencing factors. *Environment International*, 145: 106156. https://doi.org/10.1016/j.envint.2020.106156

141. Russ, C. & Yanko, W. 1981. Factors affecting salmonellae repopulation in composted sludges. *Applied and Environmental Microbiology*, 41(3): 597-602. https://doi.org/10.1128/aem.41.3.597-602.1981

142. Sánchez-Monedero, M., Aguilar, M., Fenoll, R. & Roig, A. 2008. Effect of the aeration system on the levels of airborne microorganisms generated at wastewater treatment plants. *Water Research*, 42(14): 3739-3744. https://doi.org/10.1016/j.watres.2008.06.028

143. Santos, P., Figueiredo, H., da Silva, L., da Silva, R, Cardoso, G., Moraes, C. & Rodrigues, A. 2021. Evaluation of a rapid detection method of Salmonella in comparison with the culture method and microbiological quality in fish from the Brazilian Amazon. *Food Science & Technology* (Campinas), 41(1): 151-157. https://doi.org/10.1590/fst.38719

144. Sawyer, B., Elenbogen, G., Rao, K., O`Brien, P., Zenz, D. & Lue-Hing, C. 1993. Bacterial aerosol emission rates from municipal wastewater aeration tanks. *Applied and Environmental Microbiology*, 59(10): 3183-3186. https://doi.org/10.1128/aem.59.10.3183-3186.1993

145. Scarpino, P. 1975. Human enteric viruses and bacteriophages as indicators of sewage pollutions, P. 49-61, In: Gamegon ALH (Ed.), Discharge of sewage from sea outfalls, Pergamon Press, Oxford.

146. Seedorf, J., Hartung, J., Schroder, M., Linkert, K., Phillips, V., Holden, M., Sneath, R., Short, J., White, R., Pedersen, S., Takai, H. Johnsen, J.O., Metz, J.H.M., Groot Koerkamp, P.W.G., Uenk, G.H., & Wathes, C.M. 1998. Concentrations and emissions of airborne endotoxins and microorganisms in livestock buildings in Northern Europe. *Journal of Agricultural Engineering Research*, 70(1): 97-109. https://doi.org/10.1006/jaer.1997.0281

147. Sekla, L., Gemmill, D., Monfreda, J., lysyk, M., Stackiw, W, Kay, C., Hopper C., van Buckenhoutm, L. & Eibisch, G. 1980. Sewage treatment plant workers and their environment; a health study, pp. 281-294, In: Pahren, H. & Jakubowski, W. (eds), *Wastewater Aerosols and Diseases*, US-EPA, Ohio, Cincnnati, Sept. 19-21.

148. Shaji, S., Selvaraj, R. & Shanmugasundaram, R. 2023. *Salmonella* infection in poultry: A review on the pathogen and control strategies. *Microorganisms*, 11(11): 2814. https://doi.org/10.3390/microorganisms11112814

149. Shuval, H., Fattal, B. & Yekutiel, P. 1986. State of the art review: An epidemiological approach to the health effects of wastewater reuse. *Water Science and Technology*, 18(9): 147-162. https://doi.org/10.2166/wst.1986.0087

150. Sidhu, J., Gibbs, R., Ho, G. & Unkovich, I. 2001. The role of indigenous microorganisms in suppression of *Salmonella* regrowth in composted biosolids. *Water Research*, 35(4): 913-920. https://doi.org/10.1016/S0043-1354(00)00352-3

151. Sidhu, J. & Toze S. 2009. Human pathogens and their indicators in biosolids: a literature review. *Environment International*, 35(1): 187-201. https://doi.org/10.1016/j.envint.2008.07.006

152. Skórska, C., Sitkowska, J., Krysińska-Traczyk, E., Cholewa, G. & Dutkiewicz, J. 2005. Exposure to airborne microorganisms, dust and endotoxin during processing of peppermint and chamomile herbs on farms. *Annals of Agricultural and Environmental Medicine*, 12(2): 281-288. PMID: 16457486

153. Smets, W., Moretti, S., Denys, S. & Lebeer, S. 2016. Airborne bacteria in the atmosphere: Presence, purpose, and potential. *Atmospheric Environment*, 139: 214-221. https://doi.org/10.1016/j.atmosenv.2016.05.038

154. Sorber, C. & Guter, K. 1975. Health and hygiene aspects of spray irrigation. *American Journal of Public Health*, 65(1): 47- 52. https://doi.org/10.2105/ajph.65.1.47

155. Sorber, C. & Sagik, B. 1979. Indicators and pathogens in wastewater aerosols and factors affecting survivability, pp 23-35, In: Pahren, H. & Jakubowski, W. (eds), *Wastewater Aerosols and Diseases*, US-EPA, Ohio, Cincnnati, Sept. 19-21.

156. Stackhouse, R., Faith, N., Kaspar, C., Czuprynski, C. & Wong, A. 2012. Survival and virulence of Salmonella enterica serovar Enteritidis filaments induced by reduced water activity. Applied *and* Environmental Microbiology, 78(7): 2213-2220. https://doi.org/10.1128/AEM.06774-11

157. Stärk, K. 1999. The role of infectious aerosols in disease transmission in pigs. *The Veterinary Journal*, 158(3): 164-181. https://doi.org/10.1053/tvjl.1998.0346

158. Stetzenbach, L. 2009. Airborne infectious microorganisms. *Encyclopedia of Microbiology*, 175-182. https://doi.org/10.1016/B978-012373944-5.00177-2

159. Straub, T., Pepper, I. & Gerba, G. 1993. Hazards from pathogenic microorganisms in land deposed sewage sludge. *Reviews of Environmental Contamination and Toxicology*, 132: 55-91. https://doi.org/10.1007/978-1-4684-7065-9_3

160. Tang, J. 2009. The effect of environmental parameters on the survival of airborne infectious agents**.** *Journal of the Royal Society Interface*, 6(Suppl 6): S737-46. https://doi.org/10.1098/rsif.2009.0227.focus

161. Tanner, B. 2004. Aerosolization of microorganisms and risk of infection from reuse wastewater residuals. Ph.D Dissertation, graduate school of medicine, University of Arizon, Tucson, AZ.

162. Teltsch, B., Kedmi, S., Bonnet, L., Borenzstajn–Rotem Y. & Katzenelson, E. 1980. Isolation and identification of pathogenic microorganisms at wastewater irrigated fields: ratio in air and wastewater. *Applied* and *Environmental Microbiology*, 39(6): 1183-1190. https://doi.org/10.1128/aem.39.6.1183-1190.1980

163. Thomas, G., Paradell Gil, T., Müller, C., Rogers, H., Berger, C. 2024. From field to plate: How do bacterial enteric pathogens interact with ready-to-eat fruit and vegetables, causing disease outbreaks? *Food Microbiology*, 117: 104389. https://doi.org/10.1016/j.fm.2023.104389

164. Venter, P., Lues J. F. R., Theron H. 2004. Quantification of bioaerosols in automated chicken egg production plants. *Poultry Science*, 83(7): 1226-1231. https://doi.org/10.1093/ps/83.7.1226

165. Vilanova, X. & Blanch, A. 2005. Distribution and persistence of fecal bacterial populations in liquid and dewatered sludge from a biological treatment plant. *The Journal of General and Applied Microbiology*, 51(6): 361-368. https://doi.org/10.2323/jgam.51.361

166. Vought, K. & Tatini, S. 1998. *Salmonella* enteritidis contamination of ice cream associated with a 1994 multistate outbreak. *Journal of Food Protection*, 61(1): 5-10. https://doi.org/10.4315/0362-028x-61.1.5

167. Wang, S., Yeh, D. & Wei, C. 2009. Specific PCR primers for the identification of *Salmonella enterica* serovar enteritidis in chicken-related samples. *Journal of Food and Drug Analysis*, 17(3): 183-189. https://doi.org/10.38212/2224-6614.2612

168. Wang, B., Butler D., Hamblin M. & Monack D. 2023a. One species, different diseases: the unique molecular mechanisms that underlie the pathogenesis of typhoidal Salmonella infections. *Current Opinion in Microbiology* 72: 102262. https://doi.org/10.1016/j.mib.2022.102262

169. Wang, J., Vaddu, S., Bhumanapalli, S., Mishra, A., Applegate, T., Singh, M. & Thippareddi, H. 2023b. A systematic review and meta-analysis of the sources of *Salmonella* in poultry production (pre-harvest) and their relative contributions to the microbial risk of poultry meat. *Poultry Science*, 102(5): 102566. https://doi.org/10.1016/j.psj.2023.102566

170. Wathes, C. 1995. Bioaerosols in animal houses, pp. 547-577. In: Cox, C. & Wathes, C. (eds). *Bioaerosols Handbook*, Boca Roton, FL: CRC press.

171. Wathes, C., Zidan, W., Pearson, G., Hinton, M & Todd, N. 1988. Aerosol infections of calves and mice with *Salmonella typhimurium*. *Veterinary Research*, 123(23): 590-594. PMID: 3062881

172. Werber, D., Dreesman, J., Feil, F., Van Treeck, U., Fell, G., Ethelberg, S., Hauri, A.M., Roggentin, P., Prager, R., Fisher, I.S.T., Behnke, S.C., Bartelt, E., Weise, E., Ellis, A., Siitonen, A., Andersson, Y., Tschäpe, H., Kramer, M.H. & Ammon, A. 2005. International outbreak of Salmonella Oranienburg due to German chocolate. *BMC Infectious Diseases*, 5: 7. https://doi.org/10.1186/1471-2334-5-7

173. Wéry, N. 2014. Bioaerosols from composting facilities – a review. *Frontiers in Cellular and Infection Microbiology*, 4: 42. https://doi.org/10.3389/fcimb.2014.00042

174. Xie, W., Li, Y., Bai, W., Hou, J., Ma, T., Zeng, X., Zhang, L. & An, T. 2021. The source and transport of bioaerosols in the air: A review. *Frontiers of Environmental Science & Engineering*, 15(3): 44. https://doi.org/10.1007/s11783-020-1336-8

175. Zeng, D., Chen, Z., Jiang, Y., Xue, F. & Li, B. 2016. Advances and challenges in viability detection of foodborne Pathogens. *Frontiers in Microbiology*, 7: Article 1833. https://doi.org/10.3389/fmicb.2016.01833

176. Zhang, B. 2020.The effect of aerosols to climate change and society. *Journal of Geoscience and Environment Protection*, 8(8): 55-78. https://doi.org/10.4236/gep.2020.88006

177. Zhang, J, Li, Y., Xu, E., Jiang, L., Tang, J., Li, M., Zhao, X., Chen, G., Zhu, H., Yu, X, & Zhang, X. 2019. Bacterial communities in PM2.5 and PM10 in broiler houses at different broiler growth stages in spring. *Polish Journal of Veterinary Sciences*, 22(3): 495-504. https://doi.org/10.24425/pjvs.2019.129957

178. Zhao, Y. 2011. Effectiveness of multi-stage scrubbers in reducing emissions of air pollutants from pig houses. *Transactions of the ASABE*, 54(1): 285-293. https://doi.org/10.13031/2013.36256

179. Zhao, Y., Aarnink, A., De Jong, M. & Groot Koerkamp, P. 2014. Airborne microorganisms from livestock production systems and their relation to dust. *Critical Reviews in*

Environmental Science and Technology, 44(10): 1071-1128. https://doi.org/10.1080/10643389.2012.746064

180. Zucker, B., Trojan, S. & Muller, W. 2000. Airborne gram negative bacterial flora in animal houses. *Journal of Veterinary Medicine Series B-Infectious Diseases and Veterinary Public Health*, 47(1): 37-46. https://doi.org/10.1046/j.1439-0450.2000.00308.x

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