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Molecular phylogenetics of some *Orchis* species (Orchidaceae) native to Turkey using nuclear and chloroplast DNA sequences

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Abstract

Phylogenetic relationships among nine Turkish *Orchis* species were inferred from variation in the internal transcribed spacer and maturase Kinase regions. Foreign sequences were also retrieved from NCBI to increase interspecific sampling. The topology of ITS tree was broadly congruent to that of matK tree. The trees showed two major clades; the first one included species from *Orchis* and *Neotinea* subgenera and the second included species of *Anacamptis* subgenus. Phylogenetic separation of some species found in *Neotinea* and *Anacamptis* subgenera were previously reported, and the current study also indicated that moving these species into *Neotinea* and *Anacamptis* genera could be reasonable.

1. INTRODUCTION

Orchidaceae family includes about 800 genera and 25000 species, most of which are in the tropical regions [1]. The family attracts attentions to study evolutionary patterns because of its diverse habitats and specialized pollination systems [2]. Genus *Orchis* L. is in this family and diagnosed by a basal rosette and terminal, unbranched inflorescence that is composed of various sized resupinate flowers. This genus is represented by 25 species in Turkey [3], and underground tubers of the genus are used to make hot beverage sahlab and ice-cream. Around 20 million *Orchis* corms are collected from naturally growing populations in Turkey [4].

Different researchers have studied morphological, anatomical, histological, ecological, and karyological properties of the genus [5-6-7-8]. However, only a small number of studies exists covering phylogenetic relationships among them. DNA sequences of specific regions have been used in order to indicate phylogeny among *Orchis* species [9-10-11-12-13]. In this study, nine *Orchis* species that are naturally living in Van province of Turkey were used to figure out evolutionary relationships and delimit border of the species.

We preferred two regions; internal transcribed spacer (ITS; ITS1 intergenic spacer+5.8S gene+ITS2 intergenic spacer) region located in nuclear DNA and a maturase-encoding gene (matK) region located in chloroplast DNA. ITS of 18S–26S nuclear ribosomal DNA (nrDNA) is suitable for molecular systematic studies [9] due to its variability at the species level. The matK gene region located in the intron of the transfer RNA gene for lysine is also widely used to resolve the taxonomic problems of closely related genera [14].

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Main objectives of the current study were (i) to shed further light on the systematics and evolutionary structure of nine *Orchis* species living in Van-Turkey by using sequence diversity of the ITS and matK regions (ii) to figure out phylogenetic relationships among different subgenera, sections and subsections including native and foreign *Orchis* taxa (iii) to understand usefulness of the regions for phylogeny of *Orchis* genus.

2. MATERIALS and METHODS

2.1. Plant Samples

To construct the phylogenetic relationships of the *Orchis* genus, we analyzed sequences of ITS and matK regions from a total of 18 specimens, representing 9 species. Species were included in the three different subgenera; *Orchis* L., *Neotinea* (Reichb. f.) P. Quentin, and *Anacamptis* (Rich.) P. Quentin (Table 1). Samples were collected from Van province of Turkey and identified according to the diagnostic morphological characteristics described in the Flora of Turkey and the East Aegean Islands [15]. For each species, 2 accessions were collected (Table 1) and preserved in plastic bags with silica gel until DNA extraction. To increase the interspecific sampling, additional sequences (seventeen ITS sequences and only one matK sequence; Appendix 1) were retrieved from NCBI database. These foreign sequences were intentionally included in the study to demonstrate phylogeny of them with representative taxa in Turkey and to figure out evolutionary relationships among *Orchis* species found in different subgenus, sections and subsections. Species from *Disa* genus was chosen as outgroup (AJ000131; ITS and DQ415024; matK).

Table 1. *Species of Orchis used in the study, collected number of samples for each taxon, their locations and accession numbers*

	Species	# of sample	Subgenus/ Section/ Subsection[a]	Location	Accession # (ITS)[b]	Accession # (matK)[b]
O1	*O. collina* Banks & Solander	2	*Orchis/ Orchis/ Patentes*	Van, Gevas	KU697368	KU697377
O2	*O. simia* Lamarck	2	*Orchis/ Orchis/ Orchis*	Van, Gevas	KU697369	KU697378
O3	*O. anatolica* Boissier	2	*Orchis/ Orchis/ Pusillae*	Van, Gevas	KU697370	KU697379
O4	*O. pinetorum* Boissıer & Kotsch.	2	*Orchis/ Orchis/ Masculae*	Van, Gevas	KU697371	KU697380
O7	*O. tridentata* Scopolı	2	*Neotinea/*	Van, Gevas	KU697372	KU697381
O8	*O. spitzelii* Sauter Ex W.D.J. Koch	2	*Orchis/ Orchis/ Patentes*	Van, Gevas	KU697373	KU697382
O9	*O. coriophora* L.	2	*Anacamptis/ Coriophorae/*	Van, Gevas	KU697374	KU697383
O10	*O. pseudolaxiflora* Czernıakovska	2	*Anacamptis/ Platycheilae/Laxiflorae*	Van, Gevas	KU697375	KU697384
O11	*O. palustris* Jacq.	2	*Anacamptis/ Platycheilae/Laxiflorae*	Van	KU697376	KU697385

[a]Subgenus, Section and Subsection were given according to QUENTIN P. [16]
[b]DNA sequences of 2 samples of one taxa were identical. Therefore, only one of them was submitted to the NCBI database.

2.2. DNA isolation, PCR, Sequencing, Sequence Alignment and Phylogenetic Analyses

Total genomic DNA was isolated from fresh leaf tissues using the cetyltrimethylammonium bromide (CTAB) method [17]. The purity and quantity of extracted DNA were determined by NanoDrop 2000c

UV–Vis Spectrophotometer (Thermo Scientific). Amplification of ITS region was carried out by using primer pairs ITSL 5'TCG TAA CAA GGT TTC CGT AGG TG3', (forward)/ITS4 5' TCC TCC GCT TAT TGA TAT GC 3' (reverse) [18]. Primer pair F1 5' ACT GTA TCG CAC TAT GTA TCA 3' and R3 5' GAT CCG CTG TGA TAA TGA GA 3' was used to amplify matK region [19].

DNA amplification was performed in a 25 µl volume mixture containing genomic DNA (10 ng/µl), 10X PCR Buffer, MgCl2 (25 mM), dNTP mixture (10 mM), selected primer pair (10 µM), Taq polymerase (5u/µl) and sterile water. PCR reaction of each region consisted of almost same amount of Buffer (2.5 µl), MgCl2 (2 µl), dNTP (1 µl), and Taq polymerase (0.25µl). Amount of each primer was 1 µl for ITS and 2 µl for matK region. 2 and 3 µl diluted DNA were added into PCR mixtures of ITS and matK regions, respectively. For each region, PCR amplification was started with 5 min initial denaturation at 94 °C, and terminated with 7 min at 72 °C. Each reaction ended with a final 4°C hold step and consisted of 30 cycle numbers. Each reaction cycle consisted of denaturation step at 94 °C for 30 sec (1 min), annealing step at 53 °C (55 °C) for 30 sec (1 min), and elongation step at 72 °C for 30 sec (1 min) (Values in parenthesis were used for matK region and other values were used for ITS region).

Amplicons were visualized by electrophoresis on 1–1.5 % agarose gels. After purification, products were sequenced in both directions using ABI 3730XL automated sequencer (Applied Biosystems, Foster City, CA, USA). All sequence chromatograms were opened using Finch TV (http://www.geospiza.com/finchtv/). Alibee Multiple Alignment 3.0 software from the GeneBee website (www.genebee.msu.su/genebee.html) was used to assemble complementary strands and verify software base-calling. Ambiguous sites were checked manually and corrected by comparing the strands. Sequences of ITS and matK regions have been deposited in NCBI database for further studies (accession numbers were indicated in Table 1).

Total nucleotide length (base pair, bp), GC content (%), number of deletion/insertion (indel), parsimony informative (variable) sites of both regions were calculated by using Molecular Evolutionary Genetics Analysis software (MEGA 5.0; [20]. Sequences taken from the current study were combined with the foreign sequences downloaded from NCBI database and analyzed together. The sequence data was analyzed by using the Maximum Likelihood (ML) method based on the Tamura-Nei model [21] and bootstrap analysis with 500 replications [22]. The tree with the highest log likelihood was shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then, the topology with superior log likelihood value was selected. All positions containing gaps and missing data were eliminated.

3. RESULTS and DISCUSSION

3.1. Results

The ITS alignment composed of 18 accessions of 9 native *Orchis* species and 17 foreign sequences downloaded from NCBI. The length of the ITS region ranged from 621 bp (*O. ustulata* L.*) to 643 bp (*O. pallens* L.*, *O. mascula* L.* and *O. pinetorum*) (* indicates samples taken from NCBI) with a GC content from 43% (*O. tridentata**) to 52 % (*O. spitzelii*). For Turkish *Orchis* species, which are the main focus of this paper, ITS ranged from 629 bp (*O. tridentata*) to 643 bp (*O. pinetorum*) and GC content from 46% (*O. tridentata*) to 52% (*O. spitzelii*). 213 polymorphic sites with 191 parsimony informative sites were detected in 657 bp aligned sequence (Table 2), and as expected variation sites was reduced to 193 when only Turkish *Orchis* species were analyzed. In the aligned sequence, about 60 indel positions were detected. We excluded the second sequence of the same species from the data matrices after verification that their introduction would not change the results.

Table 2. *Estimated molecular diversity parameters for ITS nrDNA and matK cpDNA regions. All of the values were estimated with foreign sequences retrieved from NCBI database*

	ITS (ITS1+5.8S+ITS2)	matK
Number of taxa	17	10
Number of sequences	35	10
Total length (bp)	657	1317
Variable sites	213	109
P. informative sites	191	109
Number of indels (bp)	61	21
G/C content (%)	48	30
Mean Distance (Divergence)	0.11	0.035

The border of the ITS subunits was determined using several sequences (HQ657131, AY699977, AY014549) retrieved from NCBI database. According to the reference sequences, ITS1 subunit length was 255 bp, 5.8S subunit was 153 bp, and ITS2 subunit was 249 bp. 5.8S subunit was the most conservative area, 6 substitutions and no indel were detected. ITS1 and ITS2 subunits showed lots of nucleotide variations and indels (data was not shown). Mean divergence was calculated as 0.11, the highest value (0.218) was observed between sequences of *O. spitzelii* and *O. collina**.

The sequences of native *Orchis* species and those of their representatives taken from NCBI were not identical; at least two point mutations were seen in the sequence of ITS region. Only two sequences [*O. anthropophora* L.* (Z94059+Z94060) and *O. anthropophora** (AY364869)] downloaded from NCBI were identical. All the taxa in the study, with the exclusion of outgroup, were nested together and the ingroup was divided into two major clades with high bootstrap values (Figure 1). The first major clade comprised 5 of Turkish species (*O. spitzelii, O. anatolica, O. pinetorum, O. simia*, and *O. tridentata*) and most of the foreign sequences. This main clade divided in two sister subclades with 100% bootstrap values. The first one included species from *Orchis* subgenus and the second one included species from *Neotinea* subgenus. The first subclade containing species from *Orchis* subgenus, divided into two groups; one included species (*O. spitzelii, O. anatolica, O. quadripunctata*, O. pallens*, O. mascula*, O. pinetorum*) from Patentes Schltr., *Pusillae* (Parl.) E. Klein and *Masculae* Rchb. f. subsections (Figure 1). The second group included species (*O. anthropophora*, O. italic* Poir.**, O. militaris* L.**, O. purpurea* Huds.**, O. simia*) from *Orchis* section and *Orchis* subsection. The second subclade consisted of species (*O. tridentata*, and *O. ustulata**) from *Neotinea* subgenus (Figure 1).

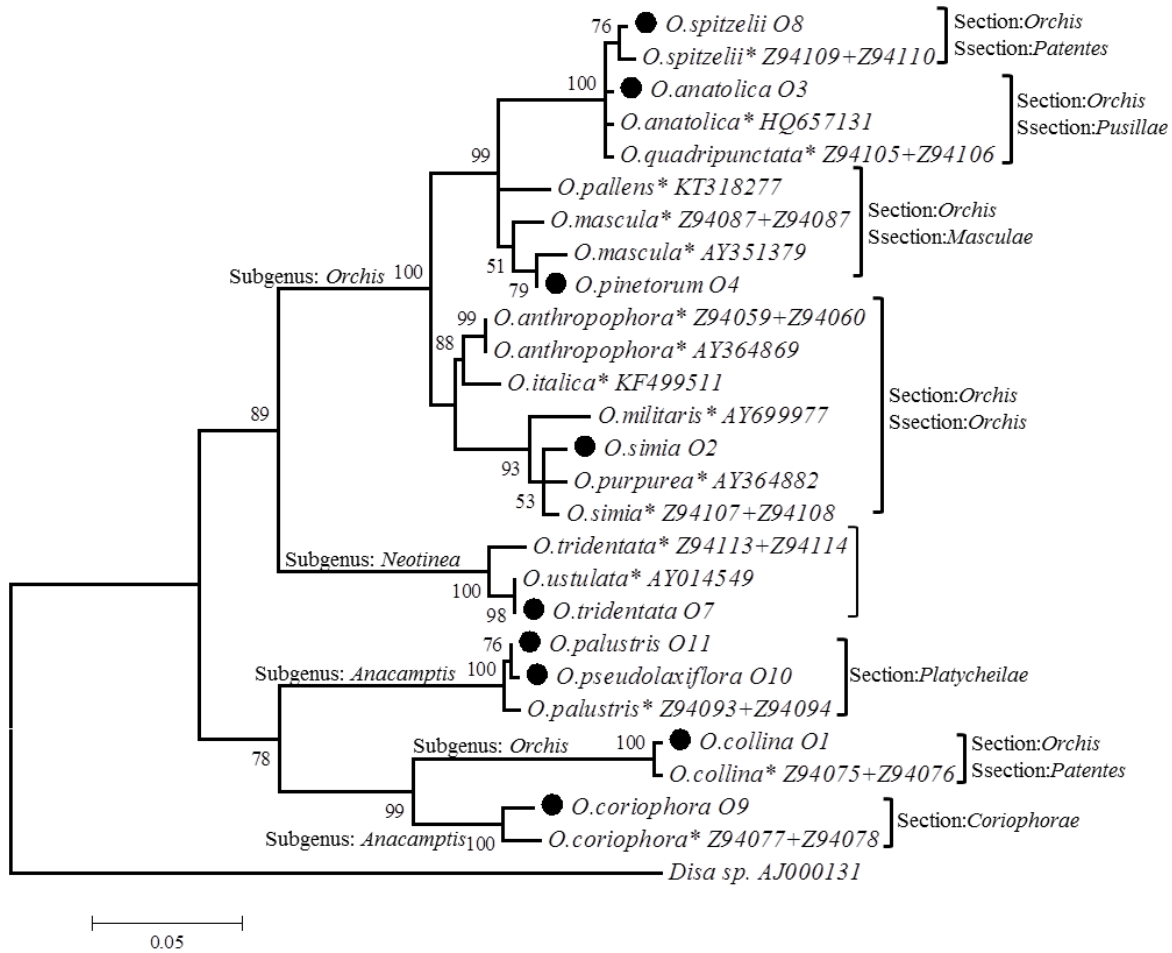

Figure 1. *Phylogenetic tree constructed based on nrDNA ITS sequence with the ML method. Numbers above/below the branches indicate bootstrap values >50% (*species retrieved from NCBI database, Disa sp. is outgroup, and black circles show Orchis species native to Turkey).*

The second major clade included 4 of Turkish *Orchis* species (*O. palustris, O. pseudolaxiflora, O. collina,* and *O. coriophora*) and 3 species from NCBI (*O. palustris*, O. collina** and *O. coriophora**). The major clade divided into two sister subclades with 100% and 99% bootstrap values, respectively. The first subclade included species (*O. palustris* and *O. pseudolaxiflora*) from *Anacamptis* subgenus, and the second included species (*O. collina*, and *O. coriophora*) from *Orchis* and *Anacamptis* subgenus, respectively (Figure 1).

For the analyses of matK region, 18 accessions of 9 native *Orchis* species and only one foreign sequence downloaded from NCBI were used. Alignment of the sequence resulted in a data matrix of 1317 bp nucleotides (Table 2). The length of the region ranged from 1299 bp to 1308 bp (*O. simia*). GC content was about 30%, which was lower compared to the value of ITS region (43-52%). 109 polymorphic sites (parsimony informative) were detected in the aligned sequence, and this number did not change when only Turkish *Orchis* species were analyzed (Table 2). In the aligned sequence 21 indel positions were detected. Mean divergence was calculated as 0.035, the highest value (0.048) was observed between sequences of *O. collina* and *O. tridentata*. As observed in the tree constructed using ITS region, *Orchis* species was divided into two major clades supported with 93% and 100% bootstrap values, respectively (Figure 2). The topology of the ITS tree was broadly congruent to that shown in matK tree (Figure 1 and 2). The first major clade included 5 of Turkish species (*O. spitzelii, O. anatolica, O. pinetorum, O. simia,* and *O. tridentata*) and one foreign sequence (*O. quadripunctata**). *Orchis anatolica* and *O. quadripunctata** showed closer relationship and left species bound this clade with a ladderized sequence of *O. spitzelii, O. pinetorum, O. simia,* and *O. tridentata* (Figure 2). The second major clade included 4 of Turkish *Orchis* species (*O. collina, O. coriophora, O. palustris, O. pseudolaxiflora*). The major clade divided into two sister subclades

with 99% and 100% bootstrap values, respectively. The first subclade included species (*O. collina*, and *O. coriophora*) and the second one included species (*O. palustris* and *O. pseudolaxiflora*) (Figure 2). Close phylogenetic relationships between these species were also seen in the ITS tree (Figure 1).

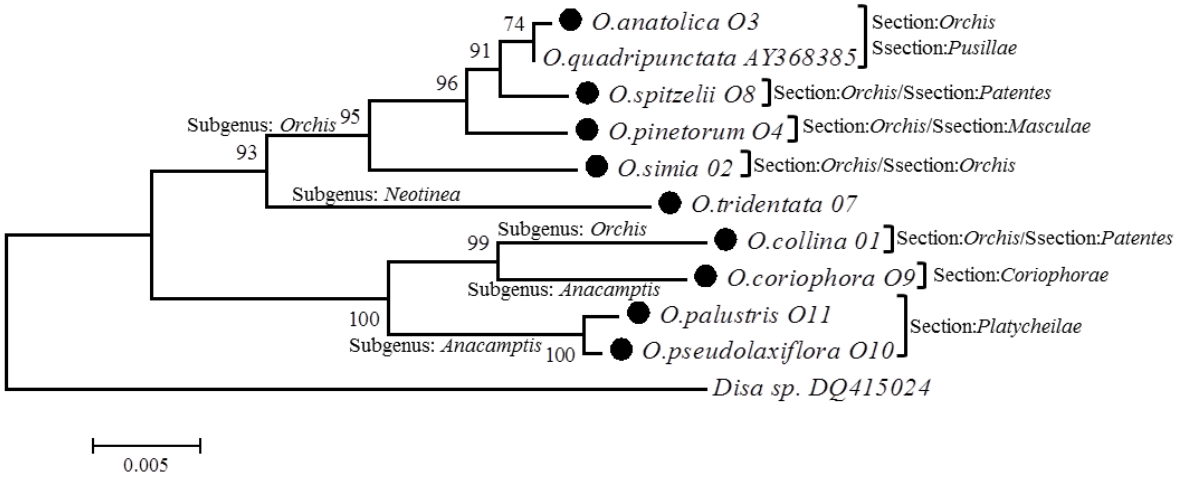

Figure 2. *Phylogenetic tree constructed based on cpDNA matK sequence with the ML method. Numbers above/below the branches indicate bootstrap values >50% (*species retrieved from NCBI database, Disa sp. is outgroup, and black circles show Orchis species native to Turkey).*

3.2. Discussion

Phylogenetic results taken from the sequences of ITS and matK regions were found to be very useful to understand the evolutionary relationships at the levels of species, subsection, section and subgenus of *Orchis*. Genetic variation observed in the matK data was much lower than that of the ITS data, but still sufficient to figure out phylogeny within the genus. There was close relationship between Turkish *Orchis* taxon and its foreign representative in the ITS tree. Bootstrap analysis yielded significant support for the majority of the clades in both trees (Figure 1 and 2). Especially, the major clades and subclades, containing species of same subgenus and sections, showed very high bootstrap values.

All species in *Orchis* subgenus except *O. collina* grouped in a major clade and supported with 95 and 100% bootstrap values in matK and ITS trees, respectively. These major clades divided into two subclades and a few small groups, each of which consisted species from different subsections of *Orchis* section (Figure 1). Phylogenetic separation of *O. collina* from the other species of the same subgenus was also indicated by Haider and his colleagues [23]. They proved distant position of *O. collina* and moved this species into the genus *Anacamptis*. This result was also observed in Figures 1 and 2 where *O. collina* took a position within subgenus *Anacamptis* (second major) clade. When positions of *O. collina* and other species were taken into account, moving this species into the *Anacamptis* genus may be more meaningful. Moreover, we can figure out phylogenetic relationships between subsections using positions of the species in the major clade. For instance, the closest position was observed between *Patentes* and *Pusillae* subsections whereas the furthest one *Orchis* subsection.

Close evolutionary relationships between species *O. spitzelii* (Patentes subsection) and *O. anatolica* / *O. quadripunctata** (*Pusillae* subsection) were observed in ITS tree. This type of relationship was also reported by Aceto and his colleagues [9]. They studied not only phylogeny of *Orchis* genus but also few allied genera based on ITS region and showed close relation between *O. spitzelii* and *O. quadripunctata*. *Orchis anatolica* was not included in their study but position of this species can be seen in another study [12]. They showed close relation between *O. anatolica* and *O. quadripunctata* located in the same subsection. Even though ITS region of *O. anatolica* was studied by these researchers, the sequence was not found in the NCBI database and it was loaded as a result the current study. Therefore, this study would be invaluable to delimit position of the *O. anatolica*. By using these results, it is safe to say that subsection *Patentes* is evolutionarily close to the subsection *Pusillae* compared to the other subsections of *Orchis*

section. Same result and phylogenetic relationship were also seen in the tree constructed by using matK region (Figure 2).

ITS sequences of *O. pallens** and *O. mascula** (*Masculae* subsection) taken from NCBI database grouped with Turkish *O. pinetorum* species. This close relationship was expected since *O. pinetorum* was accepted as a synonym of *O. mascula* (www.theplantlist.org/tpl1.1/record/kew-143077, www.gbif.org/species/2849455). Close relationship between *O. pinetorum* and *O. mascula* could not be proved based on matK region because only two useless (short) sequences of *O. mascula* were available in NCBI database. DNA sequence of *O. pinetorum* is not available in the NCBI database. Therefore, ITS and matK sequences of this species were introduced for the first time to the literature by this study.

All used species (*O. anthropophora**, *O. italica**, *O. militaris**, *O. purpurea**, *O. simia*) of *Orchis* subsection separated from the other subsections of *Orchis* section. These relationships matched those obtained by Cozzolino et al [12] on nuclear sequences. In their study, *O. italica* and *O. anthropophora* species collapsed at terminal and *O.militaris, O. purpurea*, and *O. simia* grouped together. When we summarize all of results discussed up to now, it is clear that species found in *Orchis* subgenus separated from those of *Neotinea* and *Anacamptis* subgenera with relatively long molecular branches (Figure 1 and 2). Species of *Neotinea* subgenus (*O. tridentata*, and *O. ustulata**) grouped together and located more closely to the *Orchis* clade. This position proved that *Neotinea* subgenus is phylogenetically closer to the *Orchis* subgenus according to the *Anacamptis* subgenus. Aceto et al [9] and Cozzolino et al [12] showed close relationships among *O. tridentata, O. ustulata* and *Neotinea maculata*. Bateman and his coworkers [24-25] called *O. tridentata* as the synonym of *Neotinea tridentata* (Scop.) R.M.Bateman, Pridgeon & M.W.Chase and *O. ustulata* as the synonym of *Neotinea ustulata* L. based on sequence of ITS region. Additionally, Haider et al [23] studied phylogeny of Orchidaceae species based on ISSRs and concluded that the species *O. tridentata* was the most genetically distant species in the genus. This species was reclassified by the Kew Gardens and regarded as *Neotinea tridentata*. All these results were considered, moving of *O. tridentata*, and *O. ustulata* species to the genus *Neotinea* is seen meaningful.

Four of Turkish *Orchis* species (*O. coriophora, O. palustris, O. pseudolaxiflora, O. collina*) were phylogenetically separated from species of *Orchis* and *Neotinea* subgenera. Their separation is not unexpected since they were claimed to be species of *Anacamptis* genus by Bateman et al [25]. They used lots of specimens of different genera (*Anacamptis, Serapias, Ophrys, Orchis*, and *Barlia*) and concluded that *O. coriophora* is a synonym of *Anacamptis coriophora* (L.) and *O. palustris* is a synonym of *Anacamptis palustris* (Jacquin) R.M.Bateman. *O. pseudolaxiflora* is also accepted as a synonym of *Anacamptis laxiflora* (Lam.) R.M. Bateman (http://www.theplantlist.org/tpl1.1/record/kew-143110). Moving of these species into the *Anacamptis* genus is considered essential in the light of these results.

Phylogenetic relationships among *Orchis* species and moving some of them into the *Anacamptis* and *Neotinea* genera were studied by several researchers using ITS region. The current study is significant since we preferred a region from chloroplast DNA (matK) in addition to ITS region to compare availability of chloroplast and genomic DNA regions. Studying phylogenetic relationships among *Orchis* species can be regarded as a useful tool that helps to evaluate previous classifications and decide usefulness of the nrDNA ITS and cpDNA matK regions. The congruence between topologies of ITS and matK trees may suggest that hybridization was not a dominant force in the evolution of the *Orchis* and allied genera. Higher genetic variation of ITS region compared to matK may be originated from fast concerted evolution and crossing over.

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CONFLICT OF INTEREST

No conflict of interest was declared by the authors.

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APPENDICES

Appendix 1: DNA sequences taken from NCBI database to increase the interspecific sampling

ITS region: *O.anatolica* HQ657131, *O.militaris* AY699977, *O.mascula* AY351379, *O.italica* KF499511, *O.ustulata* AY014549, *O.purpurea* AY364882, *O.anthropophora* AY364869, *O.pallens* KT318277, *O.mascula* Z94087+Z94087 (ITS1+ITS2), *O.anthropophora* Z94059+Z94060, *O.collina* Z94075+Z94076, *O.palustris* Z94093+Z94094, *O.simia* Z94107+Z94108, *O.tridentata* Z94113+Z94114, *O.coriophora* Z94077+Z94078, *O.spitzelii* Z94109+Z94110, *O.quadripunctata* Z94105+ Z94106

matK region: *O.quadripunctata* AY368385