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Morphological Variation, ITS and EF-1α Gene Nucleotide Polymorphism in *Gyromitra esculenta* (Discinaceae)

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Abstract
Gyromitra esculenta (Pers.) Fr. has many morphologically and microscopically different specimens. Sometimes these morphologically different specimens are called as a different taxon. In this study a collection of 143 *Gyromitra esculenta* specimens were analyzed using macroscopic and microscopic features. Then ten groups were obtained which are potentially confusable because of morphological and microscopical variations. nrDNA ITS and translation elongation factor (EF-1α) genes are used to determine molecular differentiations between these groups. *Morchella* Dill. ex Pers. spp. were used as outgroups. nrDNA ITS region of ten different groups give three phylogenetically distinct groups. Between these groups nucleotide polymorphism ratio is nearly % 1. EF-1α gene doesn't consist of any polymorphism and not informative. Also intraspecific ITS nucleotide polymorphism ratio in *Gyromitra infula* (Schaeff.) Quél. and *Gyromitra gigas* (Krombh.) Cooke which are taken from Genbank is nearly %1 too. So, we conclude ecological factors cause morphological and microscopical variants of *Gyromitra esculenta*.

Keyword: *Gyromitra*, ITS, EF-1α, polymorphism

INTRODUCTION

Gyromitra Fr. taxa are famous and called as "kuzugöbeği ebesi" in Turkey. They are easily recognized with brain shaped ascocarps in field. They generally grow in spring and autumn with the same habitat of morels. It is poisonous and causes gyromitrin syndrome. *Gyromitra esculenta* is known as systematically problematic because of the variable morphological and microscopical characters. But it is not known that these morphological and microscopical variations are because of environmental conditions or genetically. These morphologically different specimens cause misidentified and sometimes called as a different taxon.

Recent advances in molecular biology provide a convenient and rapid assessment of the differences in the genetic composition of the related individuals using DNA sequence data [1]. Therefore, molecular data may resolve the systematic problems or may provide a robust support to the traditional studies on the systematic problems [2]. Nuclear ribosomal RNA genes provide markers for retrieving phylogeny at a variety of taxonomic levels [3]. The internal transcribed spacer (ITS) region and many other molecular markers have been successfully used in numerous plant and fungal systematic studies at the family, generic, and specific levels [4-6].

The aim of this study is to investigate morphological and microscopical differences are meaningful in *G. esculenta* and molecular basis of this differences.

MATERIALS AND METHODS

Fungi Samples and DNA Extractions

The 143 *Gyromitra esculenta* specimens included in the present study were collected and photographed in the spring of 2010 and 2011 from different localities of Muğla province and dried for subsequent analysis. After macroscopic and microscopic studies in the fungarium, ten groups were obtained which are potentially confusable because of morphological and microscopical variations (Figure 1). Total genomic DNA was extracted following a CTAB (hexadecyltrimethyl-ammonium bromide, Sigma Chemical Co., St. Louis, MO) protocol [7]. Sample DNAs were diluted to 25 ng/µl. Stock DNAs were kept at – 20 ^{o}C.

PCR Amplifications and Sequencing

All PCR and sequencing primers are listed in Table 1. The amplification process was performed in 25 µl of PCR reaction volume. Each PCR reaction contained 2.5 µl of Taq buffer, 1.5 µl of magnesium chloride ($MgCl_2$), 0.4 µl of dNTP, 2.5 µl for primer forward and 2.5 µl for primer revers 0,3 µl of Taq DNA polymerase, 2,5 µl of total genomic DNA, and 10,8 µl of ddH_2O. Amplifications were conducted using an Applied Biosystems (ABI) veriti 96 well thermocycler using the following program: 1 cycle of 5 min. At 95 °C; 40 cycles of 30 s at 94 °C, 30 s at 53 °C, and 2 min at 72 °C; followed by 1 cycle of 10 min. at 72 °C and a 4 °C soak for rDNA region. 1 cycle of 5 min at 95 °C; 40 cycles of 30 s at 94 °C, 30 s at 58 °C, and 2 min at

72 °C; followed by 1 cycle of 10 min. at 72 °C and a 4 °C soak for EF-1α region. Gel electrophoresis in 0.8% agarose gel run in TBE buffer was used to size-fractionate amplicons. Subsequently gels were stained with ethidium bromide and visualized over a UV trans-illuminator and subsequently sequenced.

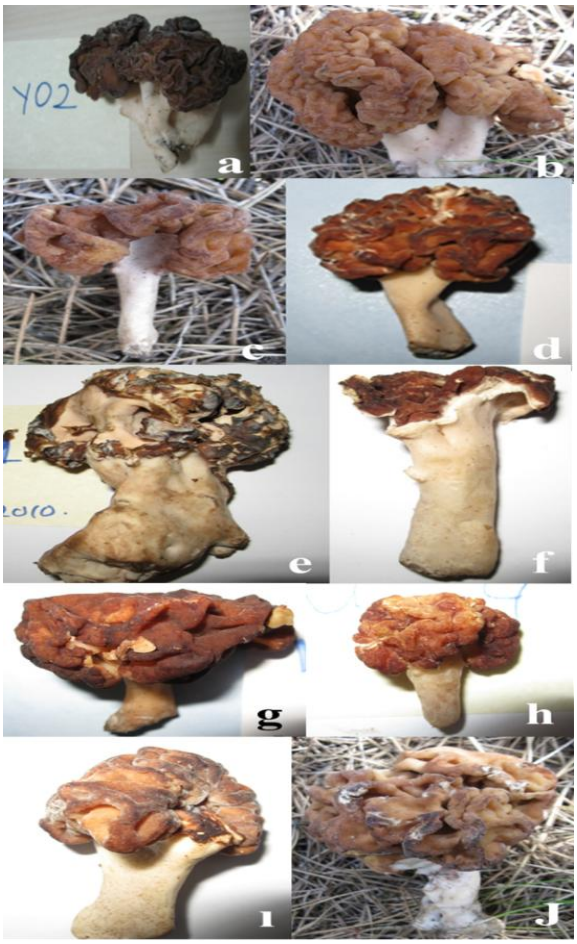

Figure 1. a-j. Morphologically different groups of *G. esculenta*.

Aligment and Phylogenetic Analysis

ITS and EF-1α sequences were manually/visually checked by using the Bioedit Version 7.0.4.1 software [11]. ITS and EF-1α sequences were aligned via ClustalW alignment software [12]. Ends of the alignment were trimmed to make all the sequences of equal length, which was a total of 807 nucleotide (nt) positions in the final dataset for ITS region and 844 for EF-1α gene. *Morchella* spp. are used as outgroup. Also ITS sequences of *G. infula* and *G. gigas* are taken from Genbank to assess intra and interspecific polimorphism ratio. The phylogenetic trees were generated with maximum parsimony and genetic distance [13] criteria, using PAUP* software's 4.0b10 beta version [14].

RESULTS

As a result of this study, three unique ITS nrDNA sequences from 10 different groups of *G. esculenta* with a length of 807 bp were obtained. Average nucleotide composition of**,** %19.0 (T), % 29.8 (C), %23.9 (A) and %27.3 (G). The total length of the aligned ITS sequence matrix were 909 nucleotides. There were a total of 232 variable characters of which 327 were parsimony informative and 350 characters were constant. Phylogenetic trees, divided into 3 main clades. Clade 1, consist of *G.esculenta* variations, and is strongly supported with a boostrap value of %.100 (Figure 2 and Figure 3). Clade 2, consist of *G. Infula* populations. This clade, is supported boostrap analyzed value of %100 (Figure 2 and Figure 3). Finally, clade 3, consist of only *G.gigas* (Figure 2). Between these groups nucleotide polymorphism ratio is nearly % 1. Also intraspecific ITS nucleotide polymorphism ratio in *G. infula* which are taken from Genbank is nearly %1 too. Interspecific ITS nucleotide polymorphism ratio between *G. infula*, *G. esculenta* and *G. gigas* is nearly % 16. Maximum parsimony analysis and genetic distance analysis using Neighbour Joining Method (Figure 2) and Bootstrap analysis (Figure 3) for ITS sequences revealed polytomies between *G. esculenta* specimens. *Gyromitra* spp. showed a good solution between species. The phylogenetic trees clearly revealed that the genus *Gyromitra* was a monophyletic taxon. However biguttulate and non apiculate spore forming members of the genus (*G. esculenta* and *G. infula*) more closely related than triguttulate and apiculated spore forming member (*G. gigas*) as seen in the trees. EF-1α gene doesn't consist of any nucleotide polymorphism between *G. esculenta* samples and not informative. Maximum parsimony analysis and genetic distance analysis using Neighbour Joining Method (NJ) for EF-1α revealed polytomies so no meaningful resolved tree was obtained. Also in Genbank nearly any EF-1α sequence for *Gyromitra* spp. Therefore, the trees were not shown here.

Table 1. ITS and EF-1α primers used in this study with their designers

Loci	Primer	References	Sequence
ITS	ITS4	(White et al. 1990)[8]	(5'-TCC TCC GCT TAT TGA TAT GC-3')
	ITS5M	(Sang et al. 1995) [9]	(5'-GGA AGG AGA AGT CGT AAC AAG G-3')
EF-1α	EF1-983F	(Rehner & Buckley, 2005)[10]	(5'-GCY CCY GGH CAY CGT GAY TTYAT-3')
	EF1-2218R	(Rehner & Buckley, 2005)[10]	(5'-AT GAC ACC RAC RGC RAC RGT YTG-3')

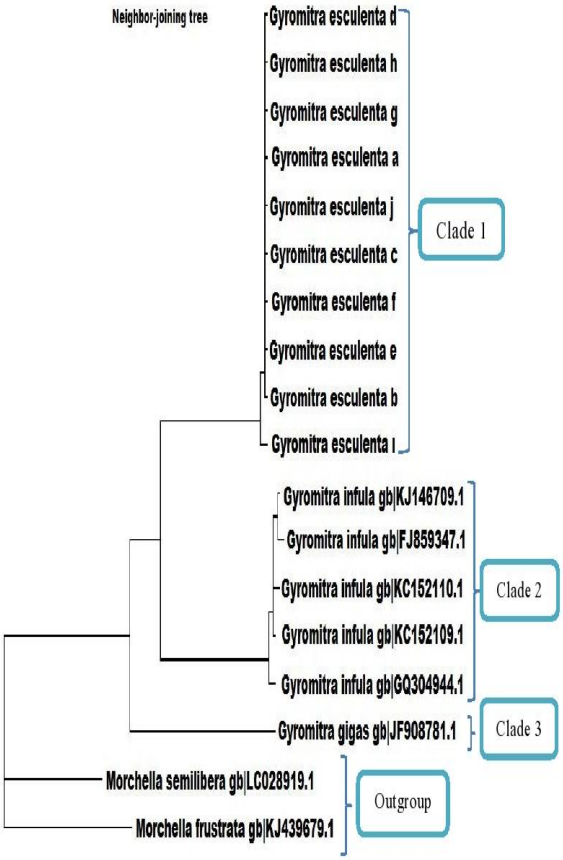

Figure 2. The neighbour joining tree generated using nrITS DNA sequences of genus *G. esculenta* specimens and the related sequences trieved from NCBI GenBank.

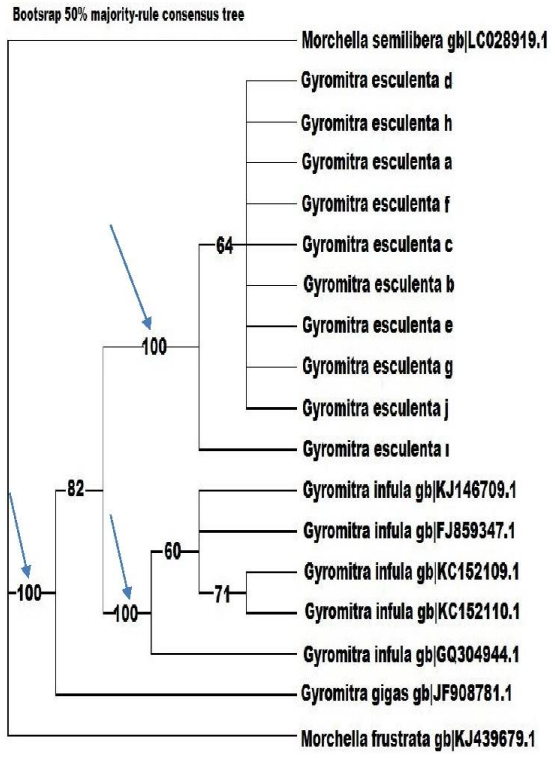

Figure 3. Bootstrap method tree generated using nrITS DNA sequences of genus *G. esculenta* specimens and the related sequences retrieved from NCBI GenBank

DISCUSSION

Recently, phylogenetic analyses based on DNA have been used for the purpose of revealing taxonomic data. These analyses are used in rebuilding phylogenetics of many taxonomically complex species, types or groups that contain a great number of taxa. Because of the fact that phylogeny is used widely and many methods were developed for reconstructing these.

EF-1α gene does not have any nucleotide polymorphism and also ITS sequences located at the nrDNA repeating units don't have high nucleotide polymorphism ratio between morphologically different *G. esculenta* specimens. Between these specimens ITS nucleotide polymorphism ratio is nearly % 1. Also intraspecific ITS nucleotide polymorphism ratio in *G. infula* which is taken from Genbank is nearly %1 too. Regard to interspecific ITS nucleotide polymorphism ratio between *G. infula, G. esculenta* and *G. gigas* is nearly % 16. we conclude that %1 nucleotide polymorphism ratio is a normal situation within *Gyromitra esculenta* samples.

That means macroscopic and microscopical variations in *G. esculenta* samples were not supported by molecular data. So we think that morphological variations are not genetically. Also a detailed study including more factors potentially causing taxonomic differentiations such as altitude, geographical distance, ecological characters as soil type, temperature, and drought is required in order to shed light on the morphological changes. But most likely morphological characters of *G. esculenta* easily influenced by environmental changes.

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