THE TAXONOMICAL CHARACTERIZATION OF SOME *HELVELLA* AND ITS RELATIVES BY MORPHOLOGICAL AND MOLECULAR DATA FROM TURKEY

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Abstract. Herein, 15 fungi species belonging to different genera of Pezizales were studied morphologically and molecularly. During the morphological analyses performed by means of Primer 7 software, 28 characters were used, mainly including the stalk, cap, asci, and spores, to assess the taxonomical relationships of the fungi species. The data matrix and nMDS plot approaches based on the morphological data were highly functional in the determination of the taxonomical relationship of very closely related fungi taxa, particularly at the genus level. Moreover, the data from the ISSR locus analyses significantly supported the current morphological relationships on a wide scale. Contrary to the morphological data expressing taxonomical relationships related to morels or early morels, a minor exceptional condition was determined inferring to the relatively higher genetic similarity among Verpa and Gyromitra in the dendrogram connected with the ISSR analyses.
Keywords: *fungi, DNA, genetic relationships, ISSR, numerical analyses*

Introduction

Helvella L. is a widespread and interesting genus of large apothecial ascomycet (Pezizomycete: *Pezizales* J. Schröt.) that are found in terrestrial biomes of the Northern and Southern Hemispheres. The genus contains many of the larger and charismatic species of the order *Pezizales* and comprises a range of elaborate apothecia, from cupulate to saddle-shaped, and convex to campanulate, including species with folded and lobed caps seated on a simple, ribbed, or furrowed stipe (Skrede et al., 2017). Compared to other close relatives, *Helvella* is a relatively more crowded group in terms of the number of taxa and it is represented by 21 species in Turkey (Sesli and Denchev, 2014).

Morchella Dill. ex Pers. taxa are known as Morels, which occur in different types of forests with different mycelial dynamics, alternating between saprotrophic and symbiotic behaviors (Stefani et al., 2010). The changes in the developmental process may induce a high degree of variability in the ascocarps (Yoon et al., 1990).

Verpa Sw. is a genus of ascomycete fungi related to the morels, resembling the latter genus; hence, they are called false or early morels. There are 5 species in the widespread genus (Kirk et al., 2008). *Verpa* comes from the Latin for erection or little rod. Thus, the 3 genera are now included in the family *Morchellaceae* Rchb. (O'Donnell et al., 1997).

Gyromitra esculenta (Pers.) Fr. is a beautiful spring time mushroom that is often encountered by morel hunters in northern and montane areas of the continent. It can be distinguished from other species of *Gyromitra* Fr. by its convoluted and brain-like, reddish-brown cap, and by the fact that its stem is not massive in proportion to its cap

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(Kuo, 2014). Additionally, the species display high similarity with morel taxa in terms of their ascocarp features.

Sarcoscypha coccinea (Jacq.) Sacc., commonly known as the scarlet elf cup, or the scarlet cup, is a species of fungus in the family *Sarcoscyphaceae* Le Gal ex Eckblad of the order Pezizales. The fungus, widely distributed in the Northern Hemisphere, has been found in Africa, Asia, Europe, North and South America, and Australia. The type species of the genus *Sarcoscypha* Roum., *S. coccinea*, has been known by many names since its first appearance in the scientific literature in 1772. The species was originally named *Helvella coccinea* by the Italian naturalist Giovanni Antonio Scopoli in 1772 (Scopoli, 1772). Other early names include *Peziza coccinea* (Jacquin, 1774) and *Peziza dichroa* (Holmskjold, 1799). Although some authors in older literature have applied the generic name *Plectania* to the taxon, following Fuckel's 1870 name change (e.g., Seaver, 1928; Kanouse, 1948; Nannfeldt, 1949; Le Gal, 1953), that name is now used for a fungus with brownish-black fruit bodies (Korf and Harrington, 1990). *S. coccinea* was given its current name by Lambotte in 1889.

In recent years, developments in molecular biology and genetic have made inevitable the emergence of new techniques in the systematic field, and these developments have also been effective in fungal systematics. Previous classifications of the fungi could be checked via molecular studies to determine whether it is natural or not. The marker system, called inter-simple sequence repeats (ISSRs), is also a polymerase chain reaction (PCR)-based technique (Wolfe, 1998) that has been successfully applied for the genetic analyses of plants (Fang et al., 1997; Prevost et al., 1999; Song et al., 2006; Uysal et al., 2012 a, b; Bozkurt et al., 2013) and fungal classification (Tang et al., 2005). In particular, ISSR markers can be highly variable within a species and have an advantage over others in utilizing longer primers that allow more stringent annealing temperatures (Camacho and Liston., 2001; Tsumura et al., 1996; Nazrul and Bian, 2010). Recently, the development of molecular systematics, thanks to ISSR and similar molecular markers, has made it possible to identify specimens of closely related species and, to some extent, it has been applied to taxonomic studies of *Helvella* (Landvik et al., 1999; Nguyen et al., 2013; Landeros et al., 2015; Ariyawansa et al., 2015; Zhao et al., 2017). In previous studies were also reported that some cryptic fungi species would be recognized by means of applying molecular markers (Nguyen et al., 2013; Balasundaram et al., 2015). Hence, ISSR has been chosen for the taxonomical characterization of some *Helvella* taxa from Turkey herein. Our aim was to first reveal the natural taxonomical positions of some *Helvella* species with their taxonomical characters and then solve the natural relationships between *Helvella* and its relatives.

Materials and methods

In this study, 15 *Ascomycota* taxa were collected from different localities in Turkey (*Table 1* and *Fig. 1)*.

Morphology

The morphological analyses of the studied fungi taxa were conducted on 15 samples from each taxa in the field from 2011–2016. A total of 28 qualitative (15) and quantitative (13) morphological characters were used (*Table 2*). The diagnostic traits at the species or genera level were measured, including the stalk, ascocarp, cap, spores, and asci. The qualitative and quantitative characters determined were measured and

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scored over the samples and finally turned into a data matrix. Morphometric data (*Table 2*) were analyzed using multivariate techniques with the PRIMER7 software package (Plymouth Marine Laboratory, Plymouth, UK; Clarke and Warwick, 1994). The Bray-Curtis similarity matrix was used to generate a 2-dimensional ordination plot applying non-metric multidimensional scaling in the PRIMER7 software (nMDS; Clarke, 1993).

Table 1. *Ascomycota taxa used for ISSR and morphological analyses*

Sample number	Taxa	Locality, Collector(s) and collector's number
HM1	*Helvella ephippium* Lév.	Adana, pine forest, 30.10.2011, Aktaş 1112; Isparta, pine forest, 17.12.2011, Aktaş 1128; Amasya, pine forest, 20.04.2012, Aktaş 1130; Amasya, pine forest, 22.04.2012, Aktaş 1145; Amasya, pine forest, 23.04.2012, Aktaş 1152; Amasya, pine forest, 11.06.2012, Aktaş 1154; Amasya, pine forest, 12.06.2012, Aktaş 1158
HM2	*H. lacunosa* Afzel.	Antalya, fir forest, 26.11.2011, Aktaş 1122; Amasya, fir-pine forest, 21.04.2012, Aktaş 1142; Amasya, fir-pine forest, 22.04.2012, Aktaş 1146
HM3	*H. leucomelaena* (Pers.) Nannf.	Adana, pine forest, 28.10.2011, Aktaş 1111; Antalya, pine forest, 25.11.2011, Aktaş 1119; Antalya, pine forest, 26.11.2011 Aktaş 1121; Isparta, pine forest, 16.12.2011, Aktaş 1125; Amasya, pine forest, 20.04.2012, Aktaş 1131; Amasya, pine forest, 21.04.2012, Aktaş 1141; Amasya, pine forest, 22.04.2012, Aktaş 1147; Amasya, pine forest, 10.06.2012, Aktaş 1153; Amasya, pine forest, 11.06.2012, Aktaş 1155
HM4	*H. queletii* Bres.	Antalya, fir forest, 27.11.2011, Aktaş 1123; Amasya, fir-pine forest, 20.04.2012, Aktaş 1132; Amasya, fir-pine forest, 12.06.2012, Aktaş 1157
HM5	*H. acetabulum* (L.) Quél.	Amasya, pine forest, 21.04.2012, Aktaş 1140; Amasya, pine forest, 22.04.2012, Aktaş 1148
HM6	*H. spadicea* Schaeff.	Isparta, among poplar woods, 16.12.2011, Aktaş 1124; Amasya, among poplar woods, 20.04.2012, Aktaş 1133; Amasya, among poplar woods, 21.04.2012, Aktaş 1139
HM13	*H. crispa* Sowerby	Antalya; Akseki, fir-pine forest, 26.11.2011, Aktaş 1121
HM14	*H. solitaria* P. Karst.	Samsun; Ladik, beech forest, 22.10.2016, Aktaş 2033
HM15	*H. leucopus* Pers.	Konya; Akşehir, near stream, 15.10.2016, Aktaş 2027
HM7	*Peziza michelii* (Boud.) Dennis	Amasya, pine forest, 23.04.2012, Aktaş 1153
HM8	*P. varia* (Hedw.) Alb. & Schwein.	Amasya, pine forest, 22.04.2012, Aktaş 1149
HM9	*Gyromitra esculenta* (Pers.) Fr.	Antalya, pine forest, 25.11.2011, Aktaş 1120; Amasya, fir-pine forest, 21.04.2012, Aktaş 1138; Amasya, pine forest, 20.04.2012, Aktaş 1134; Amasya, pine forest, 21.04.2012, Aktaş 1143; Amasya, pine forest, 12.06.2012, Aktaş 1156
HM10	*Verpa conica* (O.F. Müll.) Sw.	Amasya, among poplar woods, 21.04.2012, Aktaş 1136
HM11	*Sarcoscypha coccinea* (Gray) Boud.	Isparta, pine forest, 16.12.2011, Aktaş 1126; Isparta, pine forest, 17.12.2011, Aktaş 1127; Isparta, pine forest, 18.12.2011, Aktaş 1129; Amasya, pine forest, 23.04.2012, Aktaş 1150
HM12	*Morchella esculenta* (L.) Pers.	Amasya, pine forest, 20.04.2012, Aktaş 1135; Amasya, pine forest, 21.04.2012, Aktaş 1137; Amasya, pine forest, 22.04.2012, Aktaş 1144

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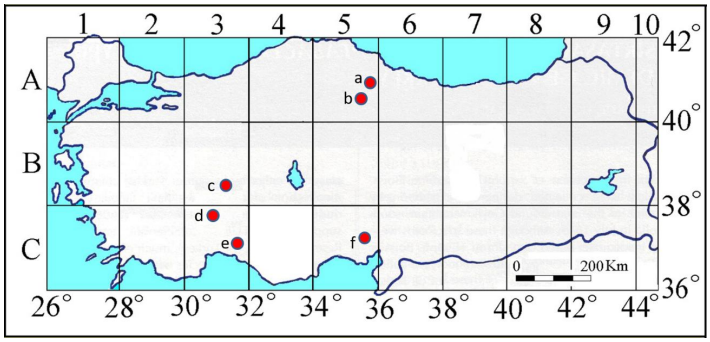

Figure 1. *The location of collected samples on map (a: Samsun; b: Amasya; c: Konya; d: Isparta; e: Antalya; f: Adana)*

Table 2. *Characters used in numerical analysis*

Diagnostic characters of the Ascomycota species		
Stalk	Absent (0), present (1)	HS1
Stalk	Whitish (0), yellowish (1), brownish (2), blackish (3), absent (4)	HS2
Stalk	Smooth (0), ribbed or veined (1), absent (2)	HS3
Stalk	Narrowed at the base (0), enlarged at the base (1), same anywhere (2), absent (3)	HS4
Stalk	Up to 20 mm (0), between 21–50 mm (1), 51–80 mm (2), more than 81 mm (3)	HS5
Stalk	Up to 15 mm (0), between 16–30 mm (1), more than 31 mm (2)	HS6
Rate of stalk length/diameter	Unit	HS7
The type of ascocarp	Apothecium (0), other (1)	HS8
Ascocarp	Saddle-shaped (0), cup-shaped (1), Globose (2), conical (3)	HS9
Ascocarp	Smooth (0), lobed (1), curved-brain (2), alveolate (3)	HS10
Ascocarp (outer surface)	Cream (0), reddish-brown (1), yellowish (2), greyish (3)	HS11
Ascocarp (inner surface; Hymenium)	Greyish brown (0) brownish-black (1) yellowish (2), reddish-brown (3), red (4), light brown (5)	HS12
Ascocarp (inner surface; Hymenium)	Glabrous (0), hairy (1)	HS13
Cap	Up to 25 mm (0), 26–60 mm (1), more than 61 mm (2)	HS14
Cap	Up to 25 mm (0), 26–60 mm (1), more than 61 mm (2)	HS15
Rate of cap length/diameter	Unit	HS16
Cap	Convex (0), concave (1), semi-convex (2)	HS17
Spores	Ellipsoidal (0), elliptic (1), weakly-elliptic (2)	HS18
Spores (oil drop number)	Only one (0), two (1), more (2)	HS19
Spores (oil drop position)	Inside of spores (0), outside of spores (1)	HS20
Spores	Between 16 and 21 µm (0), 22–27 µm (1), more than 27 µm (2)	HS21
Spores	Between 8 and 10 µm (0), 11–13 µm (1), 14-16 µm (2)	HS22
Rate of spore length/diameter	Unit	HS23
Spores (surfacial)	Smooth (0), verrucose (1)	HS24
Asci	Between 225 and 300 µm (0), 301–375 µm (1), more than 376 µm (2)	HS25
Asci	Between 14 and 18 µm (0), more than 18 µm (1)	HS26
Rate of asci length/width	Unit	HS27
Asci (number of spores)	Number	HS28

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DNA extraction

Total genomic DNA was extracted following the 2xCTAB method of Doyle and Doyle (1987) as was modified by Soltis et al. (1991) and Cullings (1992) from silica gel-dried leaves collected in the field.

ISSR-PCR

Our modified ISSR-PCR analyses were basically performed according to the method of Zietkiewicz et al. (1994). During the PCR-optimization reactions, some modifications were quantitatively carried out, particularly for the Mg and primer amounts, as well as the Tm selection. The designed ISSR primers by British Colombia University were chosen for the PCR-amplifications. Amplification products were separated by electrophoresis in 1.2% agarose gel run in a TAE buffer at 100 V. The fragment size was estimated using a 20,000–75 bp molecular size DNA ladder (Thermoscientific, SM1331).

Data analysis

The ISSR profiles were scored as present (1) or absent (0). Only reproducible bands were scored as monomorphic or polymorphic. The dendrogram was created using NTSYS-pc version 2.1 (Rohlf, 1998).

Results

Molecular results

A total of 15 taxa were tested with 20 ISSR primers and only 10 of them provided reproducible and analyzable amplification products for all taxa and a total of 477 bands ranging from 80–2500 bp were obtained to put the relativeness of the fungi species (*Fig. 2*). All of the ISSR primers showed 100% polymorphism for Ascomycota taxa (*Table 3*).

Table 3. *Number and percentage of polymorphic ISSR markers*

Primers	Primer sequences (5'-3')	Fragment size (bp)	Total number of bands	Total number of polymorphic bands
UBC827	ACA CAC ACA CAC ACA CG	1500-100	47	47
UBC812	GAG AGA GAG AGA GAG AA	1400-80	47	47
UBC810	GAG AGA GAG AGA GAG AT	1250-80	51	51
UBC847	CAC ACA CAC ACA CAC ARC	1100-80	35	35
UBC857	ACA CAC ACA CAC ACA CYG	2000-80	46	46
UBC808	AGA GAG AGA GAG AGA GC	1250-80	46	46
UBC855	ACA CAC ACA CAC ACA CYT	2000-250	45	45
UBC834	AGA GAG AGA GAG AGA GYT	2500-80	79	79
UBC840	GAG AGA GAG AGA GAG AYT	1750-80	45	45
UBC856	ACA CAC ACA CAC ACA CYA	1250-80	36	36

When we look at the dendrogram that was created using the locus analysis based on the ISSR primers, all of the *Helvella* taxa were positioned together as only one group (*Fig. 3*). However, the relationships and positions of the *Helvella* species among

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themselves did not fit exactly with the performed previously classification on a morphology basis. In the dendrogram, the *Helvella* species displayed very close genetic relationships with at least 80% similarity and they were placed in the core of dendrogram. As the furthest taxa, *Morchella esculenta* was placed in the outermost area with 75% similarity to others. Compared to the first taxa, *Sarcoscypha coccinea* showed a closer similarity with the remaining taxa and its genetic relationship was more than 76%. Another relatively smaller group (subclade B) consisted of 3 taxa representing 3 different genera and they showed at least 81% genetic similarity. Even if classified morphologically in different families, *Gyromitra esculenta* is genetically closer to *Verpa conica* when compared to *Peziza* and others analyzed herein. Naturally, *Peziza michelii* and *P. varia* are very similar genetically and the distance between them is less than 15%.

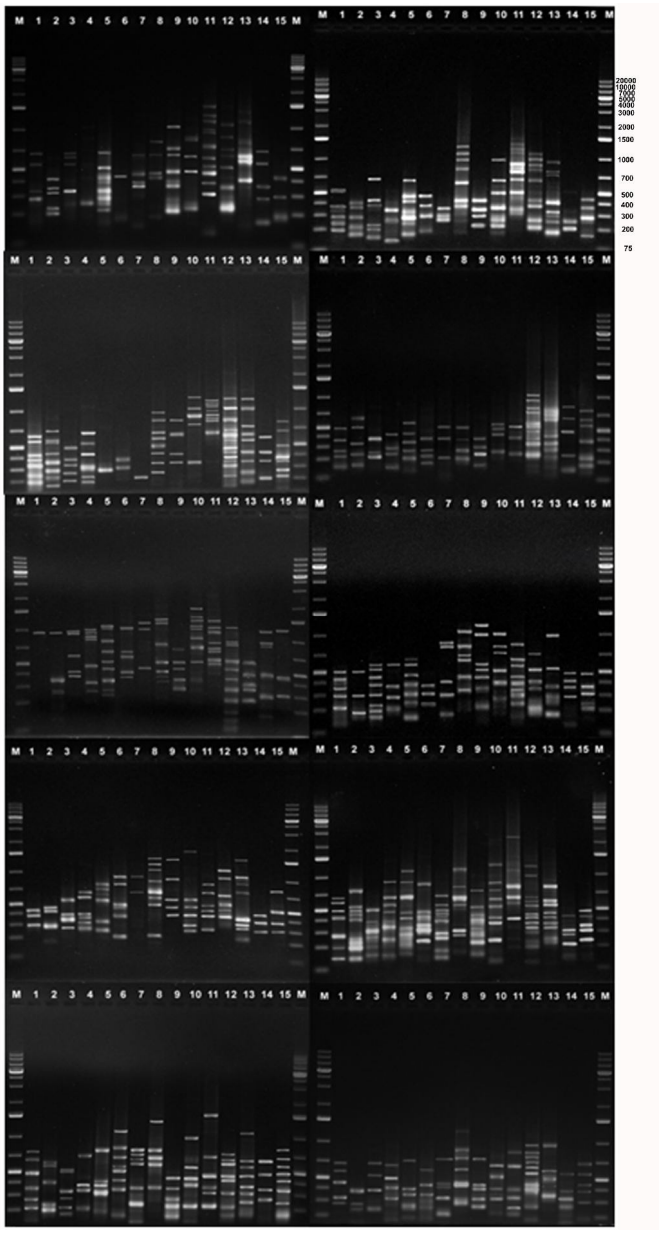

Figure 2. *Electrophoresis Patterns of ISSR products amplified with primers UBC827, 812, 810, 847, 857, 808, 855, 834, 840 and 856 for Ascomycota taxa*

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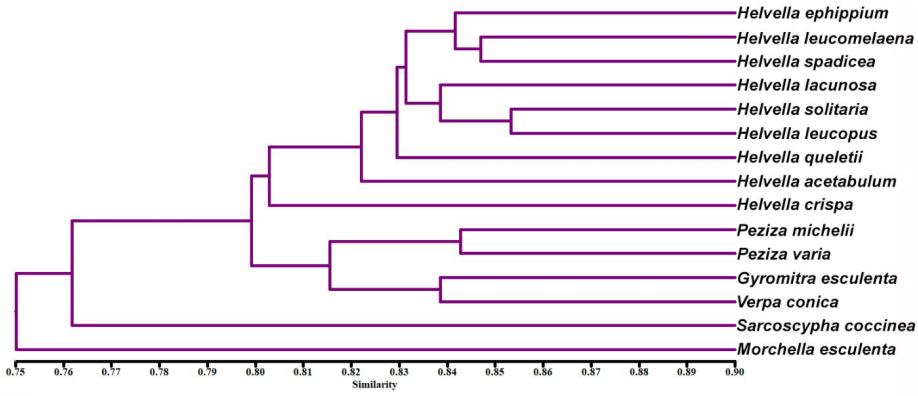

Figure 3. *The dendrogram showing the genetic relationships among Ascomycota taxa based on ISSR data*

Morphological results

The nMDS plots revealed the morphological differences among the studied taxa and helped to make prominent separations among them (*Fig. 4*). The phenogram pointed out very important characters which were very functional in classifying the fungi at different taxonomical levels (for instance, levels of species, genera, or higher). According to the phenogram (*Fig. 5*), it could be said that the characters belonging to the cap and the stalk are important at the primary level; hence, the taxa were divided in the main 2 groups according to these characters. Second, it was seen that the fungal species were divided into 2 subgroups according to whether the cap surface was smooth or not, and that this separation was largely compatible with previous classifications based on morphology. Finally, it was determined that the structural ornamentations made by the cap inside or outside were important in the classification of the fungi, especially to determine the 2 different groups of taxa located in the *Helvella* genus as saddle or cup (*Fig. 5*).

Discussion and conclusion

True morels (*Morchella* spp.) are highly popular for their edibility and appearance, and they have been examined in many broad-scale studies at different times (Kirk et al., 2011); however, other relatives have not be researched as deeply until now. We can emphasize clearly from our results that ISSR markers and morphometric analyses are very effective tools to assess close relative fungi, particularly at the genera level.

Traditionally, *Verpa* and *Gyromitra* were found to be very close to the genus *Morchella* and so, they were named false morels (Kirk et al., 2008). Our examinations indicated that these taxonomical groups are very close in terms of genetic and morphological features, and clear differences can be displayed among them. According to our findings, *Morchella esculenta* is the farthest taxa genetically and the alveolate ascocarp was not seen in any other relatives. From these, we can say that the alveolate cap is an important character for true morels to separate it from its close relative genera. Analysis of the ribosomal DNA of many of the *Pezizales* showed the genus *Verpa* to be closely related to the genus *Morchella* (Bunyard et al., 1995; O'Donnell et al., 1997). The results from the ISSR analyses did not exactly reflect this information, such that *S. coccinea* was positioned exceptionally among them.

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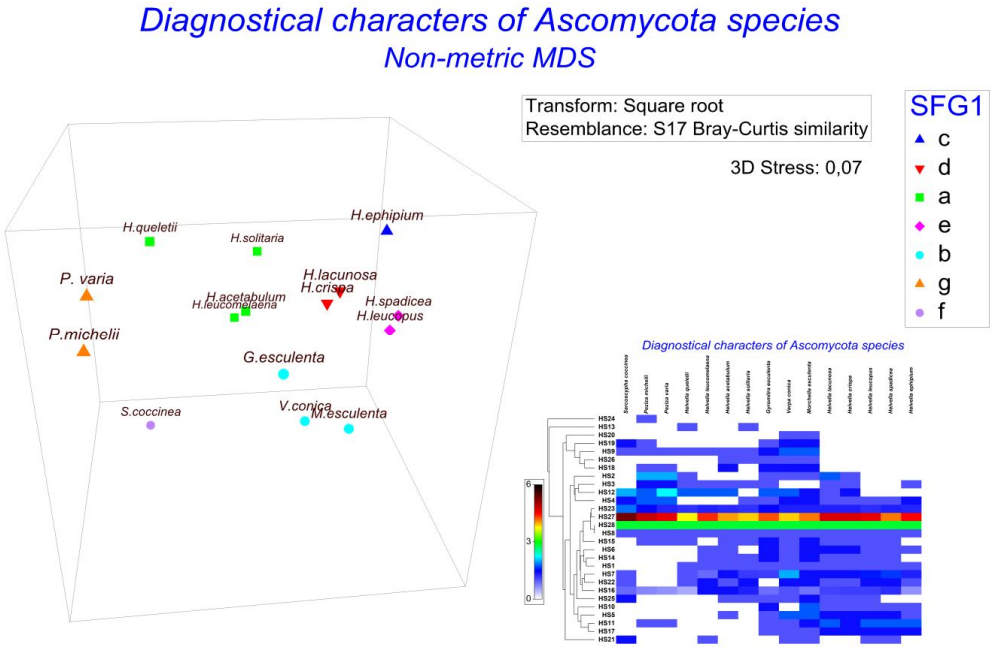

Figure 4. *Two-dimensional nMDS ordination belonging to morphological characters*

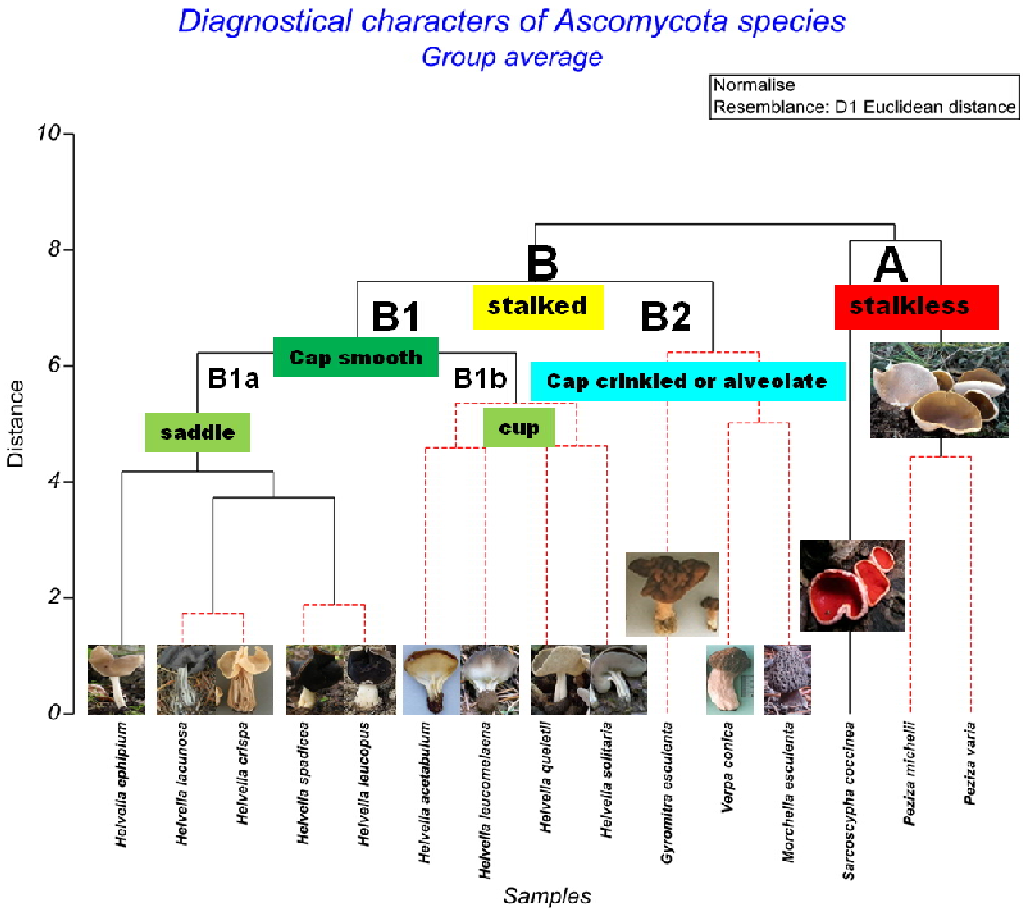

Figure 5. *The phenogram showing relationships among Ascomycota taxa according to morphological characters*

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The morphological data has been found largely correlated with the genetic information based on the ISSR locus analyses in except of a limited homoplasy and pseudocriptic variation. It is an interesting finding that species such as *H. lacunosa* and *H. crispa*, which are very close taxa morphologically within the genus *Helvella*, constituted the main body of this study, and are taken place genetically relatively remote (*Fig. 3*). This observed homoplasic similarity on their morphologies might be sourced from similar environmental conditions and habitats rather than genealogy. As together with this, Skrede et al. (2017) reported a pseudocryptic variation, in which 2 taxa could be recognized morphologically, only after molecular systematic data unveiled their existence. The authors suggested that the morphologically similar species pairs of *H. corium/H. macrosperma, H. lactea/H. pallescens,* and *H. elastica/H. panormitana*, which were initially indiscernible due to the lack of discriminating morphological characters, could be resolved as genealogically exclusive using the sequence data. This was true with our 4 fungi, such as with *H. lacunosa* and *H. crispa* or *H. spadicea* and *H. leucopus*. The results indicated that these species pairs are genetically divergent and located in relatively distant related *Helvella* lineages according to the ISSR data. The use of molecular characters is important in cases whether the morphological characters of the taxa are identical to each other or not, or may have been reduced or absent from the taxa (Blackwell et al., 2007). The genus *Helvella*, which constitutes the main body of this work, is relatively taxonomically problematic and shows a series of relationships with the other genera mentioned in this paper. Although easily separated from other macrofungi by conspicuous polymorphic apothecia, it is surprisingly difficult to distinguish between *Helvella* species. Historically, the shape, color, and outer surface characters have been emphasized in species discrimination, while the microanatomy of the sterile and fertile structures added few characters of value in species recognition. In a previous study performed on *Helvella* (Weber, 1972), it was concluded that most morphological and anatomical characters exhibited a nearly continuous variation in the genus as a whole. Unlike the expressed critics, we think that the trustable and functional characters could be revealed for the genus *Helvella* in light of the molecular or morphological data, as illustrated via the nMDS plot of Primer7, and they could be used to explain taxonomical relationships. In conclusion, it was decided that both the stalk and cap features were quite efficient when the characters were handled comprehensively.

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