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Species diversity within the *Morchella esculenta* group (Ascomycota: Morchellaceae) in Germany and France

Harald Kellner, Carsten Renker*, François Buscot

Institute of Botany, Terrestrial Ecology, University of Leipzig, Johannisallee 21, D-04103 Leipzig, Germany

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Abstract

The distribution of the *Morchella esculenta* group in Germany and France is examined based on 22 samples, a sample from Montenegro is studied as well. In the recent literature the group was often treated as a single species, *M. esculenta* sensu lato. Our study, based on the polymorphism of the internal transcribed spacer (ITS) region within the nuclear ribosomal DNA (nrDNA), indicates the presence of three distinct species: *M. esculenta* (L.) Pers., *M. crassipes* (Vent.) Pers. : Fr., and *M. spongiola* Boud. They can be identified easily by restriction fragment length polymorphisms (RFLP) of the ITS region.

Keywords: Bayesian analysis; Distribution; ITS; nrDNA; RFLP; *Morchella esculenta* group

Introduction

The debate about species concepts and diversity in the genus *Morchella* Dill. ex Pers. is very old and long-standing. After Nannfeldt (1937) had recognised the number of nuclei per spore as a valuable systematic feature in ascomycetes, Berthet (1964) used this criterion to distinguish four families within the operculate discomycetes and to separate Morchellaceae as the group with the highest coenosity level (15–60 nuclei per spore). In systematic treatments essentially based on morphological features, the species number in *Morchella* fluctuates between 3 and 30 (Seaver 1942; Korf 1973; Jacquetant 1984; Weber 1988; Gessner 1995), reflecting the enormous typological variability of the fruit bodies according to habitats and their specific abiotic and biotic conditions (Bresinsky and Stangl 1961; Bresinsky et al. 1972). Wipf et al. (1996, 1999) tried to clarify the systematics within *Morchella* based on sequence data for the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (nrDNA), and thereby confirmed the separation of *Adnatae* (yellow morels) and *Distantes* (black morels) made by Boudier (1897) in one of the first systematic treatments of the genus. O'Donnell et al. (1997) confirmed monophyletic status for Morchellaceae and the genus *Morchella*, respectively, using nrDNA of the small and large ribosomal subunit.

The huge variability of macroscopic features and the consistence of microscopic ones found for spores and hyphae (Weber 1992) hamper the distinction of more than three species in field surveys. This is reflected by the fact that most recent works (e.g. Krieglsteiner 1993) only distinguish three species within Germany: *Morchella elata* Fr. (including *M. conica* Pers., *M. costata* (Vent.) Pers., *M. deliciosa* Fr., *M. hortensis* Boud.), *Morchella esculenta* (L.) Pers. (including *M. crassipes* (Vent.) Pers. : Fr., *M. rotunda* (Fr.) Boud., *M. umbrina*

*Corresponding author.
E-mail address: crenker@uni-leipzig.de (C. Renker).

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Boud., *M. spongiola* Boud., *M. vulgaris* (Pers.) Boud.), and *Morchella gigas* (Batsch : Fr.) Pers. (including *M. hybrida* Pers., *M. rimosipes* DC., *Mitrophora semilibera* (DC. : Fr.) Lév.). For Switzerland, Breitenbach and Kränzlin (1984) keep *M. elata* and *M. conica* as distinct species and separate four varieties within *M. esculenta* (var. *rigida* Krombh., var. *rotunda* Fr., var. *umbrina* (Boud.) S. Imai, var. *vulgaris* Pers.), without giving convincing morphological features supporting their distinction. These authors separate *M. gigas* as a monotypic genus, i.e. *Mitrophora semilibera*, distinguished by only half of its cap being attached to the stipe, whereas in all *Morchella* species the cap is almost completely attached. These are representative examples highlighting the ongoing debate over species separation within the genus *Morchella*.

The present work focuses on genetic diversity in the *M. esculenta* group according to aspects of its distribution in Germany and France, based on restriction fragment length polymorphisms (RFLP) and sequencing of the ITS region within the nrDNA. Taxonomic treatment of the gathered sequences follows Wipf et al. (1999).

Material and methods

Fungal material

Fruit bodies were collected in spring 2002 and 2003, and dried on tissue-covered petri dishes at room temperature until sporulation. Spores were diluted in water, put on malt(10 g/l)–glucose(5 g/l)–agar(14 g/l) plates, and stored in darkness at 25 °C. One day later, single germinated spores were isolated and transferred onto new plates.

Herbarium material was included to widen the sampling range. In total, the ITS regions of 23 collections of *M. esculenta* group were analysed (Table 1). In addition, the majority of the ITS sequences published by Wipf et al. (1999) were retrieved from GenBank and incorporated in the study.

Voucher specimens from the authors' collections are preserved at the State Museum of Natural History, Görlitz, Germany (GLM), others at the herbaria indicated in Table 1. The ITS sequences generated in this study have been deposited in GenBank; accession numbers are given in Table 1.

DNA isolation and PCR conditions

Pieces of mycelium from the single-spore cultures and parts of the dried fruit bodies were used for DNA extraction. The fungal material was crushed in an Eppendorf tube with a micro pestle after addition of liquid nitrogen. Afterwards DNA was extracted using the DNeasy Plant Mini Kit (Qiagen; Hilden, Germany).

The internal transcribed spacer regions (ITS 1 and ITS 2) together with the 5.8S rRNA gene were amplified in a Mastercycler Gradient Thermo Cycler (Eppendorf; Hamburg, Germany), using the primer pairs ITS1 and ITS4 (White et al. 1990). PCR was performed in a total volume of 50 µl containing 1 U Taq DNA polymerase (QBiogene; Heidelberg, Germany), 5 µl of 10 × Taq polymerase reaction buffer (QBiogene), 10 nmol of each dNTP (MBI-Fermentas; St. Leon-Rot, Germany), 50 pmol of each of the two primers (Invitrogen; Karlsruhe, Germany), and 1 µl of the DNA extract. PCR was performed after 3 min of initial denaturation at 94 °C, followed by 35 cycles (60 s at 94 °C, 30 s at 50 °C, 80 s at 72 °C). A final elongation of 10 min at 72 °C followed the last cycle.

Cloning, sequencing and sequence analysis

PCR products of about 1200 bp were cloned into the pCR4-Topo Vector following the manufacturer's protocol for the TOPO TA Cloning Kit (Invitrogen), and transformed into TOP10 Chemically Competent *Escherichia coli*. Plasmids were extracted from *E. coli* using the Perfectprep plasmid mini kit (Eppendorf). Sequencing was done on a LI-COR DNA Sequencer Long Reader 4200, in both directions with the primers M13 Forward (5′-GTAAAACGACGGCCAG-3′) and M13 Reverse (5′-CAGGAAACAGCTATGAC-3′), using the Thermo Sequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham; Little Chalfont, UK).

DNA sequences of the full ITS region including the 5.8S nrDNA were submitted to the EMBL database under the accession numbers given in Table 1. Sequences were aligned with ClustalW and visually corrected. Additional sequences were taken from GenBank to augment the taxon sampling. The final alignment used for the following phylogenetic analyses can be obtained from the corresponding author. Phylogenetic analyses were performed using PAUP* 4.0b10 (Swofford 2003) and MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001). For the first analysis, MrBayes, a programme for Bayesian inference of phylogeny, was used. A Markov chain Monte Carlo (MCMC) run with four simultaneous chains and 500,000 generations was performed. As substitution model, the general time reversible model with invariable sites and gamma shape distributed substitution rates (GTR + I + G) was chosen. Every hundredth generation, the tree with the best likelihood score was saved, resulting in 5000 trees. The first 1000 trees without reaching a stable likelihood score were deleted. The remaining 4000 trees were condensed in a majority

Table 1. Overview of *Morchella* material investigated

Species	Strain	Origin (collector, year)	Source	GenBank accession and/or herbarium No.	ITS length (bp)
M. crassipes (Vent.) Pers. : Fr.	10J1	Germany, Jena (Kellner, 2002)	C, Sp	AJ539480, GLM 51663	1226
M. crassipes (Vent.) Pers. : Fr.	26J1	France, Illkirch-Graffenstaden (Buscot, 1985)	C, Sp	AJ539481, GLM 51668	1225
M. crassipes (Vent.) Pers. : Fr.	m19	Germany, Leipzig (Arnold, 1955)	C	AJ539482	1225
M. crassipes (Vent.) Pers. : Fr.	30J	Montenegro, Lovcen National Park (Lakusic, 1998)	F	AJ623265, GLM 51665	1226
M. crassipes (Vent.) Pers. : Fr.	HG1	Germany, Braunschweig (Boyle, 2001)	F	GLM 44031	>1200[a]
M. crassipes (Vent.) Pers. : Fr.	HG4	Germany, Dresden (Stilbach, 1984)	F	GLM 14120	>1200[a]
M. crassipes (Vent.) Pers. : Fr.	HG5	Germany, Wilthen (Marx, 1996)	F	GLM 37210	>1200[a]
M. crassipes (Vent.) Pers. : Fr.	HG6	Germany, Zwickau (Hallbauer, 1989)	F	GLM 23456	>1200[a]
M. crassipes (Vent.) Pers. : Fr.	HH1	Germany, Heiligenstadt (Meyer, 1993)	F	JE HK 001	>1200[a]
M. crassipes (Vent.) Pers. : Fr.	HH2	Germany, Rudolstadt (Meyer, 1991)	F	JE HK 002	>1200[a]
M. crassipes (Vent.) Pers. : Fr.	HH7	Germany, Nordhausen (Günther, 1993)	F	JE HK 003	>1200[a]
M. esculenta (L.) Pers.	A7	France, Offendorf (Buscot, 1987)	C	AJ543737	1138
M. esculenta (L.) Pers.	D0	Germany, Krenzingen, Weisweil (Kost, 1990)	C	AJ543739	1138
M. esculenta (L.) Pers.	G2	Germany, Münster (Gessner, 1994)	C	AJ543741	1138
M. spongiola Boud.	7J1	Germany, Jena (Kellner, 2002)	C, Sp	AJ539474, GLM 51664	1186
M. spongiola Boud.	24J1	Germany, Eschenbergen (Kellner, 2002)	C, Sp	AJ539477, GLM 51662	1186
M. spongiola Boud.	31J	Germany, Detmold (Blanke, 2003)	F	GLM 51692	<1200[a]
M. spongiola var. *dunensis* R. Heim	Du3	France, Merlimont (Buscot, 1995)	C	AJ539478	1186
M. spongiola Boud.	HL2	Germany, Erfurt (Otto, 1988)	F	LZ Ot 210599/1	<1200[a]
M. spongiola Boud.	HG2	Germany, Obergrombach (Reimann, 1998)	F	GLM 40115	<1200[a]
M. spongiola Boud.	HG3	Germany, Görlitz (Kloß, 1973)	F	GLM 02641	<1200[a]
M. spongiola Boud.	HH9	Germany, Sondershausen (Gassel, 1982)	F	JE HK 004	<1200[a]
M. spongiola Boud.	HH11	Germany, Creuzburg (Meyer, 1989)	F	JE HK 005	<1200[a]

C = culture, F = fruit body, Sp = spores; herbarium acronyms according to Index herbariorum (Holmgren et al. 1990).
[a]Length estimated by RFLP.

rule consensus tree using PAUP* 4.0b10. Branch supports were assigned as posterior probabilities on the consensus tree (Fig. 1).

Second, a maximum parsimony analysis was performed with PAUP*, using the Branch-and-Bound-Search mode. All characters were treated as unordered and equally weighted. Strict consensus trees were calculated including all MP trees. The confidence of branching was assessed using 1000 bootstrap resamplings in the heuristic search mode with 10 addition sequence replicates and tree bisection-reconnection branch swapping. All calculations were based on an

alignment of 813 positions, with 40 being parsimony informative (Fig. 2).

Besides sequence analysis, fungal collections (Table 1) were analysed by RFLP. The PCR products for the ITS region were restricted separately with *Alu*I, *Bsu*RI, *Eco*RI, *Hin*fI, *Msp*I, and *Taq*I (MBI Fermentas). Restriction fragments were separated and visualised (see Fig. 3) on a 2% agarose gel stained with ethidium bromide.

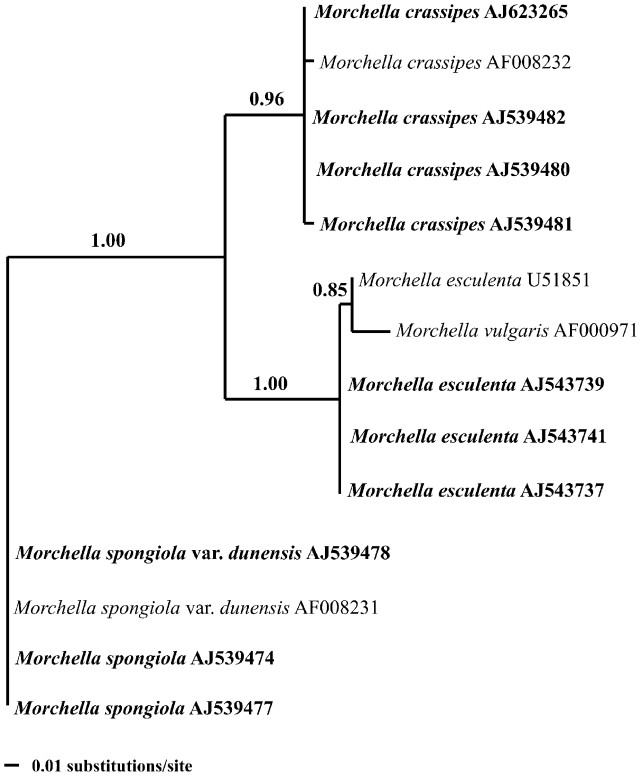

Fig. 1. Consensus tree derived from Bayesian phylogenetic inference analysis of the *M. esculenta* group, based on ten ITS nrDNA sequences obtained in the present study (shown in boldface) and four previously published sequences obtained from GenBank; taxonomic treatment of the sequences follows Wipf et al. (1999).

Results

As shown in Figs. 1 and 2, at least three different sequence types were detected within the analysed specimens of the *M. esculenta* group. Following Wipf et al. (1999), these may be named *M. esculenta* (including *M. vulgaris*), *M. crassipes*, and *M. spongiola*, respectively. Checking the ITS sequences of several *E. coli* clones within each treated *Morchella* specimen always revealed identical sequence patterns. Therefore it can be concluded that each *Morchella* specimen has only one ITS sequence. Nevertheless, considerable variation in ITS length between the different species was found (Table 1), with *M. crassipes* displaying the greatest length (1225–1226 bp), followed by *M. spongiola* (1186 bp), and *M. esculenta* (1138 bp). Sequence identity among the ITS1 regions of these three species was low (between 53 and 58%), leading to an exclusion of large parts of this region (ITS1 nucleotide exclusion for *M. crassipes*: 61–481; *M. spongiola*: 61–443; *M. esculenta*: 61–395), and resulting in a total alignment of only 813 unambiguous positions. Based on the conserved character, the included 5.8S nrDNA (alignment positions 224–389) showed only one base substitution. Despite the reduced amount of parsimony informative positions, high a posteriori probabilities were found in the Bayesian analysis (Fig. 1).

Sequence heterogeneity is also reflected by characteristic RFLP patterns (Fig. 3). The sizes of distinguishable fragments are given in Table 2. Restriction enzymes *Alu*I and *Eco*RI cut few (1–3) times, whereas the others cut mostly four times and more.

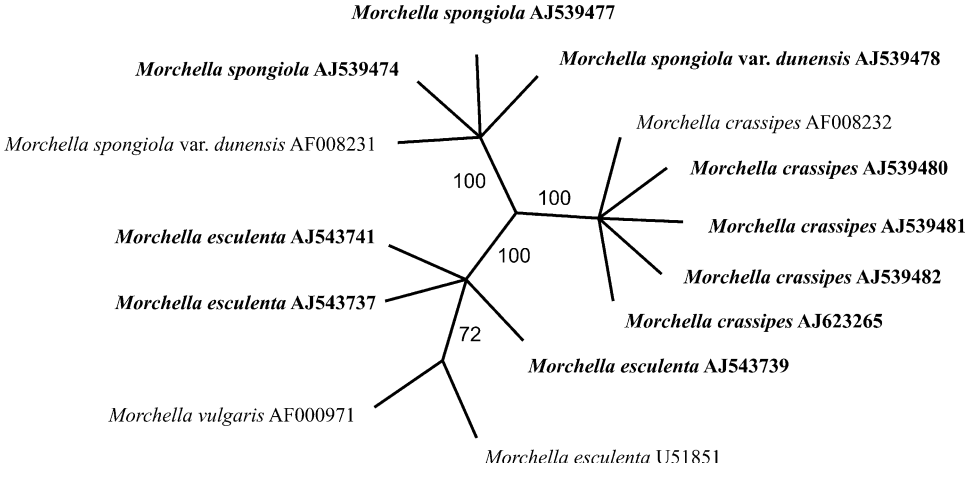

Fig. 2. Strict consensus cladogram of 26 most parsimonious trees (length = 47 steps); numbers above branches give bootstrap support derived from 1000 replicates; consistency index = 1, retention index = 1.

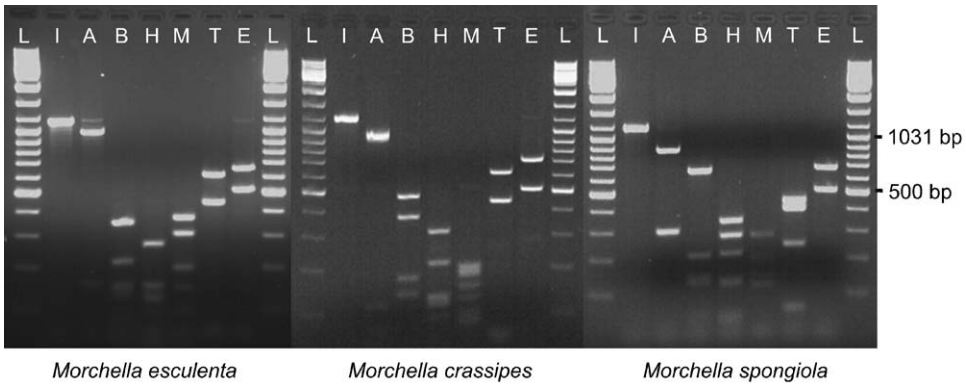

Fig. 3. RFLP patterns of the ITS region within the nrDNA of *M. esculenta*, *M. crassipes*, and *M. spongiola*; fragments were separated on 2% agarose gels after restriction with: *Alu*I (lanes A), *Bsu*RI (B), *Hin*fI (H), *Msp*I (M), *Taq*I (T); and *Eco*RI (E); original PCR products of the uncut ITS region are shown in lanes I; Gene Ruler DNA Ladder Mix was used in lanes L.

Table 2. Lengths of restriction fragments after digestion with different restriction enzymes, based on reconstructions with sequences of (I) *Morchella esculenta*, (II) *M. crassipes*, (III) *M. spongiola*

Restriction enzyme	Species	Length (bp) of restricted ITS fragment
*Alu*I	I	**1004**, 134
	II	**979**, 119, 75, 53
	III	**884**, **302**
*Bsu*RI	I	**351**, 214, 142, 130, 128, 77, 53, 43
	II	**483**, **379**, 180, 141, 43
	III	**666**, 207, 142, 128, 43
*Hin*fI	I	269, 151, 146, 129, 119, 118, 92, 35, 35, 34, 8
	II	**317**, 211, 131, 129, 118, 116, 92, 35, 35, 34, 8
	III	**338**, **278**, 208, 129, 119, 36, 35, 35, 8
*Msp*I	I	**345**, **306**, 200, 143, 94, 18, 14, 10, 8
	II	206, 188, 179, 151, 126, 99, 91, 84, 80, 14, 8
	III	272, 203, 180, 141, 117, 115, 110, 22, 16, 10
*Taq*I	I	**584**, **442**, 59, 53
	II	**614**, **439**, 61, 59, 53
	III	**440**, **397**, 237, 59, 53
*Eco*RI	I	**628, 510**
	II	**719, 507**
	III	**678, 508**

Fragments clearly distinguishable on agarose gels (see Fig. 3) are given in boldface; fragments shorter than 80 bp are not visible on agarose gels.

Based on the available dataset, it is impossible to conclude a species-specific distribution in central and western Europe. However, as shown in Fig. 4, there is so far only one region (the Upper Rhine Valley) in which all three types were found within the present study. Although a large number of samples came from eastern Germany, especially from Thuringia, *M. esculenta* was not observed there, and overall was found only twice in samples from western Germany.

Discussion

The results of this study indicate that the species concept for *M. esculenta* of most German authors (e.g. Bresinsky and Stangl 1961; Krieglsteiner 1993; Hardtke and Otto 1998) probably is too simplistic. To treat the different sequence types taxonomically as subspecies or varieties seems inadequate because of the marked differences found in their ITS sequences and a clear separation in the phylogenetic analyses (Figs. 1 and 2). The 7% difference in sequence length between *M. crassipes* and *M. esculenta*, leading to an overall sequence identity of only 80%, should justify treatment as distinct species. Even within the highly variable Glomeromycota, a species border based on ITS sequence data was proposed at a level of 93% sequence identity (Wubet et al. 2003). Common growth of all three species within an area and similar habitats additionally encourages us to treat the three discovered types as distinct species. As mentioned by Percudani et al. (1999), sole analysis of small (SSU) or large subunit (LSU) rDNA, as used to estimate phylogeny in fungi, is often not sufficient at or below the genus level, for which analysis of the more rapidly evolving ITS might help. Also in other groups of Pezizales, especially truffles, ITS has been used successfully to clarify the phylogeny of closely related species (e.g. Díez et al. 2002).

Based on our data, and following the taxonomic treatment of Wipf et al. (1999), it seems justified to

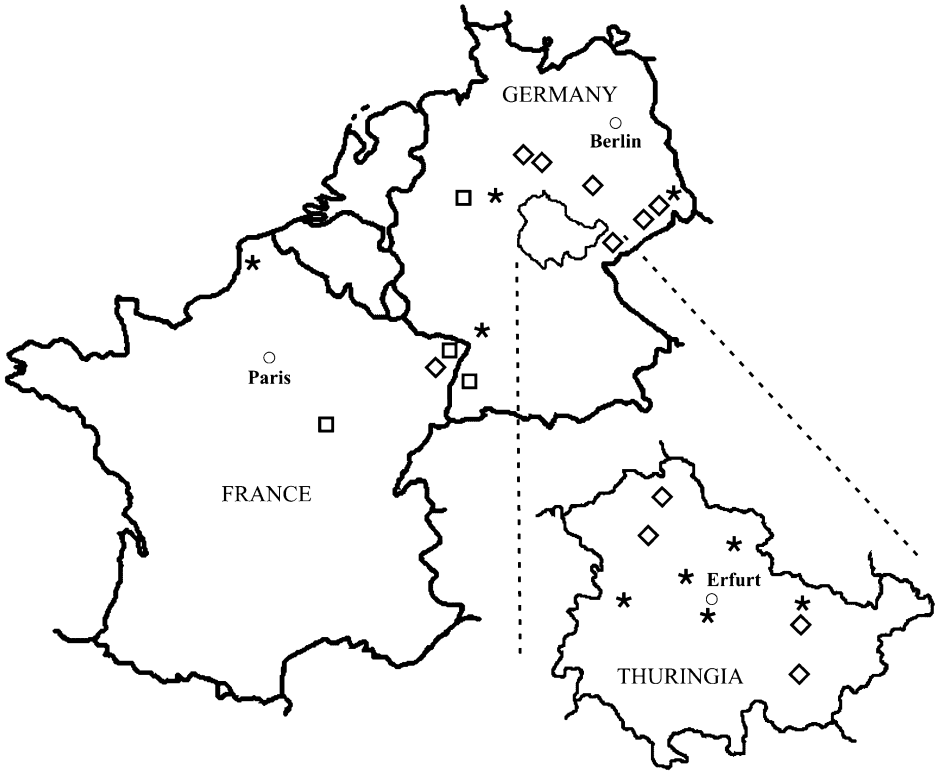

Fig. 4. Distribution of species belonging to the *M. esculenta* group in Germany and France, based on ITS nrDNA sequences and RFLP; symbols indicate species as follows: squares = *M. esculenta*, asterisks = *M. spongiola*, diamonds = *M. crassipes*.

distinguish three different species within the *M. esculenta* group: *M. esculenta* s.str., *M. crassipes*, and *M. spongiola*. They can be separated easily by the marked differences in ITS length, resulting in polymorphic restriction patterns (see also Buscot et al. 1996). Restriction enzymes appropriate for species separation using restriction fragments on agarose gels are: *Alu*I, *Bsu*RI, *Eco*RI, and *Taq*I. Much of this ITS heterogeneity is due to remarkable indels, especially in the ITS1 region.

To support this separation one has to consider that, if there are only few species of morels (which would lead to a grouping of the three types separated here in the single taxon *M. esculenta*), these display high intraspecific variability in the ITS region. If this is so, it is difficult to explain how three clear clades (showing nearly no internal variation and no transitions to the other clades) can be separated within *M. esculenta*, and how within one clade individuals gathered at 1000 km distance, i.e. in Merlimont (France) and Thuringia (Germany), can have exactly the same ITS sequence (see below). This should not be the case if ITS variation reflects intraspecific variation. Moreover, Buscot et al. (1996) showed that within one ITS type, individuals cannot even be separated by microsatellite PCR, indicating a high degree of ITS homogeneity within a species.

Following the treatment of these latter authors, *M. vulgaris* can be seen as a synonym of *M. esculenta* (Figs. 1 and 2), based on the identity displayed by our *M. esculenta* s.str. sequences with one sequence of *M. vulgaris* in GenBank. The weak support for the subclade within *M. esculenta* s.str. (Figs. 1 and 2) is only due to some substitutions in the otherwise homogeneously alignable sequence data for this species. According to Larget and Simon (1999), branch supports less then 0.95 using Bayesian posterior probabilities (Fig. 1) are not significant.

In conclusion, it can be proposed that, rather than one variable *M. esculenta*, three different species of this group, as mentioned above, occur in Germany and France.

Based on this result it would be necessary to revise the treatment of morels in German textbooks on fungi, although this seems impossible as long as no appropriate morphological or ecological features separating these species are available. Studies integrating morphological, ecological and molecular characterisations of a large number of samples could help to find typological factors consistent with the molecular separation.

An interesting aspect concerns the distribution of *Morchella spongiola*. While Wipf et al. (1999) had all their samples of this species from a dune region in

France (hence the name *Morchella spongiola* var. *dunensis* R. Heim), we found it frequently in Thuringia on limestone and far away from any Atlantic influence or acidic sandy soils. Sequence comparison and re-sequencing of the Dune Morel, *Morchella spongiola* var. *dunensis*, revealed no nucleotide difference to the Thuringian material. This indicates that the occasional practice of separating morel species by their supposed ecological features may have led to an artificial classification. Abiotic factors easily influence morphological characters in morels, which do not reflect their genetic distance.

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