Data Partitions, Bayesian Analysis and Phylogeny of the Zygomycetous Fungal Family Mortierellaceae, Inferred from Nuclear Ribosomal DNA Sequences

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Abstract

Although the fungal order Mortierellales constitutes one of the largest classical groups of Zygomycota, its phylogeny is poorly understood and no modern taxonomic revision is currently available. In the present study, 90 type and reference strains were used to infer a comprehensive phylogeny of Mortierellales from the sequence data of the complete ITS region and the LSU and SSU genes with a special attention to the monophyly of the genus Mortierella. Out of 15 alternative partitioning strategies compared on the basis of Bayes factors, the one with the highest number of partitions was found optimal (with mixture models yielding the best likelihood and tree length values), implying a higher complexity of evolutionary patterns in the ribosomal genes than generally recognized. Modeling the ITS1, 5.8S, and ITS2, loci separately under certain constraints, but that significant heterogeneity can be found within these loci also. The phylogenetic analysis indicated that the genus Mortierella is paraphyletic with respect to the genera Dissophora, Gamsiella and Lobosporangium and the resulting phylogeny contradict previous, morphology-based sectional classification of Mortierella. Based on tree structure and phenotypic traits, we recognize 12 major clades, for which we attempt to summarize phenotypic similarities.

Introduction

The order Mortierellales constitutes one of the largest groups of Zygomycota. Most of the taxa are oligo– or mesotrophic and occur typically as saprobes in soil, dung or other decaying organic material. Many of them, such as Mortierella alpina and related species, are able to convert various carbon sources into lipids and are of great biotechnological potential as producers of polyunsaturated fatty acids [1–3], while others are used as biotransforming agents of various organic compounds [4,5]. The genus Mortierella also contains an animal pathogen, M. iciflora, which differs from the other species in its thermophilic nature [6].

Members of the order generally form unusually delicate, cottony mycelia, which are coenocytic when young, but often become septate on ageing. In most cases, the colonies are white to grayish–white and display a rosette–like (zonate) surface on several types of medium (Fig. 1a), pure colonies may produce a characteristic garlic–like odor. These fungi produce sporangia or sporangiola, where columellae are often absent or rudimentary and never protrude into the sporangium, as well as collarettes (Fig. 1i), collar–like structures on the sporangiophores, which are formed by remnants of the sporangial envelope [7–9]. The lack of pronounced columellae and the production of non–apophysate sporangia distinguish members of the Mortierellales from those of the Mucorales [10]. They may form smooth or ornamented, intercalary and/or terminal chlamydospores (Fig. 1c, e–g, j, l). Zygospores (sexually produced spores), when present, are formed between apposed suspensors and are often covered by a thick hyphal sheath [6,9]. As zygospores have been observed for only some of the species [11], the characterization of Mortierellales is based entirely on asexual characteristics [12].

The phylogeny of Mortierellales is poorly understood and no modern taxonomic revision of the order is available. Originally, this fungal group was considered as a family of the Mucorales, named Mortierellaceae [13] containing two genera, Herpocladium [14] and Mortierella [15]. Subsequently, several genera were added...
to the family or segregated from the genus Mortierella, such as Dissophora, Lobosporangium [16], Azygosporangium [17], Umbelopsis [18], Aquamortierella [19], Echinosporangium [20], Actinomortierella [21], Modicella and Gamsiella [22]. Echinosporangium is now regarded as a synonym of Lobosporangium [22]. The order Mortierellales was proposed by Cavalier-Smith [23] and molecular phylogenetic studies based on ribosomal and protein coding gene sequences reinforced the need for the separation of the Mortierellaceae from the Mucorales [8,24–26].

Although several studies have been performed in attempt to clarify the relationships of zygomycete fungi at the family and ordinal levels, the lack of information concerning the phylogeny of the largest genus, Mortierella, is surprising. At present, Mortierellales contains one family, Mortierellaceae, which comprises six genera, Aquamortierella, Dissophora (Fig. 1k–m), Gamsiella (Fig. 1o), Lobosporangium, Modicella and Mortierella [8,9]. Among them, Dissophora and Modicella contain only two species each, while Aquamortierella, Lobosporangium and Gamsiella are monotypic. At the same time, Mortierella is considered the largest genus of Zygomycota, with about 100 recognized species [9]. Earlier, two subgenera, Mortierella and Micromucor, were distinguished within it [29]. Based on ITS sequence data, members of the Micromucor subgenus, which was also known as the Mortierella isabellina group, were transferred to the genus Umbelopsis and placed in a newly proposed family, Umbelopsisidae, within the order Mucorales [30]. Several molecular phylogenetic studies have shown that Umbelopsis form a basal sister–group to the Mucorales [8,24–26].

Based on morphological similarities and previous classifications, Gams [29] divided the subgenus Mortierella into the following nine sections: Simplex, Alpina, Schneckii, Mortierella, Actinomortierella, Hygrophila, Stylopora, Spinosa and Haplosporangium. In view of the differences and contradictions that can be found among the different classifications [7,29,31], it appears reasonable to revise the taxonomy of the genus, which requires an exploration of its phylogenetic structure.

The aim of the present study was to infer the phylogeny of the order Mortierellales from sequence data of three nuclear ribosomal regions (the complete ITS region and the LSU and the SSU genes) with special emphasis on the monophyly of Mortierella with respect to Dissophora, Gamsiella and Lobosporangium, since this has been brought into question by preliminary analyses [8]. Hence, ribosomal genes from 90 strains including representatives of the genera Dissophora, Gamsiella, Lobosporangium, Mortierella and Umbelopsis were sequenced, and phylogenetic analyses involving partitioned and mixture models were undertaken. The monophyly of previously morphologically distinguished sections of Mortierella were also tested on the basis of the resulting phylogeny.

Finding the balance between model complexity and the variance of the estimation, or the ease of convergence in Bayesian MCMC analyses is an important and highly debated aspect in phylogenetics [32–36]. While it is evident that oversimplified models lead to severely biased estimates, including unreliable posterior probabilities, the effects of overly complex models is not as straightforward. Advocating the power of Bayesian estimation for converging to the right posterior distribution under complex parameter spaces, several recent studies have used highly partitioned complex models (e.g. [35,37,38]) and simulation studies support the legibility of highly complex models in molecular evolution. On the other hand, overpartitioned models have been shown to negatively affect MCMC convergence [32,39] and the computation of Bayes Factors on the basis of harmonic mean likelihoods results in a strong preference of overpartitioned models. How partition boundaries should optimally be defined, and how optimal partitioning relates to commonly recognized, biologically meaningful features within the phylogenetic markers represents an intriguing question with very few experimental analyses so far [35]. Therefore, in this study we examined the effects of increasing model complexity in nearly contiguous spans of the nuclear ribosomal SSU, ITS1, 5.8S, ITS2 and LSU genes and the relationship between a priori defined biologically recognized partition boundaries and another partitioning regime, in which homogeneously evolving sites are identified during the MCMC runs [40].
Results

Model testing of single gene alignments and tests of congruence

Substitution models for the single-gene alignments were selected by using jModelTest based on the sample-size corrected AIC criterion. When the model suggested by the program was not available in subsequently used software (MrBayes, BayesPhylogenies and RaxML), the next more complex model was chosen for the alignment. The GTR+Γ substitution model was therefore selected for all single-gene alignments for subsequent analyses. Since we did not find any significant conflict between the single-gene alignments, all the data were concatenated and subjected to supermatrix analyses. The concatenated alignment is available on TreeBase (accession no.: S11367).

Evaluation of alternative partitioned models

Altogether 15 different partitioned models were compared on the basis of Bayes factors. The Bayesian runs converged quickly to the stationary distributions, well before the specified burn-in values. Pairs MrBayes and BayesPhylogenies runs converged to the same region of the posterior based on Bayes factor tests. The mean of the obtained log likelihood values and Bayes factors of pairwise model comparisons are presented in Table 1. As expected, the more complex models fitted the data better in all cases, but the Bayes factor tests revealed an interesting pattern. Increasing the number of partitions always caused significant improvements in the likelihood values (Fig. 2a). Analyses in which the three ribosomal regions (ITS, SSU and LSU) were treated as one contiguous partition and the indel matrix as a second one (“2 partitions A”, “B” and “C”) returned the poorest likelihoods of all runs, regardless of the model used for the specific partitions (conventional GTR+Γ in “A” and “B” and mixture model in “C”) and are significantly rejected against more sophisticated partitioning regimes. Runs with ITS, SSU and LSU as three partitions returned transitional likelihood scores (“3 partitions A” to “C”), whereas the best results were obtained when the ITS1, 5.8S and ITS2 genes were modeled separately in addition to the SSU, LSU and indel matrices (“6 partitions A” to “I”). It is interesting that increasing the number of parameters for the indel matrix had a negligible effect on the likelihoods (log BF: 0.315–1.369, partitioning schemes “A” versus “B”).

Of the analyses with 6 partitions, better results were obtained when mixture models were used to model the specific partitions. Increasing the number of GTR+Γ matrices caused improvements in the likelihoods, but the improvements between subsequent models decreased until the likelihoods became saturated (Fig. 2b). This saturation was observable when 5 GTR+Γ matrices were fitted to the data. Six and seven such matrices did not improve the results appreciably, whereas the computational burden for adding extra matrices to the model increased linearly. Therefore, in subsequent Bayesian analyses we used the “6 partitions G” partitioning and modeling regime as optimal for our data.

It is noteworthy that the total tree lengths recovered by the models also increased when the complexity of partitioning and modeling was increased (Table 2), which suggests that more complex models are able to recover more hidden substitutions than simpler ones. Exceptions from this were “6 partitions G” and “D” which returned much higher TL values than would be expected on the basis of their likelihood-based ranking. This might be caused by differences in branch-length priors between MrBayes and BayesPhylogenies and/or being trapped in regions of the posterior with unrealistically long branches due to poor convergence [41–43].

Phylogenetic analyses and relationships within the Mortierellales

All three replicates of the final BayesPhylogenies analysis converged to the same posterior distribution, hence, the trees were pooled and a consensus tree was computed. The majority rule consensus tree computed from 2×105 trees sampled after stationarity is presented in Fig. 3 with MPBS, MLBS and BPP values on the branches.

At the genus level, two large clades can be recognized, Umbelopsis (MPBS: 100%, MLBS: 100%, BPP: 0.99) and Mortierella (MPBS: ~, MLBS: 100%, BPP: 0.92). As expected, Umbelopsis, including representatives of Microoncur, clusters with Rhizopus oryzae, a representative of the Mucorales. The genera Dissophora, Lobosporangium and Ganssella are nested within the genus Mortierella with significant support. With the exception of Mortierella longicollis, a monophyletic Mortierellaceae can be recognized if the above-mentioned genera are included. M. longicollis is placed in a basal position, closest to the outgroup taxon R. oryzae (Mucorales).

Within the Mortierella clade, 12 large clades were distinguished and named after a representative or well-known species of the genus. The /selenospora clade (MPBS: 100%, MLBS: 100%, BPP: 1.00) includes the M. wolfii strain CBS 614.70 and the type strain of M. selenospora. The position of the other isolates of M. wolfii suggests that strain CBS 614.70 was misidentified, which is reinforced by the thermophilic nature of this strain with an optimal growth temperature of 24 °C. The thermophilic M. wolfii isolates were found in the /wolfii clade (MPBS: 100%, MLBS: 100%, BPP: 1.00) together with M. capitata. The /angusta clade (MPBS: 100%, MLBS: 92%, BPP: 1.00) contains three closely related taxa, M. alpina, M. anamboidea and M. antarctica, all with preference for low temperatures. The /parvispora clade (MPBS: 94%, MLBS: 85%, BPP: 0.88) contains six taxa, of which M. cystojenkinii, M. elongatula, M. tafiocola and M. pulchella form a closely related subclade. The /verticillata–humilis clade (MPBS: 98%, MLBS: 96%, BPP: 0.98) includes eight taxa, among which isolates of M. verticillata and M. humila form one strongly supported (MPBS: 100%, MLBS: 99%, BPP: 1.00) species-level group. M. minutissima and M. harticola also seem to be very closely related. The /mutabilis clade (MPBS: 100%, MLBS: 100%, BPP: 1.00) contains M. rostafinski and M. strangulata. Lobosporangium versans is found as a sister group of this clade with low support (MPBS: ~, MLBS: ~, BPP: 0.69). The seven species in the well-defined /ligicola clade (MPBS: 100%, MLBS: 98%, BPP: 1.00) are distributed in four similarly well-supported subclades. The /globulifera clade (MPBS: 100%, MLBS: 100%, BPP: 1.00) only includes representatives of M. globulifera. The /angusta (MPBS: 100%, MLBS: 96%, BPP: 1.00) and /mutabilis (MPBS: 100%, MLBS:100%, BPP: 1.00) clades contain the two non-mortierella-lean genera, where Dissophora belongs to the former and Ganssella to the latter. The type species of the genus Mortierella, M. polycephala, is included in the /polycephala clade (MPBS: 100%, MLBS: 91%, BPP: 1.00), together with M. polygonia, M. indohii, M. hyspicalda and M. hyalina. The most species-rich and heterogeneous group is the /ganssiae clade (MPBS: 97%, MLBS: 82%, BPP: 1.00), consisting of several species and subgroups. The gross topology and strongly supported nodes of the trees were affected neither by GBlocks-curation of the ITS alignment nor the exclusion of the complete ITS1-5.8S-ITS2 region or the indel-matrix (Fig. S1, S2, S3, S4). Table 3 shows the posterior probability values of the 12 main clades inferred from the different analyses. Because the different exclusion strategies have not influenced the resolvability of the backbone of the tree, it is likely that the polytomies there were not caused by intra-alignment conflict or alignment noise. However, the position of some clades
Table 1. Comparison of alternative partitioning regimes on the basis of Bayes factors.

| Partitioned Model | -lnL  | 95% HPD | 2 partitions A | 2 partitions B | 6 partitions C | 2 partitions D | 4 partitions A | 4 partitions B | 6 partitions A | 4 partitions C | 6 partitions E | 6 partitions F | 6 partitions G | 6 partitions H | 6 partitions I | TL |
|-------------------|-------|---------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|---|
| 2 partitions A    | 34155.6 | +/- 0.28 | -              | -              |                |                |                |                |                |                |                |                |                |                |                | 7.51 |
| 2 partitions B    | 34154.7 | +/- 0.26 | 0.4            | -              |                |                |                |                |                |                |                |                |                |                |                | 7.48 |
| 6 partitions C    | 33982.8 | +/- 1.00 | 75.0           | 74.7           |                |                |                |                |                |                |                |                |                |                |                | 10.76 |
| 2 partitions C    | 33711.3 | +/- 1.73 | 192.9          | 192.5          | 117.9          | -              |                |                |                |                |                |                |                |                |                | 7.13 |
| 6 partitions D    | 33373.2 | +/- 1.59 | 339.8          | 339.4          | 264.7          | 146.9          | -              |                |                |                |                |                |                |                |                | 15.78 |
| 4 partitions A    | 33358.8 | +/- 0.39 | 346.0          | 345.7          | 271.0          | 153.1          | 6.3            | -              |                |                |                |                |                |                |                | 8.47 |
| 4 partitions B    | 33358.0 | +/- 0.42 | 346.0          | 346.0          | 271.3          | 153.4          | 6.6            | 0.3            | -              |                |                |                |                |                |                | 8.53 |
| 6 partitions B    | 33142.2 | +/- 0.27 | 440.1          | 439.7          | 365.0          | 247.2          | 100.3          | 94.0           | 93.7           | -              |                |                |                |                |                | 10.32 |
| 6 partitions A    | 33139.1 | +/- 0.29 | 441.4          | 441.1          | 366.4          | 248.5          | 101.7          | 95.4           | 95.1           | 1.4            | -              |                |                |                |                | 10.18 |
| 4 partitions C    | 33114.3 | +/- 1.84 | 452.2          | 451.8          | 377.2          | 259.3          | 112.4          | 106.2          | 105.9          | 12.2           | 10.8           | -              |                |                |                | 16.20 |
| 6 partitions E    | 33058.4 | +/- 1.04 | 476.5          | 476.1          | 401.5          | 283.6          | 136.7          | 130.5          | 130.1          | 36.4           | 35.1           | 24.3           | -              |                |                | 19.54 |
| 6 partitions F    | 33003.7 | +/- 0.98 | 500.2          | 499.9          | 425.2          | 307.3          | 160.5          | 154.2          | 153.9          | 60.2           | 58.8           | 48.0           | 23.7           | -              |                | 20.63 |
| 6 partitions G    | 32923.1 | +/- 2.09 | 535.2          | 534.9          | 460.2          | 342.3          | 195.5          | 189.2          | 188.9          | 95.2           | 93.8           | 83.0           | 58.7           | 35.0           | -              | 21.3 |
| 6 partitions H    | 32915.6 | +/- 2.04 | 538.5          | 538.1          | 463.4          | 345.6          | 198.7          | 192.4          | 192.1          | 98.4           | 97.0           | 86.3           | 62.0           | 38.2           | 3.2            | -              | 21.14 |
| 6 partitions I    | 32894.6 | +/- 2.48 | 547.6          | 547.2          | 472.6          | 354.7          | 207.8          | 201.6          | 201.3          | 107.5          | 106.2          | 95.4           | 71.1           | 47.4           | 12.4           | 9.1            | 22.36 |

The means of the ln likelihood values (-lnL), the 95% highest posterior densities (HPD) and the tree lengths (TL) averaged over all post-burn-in samples are also shown.

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(a)

- $\ln L$

Partitioning scheme

(b)

- $\ln L$

Number of GTR+\(\Gamma\) matrices
Figure 2. Saturation of log likelihood (InL) values as a function of model complexity, calculated as a mean of the likelihoods of post-burn-in trees. (a) Comparison of all 15 partitioned models. Figure shows that the indel matrix is best described by one-parameter models, which show up as local plateaus (see arrowheads) in the saturation of likelihoods. (b) Comparison of MCMC analyses performed by using the mixture models (in BayesPhylogenies) only with 1 to 7 GTR+Γ matrices.

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Discussion

Model selection

For the data set used here, we observed increases in the likelihood values when the complexity of the model was increased. Although this accords with expectations based on the properties of likelihood approaches, it has important implications for the handling of ribosomal genes in phylogenetic analyses. There has been some concern regarding the independence of ribosomal loci and the evolutionary processes acting on them (apart from concerted evolution). Some authors argue that the SSU, ITS and LSU genes are not independent and, among others, cannot be listed as different loci in phylogeny papers [44]. We found that separate modeling of the three regions improved the fit of the model to the data significantly, which suggests that the three loci evolve not only at different rates, but also under different constraints. In addition, we found that dividing the ITS regions into three partitions, ITS1, 5.8S and ITS2, also improved the results significantly. We therefore suggest independent modeling of these three regions in phylogenetic analyses when the amount and quality of the phylogenetic signal contained within them can support such parameter–rich models.

We observed extensive rate heterogeneity within the individual loci by using mixture models. These models are able to recover hidden evolutionary patterns in the data by iterating several (1 to 7 in our case) GTR+Γ matrices for each site of the alignment during the MCMC run. In this manner, each site in the alignment is assigned the most appropriate GTR+Γ matrix during the analysis, which can be considered a means of automatic partitioning of the alignments. For most of our partitions, five different matrices proved optimal for a description of the underlying patterns of evolution. For ribosomal coding regions, such models can be used as a surrogate for more parameter–rich secondary structure models, such as the doublet model in MrBayes [45]. In such cases, the mixture model does not require prior knowledge of the secondary structure and a priori partitioning of the alignment, which is often difficult, especially in groups with poor taxonomic coverage of experimentally established secondary rRNA structures.

It is also noteworthy that the exclusion of the complete ITS region strongly decreases tree resolution, which suggests that the ITS region contributes valuable phylogenetic signal congruent with that in the LSU and SSU genes, which is expected when the inference of positional homologies and indel placement in the ITS locus is accurate (see Fig. S1, S2, S3, S4).

Phylogenetic relationships

Our study included all genera of Mortierellales accepted to date, with the exceptions of the monotypic Aquamortierella and the bitypic

| Partitioning | ITS1 | 5.8S | ITS2 | LSU | SSU | Indel matrix | Software* | No. of GTR+Γ matrices |
|--------------|------|------|------|-----|-----|--------------|-----------|-----------------------|
| 2 partitions A | GTR+Γ | Mk1 | MB | 1 |
| 4 partitions A | GTR+Γ | GTR+Γ | GTR+Γ | Mk1 | MB | 1 |
| 6 partitions A | GTR+Γ | GTR+Γ | GTR+Γ | GTR+Γ | Mk1 | MB | 1 |
| 2 partitions B | GTR+Γ | Mk2 | MB | 1 |
| 4 partitions B | GTR+Γ | GTR+Γ | GTR+Γ | Mk2 | MB | 1 |
| 6 partitions B | GTR+Γ | GTR+Γ | GTR+Γ | GTR+Γ | Mk2 | MB | 1 |
| 2 partitions C | GTR+Γ | Nst1 | BP | 1 |
| 4 partitions C | GTR+Γ | GTR+Γ | GTR+Γ | Nst1 | BP | 1 |
| 6 partitions C | GTR+Γ | GTR+Γ | GTR+Γ | GTR+Γ | Nst1 | BP | 1 |
| 6 partitions D | GTR+Γ | GTR+Γ | Nst1 | BP | 2 |
| 6 partitions E | GTR+Γ | GTR+Γ | Nst1 | BP | 3 |
| 6 partitions F | GTR+Γ | GTR+Γ | Nst1 | BP | 4 |
| 6 partitions G | GTR+Γ | Nst1 | BP | 5 |
| 6 partitions H | GTR+Γ | Nst1 | BP | 6 |
| 6 partitions I | GTR+Γ | Nst1 | BP | 7 |

*Mb: MrBayes 3.1.2, BP: BayesPhylogenies 1.0.
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Dissophora species of the Mortierellales and do not fit the sections of the genus Mortierella established by different authors [7,29,31] (Fig. 3). Species of Dissophora, Gamsiella and Lobosporangium are nested in well-supported Mortierella clades, indicating the paraphyly of the latter genus. This result supports the suspicion of White et al. [8] based on a preliminary two-locus phylogenetic analysis. Moreover, both Dissophora and Gamsiella species were proven to be closely related to certain Mortierella species, i.e. M. angusta and M. mutabilis, respectively. Benjamin originally described G. multitubicata as M. multivaricata [46], as the only member of the newly established subgenus Gamsiella. Later, Benny and Blackwell elevated this subgenus to a generic level [22] because of the presence of characteristic, repeatedly divaricately branching sporangiophores (Fig. 1o), two-spored sporangia and terminal, ornamented chlamydospores. M. mutabilis was described as a member of the section Mutabilis of Mortierella with long, monopodially or synpodially branched sporangiophores and rarely observable chlamydospores [7,31]. Although the strain CBS 308.52, syntype of M. mutabilis, showed this morphology on MEA (Fig. 1p), on certain media, such as OA, we observed sporangiophores, sporangia and, what is more, terminal chlamydospores very similar to those of G. multitubicata (Fig. 1q). It is worth mentioning that, our observations indicated that the presence or absence and the shape of the sporangiophores, the sporangia and the chlamydospores depend to a considerable extent on the culturing medium applied (cf. Fig. 1f-g, p-q). For these traits, we demonstrated large differences between closely related species in many cases and high levels of homoplasy across the tree, which suggests that phenotypic characters have been evolving at fast rates in the Mortierellales. Moreover, only one or a few environmental isolates and relatively old morphological descriptions are available for several species, and reliance on these descriptions without explicit information on the culturing conditions may confound comparisons between different descriptions. These facts certainly contribute to the difficulties inherent in the morphology-based identification of Mortierellales species and to the prevalence of misidentifications in this group. We therefore believe that a standardized technique should be developed for the culturing and description of Mortierella species.

M. longicollis was initially considered a member of the M. isabellina group and the Micronucor subgenus [7,29,31]. With regard to ITS RFLP and sequence data, this group was later transferred into the genus Umbelopsis in the Mucorales [30]. However, that analysis found M. longicollis falling outside the genus Umbelopsis and more closely related to Mortierella. In our multilocus phylogeny, this species is situated far from the Mortierellales and also out of the Mucorales clade, being a sister group of the core Mucorales represented by Rhizopus oryzae (Mucorales, Mucoraceae), which suggests that M. longicollis is actually a mucoralean fungus, whose taxonomic position demands further analysis and the inclusion of more taxa from the Mucorales.

The ITS sequence of a M. turficola strain (GenBank accession no.: AJ870784) led Kwasna et al. [47] to conclude that this species may also belong in Umbelopsis. Linemann [31] and Gams [29] placed M. turficola in the section Hygrophila of Mortierella, while Zycha et al. [7] classified it in the section Isabellina. This section contained other species that were later transferred to Umbelopsis by Meyer and Gams [30], but they did not include M. turficola in their study. In our analysis, the neotype strain of M. turficola (Fig. 1n) was found to be closely related to M. pulchella, in the /parvispora clade of our tree, indicating that the strain used by Kwasna et al. [47] must have been misidentified.

M. humilis and M. verticillata (Fig. 1f-b) form a subclade within the clade /verticillata-humilis. The clade of M. verticillata includes syntype strains of M. humilis and M. marburgensis, which suggests that these three species are conspecific. Sexual compatibility tests between M. humilis and M. verticillata support this finding [48].

Table 3. Bayesian posterior probability values of the 12 larger Mortierella clades inferred from different datasets.

|             | ITS-nrLSU-nrSSU-gap | gbITS-nrLSU-nrSSU-gap | gbITS-nrLSU-nrSSU | nrLSU-nrSSU-gap | nrLSU-nrSSU |
|-------------|---------------------|-----------------------|------------------|----------------|-------------|
| /gamsii     | 1.00                | 0.96                  | 1.00             | 1.00           | 1.00        |
| /polycephala| 1.00                | 1.00                  | 1.00             | 1.00           | 1.00        |
| /angusta    | 1.00                | 1.00                  | 0.70             | 1.00           | 1.00        |
| /globulifera| 1.00                | 1.00                  | 1.00             | 1.00           | 1.00        |
| /mutabilis  | 1.00                | 1.00                  | 1.00             | 1.00           | 1.00        |
| /lignicola  | 1.00                | 1.00                  | 1.00             | 1.00           | 1.00        |
| /strangulata| 1.00                | 1.00                  | 1.00             | 1.00           | 1.00        |
| /verticillata-humilis | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| /parvispora | 1.00                | 1.00                  | 1.00             | 1.00           | 0.61        |
| /alpina     | 1.00                | 1.00                  | 0.94             | 1.00           | 0.95        |
| /volvii     | 1.00                | 1.00                  | 1.00             | 1.00           | 1.00        |
| /elenospora | 1.00                | 1.00                  | 1.00             | 1.00           | 1.00        |

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However, the distinction between the two species has been maintained on the basis of the differences in the ornamentation of sporangiogspores observed on scanning electron microscopy. We consider that the evidence provided by the phylogenetic structure and the interfertility of *M. humilis* and *M. verticillata* is sufficiently unequivocal for them to be regarded as a single species. *M. polycephala* (Fig. 1d, e), *M. polygyna* (Fig. 1f, g) and *M. indohii* (Fig. 1j) were earlier placed in various sections [29], whereas our phylogeny has furnished evidence of their high affinity for each other (*polycephala* clade). Further, these species exhibit the special feature of producing characteristic terminal, stalked chlamydospores, also known as stylospores (Fig. 1e, j). Relationship of *M. polycephala* and *M. indohii* has already been proposed on the basis of these structures [48]. Similar terminal chlamydospores have been observed in the case of *M. polygyna* (Fig. 1j).

This study has addressed the monophyly of *Mortierella* and the phylogenetic affinities of several segregate genera, such as *Gamsiella, Labosporangium* or *Disphora* for the first time. We found that a large monophyletic *Mortierella s. str. clade* can be discerned, which contains the type species of the genus *Mortierella, M. polycephala* Coem. 1863. The results necessitate either that the above-mentioned genera be placed within the genus *Mortierella* or that several distinct genera should be described in order to achieve a natural classification of the Mortierellales. Our results suggest that the phenotypic traits of *Mortierella* species depend strongly on the culturing conditions, which makes the search for synapomorphic generic criteria difficult. Thus, taxonomic descriptions should follow a standardized procedure for the reporting of the phenotype. We consider the main source of taxonomic disagreement and confusion between earlier monographic treatments of *Mortierella* to be the lack of standards, which is also the major cause of the frequent misidentifications in strain collections and environmental studies [49]. Our results underline the need for a new classification of Mortierellales, where molecular phylogenetic analysis should play a decisive role, and for a careful taxonomic and phylogenetic revision of the described species and sections within the genera.

**Materials and Methods**

**Taxon sampling and culturing conditions**

A total of 90 strains were obtained from the Centraalbureau voor Schimmelcultures (CBS–KNAW, Utrecht, the Netherlands) and the Jena Microbial Resource Collection (University of Jena, Germany) and examined in this study (Table S1). Based on previously published classifications and phylogenies, 85 strains of the Mortierellales were selected, representing the genera *Mortierella* (*61* taxa), *Gamsiella* (*1* species), *Disphora* (*2* species) and *Labosporangium* (*1* species). In addition, 4 representatives of the Umbelopsidaceae (*Umbelopsis* and the synonymous *Micromucor*, *2* taxa each) were analyzed. Two sequences of *Rhizopus oryzae* NRRL 28631 were downloaded from GenBank (LSU, *AY213626* and SSU, *AF113440*) and used as outgroup. An attempt was made to obtain the type strain of the respective species, whenever possible. We included multiple specimens of *M. wolffi* and *M. gamsii*, since preliminary analyses split them into different clades (results not shown).

Strains were grown in liquid malt-extract medium (5% malt extract, 1% glucose) for DNA extraction and on malt-extract agar (MEA, 5% malt extract, 1% glucose, 2% agar), oatmeal agar (OA, Difco, Becton, Dickinson, MD, USA) or cornmeal agar (CMA, 6% cornmeal, 1.5% agar) for morphological examinations. Cultivations were performed at 20–37°C for 7–12 days, depending on the requirements of the fungus.

**DNA sequencing**

Genomic DNAs were prepared from 10 mg of mycelia ground to a fine powder in liquid nitrogen and purified by using the MasterPure Yeast DNA purification kit (Epicentre, Madison, WI, USA) according to the instructions of the manufacturer. For all strains, ITS, LSU and SSU regions of the nuclear ribosomal rDNA were amplified by PCR, using the following primers: ITS1 and ITS4 for the complete ITS1 – 5.8S – ITS2 region, LR0R and LR7 for the first 1.5 kb of the nuclear LSU gene, and NS1 and NS4 for an approximately 1.0 kb long portion of the nuclear SSU gene [30,51]. Reactions were performed in a final volume of 20 µl, according to standard protocols [50]. Amplicons were sequenced on an ABI 3730xl automatic DNA sequencer (Applied Biosystems, Carlsbad, CA, USA) from both directions with the same primers, except for the LSU gene, for which the primer LR5 was used in some cases. Individual readings were assembled to contigs by using the PreGap and Gap4 programs of the Staden Package [52]. All sequences have been deposited in GenBank (Table S1).

**Alignments, model testing and tests of congruence**

Three alignments were compiled for this study. After the exclusion of non-overlapping leading and trailing gaps, the lengths of the SSU, ITS1 – 5.8S – ITS2 and LSU alignments were 1,018, 1,362 and 1,504 bp, respectively, of which the numbers of parsimony informative characters were 218, 470 and 398, respectively. Of the ITS1 – 5.8S – ITS2 regions, the ITS1 contained 659 nucleotide sites, the 5.8S gene contained 165 sites, and the ITS2 contained 538 sites. Gaps in the ITS1 – 5.8S – ITS2 alignment were recoded as a binary partition, resulting in 553 characters, of which 349 were parsimony informative. The concatenated alignment consisted of 3884 nucleic acid sites, plus 553 characters obtained by indel coding.

The alignments of the LSU and the SSU sequences were computed by ClustalX [53], followed by manual refinement where necessary. Because of the high number of indels, for the alignment of the ITS1 – 5.8S – ITS2 sequences we used the Probalign algorithm [54] with default settings. Leading and trailing gaps were deleted from the alignments. Indels in the ITS alignment were recoded as a binary matrix by means of the simple indel coding algorithm [55] as implemented in FastGap 1.21 [56]. This “indel matrix” was used in all Bayesian analyses.

Best-fit substitution models including rate heterogeneity were selected for each alignment, using jModeltest [57], the results of the sample-size corrected Akaike Information Criterion (AIC,) being preferred. Models with a proportion of invariant sites (I) in addition to Γ were excluded from the comparisons, since “Γ” accounts basically for the same phenomenon as “I” and the non-identifiability and interdependence of these two parameters have been reported [32,38].

Congruence of the phylogenetic signals in the single-gene alignments was tested by comparing Maximum Likelihood (ML) trees, using the approximately unbiased test in CONSEL 0.1 [59,60]. ML trees and single-site likelihoods were estimated from each single-gene alignment in 10 replicates, using the model selected by AIC, in RaxML 7.0.3 [61].

**Evaluation of alternative partitioned models**

To identify the best strategy for partitioning our concatenated alignment, we compared a series of partitioned and/or mixture models. Fifteen different partitioned models (Table 2) were set up as follows. Partitioning was designed on the basis of the gene function and borders. The first set of analyses considered the complete ITS region and the partial LSU and SSU genes as the first partition, and the indel matrix as the second partition
Data Partitions and Phylogeny of Mortierellaceae

Phylogenetic analyses

Bayesian MCMC, ML bootstrap (MLBS) and Maximum Parsimony bootstrap (MPBS) analyses were performed on the concatenated alignment.

Bayesian inference was performed with the model selected by the Bayes factor tests in BayesPhylogenies. Three replicates of 2 × 10^9 generations were run, every 1000th state being saved. For each partition, the model selected by jModelTest (GTR+Γ) was invoked. For the indel matrix, a one–state Markov model was used, with a correction for invariant sites not included in the matrix. The burn–in value was determined by checking likelihood and topological convergence. The convergence of likelihood values was checked by using Tracer 1.4, while topological convergence was inspected by using AWTY [67]. To obtain posterior probabilities, post–burn–in trees of the three runs were pooled and a 50% Majority Rule phylogram was generated by using the CONSENSE program of the PHYLIP package [68]. Clades receiving Bayesian posterior probabilities (BPPs) ≥0.95 were considered significantly supported.

MLBS analysis was run by using the parallel version of RaxML 7.0.3, with five partitions, each modeled by the GTR+Γ model of evolution. Gaps were treated as missing data. One thousand nonparametric bootstrap replicates were run. Clades receiving 70% or higher bootstrap support were considered to be significantly supported.

Equally weighted MP searches were executed in PAUP v. 4.0b10 [69] according to the following strategy: initial heuristic searches were performed in 1000 replicates to identify tree islands with saving of a maximum of 5 trees per replicate (nchuck = 5, chuckscore = 1, TBR branch-swapping, MAXTREES set to autoincrease). Subsequently, more thorough branch swapping was conducted on the trees resulting from the search outlined above (start = current, nchuck = 0, chuckscore = 0). Gaps were treated as missing data. Nodal support was estimated through 1000 bootstrap replicates with 10 random sequence additions per replicate.

Analysis of the exclusion of insertion-deletions in the ITS region

Both, the ITS 1 and ITS2 regions contain a large number of insertions–deletions (indels) which makes them difficult to align. To examine the noise coming from potentially misaligned sites the ITS alignment and the reliability of the indel-matrix, we set up four additional datasets as follows: (1) exclusion of the ambiguously aligned positions of the ITS region but leaving the indel-matrix, (2) exclusion of the ambiguously aligned positions of the ITS region and the indel-matrix, (3) exclusion of the complete ITS1-5.8S-ITS2 but keeping the LSU and SSU data together with the indel-matrix, and (4) exclusion of the complete ITS1-5.8S-ITS2 and the indel matrix keeping only the LSU and SSU data. Poorly aligned positions were eliminated by using the GBlocks server running version 0.91b of the program [70]. During this process we set the following parameters to the program: "Allow smaller final blocks", "Allow gap positions within the final blocks", and "Allow less strict flanking positions". The four resulting datasets were then analyzed by BayesPhylogenies as described above. Consensus trees were computed for each analysis by using the SumTrees script of the DendroPy package [71] in each case. The resulting trees can be found in the Supplementary Material (Fig. S1–S4).

Supporting Information

Figure S1 Consensus tree computed from 4000 post-burn-in trees sampled by using the GBlocks curated ITS region, the nuclear ribosomal large (LSU) and small (SSU) subunits and the indel-matrix. (PDF)

Figure S2 Consensus tree computed from 4000 post-burn-in trees sampled by using the GBlocks curated ITS region, the nuclear ribosomal large (LSU) and small (SSU) subunits without the indel-matrix. (PDF)

Figure S3 Consensus tree computed from 4000 post-burn-in trees sampled by using the nuclear ribosomal large (LSU) and small (SSU) subunits without the indel-matrix. (PDF)

Figure S4 Consensus tree computed from 4000 post-burn-in trees sampled by using the nuclear ribosomal large (LSU) and small (SSU) subunits and the indel-matrix. (PDF)
Table S1  Fungal strains included in this study, their collection numbers and type status, and the accession numbers of the sequences deposited in GenBank.

(PDF)

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