Phylogeny and character evolution of the coprinoid mushroom genus Parasola as inferred from LSU and ITS nrDNA sequence data

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species concept

Abstract
Phylogenetic relationships, species concepts and morphological evolution of the coprinoid mushroom genus Parasola were studied. A combined dataset of nuclear ribosomal ITS and LSU sequences was used to infer phylogenetic relationships of Parasola species and several outgroup taxa. Clades recovered in the phylogenetic analyses corresponded well to morphologically discernable species, although in the case of P. leiocephala, P. lilatincta and P. plicatilis amended concepts proved necessary. Parasola galericuliformis and P. hemerobia are shown to be synonymous with P. leiocephala and P. plicatilis, respectively. By mapping morphological characters on the phylogeny, it is shown that the emergence of deliquescent Parasola taxa was accompanied by the development of pleurocystidia, brachysbasidia and a plicate pileus. Spore shape and the position of the germ pore on the spores showed definite evolutionary trends within the group: from ellipsoidal the former becomes more voluminous and heart-shaped, the latter evolves from central to eccentric in taxa referred to as ‘crown’ Parasola species. The results are discussed and compared to other Coprinus s.l. and Psathyrella taxa. Homoplasy and phylogenetic significance of various morphological characters, as well as indels in ITS and LSU sequences, are also evaluated.

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INTRODUCTION

Within Psathyrellaceae, the genus Parasola is considered a fairly homogeneous group of mushrooms, with a small umbrella-like pileus, which is deeply plicate (= grooved, Fig. 1f) up to the centre. Their fruiting bodies are completely devoid of veil (velum universale), hence the sectional name ‘Glabri’ was formerly applied to the group. They are common decomposers of different types of organic substrates (leaf-litter, wood, herbivore dung) and are distributed world-wide with most of the records being from Europe and North America, including scattered notes from Asia, Venezuela, Australia, Lesser Antilles and Africa (Pegler 1966, 1983, 1986, Dennis 1970, Grgruinovic 1997).

Much attention was paid to species delimitation within the group (Orton & Watling 1979, Uljé & Bas 1988, Uljé & Bender 1997, Roux 2006). However, problems pertaining to species identification still exists which can either be attributed to the excessive morphological variability of taxa or to incorrect circumscription of some species (Nagy 2005). Characters used to delimit species include colour and size of fruitbodies, shape and size of spores, pleuro- and cheilocystidia, position of the germ-pore on the spores, and habit. For instance, according to the most recent treatment of the group (Uljé 2005), P. kuehneri is defined as having smaller spores, a more redish pileus and more cylindrical cheilocystidia than the closely related P. leiocephala. An important role was attributed to the angle of the germ pore with regard to the longitudinal axis of the spores. In Psathyrella conopilus and P. auricoma the germ pore is central (i.e. this angle is c. 90°) whereas in other taxa it is eccentric (< 90°). Parasola megasperma and P. plicatilis can have transitional states as well. Parasola lilatincta is considered the only species with lilaceous colours, caused by the presence of a pigment in tissues (Uljé & Bender 1997). It was suggested that these characters do not always co-occur and that therefore the presence of lilaceous tints is not diagnostic for P. lilatincta (Nagy 2005).

Recent phylogenetic studies drew attention to the contradictory phylogenetic position of Psathyrella conopilus. This taxon recurrently appeared in a sister position to other Parasola taxa (Walther et al. 2005, Larsson & Örstedius 2008, Padamsee et al. 2008, Vasutova et al. 2008). Possible support for this rather unexpected relationship of a non-deliquescent species with coprinoid fungi is, as suggested by Walther et al. (2005), provided by the presence of brown setae on the pileus of both Psathyrella conopilus and P. auricoma. However, this suggestion should be tested as in none of the foregoing studies did these two species cluster together. Summarising phylogenies published to date, Larsson & Örstedius (2008) introduced the name Parasola conopilus. Inconsistent with this is the opinion of Padamsee et al. (2008): they stressed the need for a separate genus that would accommodate P. conopilus. However, their morphological arguments for a new genus are incorrect (as noted also by Larsson & Örstedius 2008), because P. conopilus has no thin-walled pleocystidia at all, but thick-walled hairs, equal to those of P. auricoma. The possible relationship of P. conopilus to P. auricoma raises fundamental questions about the new classification of psathyrelloid and coprinoid fungi, i.e. how Psathyrella and its allies can be taxonomically separated from the phylogenetically related Coprinus-like genera (Redhead et al. 2001, Padamsee et al. 2008).

Within Parasola, the placement of P. auricoma is also controversial: it was often segregated in subsect. Auriconi from the rest of the group (subsect. Glabri) (Uljé & Bas 1988, Uljé & Bender 1997), while admitting its affinity to these taxa by being classified in sect. Setulosi, e.g. Coprinellus disseminatus. Phylogenetic

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studies published so far agree that *P. auricoma* is a member of the *Parasola* clade (Hopple & Vilgalys 1999, Moncalvo et al. 2002, Walther et al. 2005, Padamsee et al. 2008), but its position within that group is still obscure due to limited sampling of *Parasola* taxa.

Studies on fungal trait evolution mainly concentrated on features that are conserved at the family-phylum level (Lutzoni et al. 2001, Hibbett & Donoghue 2001, Hibbett 2004, Aanen & Eggleton 2005). Hence, very little is known about the phylogenetic value of morphological characters at or below genus level. However, before extrapolating phylogenetic results to classification, we think it is essential to know how morphological traits evolve. Upholding classification, comparative phylogenetic methods may facilitate selection of morphological features that are less homoplasious or show definite trends. Within dark-spored agarics, Frøslev et al. (2007) reported extensive conservation of distribution of certain chemical characters at species or group level in callochroid taxa of the genus *Cortinarius*. Padamsee et al. (2008) mapped a series of morphological features on
the phylogeny (e.g., cystidal wall, presence of brachybasidia or pleurocystidia) of coprinoid fungi, but found most of them strongly homoplasious, which suggests characters in this group should be mapped on a smaller scale to get more reliable estimates of the nature of these traits.

In this study we address generic limits of Parasola based on a broad sampling of nrLSU and ITS sequences. Data from the two genes (nrLSU and ITS) and indel characters of 38 Parasola specimens, representing all but one morphologically distinct species, are combined in order to investigate species limits and intrageneric relationships. Specimens identified as P. galericuliformis and P. hemerobia were also included. Special attention is paid to the limits of P. lilatincta as compared to P. leiocephala and P. Schroeteri, in order to ascertain the diagnostic utility of ilaceous coloration and spore size. Homoplasy and the extent of conservation exhibited by the morphological characters used to delineate species are estimated by mapping them onto the phylogeny of Parasola taxa. A resulting hypothesis of evolution of morphological traits in Parasola is presented and discussed.

MATERIALS AND METHODS

Taxon sampling

Specimens for this study were either field-collected or loaned from public herbaria. Identifications were based on type studies and two revisions (Uljé & Bas 1988, Uljé & Bender 1997) supplemented with experiences of a revision of > 500 herbarium specimens of all Parasola taxa by L.N. Freshly collected fruit-bodies were dried in Silica gel to prevent tissues from collapsing. Collections within one species were chosen preferably from diverse geographical regions to reflect potential intraspecific differences.

### Table 1 Origin, herbarium number, and GenBank accession numbers of specimens used in this study.

| Species                  | Locality          | Voucher No.       | Identifier       | GenBank No. |
|--------------------------|-------------------|-------------------|------------------|-------------|
|                          |                   |                   |                  | LSU         | ITS         |
| Coprinopsis impatiens    | Hungary, Alföld   | SZMC-NL-1164      | L. Nagy          | FM160732    | FM163177    |
| Coprinus heptemerus      | Hungary, Alföld   | SZMC-NL-2144      | L. Nagy          | FM160731    | FM163176    |
| Coprinopsis lagopus      | Hungary, Alföld   | SZMC-NL-2143      | L. Nagy          | FM160730    | FM163179    |
| Coprinopsis naricola     | Hungary, Alföld   | SZMC-NL-2342      | L. Nagy          | FM160729    | FM163180    |
| Coprinopsis pseudonuneus | Hungary, Alföld   | SZMC-NL-2340      | L. Nagy          | FM160728    | FM163181    |
| 'Coprinus' pollomallus   | Hungary, Alföld   | SZMC-NL-2336      | L. Nagy          | FM160727    | FM163182    |
| 'Coprinus' bellulus      | Hungary, Alföld   | SZMC-NL-2341      | L. Nagy          | FM160680    | FM163176    |
| Lacymania lacymabunda    | Sweden, Stockholm | SZMC-NL-0082      | L. Nagy          | FM160726    | FM163183    |
|                          | Sweden, Öland     | SZMC-NL-2140      | L. Nagy          | FM160725    | FM163184    |
| Parasola auricoma        | Hungary, Alföld   | SZMC-NL-0087      | L. Nagy          | FM160741    | FM163185    |
| Parasola conopilus       | Hungary, Alföld   | SZMC-NL-0268      | L. Nagy          | FM160684    | FM163222    |
| Parasola galericuliformis| Hungary, Alföld   | SZMC-NL-0285      | L. Nagy          | FM160684    | FM163225    |
| Parasola hemerobia       | Hungary, Alföld   | SZMC-NL-0284      | L. Nagy          | FM160721    | FM163188    |
| Parasola hercules        | Netherlands, Rijswijk | Uljé 1269 (L) | C.B. Uljé           | FM160719    | FM163190    |
| Parasola kuehneri        | Netherlands, Alphen aan den Rijn | Uljé 904 (L) | C.B. Uljé           | FM160718    | FM163191    |
| Parasola leiocephala     | Hungary, Alföld   | SZMC-NL-0466      | L. Nagy          | FM160717    | FM163192    |
|                          | Sweden, Öland     | SZMC-NL-0228      | L. Nagy          | FM160716    | FM163193    |
|                          | Germany, Tübingen | SZMC-NL-0283      | L. Nagy          | FM160715    | FM163194    |
| Parasola lilatincta      | Hungary, Alföld   | SZMC-NL-0660      | L. Nagy          | FM160714    | FM163195    |
|                          | Hungary, Alföld   | SZMC-NL-0296      | L. Nagy          | FM160713    | FM163196    |
|                          | Hungary, Alföld   | SZMC-NL-0281      | L. Nagy          | FM160712    | FM163197    |
|                          | Hungary, Alföld   | SZMC-NL-0667      | L. Nagy          | FM160711    | FM163198    |
|                          | Hungary, Alföld   | SZMC-NL-0472      | L. Nagy          | FM160710    | FM163199    |
|                          | Hungary, Alföld   | SZMC-NL-0468a     | L. Nagy          | FM160709    | FM163200    |
|                          | England, Perthshire | D. Schafer 2382004 | L. Nagy         | FM160708    | FM163201    |
|                          | Netherlands, Leiden | Arnold 6939 (L) | C.B. Uljé           | FM160707    | FM163202    |
| Parasola aff. lilatincta | Hungary, Alföld   | SZMC-NL-0083      | L. Nagy          | FM160706    | FM163203    |
|                          | Sweden, Öland     | SZMC-NL-0096      | L. Nagy          | FM160705    | FM163204    |
| Parasola megasperma      | Denmark, Jutland  | C 19633 (C)       | L. Nagy          | FM160703    | FM163206    |
|                          | Spain, Gurmá Zu zones | AH 13089 | L. Nagy | FM160702 | FM163207 |
|                          | Sweden, Öland     | SZMC-NL-1924      | L. Nagy          | FM160701    | FM163208    |
| Parasola misera          | Hungary, Alföld   | SZMC-NL-0490      | L. Nagy          | FM160700    | FM163209    |
|                          | Hungary, Alföld   | SZMC-NL-0280      | L. Nagy          | FM160699    | FM163210    |
|                          | Hungary, Alföld   | SZMC-NL-0677      | L. Nagy          | FM160698    | FM163211    |
|                          | Hungary, Alföld   | SZMC-NL-0075a     | L. Nagy          | FM160697    | FM163212    |
|                          | Hungary, Alföld   | SZMC-NL-0075b     | L. Nagy          | FM160696    | FM163213    |
|                          | Hungary, Alföld   | SZMC-NL-0097      | L. Nagy          | FM160695    | FM163214    |
|                          | Sweden, Öland     | SZMC-NL-0298      | L. Nagy          | FM160694    | FM163215    |
| Parasola spinulosa       | Denmark, Jutland  | Klamer 061996 (C) | L. Nagy         | FM160693    | FM163216    |
|                          | Sweden, Öland     | SZMC-NL-0287      | L. Nagy          | FM160691    | FM163218    |
| Parasola schroeteri      | Netherlands, Hilversum | Brilir 1051999 (L) | C.B. Uljé           | FM160690    | FM163219    |
| Psathyrella ammophila     | Hungary, Alföld   | SZMC-NL-2151      | L. Nagy          | FM160689    | FM163220    |
| Psathyrella bipellis      | Hungary, Alföld   | SZMC-NL-2535      | L. Nagy          | FM160688    | FM163221    |
| Psathyrella prona var. utiformis | Hungary, Alföld | SZMC-NL-2534      | L. Nagy          | FM160687    | FM163222    |
| Psathyrella leuc opcodeia | Hungary, Alföld   | SZMC-NL-1953      | L. Nagy          | FM160683    | FM163226    |
| Psathyrella magnispora    | Hungary, Alföld   | SZMC-NL-1954      | L. Nagy          | FM160682    | FM163227    |
| Psathyrella phaseolispbra | Hungary, Alföld   | SZMC-NL-1952      | L. Nagy          | FM160681    | FM163228    |

1 Herbarium of Szeged Microbiological Collection.
In order to infer species limits, it was intended to sample at least three independent specimens from each morphological species of *Parasola* recognised by us during morphological studies (Nagy et al. unpubl.); thus we sampled 38 collections of the following 11 taxa: *Parasola auricoma*, *P. galericuliformis*, *P. hemerobia*, *P. hercules*, *P. kuehneri*, *P. leiocephala*, *P. lila-tincta*, *P. megasperma*, *P. ninerea*, *P. plicatilis* and *P. Schroeteri* (Table 1). Other taxa combined in *Parasola* were ignored in this study either because they are widely accepted synonyms of other taxa we included (*P. nudiceps*) or are considered dubious or insufficiently known (Uljé & Bas 1988, Nagy et al. unpubl.).

To root all coprinoid taxa, we used LSU and ITS sequences of *Psathyrellaceae* (Moncalvo et al. 2002, Matheny et al. 2006, Padamsee & Ochoterena 2000) in a separate, binary data partition. Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian analyses of resulting alignments were performed to infer phylogenetic relationships of the group. Equally weighted MP searches were executed in PAUPv. 4.0b10 (Swofford 2003) according to the following strategy: initial heuristic searches were performed in 1,000 replicates to identify tree islands with saving a maximum of 5 trees per replicate (nchuck = 5, chuckscore = 1, TBR branch-swapping, MAXTREES set to autoincrease). Subsequent, 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 by 1,000 bootstrap replicates with 10 random sequence additions per replicate. To assess the phylogenetic utility of gaps when coded as separate characters, a separate matrix containing only binary gap data was subjected to the same MP analyses as described above for the DNA matrix. Gaps were coded by means of the simple indel coding regime (Simmons & Ochoterena 2000) excluding leading and trailing gaps, with the expectation to provide more resolving power and nodal support (Simmons & Ochoterena 2000, Simmons et al. 2001, Kawakita et al. 2003). Rescaled Consistency Index (RCI) and Retention index (RI) (Farris 1989) were calculated in PAUP for indel data in order to describe the homoplasy exhibited by this character type.

Best-fit substitution models used in the likelihood-based analyses were selected by the model testing algorithm implemented in Topali v. 2.19 (Milne et al. 2004). During model selection, results of the AIC criterion was considered, with sample size set to alignment size, as suggested by Posada & Buckley (2004).

ML estimation was performed in PhyML v. 2.4.4 (Guindon & Gascuel 2003) with 1,000 non-parametric bootstrap replicates. To discover tree space effectively, the program was run several times by using parsimony trees, recovered in the initial MP searches described above, as user-defined starting trees. Thus in case of the ITS+LSU dataset this number was 88 as the initial MP search recovered these trees. In all bootstrap analyses, values above 70 % were considered significant (Fig. 2).

Bayesian p(MC)² analyses were run in MrBayes v. 3.1 (Ronquist & Huelsenbeck 2003). Metropolis Coupled Markov Chains with Monte Carlo simulation were run until likelihoods reached stationarity and the 2 independent runs converged (as deduced from the average standard deviation of split frequencies, i.e. < 0.01). Accordingly the chains were run three million generations in case of the ITS+LSU dataset, while the LSU+ITS+binary matrix required eight million generations (in this case burn-in was established in 5.5 × 10⁶). By sampling every 100th generation from the 2 independent runs in MrBayes, the analyses resulted in 45,002 and 50,002 trees, respectively (after the first 25 % was discarded as burn-in for the ITS+LSU dataset and the first 5.5 × 10⁶ generations for the LSU+ITS+binary matrix), which were used to construct 50 % majority rule consensus phylograms. The phylogeny inferred from the ITS+LSU+binary dataset is presented on Fig. 3. The Binary model implemented in MrBayes for restriction sites was used for the binary (gap) dataset with the command coding = variable to adjust for characters not included in this matrix, as suggested by Ronquist et al. (2005). Clades that received posterior probabilities > 0.95 were considered strongly supported.
Various morphological characters were mapped onto the phylogeny of Parasola taxa under the parsimony principle. Parsimony mapping was performed in Mesquite v. 2.01 (Maddison & Maddison 2007) by using both the ML tree and the 50% majority rule MP and Bayesian trees. The following traits were coded in 2005. By all means, to some extent (Orton & Watling 1979, Uljé & Bas 1988, Uljé et al. 2008) while others claim that they show deliquescence psathyrelloid taxa (including Parasola conopilus) in that their fruitbodies do differ from closely related taxa under the parsimony principle. Parsimony as characters in phylogenetic analyses

RESULTS

Sequence data, alignments and utility of gaps as characters in phylogenetic analyses

The ITS1-5.8S-ITS2 regions and approximately the first 1 500 bp of the LSU gene were successfully sequenced for 38 specimens of the ingroup taxa. Old or extremely minute specimens often proved difficult to use for DNA extraction, accordingly, P. hercules and P. kuehneri are represented by one sequence each in the alignment. Additional 15 taxa from the genera Psathyrella, Coprinillus and Coprinopsis were sequenced for the ITS and LSU regions to serve as outgroups. To root Psathyrellaceae eight sequences of Agaricus bisporus, Chlorophyllum molybdates, Coprinus comatus and Myceticulture cuneipes (AY635775, DQ403488, AY700187, DQ200928, AY635772, AY854066, AY745707, DQ403933, LSU followed by ITS in order of the taxa mentioned, respectively) were retrieved from GenBank. We used Agaricaceae as outgroup instead of choosing one of the genera within Psathyrellaceae because we were interested in the hitherto poorly understood deep branchings of the phylogeny as well (Hopple & Vilgalys 1999, Walther et al. 2005, Padamsee et al. 2008).

After exclusion of ambiguously aligned regions, the combined ITS+LSU dataset comprised 57 taxa and 2 114 characters of which 1 529 were constant, 155 were parsimony-uninformative.
and 430 sites were parsimony-informative. The ITS alignment contained large numbers of gaps, due to the high divergence of this region within *Psathyrellaceae*. By using more rigorously edited alignments of both LSU and ITS sequences, 397 binary coded gap characters were appended at the end of the combined matrix. Homoplasies of gap data in our case proved lower than in DNA sequence data: RCigapmatrix: 0.6849 and Rigaapmatrix: 0.906 vs RCIDN: 0.3871 and RIDNA: 0.7748.

Unfortunately, vast majority of gaps in the ITS and LSU alignments were confined to the connection of large clades (*Coprinopsis*, *Coprinellus*, *Parasola* and *Psathyrella* clades) and only few informative gaps reflected relationships within *Parasola*. The only exception is the pair *P. conopilus* – *P. auricoma*, which relationship was reflected in high degree by gap characters. For instance, at position 510 of the LSU alignment, there was a long gap unique to *P. auricoma* and *P. conopilus*, but lacking in all other *Parasola* taxa. Positions 82 (T), 126 (G), 188-9 (CA) and 297 (T) of the ITS alignment were represented by nucleotides in *P. conopilus*, while all other taxa of the alignment had gaps in these sites. The relationship of these two species to the rest of *Parasola* was also supported by gap characters.

Maximum parsimony analyses of gap data only, recovered congruent topologies as the combined ITS+LSU dataset, with 100 % bootstrap support for *P. conopilus* as sister group to all deliquescent (= collapsing) *Parasola* taxa (data not shown). In contradiction with this, Bayesian analysis of the ITS+LSU+gap dataset failed to recover this relationship with significant support (Fig. 3). Similarly, the Bayesian consensus tree of ITS+LSU data placed *P. auricoma*, *P. conopilus* and the ‘crown’ *Parasola* taxa in a tritomy. With regard to the topology within the genus *Parasola*, this tree (ITS+LSU) was congruent with those inferred from the ITS+LSU+gap dataset. Relationships of *Coprinopsis* and *Coprinellus* were better resolved when gap characters were neglected, probably due to alignment difficulties in these genera.

In our alignments, the large groups *Coprinopsis*, *Coprinellus* and *Psathyrella* clades were represented only by few taxa which – in some cases – hampered unambiguous interpretations of homology of certain indels during alignment. However, with denser sampling of taxa in these clades, this should be easier, thereby saving more characters to phylogeny inference.
Phylogenetic analyses

Model selection for likelihood-based analyses suggested the GTR+I+G and GTR+G as best-fit models for the LSU and the ITS datasets, respectively. The Maximum Likelihood tree is presented on Fig. 2. For Bayesian analyses all data (LSU+ITS+gap) were combined into a single file, resulting in a matrix of 2,511 characters. Convergence of runs in this case was difficult to get, only after 5.5 × 10^5 generations did the runs converge enough. Inclusion of gap characters did not influence the inferred branching order of Parasola specimens, only support values were increased to some extent. The 50 % Majority Rule Bayesian phylogram (Fig. 3) shows the same large clades as ML (Fig. 2) and MP (data not shown) trees. On this tree, however, the genus Coprinopsis was split into smaller clades, which may be attributed to the high divergence of ITS fragments of Coprinopsis, making the interpretation of positional homology often doubtful in our case (due to limited number of sequences).

All trees recovered provide evidence for the monophyly of Parasola including P. conopilus with strong support from all analyses (MLBS: 100 %, MPBS: 100 %, BPP: 1.00). Clades corresponding to the genera Coprinopsis and Coprinellus were also recovered.

Within Parasola, individual clades for the following taxa received strong support: P. conopilus (MLBS: 100 %), P. auricoma (100 %), P. plicatilis (100 %), P. leiocephala (100 %), P. misera (100 %), P. lilatincta (96 %) and P. aff. lilatincta (99 %). Parasola hercules and P. kuehneri were represented in the analyses by a single sequence, so their monophyly cannot be addressed. Parasola conopilus was recovered as sister taxon of the clade containing all other Parasola taxa in MP and ML analyses with low bootstrap values (MP: 55 %, ML: 56 %), whereas Bayesian analysis groups P. conopilus with P. auricoma with no support (BPP: 0.66). In support of the ML and MP trees is the dataset comprised of gaps only (MPBS: 100 %).

The clade containing P. plicatilis specimens forms an early diverging lineage with regard to the rest of ‘crown’ Parasola taxa (excluding P. auricoma = subsect Glabrati sensu Uljé 2005) on the ML and Bayesian trees (MLBS: 100 %, BPP: 1.00). Parasola hemerobia is nested in the P. plicatilis clade (MPBS: 100 %, MLBS: 100 %, BPP: 1.00) indicating that these species should be synonymous. This agrees well with results of morphological revisions (Uljé 2005, Nagy et al. unpubl.). Parasola plicatilis is characterised by lacking thick-walled hairs on the pileus, narrowly ovoid to almost ellipsoid spores measuring 10–13 μm in length. It colonises roadides, lawns and other habitats rich in nutrients and is among the common species of Parasola, although it is far less common than was formerly supposed. Coprinus plicatilis was the name most commonly assigned to Parasola taxa, but vast majority of such specimens are misidentified (Nagy et al. unpubl.).

Parasola leiocephala includes P. galericuliformis, a species that should differ in having more subglobose spores (Orton & Watling 1979, Uljé & Bas 1988, Uljé 2005). Our morphological observations on the holotype of P. galericuliformis support this finding: it contains immature fruitbodies, so aberrant shape of spores is a consequence of incomplete ripening (Nagy et al. unpubl.). Parasola leiocephala is the most common species in the genus and is characterised by ovoid to rounded triangular spores, measuring 9–12 × 7–11 μm on average and the lack of granules in tramal tissues. It colonises various habitats, roadides, lawns, but can be found on dung as well.

The clade containing P. Schroeteri and P. megasperma is also remarkable. These species are characterised by large spores, medium-sized fruitbodies and occasional occurrence on dung. Parasola Schroeteri is stated to differ from P. megasperma in having 12–15 μm long, rounded triangular (‘heart-shaped’) spores, whereas P. megasperma has larger (13–20 μm), ellipsoid spores (see Fig. 2).

Three taxa, P. lilatincta, P. misera and P. kuehneri, cluster together on a moderately supported (MPBS: 60 %, BPP: 0.85) clade in all analyses. Parasola misera is the smallest taxon in the genus (cap 3–15 mm broad), the only obligate coprophilous one, lacking pleurocystidia. According to the original description, P. lilatincta is characterised by having lilaceous colours on the pileus at least in young stages and cells that contain large amounts of oily, yellowish granules ('pigment') (Uljé & Bender 1997). However, our results clearly demonstrate that these characters are not linked to each other. Six of nine collections in the P. lilatincta clade lacked lilaceous coloration, but contained the oily granules mentioned in the original description.

The position of P. kuehneri within this clade is unclear. On the ML tree (Fig. 2) it is nested in the P. lilatincta clade, but the relatively long branch it occupies implies some kind of error in the phylogenetic reconstruction. Consistent with this, Bayesian and MP analyses place this taxon outside the P. lilatincta clade with significant support.

Two specimens that were initially identified as P. lilatincta based on spore shape and the presence of (few) granules, were grouped together in a separate clade (MLBS: 99 %, MPBS: 100 %, BPP: 1.00), but their position is not well resolved. The LSU dataset strongly supports a sister position to the P. lilatincta clade (BPP: 1.00, results not shown). ITS sequences of these specimens contain a large number of unique characters which could be a reason of being placed in diverse (mainly basal) positions in various analyses. Unfortunately, no morphological differences were found between these two and other P. lilatincta specimens.

Evolution of morphological traits

Results from mapping morphological characters including extent of homoplasy are summarized in Table 2. Measures of homoplasy are calculated only for the Parasola clade (including P. conopilus) so as to exclude effects of outgroup taxa.

Presence of veil, hairs on pileus, plicate pileus surface, presence of brachybasidia and the ability of fruitbodies to collapse showed no homoplasy within the Parasola clade, which indicates that these characters can be phylogenetically informative in Parasola and may be used for defining this genus in a phylogenetic context. Of these traits, presence of brachybasidia, pleurocystidia, spore shape and deliquescence were found strongly homoplasious on a much larger scale (Padamsee et al. 2008) in coprinoid fungi, with variable numbers of gains and reversals across different trees. Within Parasola, these traits were found highly informative.

On all trees, most parsimonious reconstructions of the presence or absence of veil imply a single loss before the Parasola clade, viz., on the branch leading to P. conopilus with no

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**Table 2** Summary of gains and losses of individual characters in the Parasola clade (Homoplasy measures are calculated only for Parasola taxa).

| Character                        | Gains ML/MP/B | Losses ML/MP/B | Rescaled Consistency Index | Retention Index |
|---------------------------------|---------------|----------------|---------------------------|-----------------|
| Veil                            | 0/0/0         | 1/1/1          | 1.0000                    | 1.0000          |
| Hairs on pileus                 | 1/1/1         | 1/1/0          | 1.0000                    | 1.0000          |
| Spore shape                     | 1/1/1         | 3/3/3          | 0.1429                    | 0.7143          |
| Pileus plicate                  | 1/1/2         | 0/0/0          | 1.0000                    | 1.0000          |
| Fruitbody collapsing            | 1/1/2         | 0/0/0          | 1.0000                    | 1.0000          |
| Brachybasidia                   | 1/1/2         | 0/0/0          | 1.0000                    | 1.0000          |
| Granules in cells               | 2/2/2         | 0/0/0          | 0.2667                    | 0.8000          |
| Germ pore                       | 1/1/1         | 2/2/2          | 0.1429                    | 0.5714          |
| Pleurocystidia                  | 1/1/2         | 1/1/1          | 0.4000                    | 0.8000          |

1 ML = Maximum Likelihood, MP = Maximum Parsimony, B = Bayesian Posterior Probabilities.
homoplasy (RCi: 1.0000). According to our results, thick-walled hairs evolved on the same branch where the veil was lost. Plicate pileus, presence of brachybasidia, pleurocystidia and the ability of fruitbodies to collapse evolved only once, on the branch leading to P. auricoma, showing no reversals in Parasola (RCi: 1.0000), except for the presence of pleurocystidia (RCi: 0.4000). Reconstruction on all trees is consistent in that rounded triangular shape of spores evolved in the ‘crown’ Parasola clades. In the P. plicatilis and P. megasperma/schroetori clades this character is variable yielding relatively low RCI and Ri (0.1429 and 0.7143). Position of the germ pore correlates with spore shape, except that only two losses are assumed in P. megasperma due to the variable nature of this character within this taxon. Granular content of the cells was reconstructed as having emerged two times; however, as mentioned above the position of the aff. lilatincta clade is dubious due to excessive variability in the ITS region (long branch attraction). If only LSU data are considered, it becomes a sister group of the P. lilatincta clade reducing the number of gains to 1 and the losses to 0. Pleurocystidia are absent in P. conopilus but are present in all other Parasola taxa except P. misera, so on the ML and MP trees one gain and one loss of this character is inferred.

DISCUSSION

Our study illustrates the potential of gap characters in non-coding DNA sequences to serve as phylogenetic characters. Although generally much fewer in number, they seem to provide more reliable estimates of the phylogeny as judged from measures of homoplasy and the number of parsimony informative sites. In our case, gaps of the ITS dataset turned out to be informative at the genus level with very low resolving power among species of Parasola. This is in concordance with former studies (Simmons & Ochoterena 2000, Simmons et al. 2001, Müller 2006, Egan & Crandall 2008) which emphasised the role of gap characters in family-genus level phylogenetic studies. Some cases were also reported where distribution of gaps is diagnostic for species, however (Kovács & Jakucs 2006). ITS alignments are often characterised by being interrupted by several gaps. Indel regions are treated by several authors as ‘ambiguously’ aligned regions and are excluded from phylogenetic analyses (Álvarez & Wendel 2003). However, the information they encode can and, in our opinion, should be incorporated in phylogenetic analyses with holding positional homology in view (Lutzoni et al. 2000). In this manner, more attention should be paid to the resolving power of ITS regions at the genus-family level when indels are integrated in phylogenetic analyses. From an analytical point of view, it is favourable to include gaps characters as a binary matrix in combined analyses, after testing for significant incongruence (which – to our knowledge – can be done at the moment only under the parsimony principle). Unfortunately, there are very few phylogeny softwares that can handle mixed data (e.g. MrBayes).

Our MP analyses of both the nucleotide and binary matrices as well as the ML tree resolve P. conopilus as an early diverging taxon of the Parasola clade, although uncertainty remains about this topology with respect to the support values and the contradicting topology of the Bayesian phylogram (Fig. 3). Former studies support the basal position of P. conopilus with significant support (Walther et al. 2005, Larsson & Orstadius 2008, Padamsee et al. 2008, Vasutová et al. 2008), so it seems likely that this relationship is correct. Analysis of more genes seems necessary to address the position of P. conopilus. Different classifications of P. conopilus have been proposed: either as a separate, monotypic genus (Padamsee et al. 2008) or as a member of Parasola (Larsson & Orstadius 2008). However, we think inclusion in Parasola is better justified so as to keep the number of new genera as low as possible. Results of the Bayesian analyses also entail a similar viewpoint, as on the Bayesian 50 % majority rule tree (Fig. 3). Parasola auricoma and P. conopilus clades form a clade containing all other Parasola taxa. Parasola auricoma and P. conopilus are morphologically united by the presence of thick-walled hairs on the pileus, warm brown colour of fruitbodies, and ellipsoid spores with central germ-pore.

Specimens identified by us as P. lilatincta based on spore shape and presence of granules in tissues, but neglecting lilaceous coloration, grouped together with strong support. This supports the assumption that lilaceous colour of fruitbodies is not linked to the presence of granules (Nagy 2005) and is of limited value in identification. Instead, it clarifies the situation of taxa found in collections labelled as P. mf. leiocephala or P. Schroetori: most specimens lacking lilac colour with larger spores and granules were assigned to these species, whereas now it is evident that the presence of granules and spore size are diagnostic enough to assign such collections to P. lilatincta.

Two specimens (NL-0096 and NL-0086) identified morphologically as P. lilatincta are clustered on a separate, strongly supported branch appearing in contradicting positions on the trees inferred, which could be attributed to long branch attraction. Oily granules are present in both specimens, in agreement with the (hereby) amended definition of P. lilatincta as well as spore shape and size, which are also similar. Although no morphological differences were found by us, considering the results of the phylogenetic analyses we label these ‘P. aff. lilatincta’, awaiting more specimens to see if they truly represent a unique lineage.

In the present study the taxonomic value of several morphological characters is amended. We found that spore characteristics, such as shape, size and the position of germ pore, are most useful for delimitation of taxa in Parasola. In certain taxa (P. plicatilis, P. megasperma), however, spore shape may vary from almost ellipsoid to rounded triangular. The issue of P. megasperma is further complicated by P. Schroetori, which phylogenetic analyses failed to separate unequivocally from P. megasperma. Of these two species, there are collections, indeed, which show transitional spore shape and size. However, the limitation of ITS to discriminate some species has also been observed in Boletus by Beugelsdijk et al. (2008). More material should be sequenced to clarify whether they should be synonymised or considered to be separate taxa. Other ‘crown’ Parasola taxa (P. hercules, P. kuehneri, P. leiocephala, P. lilatincta, P. misera) have rounded triangular spores, differences can be found in their sizes only. Of these, P. misera is remarkable in lacking pleurocystidia and being obligate coprophilous.

In the present study we evaluated the phylogenetic utility of several morphological characters. Our results revealed a number of characters which appear to be associated with the emergence of the parasoloid lineage: loss of veil (in P. conopilus), appearance of plicate pileus, pleurocystidia, brachybasidia and the ability of fruitbodies to collapse upon maturity (in P. auricoma). This group of characters could be considered diagnostic not only for the emergence of Parasola taxa, but we think also for other coprinoid lineages as well. In line with the loss of veil, thick-walled hairs evolved on the same branch. These two structures may stand for the same function, i.e. protecting the pileus from water droplets or insects. Another species, not included in the phylogenetic analyses, P. setulosa shares the thick-walled hairs with P. auricoma, but has broad, rounded triangular spores with central germ-pore. Hence this can be regarded as an intermediate between ‘crown’ Parasola taxa and P. auricoma. A next step might be P. plicatilis, in which thick-walled hairs are lost,
but the spores are (narrowly) rounded triangular and the germ pore is eccentric (although often only slightly). The remainder of the taxa possess rounded triangular spores (except *P. megasperma* in which this trait is variable) equipped with an eccentric germ-pore and lack hairs on the pileus.

Granules in cells (pleipellis elements, cystidia, basidia) were considered unique to *P. lilacina*. However, our phylogenetic analyses recovered another clade of morphologically very similar specimens which shares this character with *P. lilacina*. We refer to this clade as *P. aff. lilacina*. Hence, for the time being it cannot be concluded with certainty that this character (oil granules) evolved twice in the *Parasola* clade as suggested by the most parsimonious reconstructions.

The ability of fruitbodies to collapse showed one gain and no losses across *Parasola* regardless of the tree the character was traced over. In this respect our study should complement to the paper of Padamsee et al. (2008), who examined (among others) the evolution of deliquescence and recovered 3–6 gains across all coprinoid fungi. However, they explicitly treated *Parasola* as non-deliquescent. In the literature, there is no consensus as to whether *Parasola* is deliquescent or not. Some authors treat them non-deliquescent and maintain the genus *Pseudocoprinus* for these taxa (Kühner 1928, McKnight & Allison 1970), while others did not distinguish this way (e.g. Uljé 2005). The phenomenon observed in *Parasola* (excluding *P. conopilus*) might be interpreted as incomplete deliquescence, or an ability of the fruitbodies to collapse upon maturity, but anyway, it is markedly different from psathyrelloid taxa (e.g. *P. conopilus*). Moreover, this study illustrates that certain characters, like presence of veil, hairs on pileus, plicate pileus surface, presence of brachybasidia and pleurocystidia, found highly homoplastic by Padamsee et al. (2008) can be phylogenetically informative when mapped on a smaller scale.

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