

Article

An Integrative Taxonomic Study of *Parasola* (Psathyrellaceae, Fungi) Reveals a New Saprotrophic Species from European Temperate Deciduous Forests

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Abstract: Seventeen collections of the genus *Parasola* from Croatia were studied with integrative taxonomic methods. *Parasola papillatospora* is described as a new species, based on morphology and multigene phylogenetic analyses. Its basidiomata were growing on soil in temperate deciduous forests (*Quercus petraea*, *Fagus sylvatica*, and *Carpinus betulus*) on two different localities in NW Croatia. Based on publicly available molecular data, the species is also recorded in Hungary. The most distinctive morphological features of the new species are the characteristics of its basidiospores, (1) the papillate apex and (2) central germ pore (both present in most spores), as well as (3) a highly variable shape. A morphological description of *P. papillatospora* is accompanied by colour photographs of basidiomata, basidiospores, and cystidia. In this study, a total of 64 DNA sequences from 17 specimens belonging to 10 *Parasola* species were newly generated. As a result of Bayesian Inference and Maximum Likelihood phylogenetic analyses of the concatenated ITS, LSU, *tef-1α*, and *β-tub* gene alignment of *Parasola* species, *P. papillatospora* was resolved as an independent clade, a sister to the clade comprising the *P. plicatilis* species complex. Eight *Parasola* species (*P. auricoma*, *P. crataegi*, *P. cuniculorum*, *P. kuehneri*, *P. malakandensis*, *P. megasperma*, *P. nudiceps*, *P. plicatilis-similis*) are reported for the first time for Croatia and *P. malakandensis* also for Europe. Colour photographs of basidiomata are provided for all *Parasola* species new to Croatia except *P. kuehneri*.

Keywords: 1 new taxon; Agaricales; Basidiomycota; biodiversity; biogeography; molecular phylogeny



Citation: Pošta, A.; Tkalčec, Z.; Kušan, I.; Matočec, N.; Pole, L.; Čerkez, M.; Mešić, A. An Integrative Taxonomic Study of *Parasola* (Psathyrellaceae, Fungi) Reveals a New Saprotrophic Species from European Temperate Deciduous Forests. *Forests* **2023**, *14*, 1387. <https://doi.org/10.3390/f14071387>

Academic Editors: Nian-Kai Zeng, Li-Ping Tang and Dong-Qin Dai

Received: 7 June 2023

Revised: 30 June 2023

Accepted: 4 July 2023

Published: 7 July 2023



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1. Introduction

The traditional polyphyletic concept of the genus *Coprinus* Pers. *sensu lato* was re-defined by Redhead et al. in 2001 [1] based on molecular evidence [2–4]. The newly proposed taxonomic concept comprised the genus *Coprinus* in the narrow sense including *C. comatus* (O.F. Müll.) Pers. and a few related taxa nested in a monophyletic clade within the family *Agaricaceae* Chevall. Other coprinoid species, more closely related to *Psathyrella* (Fr.) Quél., were transferred to the newly proposed family *Psathyrellaceae* Vilgalys, Moncalvo & Redhead [1] and arranged into the genera *Parasola* Redhead, Vilgalys & Hopple, *Coprinellus* P. Karst., and *Coprinopsis* P. Karst. The genus *Parasola* comprised all species from the subsections *Glabri* and *Auricomi* of the former genus *Coprinus* s.l. These are characterized by a delicate, non-deliquestent, coprinoid basidiomata (dry, plicate–sulcate pileus and dark basidiospores), and by the absence of the veil and caulocystidia. Based on morphology, Uljé in 2005 [5] distinguished 10 species from Europe in this group. Nagy et al. in 2010 [6] studied all available type material of *Parasola* and related taxa worldwide and recognized 10 species in the genus, mostly confirmed with molecular data as well. In addition, a psathyrelloid species (with smooth pileus), *Psathyrella conopilea* (Fr.) A. Pearson & Dennis, was transferred to *Parasola* by Larsson and Örstadius [7] as a result of a molecular

phylogenetic analysis. In the following years, extensive taxonomic research on *Parasola* (based on biological material or protologue information) led to the description of as many as 18 new species [8–16]. Furthermore, based on molecular and morphological analyses, Malysheva et al. in 2019 [17] transferred *Galeropsis aporos* Courtec. to *Parasola*.

Our taxonomic research on coprinoid fungi in Croatia has already led to the publication of a new species, *Coprinopsis cerkezii* Tkalčec, Mešić, I. Kušan & Matočec [18]. Mešić and Tkalčec [19] presented an annotated checklist of all species of the former family *Coprinaceae* Overeem & Weese from Croatia, which included all coprinoid and psathyrelloid fungi. It was based on all published sources and unpublished records before 2000 and included only three *Parasola* species: *P. conopilea* (Fr.) Örstadius & E. Larss., *P. plicatilis* (Curtis) Redhead, Vilgalys & Hopple, and *P. misera* (P. Karst.) Redhead, Vilgalys & Hopple. Afterwards, the only species of *Parasola* published for Croatia was *P. lactea* (A.H. Sm.) Redhead, Vilgalys & Hopple (= *P. leioccephala* (P.D. Orton) Redhead, Vilgalys & Hopple) [20].

Seventeen collections of the saprotrophic genus *Parasola* from Croatia were studied with an integrative taxonomic approach, which led to the identification of 10 species. *Parasola papillatospora* sp. nov. from European temperate deciduous forests is described based on four-gene molecular phylogenetic and morphological analyses. Moreover, eight *Parasola* species are recorded for the first time for Croatia and a single species is recorded as new to Europe.

2. Materials and Methods

2.1. Fieldwork, Sampling, and Morphological Study

Seventeen specimens of the genus *Parasola* were collected throughout Croatia from 2004 to 2022. The methods of sampling and morphological examination were similar in all collections, as follows. The basidiomata were photographed on site with a Canon digital camera (EOS 30D, 50D, or 5D; Canon Europe, Uxbridge, UK) equipped with a Canon MR-14EX macro ring flash, collected, macromorphologically described, and preserved by drying. All collections were deposited in the Croatian National Fungarium (CNF), Zagreb, Croatia. The description of *P. papillatospora* is based on six collections consisting of 18 basidiomata. In a macroscopic description, L denotes the number of entire lamellae and l denotes the number of lamellulae between each pair of entire lamellae. Microscopic characters were observed using an Olympus BX51 optical microscope (Olympus, Hamburg, Germany) in the brightfield technique under a magnification up to 1500× and photographed with a Canon EOS M50 digital camera. The description and images of microscopic characters were obtained from rehydrated pieces of specimens mounted in 2.5% or 5% potassium hydroxide (KOH), except for basidiospores, which were observed in 3% ammonium hydroxide (NH₄OH). Basidiospores from photographs of lamellae mounts were randomly selected and measured using Motic Images Plus 2.0 software (Motic Europe, Barcelona, Spain). The length/breadth ratio of basidiospores in the frontal view is given as a Q_f value and the length/width ratio of basidiospores in the side view is given as a Q_s value. Average basidiospore lengths, widths, and Q values are shown in italics. The numbers in square brackets [X/Y/Z] denote X elements measured in Y basidiomata from Z collections.

2.2. DNA Extraction, PCR Amplification, and Sequencing

Genomic DNA was extracted from dried specimens of *Parasola* using the EZNA[®] HP Fungal DNA Kit (Omega Bio-tek, Norcross, GA, USA) following the manufacturer's protocol. Four gene regions were sequenced and analyzed in this study: two nuclear gene regions, ITS (internal transcribed spacer region) and LSU (28S large subunit of ribosomal DNA), and two protein coding regions, *tef-1α* (translation elongation factor 1-alpha) and *β-tub* (beta-tubulin). For PCR amplification and sequencing of ITS and LSU, primer pairs ITS1F/ITS4 [21,22] and LR0R/LR5 [23] were used, respectively. The primer pair EF1-983F/EF1-2218R, with the addition of 1567R and 1577F [24,25], was used for PCR amplification of the *tef-1α* gene region. The *Psathyrellaceae*-specific primer pair B36F-PSA/B12R-PSA [26] was used to amplify the *β-tub* gene region. PCR amplification for

ITS and LSU gene regions was performed using a touchdown program: initial denaturation at 95 °C for 2 min, followed by 5 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 45 s (add −1 °C per cycle), and extension at 72 °C for 1.5 min; 30 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 45 s, and extension at 72 °C for 1.5 min; and a final extension at 72 °C for 5 min. PCR amplification of *tef-1α* was performed as described by Rehner and Buckley [25], with modification of the maximum annealing temperature to 64 °C. The *β-tub* gene region was amplified as described by Nagy et al. [26], with the number of amplification cycles increased to 35.

Successful PCR products were purified using an ExoSAP-IT™ (Thermo Fisher Scientific, Waltham, MA, USA) reagent according to the manufacturer's protocol and sent to Macrogen Europe (Amsterdam, the Netherlands) for bidirectional Sanger sequencing.

2.3. Sequence Alignment and Phylogenetic Analysis

Sequence reads were assembled and edited using Geneious Prime 2023.0.4. (<https://www.geneious.com>, accessed on 31 January 2023, Biomatters, Auckland, New Zealand) and obtained sequences were deposited in the National Center for Biotechnology Information (NCBI) GenBank database.

The phylogenetic dataset comprising 179 sequences of four gene regions from 31 taxa was selected for further analyses (Table 1). Sequences were aligned by each locus using MAFFT v7.450 [27,28] available as a Geneious Prime plugin. After being aligned and trimmed, concatenation of ITS, LSU, *tef-1α*, and *β-tub* was performed using Geneious Prime 2023.0.4. Concatenated alignment contained 3034 characters including gaps, with 675 characters for ITS, 888 characters for LSU, 1006 characters for *tef-1α*, and 465 characters for *β-tub*. Four *Coprinopsis* species (*C. picacea*, *C. lagopus*, *C. marcescibilis*, and *C. pseudonivea*) were selected as the outgroup for phylogenetic analyses following Szarkandi et al. [10].

Phylogenetic analyses were conducted using the Maximum Likelihood (ML) method in IQTREE v1.6.12 [29,30] and a Bayesian Inference (BI) method in MrBayes 3.2.6 (Geneious plugin, [31]). The best model was selected by ModelFinder implemented in IQ-TREE, separately considering the corrected Akaike, and the Bayesian Information Criterion (cAIC, BIC). GTR+F+I+G4 was selected as the best model for both phylogenetic datasets. ML analyses were executed by applying the ultrafast bootstrap approximation with 1000 replicates. BI analyses were executed for 10,000,000 generations, sampling trees and other parameters every 10,000 generations. The default number of chains (four) and heating parameters were used. Posterior probabilities (BPP) were calculated after burning the first 25% of the posterior sample. Phylogenetic trees were visualized and annotated using iTOL v6.5.4 [32] and FigTree 1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>, accessed on 10 February 2023). Alignments and phylogenetic trees generated from BI and ML analyses are available at DOI 10.5281/zenodo.8099476.

Table 1. Species included in this study, associated countries of origin, voucher numbers, and GenBank accession numbers. Newly generated sequences are marked in bold.

Taxon	Country	Voucher	ITS	LSU	<i>tef-1α</i>	<i>β-tub</i>	Ref.
<i>Coprinopsis lagopus</i>	Hungary	NL-2143	FM163179	FM160730	—	—	[33]
<i>Coprinopsis marcescibilis</i>	Hungary	NL-2140	FM878020	FM876277	—	—	[34]
<i>Coprinopsis picacea</i>	Hungary	NL-0174	FN396115	FN396166	—	—	[26]
<i>Coprinopsis pseudonivea</i>	Hungary	NL-2340	FM163181	FM160728	—	—	[33]
<i>Parasola aporos</i>	France	RC-F92.191 holotype	MK397584	MK397604	—	—	[17]
<i>Parasola aporos</i>	France	CL-F09.005	MK397586	MK397606	—	—	[17]
<i>Parasola auricoma</i>	Hungary	NL-0087	JN943107	JQ045871	FM897236	FN396252	[35]
<i>Parasola auricoma</i>	Croatia	CNF 1/4718	OQ842767	OQ842768	OQ850152	OQ850168	This study
<i>Parasola auricoma</i>	Croatia	CNF 1/4618	OQ845889	OQ845835	OQ850153	OQ850169	This study

Table 1. Cont.

Taxon	Country	Voucher	ITS	LSU	<i>tef-1α</i>	<i>β-tub</i>	Ref.
<i>Parasola conopilea</i>	The Netherlands	CBS 325.39	MH856033	MH867531	—	—	[36]
<i>Parasola conopilea</i>	Croatia	CNF 1/5310	OQ845887	OQ845888	OQ850154	OQ850170	This study
<i>Parasola conopilea</i>	Croatia	CNF 1/5735	OQ845890	OQ843455	OQ850155	OQ850171	This study
<i>Parasola crataegi</i>	Germany	SSt08-154 holotype	KY928605	—	—	—	[10]
<i>Parasola crataegi</i>	Hungary	NL-4175 paratype	KY928603	KY928631	—	—	[10]
<i>Parasola crataegi</i>	Croatia	CNF 1/8905	OQ852892	—	—	—	This study
<i>Parasola cuniculorum</i>	United Kingdom	K(M)191984 holotype	OL630105	—	—	—	[16]
<i>Parasola cuniculorum</i>	Croatia	CNF 1/5143	OQ848756	OQ848757	OQ850156	OQ850172	This study
<i>Parasola galericuliformis</i>	Hungary	NL-6601	FM163187	FM160722	—	—	[33]
<i>Parasola galericuliformis</i>	Sweden	NL-0095	FM163188	FM160721	—	—	[33]
<i>Parasola glabra</i>	Pakistan	LAH-SHP-5 holotype	KY461717	KY621806	KY461735	—	[12]
<i>Parasola glabra</i>	Pakistan	HUP-SHP-23 paratype	KY461718	KY621805	—	—	[12]
<i>Parasola hercules</i>	The Netherlands	Uljé 10.8.1984 (L146) holotype	HQ847027	HQ847112	—	—	[37]
<i>Parasola kuehneri</i>	The Netherlands	Uljé 31.5.1987 holotype	KY928608	KY928633	—	—	[10]
<i>Parasola kuehneri</i>	The Netherlands	Uljé 1241 (L133)	HQ847026	HQ847111	—	—	[37]
<i>Parasola kuehneri</i>	Croatia	CNF 1/4334	OQ849153	OQ849154	OQ850157	OQ850173	This study
<i>Parasola lactea</i>	Hungary	NL-0283	JN943113	JQ045887	FM897239	FN396248	[35]
<i>Parasola lactea</i>	Hungary	NL-0288	JN943106	JQ045872	FM897233	FN396250	[35]
<i>Parasola lilatincta</i>	Hungary	NL-0468a	FM163200	FM160709	—	—	[33]
<i>Parasola lilatincta</i>	Hungary	NL-0281	FM163197	FM160712	—	—	[33]
<i>Parasola lilatincta</i>	Hungary	NL-0296	FM163196	FM160713	—	—	[33]
<i>Parasola lilatinctoides</i>	Pakistan	LAH-SHP-8 holotype	KY461722	KY461725	KY461731	—	[12]
<i>Parasola litoralis</i>	Cyprus	K(M)264814 holotype	OL630108	—	—	—	[16]
<i>Parasola litoralis</i>	Cyprus	DJS20130125001 paratype	OL630107	—	—	—	[16]
<i>Parasola malakandensis</i>	Pakistan	HUP 17501 holotype	KP738713	KU599829	KU599831	—	[9]
<i>Parasola malakandensis</i>	Pakistan	LAH-SHP-17 paratype	KU599827	KU599830	KU599832	—	[9]
<i>Parasola malakandensis</i>	Croatia	CNF 1/8698	OQ849158	OQ849167	OQ850158	OQ850174	This study
<i>Parasola megasperma</i>	United Kingdom	E:Orton 4132 holotype	OL630101	—	OL630935	—	[16]
<i>Parasola megasperma</i>	The Netherlands	Ulje 1275	KY928618	KY928637	—	—	[10]
<i>Parasola megasperma</i>	Croatia	CNF 1/5704	OQ849166	OQ849224	OQ850159	OQ850175	This study
<i>Parasola misera</i>	Hungary	NL-0280 neotype	FM163210	FM160699	—	—	[33]
<i>Parasola misera</i>	Hungary	NL-0677	FM163211	FM160698	FM897240	FN396249	[26,33]

Table 1. Cont.

Taxon	Country	Voucher	ITS	LSU	<i>tef-1α</i>	<i>β-tub</i>	Ref.
<i>Parasola nudiceps</i>	United Kingdom	E:Orton 4133 holotype	OL630102	—	—	—	[16]
<i>Parasola nudiceps</i>	Germany	HB19870911A	MK063783	—	—	—	[16]
<i>Parasola nudiceps</i>	Croatia	CNF 1/4804	OQ849230	OQ849229	OQ850160	OQ850176	This study
<i>Parasola nudiceps</i> (as <i>P. ochracea</i>)	Norway	NL-3621, holotype of <i>P. ochracea</i>	JN943134	JQ045875	—	—	[38]
<i>Parasola nudiceps</i> (as <i>P. ochracea</i>)	Sweden	NL-3167, paratype of <i>P. ochracea</i>	JN943136	JQ045865	—	—	[38]
<i>Parasola papillatospora</i> sp. nov.	Croatia	CNF 1/3473	OQ862758	OQ862756	OQ850161	—	This study
<i>Parasola papillatospora</i> sp. nov.	Croatia	CNF 1/5428	OQ862789	OQ862577	OQ850162	OQ850177	This study
<i>Parasola papillatospora</i> sp. nov.	Croatia	CNF 1/7600	OQ862790	OQ862578	OQ850163	OQ850178	This study
<i>Parasola papillatospora</i> sp. nov.	Croatia	CNF 1/7858 holotype	OQ862770	OQ862755	OQ850164	OQ850179	This study
<i>Parasola papillatospora</i> sp. nov.	Croatia	CNF 1/7861	OQ862757	OQ862771	OQ850165	OQ850182	This study
<i>Parasola papillatospora</i> sp. nov.	Croatia	CNF 1/7902	OQ862788	OQ862772	OQ850167	OQ850181	This study
<i>Parasola papillatospora</i> sp. nov.	Hungary	SZMC-NL-2952	HQ847028	HQ847113	—	—	[10]
<i>Parasola parvula</i>	India	CAL 1667 holotype	MH379796	MH393599	—	—	[11]
<i>Parasola plicatilis</i>	Hungary	NL-0075 epitype	FM163214	FM160695	—	—	[33]
<i>Parasola plicatilis</i>	Hungary	NL-0284	FM163189	FM160720	FM897235	FN396251	[26,33]
<i>Parasola plicatilis</i> aff.	China	HMJAU46405	OL355167	OL376339	—	—	[39]
<i>Parasola plicatilis-similis</i>	Sweden	NL-2125 holotype	KY928620	—	—	—	[10]
<i>Parasola plicatilis-similis</i>	Sweden	NL-0287 paratype	FM163218	FM160691	—	FN396245	[26,33,35]
<i>Parasola plicatilis-similis</i>	Croatia	CNF 1/5484	OQ850018	OQ850017	OQ850166	OQ850180	This study
<i>Parasola psathyrelloides</i>	India	CAL 1753 holotype	MK682756	MK682754	—	—	[13]
<i>Parasola psathyrelloides</i>	India	AMH 10119 paratype	MK682752	MK682759	—	—	[13]
<i>Parasola pseudolactea</i>	Pakistan	HUP-SU-412 holotype	KY461719	KY621799	KY461733	—	[12]
<i>Parasola pseudolactea</i>	Pakistan	HUP-SU-413 paratype	KY461720	KY621800	KY461734	—	[12]
<i>Parasola schroeteri</i>	Germany	Dähncke 1502	KY928616	KY928635	—	—	[10]
<i>Parasola schroeteri</i>	The Netherlands	Brier 10.5.1999	FM163219	FM160690	—	—	[33]
<i>Parasola schroeteri</i>	The Netherlands	Ulje 1067	KY928627	—	—	—	[10]
<i>Parasola schroeteri</i>	The Netherlands	Vellinga 1140	KY928629	KY928645	—	—	[10]
<i>Parasola setulosa</i>	Hungary	Maruyama 14.7.1999/ L32	HQ847030	HQ847115	—	—	[37]
<i>Parasola setulosa</i>	China	HMJAU46367	MW822929	OL376319	—	—	[39]

3. Results

3.1. Molecular Phylogenetic Analyses

In this study, a total of 64 DNA sequences (17 ITS, 16 LSU, 16 *tef-1 α* , and 15 *β -tub*) from 17 collections were newly generated. Six collections were identified as *P. papillatospora* sp. nov. (CNF 1/3473, 1/5428, 1/7600, 1/7858, 1/7861, 1/7902), two as *P. auricoma* (Pat.) Redhead, Vilgalys & Hopple (CNF 1/4618 and 1/4718) and *P. conopilea* (CNF 1/5310 and 1/5735), and one each as *P. crataegi* Schmidt-Stohn (CNF 1/8905), *P. cuniculorum* D.J. Schaf. (CNF 1/5143), *P. kuehneri* (Ulje & Bas) Redhead, Vilgalys & Hopple (CNF 1/4334), *P. malakandensis* S. Hussain, Afshan & H. Ahmad (CNF 1/8698), *P. megasperma* (P.D. Orton) Redhead, Vilgalys & Hopple (CNF 1/5704), *P. nudiceps* (P.D. Orton) Redhead, Vilgalys & Hopple (CNF 1/4804), and *P. plicatilis-similis* L. Nagy, Szarkándi & Dima (CNF 1/5484). The accession numbers of all newly generated sequences are presented in bold in Table 1.

All six Croatian collections of *P. papillatospora* showed a strong genetic homogeneity with a 99.86–100% identity in ITS, 98.96–100% in LSU, 100% in *tef-1 α* , and 100% in the *β -tub* gene region. Based on GenBank nucleotide BLAST results, the Croatian collections of *P. papillatospora* showed a high percentage identity (99.39–99.54% in ITS and 99.84–99.88% in LSU) with the previously sequenced sample from Hungary (SZMC-NL-2952, acc. no. HQ847028, HQ847113, *Parasola* sp. 1. in Szarkandi et al. [10]). The next closest hit using the ITS sequence of the holotype of *P. papillatospora* had a similarity of 95.13% to the collection from China identified as *P. plicatilis* (HMJAU46405, acc. no. OL355167, [39]). The identity between the ITS sequences of the Chinese collection and the epitype of *P. plicatilis* (NL-0075, acc. no. FM163214) was 94.85%. The results of BI and ML phylogenetic analyses of the concatenated alignment of *Parasola* species (Figure 1) showed that the Hungarian sample (SZMC-NL-2952) nested together with six Croatian *P. papillatospora* collections in an independent strongly supported clade (BI-PP = 1, ML-BP = 100), a sister to the clade of the *P. plicatilis* species complex (BI-PP = 1, ML-BP = 100). The Chinese collection *P. plicatilis* aff. (HMJAU46405) formed a well-supported clade (BI-PP = 1, ML-BP = 100) with Hungarian collections, the epitype of *P. plicatilis* (NL-0075), and *P. plicatilis* (NL-0284).

Two Croatian collections of *P. conopilea* (CNF 1/5310 and 1/5735) clustered together with the *P. conopilea* collection (CBS 325.39) from the Netherlands and formed a strongly supported (BI-PP = 1, ML-BP = 99) joint clade with its sister species *P. psathyrelloides* K.G.G. Ganga & Manim. Croatian collections of *P. auricoma* (CNF 1/4618 and 1/4718) and the Hungarian collection of *P. auricoma* (NL-0087) were recovered in a monophyletic clade (BI-PP = 1, ML-BP = 99) with its closely related species *P. setulosa* (Berk. & Broome) Redhead, Vilgalys & Hopple. Collection of *P. malakandensis* (CNF 1/8698) from Croatia was resolved in a strongly supported (BI-PP = 1, ML-BP = 100) clade with two *P. malakandensis* collections from Pakistan (HUP 17501 (holotype) and LAH-SHP-17). Collections of *P. papillatospora*, *P. crataegi*, *P. cuniculorum*, *P. kuehneri*, *P. megasperma*, *P. nudiceps*, and *P. plicatilis-similis* from Croatia (CNF samples) were recovered with the remaining *Parasola* collections from the phylogenetic dataset into a single large clade (*Parasola* section in Szarkandi et al. [10]) with maximum support (BI-PP = 1, ML-BP = 100) (Figure 1).

3.2. Taxonomy

Parasola papillatospora Tkalčec, Mešić, Pošta, I. Kušan, Čerkez, sp. nov. (Figures 2 and 3)
Mycobank MB848624

Typification: Croatia, City of Zagreb: The Cmrok park area (near the Dubravkin put street), 207 m a.s.l., 45.83214° N, 15.97409° E, on soil in deciduous forest dominated by *Quercus petraea*, *Fagus sylvatica*, and *Carpinus betulus*, leg. M. Čerkez, 22 September 2009, holotype CNF 1/7858. GenBank (ex-holotype DNA isolate): ITS = OQ862770, LSU = OQ862755, *tef-1 α* = OQ850164, *β -tub* = OQ850179.

Etymology: Referring to the basidiospores with developed apical papilla.

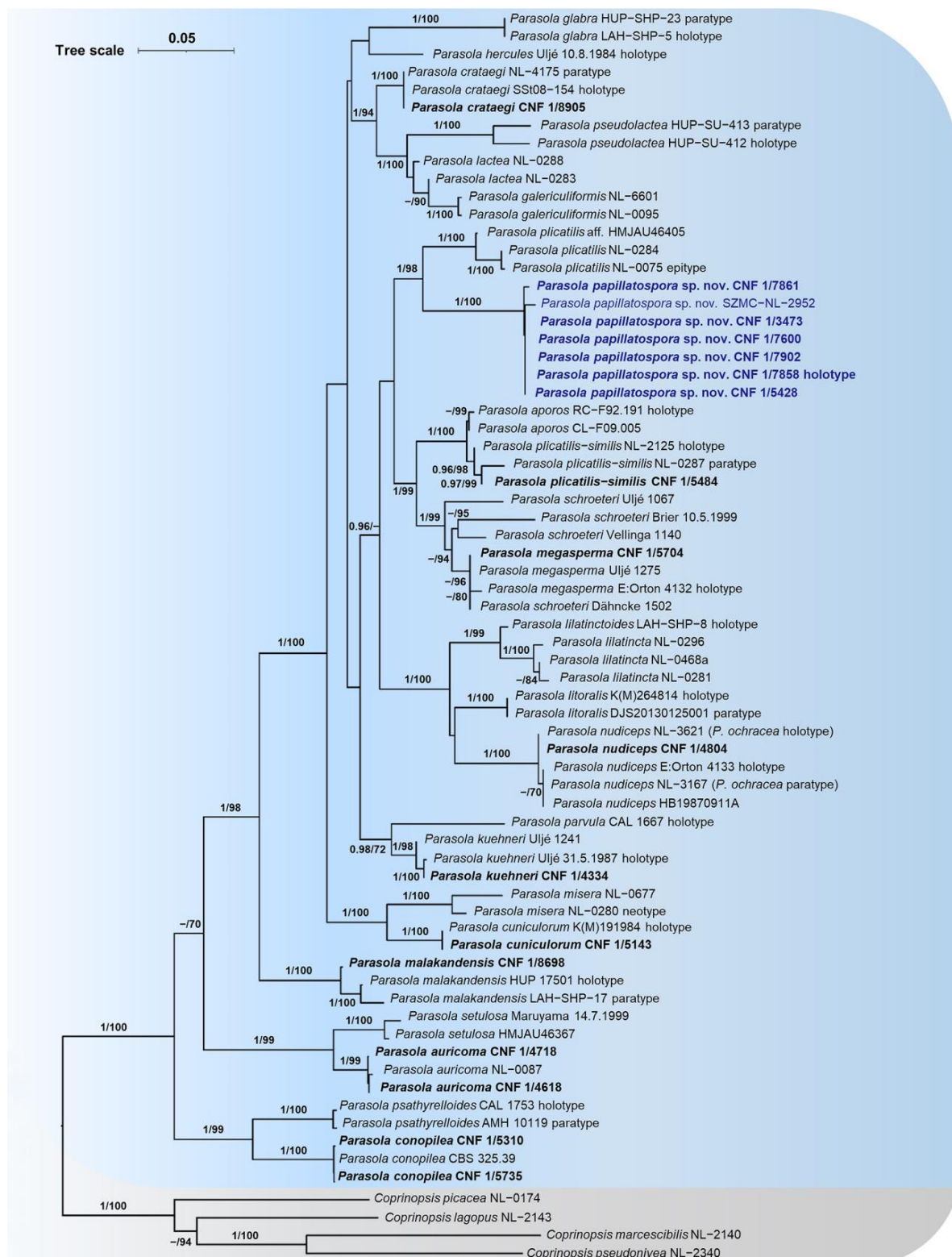


Figure 1. Phylogenetic tree of *Parasola* species based on Bayesian Inference (BI) and Maximum Likelihood (ML) analyses of the concatenated four-gene (ITS, LSU, *tef-1 α* , *β -tub*) sequence alignment. Significant branch support values, Bayesian posterior probability (BI-PP ≥ 0.95), and ultrafast bootstrap support (ML-BP $\geq 70\%$) are presented at the nodes. Collections with newly generated sequences are marked in bold and the newly proposed species *P. papillatospora* is marked in a blue color.

Description: Pileus 12–20 mm wide, subcylindrical when young, later expanding, but with flattened center and outer part directed downwards for a prolonged time, finally hemispherical, convex or broadly conical, with truncate, often $\pm$ depressed center, surface dry, matte, densely radially plicate-sulcate (ca. (60–)75–90% of radius) except in smooth central zone, without a veil, hygrophanous, when moist pale to medium brown or orange(ish)-brown at first, becoming light grey in its external (sulcate) zone at maturity, when dry whitish to cream, sometimes brownish at center, often greyish to light brown–grey in its external (sulcate) zone at maturity. Lamellae free, rather distant to medium spaced, $L = 32\text{--}36$, $l = 1\text{--}3$, white or whitish at first, then grey, dark grey-brown or almost black, with whitish, entire edge, non-deliquescent. Stipe $44\text{--}73 \times 1.2\text{--}2.3$ mm, central, $\pm$ cylindrical or gradually slightly broadening downwards, mostly with slightly to distinctly broadened base, dry, hollow, white to light brown, mostly glabrous in upper half (finely silky fibrillose under magnifying glass), often finely and sparsely flocculose in lower half or near the base. Context thin, fragile, whitish in pileus, whitish to brownish in stipe. Smell and taste not recorded.

Basidiospores $[700/7/5]$ $(7.0\text{--})7.4\text{--}8.5\text{--}10(\text{--}10.8) \times (5.8\text{--})6.2\text{--}7.4\text{--}8.7(\text{--}9.8) \times (4.4\text{--})4.6\text{--}5.2\text{--}6.1(\text{--}6.5)$ μm , averages of different basidiomata $8.2\text{--}8.8 \times 7.0\text{--}7.6 \times 5.1\text{--}5.3$ μm , $Q_f = (0.92\text{--})1.00\text{--}1.17\text{--}1.40(\text{--}1.50)$, $Q_s = (1.43\text{--})1.47\text{--}1.63\text{--}1.81(\text{--}1.96)$, av. $Q_f = 1.14\text{--}1.18$, av. $Q_s = 1.58\text{--}1.68$, strongly flattened, highly variable in shape (within the same basidioma) in frontal view, broadly to elongated (sub)limoniform, subpyriform, broadly ovoid to ovoid, lacrymoid, rounded 3-(heart-shaped) to 6-(sub)angular, broadly fusiform, globose, subglobose or broadly ellipsoid to ellipsoid, mostly with small to strongly developed apical papilla, or with convex to acute apex, and with convex, flattened, subconical, or conical base, amygdaliform to ellipsoid with rounded to conical base and rounded to $\pm$ narrowed apex in side view, often with well-developed and pigmented basal part of apiculus, smooth, moderately thick-walled (up to 1 μm), with distinct, central (in ca. 60% to >90% of spores, depending on collection) or slightly to moderately eccentric germ pore (inner diameter up to 1.6 μm wide, outer diameter up to 2.2 μm wide), red-brown to very dark red-brown in H_2O and NH_4OH , grey-brown to very dark grey-brown in KOH. Basidia $15\text{--}45 \times 6\text{--}11$ μm , mostly narrowly clavate to clavate, sometimes constricted in the upper part, mostly 4-spored, not uncommonly 3- or 2-spored in some collections, thin-walled, hyaline, mostly with granular contents, surrounded by (3–)4–6 hyaline, thin-walled, 7–25 μm broad hymenophysalides (pseudoparaphyses). Cheilocystidia $15\text{--}50(\text{--}70) \times 12\text{--}35$ μm , crowded, forming a sterile lamellar edge, clavate to broadly clavate (predominant towards the pileus margin), ovoid, ellipsoid, subglobose, rounded (sub)fusiform, (sub)utriform or broadly conical with rounded apex (the latter three shapes are the most common towards the stipe), hyaline, mostly thin-walled (up to 0.5 μm), sometimes moderately thick-walled (up to 1 μm). Pleurocystidia $45\text{--}75 \times 17\text{--}25$ μm , scattered, sometimes very scarce, (sub)utriform, oblong, ellipsoid or conical with rounded apex, mostly with pedicel, hyaline, thin-walled to moderately thick-walled. Pileipellis, a hymeniderm, composed of mostly clavate to broadly clavate, sometimes also subglobose, spheropedunculate, ellipsoid or ovoid, thin-walled, hyaline cells, $22\text{--}65 \times 12\text{--}32$ μm in size. Pileocystidia and caulocystidia absent. Clamp connections present, best developed and rather abundant in trama hyphae.

Distribution and Ecology: Known from six collections on two localities in NW Croatia, the City of Zagreb, and the Žumberak mountain in Zagreb County. Based on a high similarity of ITS sequences (99.39–99.54%) in the GenBank BLAST search between the collection SZMC-NL-2952 (GenBank, acc. no. HQ847028) and Croatian collections, the species was also found in Hungary. Basidiomata of all Croatian collections were growing on soil, from August to October, in temperate deciduous forests dominated by *Quercus petraea*, *Fagus sylvatica*, and *Carpinus betulus*. This part of Croatia has a continental climate with an average annual temperature of 9–11 °C and annual precipitation of 840–1100 mm. Unfortunately, at the holotype site in the City of Zagreb (Cmrok park forest) where five collections were gathered, intensive forestry works were subsequently carried out, which devastated the microhabitat.



Figure 2. *Parasola papillatospora* sp. nov. Basidiomata. (A,B) CNF 1/7858, holotype. (C,D) CNF 1/5428. (E) CNF 1/7600. (F,G) CNF 1/7902. (H,I) CNF 1/7861. Bars: (A,C,E,G,H) = 10 mm; (B,D,F,I) = 5 mm. Photos: M. Čerkez.

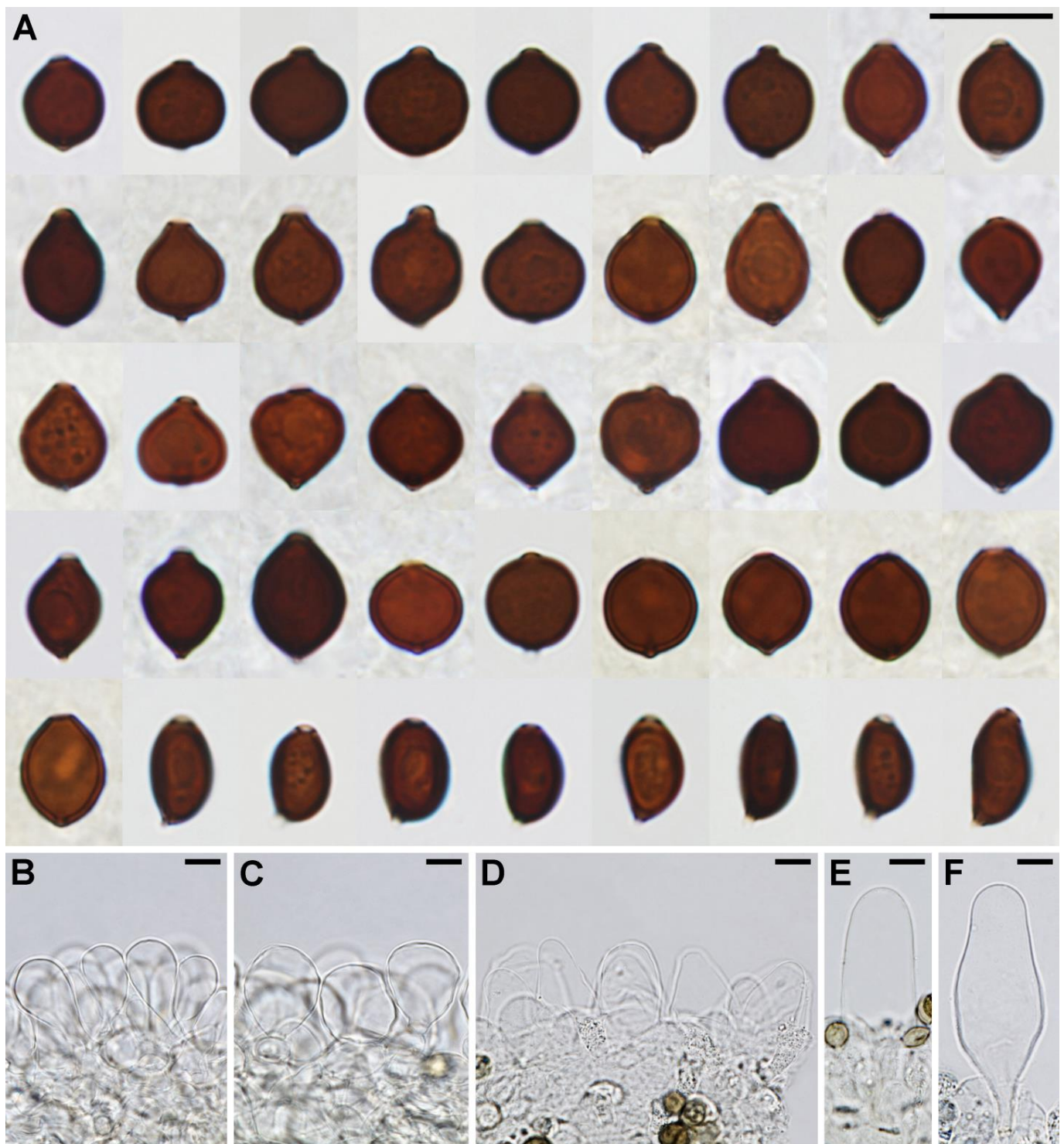


Figure 3. *Parasola papillatospora* sp. nov. (A) Basidiospores in NH_4OH . (B–D) Cheilocystidia. (E,F) Pleurocystidia. Bars = 10 μm . Photos: Z. Tkalčec and A. Mešić.

Additional material examined: Croatia, City of Zagreb: Cmrok park area (near the Dubravkin put street), on soil in deciduous forest dominated by *Quercus petraea*, *Fagus sylvatica*, and *Carpinus betulus*, leg. M. Čerkez, 207 m a.s.l., 45.83217° N, 15.97378° E, 30 September 2008, CNF 1/5428; 45.83214° N, 15.97409° E, 5 September 2009, CNF 1/7600; 45.83197° N, 15.97427° E, 23 September 2009, CNF 1/7861; 28 October 2009, CNF 1/7902; Zagreb County: Žumberak Mountain, vicinity of Novo Selo Okičko village, near Gornji

Gorički hamlet, 432 m a.s.l., 45.74826° N, 15.70050° E, on soil, near the edge of deciduous forest dominated by *Quercus petraea*, *Fagus sylvatica*, and *Carpinus betulus*, leg. M. Čerkez, 21 August 2004, CNF 1/3473.

3.3. Additional Data on *Parasola* from Croatia

An additional 11 Croatian collections of nine *Parasola* species were sequenced, used in phylogenetic analyses, and their morphological characters were examined to confirm the taxonomic identification [5,6,8–16,40,41]. Eight of the nine identified *Parasola* species (all except *P. conopilea*) were recorded for the first time for Croatia in this study, while *P. malakandensis* was also recorded for the first time for Europe (hitherto known only from Pakistan).

Additional materials examined: Parasola auricoma. Croatia, Zagreb County: Žumberak Mountain, vicinity of Kostanjevec Podvrški village, 240 m a.s.l., 45.83093° N, 15.58817° E, on grassy soil, courtyard near deciduous forest, leg. M. Čerkez, 14 July 2007, CNF 1/4618 (Figure 4B); 2 September 2007, CNF 1/4718 (Figure 4A).

Notes: The collections of *P. auricoma* from Croatia (CNF 1/4618 and 1/4718) and *P. auricoma* (NL-0087) from Hungary [35] clustered together in a monophyletic clade (BI-PP = 1, ML-BP = 99). The species most closely related to *P. auricoma* (CNF 1/4618 and 1/4718) clustered in a sister clade (BI-PP = 1, ML-BP = 99) was *P. setulosa* (Maruyama 14.7.1999 and HMJAU46367).

Parasola conopilea. Croatia, Šibenik-Knin County: Krka National Park, Skradinski buk area, 10 m a.s.l., 43.80478° N, 15.96353° E, on litter, forest of *Ostrya carpinifolia*, leg. Z. Tkalčec & A. Mešić, 14 December 2007, CNF 1/5310; Dubrovnik-Neretva County: island of Lokrum, 25 m a.s.l., 42.62533° N, 18.12239° E, on heap of litter, forest of *Quercus ilex* and *Pinus halepensis*, leg. Z. Tkalčec & A. Mešić, 12 November 2009, CNF 1/5735.

Notes: Croatian collections of *P. conopilea* (CNF 1/5310 and 1/5735) formed a monophyletic clade (BI-PP = 1, ML-BP = 100) with *P. conopilea* (CBS 325.39) from the Netherlands [36]. The species most closely related to *P. conopilea* (CNF 1/5310 and 1/5735) was *P. psathyrelloides* (CAL 1753, holotype; AMH 10119, paratype), forming a strongly supported sister clade (BI-PP = 1, ML-BP = 99).

Parasola crataegi. Croatia, Požega-Slavonia County: vicinity of the town of Pleternica, near the village of Gradac, 135 m a.s.l., 45.31658° N, 17.79921° E, on soil and litter of *Crataegus monogyna*, under *C. monogyna* on the edge of deciduous forest dominated by *Quercus petraea* and *Carpinus betulus*, leg. M. Čerkez, 4 October 2022, CNF 1/8905 (Figure 4C).

Notes: The collection of *P. crataegi* from Croatia formed a monophyletic clade with the holotype (SSt08-154) and the paratype (NL-4175) of *P. crataegi* with full support (BI-PP = 1, ML-BP = 100). In our phylogram, the *P. crataegi* clade was a sister to a clade comprising *P. pseudolactea* (HUP-SU-412, holotype; HUP-SU-413, paratype), *P. lactea* (NL-0288 and NL-0283), and *P. galericuliformis* (NL-6601 and NL-0095) with strong support (BI-PP = 1, ML-BP = 94).

Parasola cuniculorum. Croatia, Primorje-Gorski Kotar County: vicinity of the village of Skrad, near Rogi hamlet, 725 m a.s.l., 45.42022° N, 14.88305° E, on dung of *Cervus elaphus* (red deer), forest of *Abies alba* and *Fagus sylvatica*, leg. M. Čerkez, 10 April 2008, CNF 1/5143 (Figure 4D,E).

Notes: The collection of *P. cuniculorum* from Croatia formed a monophyletic clade (BI-PP = 1, ML-BP = 100) with the holotype of *P. cuniculorum* (K(M)191984) [16]. In our analysis, the most closely related species was *P. misera* (NL-0280, neotype; NL-0677), which formed a sister clade to *P. cuniculorum* with full support (BI-PP = 1, ML-BP = 100).

Parasola kuehneri. Croatia, Sisak-Moslavina County: vicinity of the village of Letovanić, near Palanjek Pokupski hamlet, 155 m a.s.l., ca. 45.5178° N, 16.1378° E, on grassy soil, deciduous forest of *Fagus sylvatica*, *Carpinus betulus*, and *Quercus* sp., leg. M. Čerkez, 24 August 2003, CNF 1/4334.

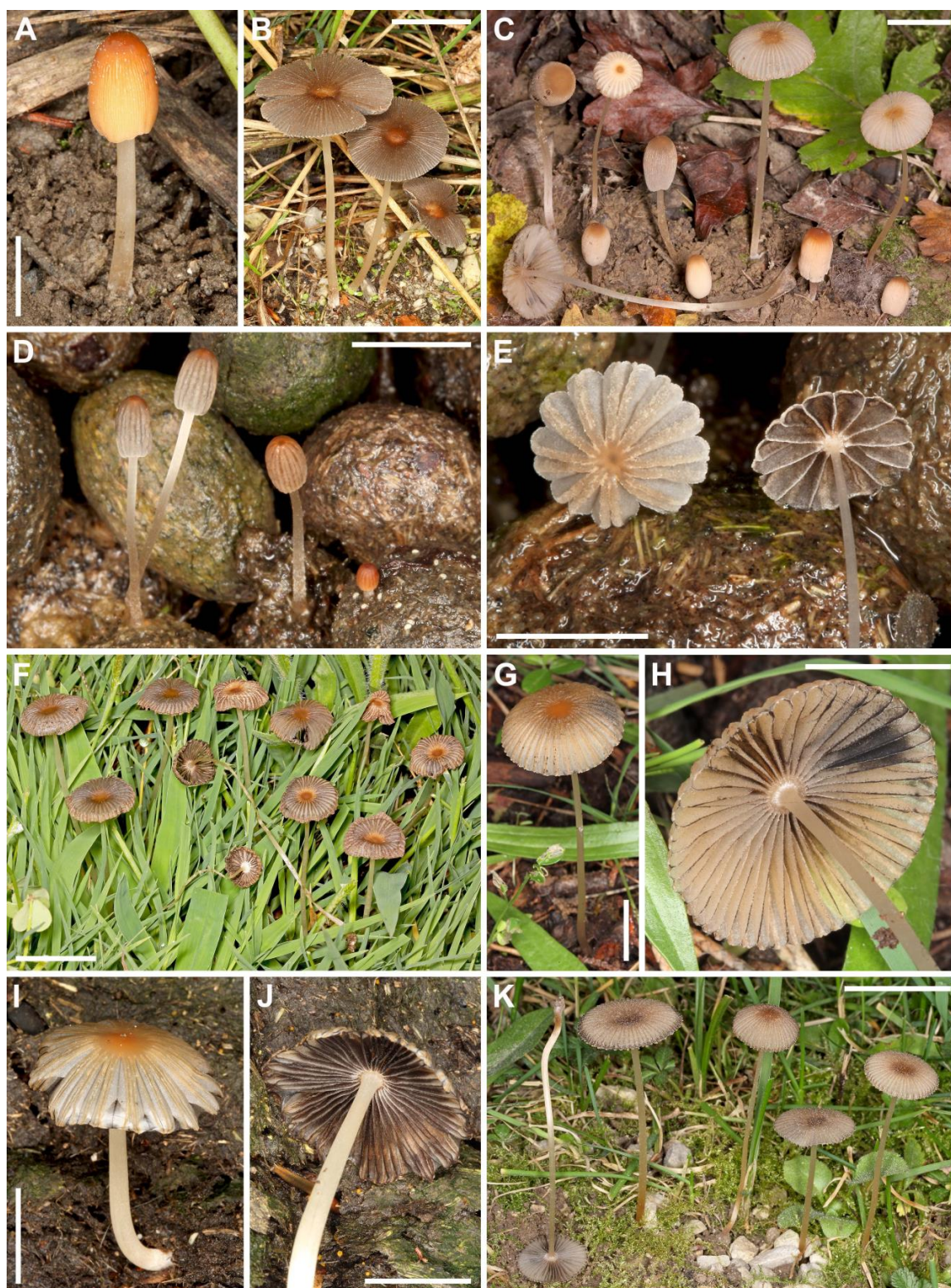


Figure 4. Basidiomata of *Parasola* species new to Croatia. (A,B) *P. auricoma* CNF 1/4718, 1/4618. (C) *P. crataegi* CNF 1/8905. (D,E) *P. cuniculorum* CNF 1/5143. (F) *P. malakandensis* CNF 1/8698. (G,H) *P. megasperma* CNF 1/5704. (I,J) *P. nudiceps* CNF 1/4804. (K) *P. plicatilis-similis* CNF 1/5484. Bars: (A,D,E) = 5 mm; (B,F,K) = 20 mm; (C,G–J) = 10 mm. Photos: M. Čerkez.

Notes: The collections of *P. kuehneri* from the Netherlands (Ulje 31.5.1987, holotype; Ulje 1241) formed a monophyletic clade with the Croatian collection of *P. kuehneri* with

strong support (BI-PP = 1, ML-BP = 98). *Parasola parvula* Ganga & Manimohan was recovered as its sister species in our analysis (BI-PP = 0.98, ML-BP = 72).

Parasola malakandensis. Croatia, Split-Dalmatia County: island of Hvar, near the town of Jelsa, 20 m a.s.l., 43.16452° N, 16.68287° E, on soil, grassy football field, leg. M. Čerkez, 20 July 2021, CNF 1/8698 (Figure 4F).

Notes: The collection of *P. malakandensis* from Croatia clustered in a monophyletic clade (BI-PP = 1, ML-BP = 100) with the holotype (HUP 17501) and the paratype (LAH-SHP-17) of *P. malakandensis* from Pakistan [9].

Parasola megasperma. Croatia, Dubrovnik-Neretva County: island of Mljet, near the village of Prožurski Porat, 35 m a.s.l., 42.73075° N, 17.64633° E, on soil, Mediterranean scrubland (maquis) dominated by *Ceratonia siliqua*, *Cupressus sempervirens*, *Pistacia lentiscus*, and *Cistus* sp., leg. M. Čerkez, 9 November 2009, CNF 1/5704 (Figure 4G,H).

Notes: The collection of *P. megasperma* from Croatia formed a monophyletic clade (ML-BP = 96) together with two collections of *P. megasperma* (E:Orton 4132, holotype; Ulje 1275) and the collection identified as *P. schroeteri* (P. Karst.) Redhead, Vilgalys & Hopple (Dähncke 1502) [10].

Parasola nudiceps. Croatia, Krapina-Zagorje County: Medvednica Mountain, 750 m a.s.l., 45.91905° N, 15.96917° E, on old cow dung, pasture, leg. M. Čerkez, 29 September 2007, CNF 1/4804 (Figure 4I,J).

Notes: The morphological and phylogenetic analyses performed in this study confirmed the conclusion of Schafer et al. [16], who considered *P. ochracea* to be a later synonym of *P. nudiceps*. Our measurements of the basidiospore size (ca. $12\text{--}14.5 \times 10\text{--}12.5 \times 8\text{--}10 \mu\text{m}$) from the holotype of *P. ochracea* NL-3621 revealed that Szarkandi et al. [10] had mistakenly reported an erroneous spore size ($10\text{--}11 \times 6\text{--}8.5 \mu\text{m}$) in the protologue. On the other hand, the size of basidiospores in the holotype of *P. ochracea* is within the size range of spores from the holotype of *P. nudiceps* ($11.6\text{--}16.0 \times 10.6\text{--}14.0 \times 6.7\text{--}9.5 \mu\text{m}$) reported by [6,42,43]. Based on morphological studies alone [6,43], *P. nudiceps* could be considered a later synonym of *P. schroeteri*, but further research is needed (see Schafer et al. [16] for a discussion). The collection of *P. nudiceps* from Croatia formed a monophyletic group (BI-PP = 1, ML-BP = 100) along with another four collections of *P. nudiceps* (E:Orton 4133, holotype; HB19870911A; *P. ochracea* NL-3621, holotype; and *P. ochracea* NL-3167, paratype). *Parasola litoralis* was recovered as a sister clade to *P. nudiceps* in our analysis.

Parasola plicatilis-similis. Croatia, Zagreb County: Žumberak Mountain, near the village of Stojdraga, 240 m a.s.l., 45.83774° N, 15.61053° E, on grassy soil, grassland, leg. M. Čerkez, 26 October 2008, CNF 1/5484 (Figure 4K).

Notes: The collection of *P. plicatilis-similis* from Croatia clustered in a monophyletic clade (BI-PP = 0.96, ML-BP = 98) together with the holotype (NL-2125) and the paratype (NL-0287) of *P. plicatilis-similis* from Sweden [10]. Two collections of *P. aporos* (RC-F92.191, holotype; CL-F09.005) formed a sister clade to *P. plicatilis-similis* (BI-PP = 1, ML-BP = 100) in agreement with Malysheva et al. [17]. Based on GenBank BLAST results, the sequence similarities between *P. plicatilis-similis* (CNF 1/5484) and *P. aporos* collections (RC-F92.191, holotype; CL-F09.005) were 99.55% and 99.31–99.54% in ITS and LSU gene regions, respectively. Considering important morphological differences between both taxa, e.g., basidiospore features, an absence/presence of pleurocystidia, and the habit of basidiomata, we followed the taxonomic concept from Malysheva et al. [17] and treated *P. aporos* and *P. plicatilis-similis* as separate species.

4. Discussion

Ten species of the genus *Parasola* from Croatia were identified using a combination of morphological and molecular characteristics. *Parasola papillatospora*, a species occurring in temperate deciduous forests of Europe (Croatia, Hungary), was described as new to science. Eight species, *P. auricoma*, *P. crataegi*, *P. cuniculorum*, *P. kuehneri*, *P. malakandensis*, *P. megasperma*, *P. nudiceps*, and *P. plicatilis-similis*, were reported for the first time from Croatia. *Parasola malakandensis* was also reported for the first time outside Pakistan. *Parasola conopilea*,

a species previously reported from Croatia based on morphological identification [19], was confirmed with multigene phylogenetic analyses. The collections of seven species new to Croatia (all except *P. auricoma*) were clustered with the type collection of the same species in our phylogenetic analyses.

Parasola papillatospora sp. nov. is phylogenetically well distinguished from all other published *Parasola* species included in the GenBank database. In the phylogram, *P. papillatospora* recovered as an independent clade, a sister to the *P. plicatilis* species complex (BI-PP = 1, ML-BP = 100). The *P. papillatospora* clade consisted of six Croatian collections and a single one from Hungary (*Parasola* sp. 1 (SZMC-NL-2952) in Szarkandi et al. [10]). The Hungarian collection of *P. papillatospora* was analyzed by Szarkandi et al. [10] but was not introduced as a new species due to a lack of sufficient sample information and/or molecular data.

Six species of *Parasola* may have (sub)papillate basidiospores (at least sometimes), but always in combination with eccentric germ pores: *P. lactea* (= *P. leiocephala*), *P. lilatincta* (Bender & Uljé) Redhead, Vilgalys & Hopple, *P. lilatinctoides* P. Voto, *P. litoralis* Loizides, D.J. Schaf. & P. Alvarado, *P. misera*, and *P. parvula*. DNA sequences from the type material of the latter four species were deposited in GenBank and were included in phylogenetic analyses conducted here. In addition to the presence of exclusively eccentric germ pores, the above species can be distinguished from *P. papillatospora* by other morphological characteristics as well. None of these species have such a great variety of basidiospore shapes, including a strongly developed apical papilla and a distinctly conical base, which are not uncommon in *P. papillatospora*. *Parasola lactea* is the most similar species but differs by somewhat larger basidiospores (average length, 9.0–10.7 µm; average breadth, 8.1–9.8 µm) and mostly utriform to lageniform cheilocystidia [5]. *Parasola lilatincta* has a larger pileus (20–50 mm wide), larger basidiospores (average length, 10.7–12.3 µm; average breadth, 9.5–10.1 µm), and basidia surrounded by (4–)5–8(–9) hymenophysalides [5]. *Parasola lilatinctoides* differs by much larger basidiospores (average length, 14.2–14.5 µm; average breadth, 12.5 µm) which are only sometimes subpapillate and by having 5–8 hymenophysalides around the basidium [14]. *Parasola litoralis* has much larger basidiospores (average length, 16.3 µm; average breadth, 11.7 µm), only sometimes with a slight apical protrusion, much wider basidia (12–20 µm), and longer pleurocystidia (80–96 µm) [16]. *Parasola misera* differs by more isodiametric basidiospores ($Q_f = 0.96$ –1.12) which are only sometimes papillate, by the absence of pleurocystidia, and the fimicolous habitat [5,6]. Finally, *P. parvula* differs in having a smaller pileus (3–11 mm) with violet or purplish color tones present in most basidiomata, pinkish lamellae when young, a yellowish or purplish black base of the pileipellis elements, and the fimicolous habitat [11].

5. Conclusions

Until 2013, *Parasola* was considered a rather small fungal genus with up to 12 accepted species. However, subsequent extensive taxonomic research led to the description of 19 additional new species in the genus to date. Prior to this work, only four species of *Parasola* were known from Croatia.

We performed an integrative taxonomic study of 17 *Parasola* specimens collected in different parts of Croatia and identified 10 species. A new saprotrophic soil species from temperate deciduous forests, *P. papillatospora*, was described based on morphology, ecology, and multigene (ITS, LSU, *tef-1α*, and *β-tub*) phylogenetic analyses. Eight species were reported for the first time from Croatia and *P. malakandensis* also for Europe.

The integrative taxonomic approach used in this study to distinguish species within *Parasola* is highly recommended to be applied in future studies of the genus.

Author Contributions: Conceptualization, A.P., Z.T. and A.M.; methodology, A.P., Z.T. and A.M.; formal analysis, A.P., Z.T., A.M. and L.P.; investigation, A.P., Z.T., A.M., M.Č., I.K. and N.M.; resources, A.M., Z.T., I.K. and N.M.; data curation, A.P., Z.T., A.M. and M.Č.; writing—original draft preparation, Z.T., A.P. and A.M.; writing—review and editing, A.P., Z.T., A.M., M.Č., L.P., I.K. and

N.M.; visualization, A.P. and Z.T.; supervision, Z.T. and A.M.; project administration, A.M.; funding acquisition, A.M. All authors have read and agreed to the published version of the manuscript.

Funding: This work was fully supported by the Croatian Science Foundation under the ForFungiDNA project grants HRZZ-IP-2018-01-1736 (to A.P., A.M., L.P., I.K., N.M., Z.T.), HRZZ-DOK-2018-09-7081 (to A.P.), and HRZZ-DOK-2021-02-4010 (to L.P.).

Data Availability Statement: Sequences generated in this study were submitted to the GenBank database of NCBI (<https://www.ncbi.nlm.nih.gov/genbank/> accessed on 16 May 2023). Alignments and phylogenetic trees generated in this study are available at Zenodo (DOI 10.5281/zenodo.8099476).

Acknowledgments: We are very grateful to László G. Nagy for the loan of the type material of *Parasola ochracea* deposited in BP, which was used for the morphological analyses in this study.

Conflicts of Interest: The authors declare no conflict of interest. The funder had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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