News from the Sea: A New Genus and Seven New Species in the Pleosporalean Families Rousselloellaceae and Thyridariaceae

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Abstract: Nineteen fungal strains associated with the seagrass Posidonia oceanica, with the green alga Flabellia petiolata, and the brown alga Padina pavonica were collected in the Mediterranean Sea. These strains were previously identified at the family level and hypothesised to be undescribed species. Strains were examined by deep multi-loci phylogenetic and morphological analyses. Maximum-likelihood and Bayesian phylogenies proved that Parathyridariella gen. nov. is a distinct genus in the family Thyridariaceae. Analyses based on five genetic markers revealed seven new species: Neoroussella lignicola sp. nov., Roussella margidorensis sp. nov., R. mediterranea sp. nov., and R. padinae sp. nov. within the family Rousselloellaceae, and Parathyridaria flabelliae sp. nov., P. tyrrhenica sp. nov., and Parathyridariella dematiacea gen. nov. et sp. nov. within the family Thyridariaceae.

Keywords: marine fungi; new taxa; phylogeny; lignicolous fungi

1. Introduction

Marine fungi are a relevant and active component of the microbial communities that inhabit the oceans [1]. Fungi in the marine environment live as mutualists, parasites, pathogens and saprobics, and are pivotal to marine food webs because of the recycling of recalcitrant substrata [2]; besides, these widely dispersed organisms are a source of novel bioactive compounds [3].

Marine fungi have been recovered worldwide from a broad range of biotic and abiotic substrata, such as driftwood algae, sponges, corals, sediments, etc. [4,5]. Following the definition of Pang et al [6] that considered “a marine fungus” to be any fungus retrieved repeatedly from marine environment and that reproduces in the marine environment, Jones et al. [7] listed 1680 fungal species belonging to 693 genera, 223 families, 87 orders, 21 classes and six phyla. However, considering that the total number of marine fungi has been estimated to exceed 10,000 taxa [8], fungal diversity remains largely undescribed. With more than 900 species [9], the Ascomycota are the dominant fungal phylum in the sea; the most represented lineages include the order Pleosporales (class Dothideomycetes) with 36 families, 95 genera and 194 species described to date (www.marinefungi.org).

In recent surveys aimed to uncover the underwater fungal diversity, 19 unidentified Rousselloellaceae were isolated from several substrates, as follows: 12 from the brown alga Padina pavonica (L.) Thivy [10], 4 from the green alga Flabellia petiolata (Turra) Nizamuddin [11], 2 from the seagrass Posidonia oceanica (L.) [12] Delile, and 1 from the Atlantic sponge Dysidea fragilis (Montagu) [13]. The Rousselloellaceae is a well-resolved family in the Pleosporales [14]. Others [15] have treated the family Rousselloellaceae as a synonym of Thyridariaceae, based on phylogenetic affinities.
However, following the discovery of new genera in this group, delineated by high resolution multi-locus phylogenetic analyses, the Roussoellaceae and Thyridariaceae are now recognized as two distinct but closely related families [16–20].

Many new species of Roussoellaceae and Thyridariaceae have recently been described on terrestrial plants including bamboo, palms and mangroves [14,17,20,21]. This paper provides a more precise phylogenetic placement of the 19 strains isolated from marine substrata together with morphological insights of those strains that represent new species within these two families.

2. Materials and Methods

2.1. Fungal Isolates

The fungal isolates analyzed in this paper were retrieved in the Mediterranean Sea from P. oceanica (2), collected in Riva Trigoso bay and Elba island, P. pavo
cica (12), and F. petiolata (3) from the coastal waters of Elba island [10–12]. A single isolate was previously retrieved in association with D. fragilis in the Atlantic Ocean [13] (Table 1).

The strains investigated were originally isolated on Corn Meal Agar medium supplemented with sea salts (CMASS; 3.5% w/v sea salt mix, Sigma-Aldrich, Saint Louis, USA, in ddH2O) and are preserved at the Mycotheca Universitatis Taurinensis (MUT), Italy.

2.2. Morphological Analysis

All isolates were pre-grown on Malt Extract Agar-sea water (MEASW; 20 g malt extract, 20 g glucose, 2 g peptone, 20 g agar in 1 L of sea water) for one month at 24 °C prior to inoculation in triplicate onto new Petri dishes (9 cm Ø) containing i) MEASW, ii) Oatmeal Agar-sea water (OASW; 30 g oatmeal, 20 g agar in 1 L of sea water), or iii) Potato Dextrose Agar-sea water (PDASW; 4 g potato extract, 20 g dextrose, 20 g agar in 1 L of sea water). Petri dishes were incubated at 15 and/or 24 °C. The colony growth was monitored periodically for 28 days. Macroscopic and microscopic traits, were assessed for strains grown on MEASW at the end of the incubation period.

In an attempt to induce sporulation, sterile pieces of Quercus ruber cork and Pinus pinaster wood (species autochthonous to the Mediterranean area) were placed on 3 week old fungal colonies grown on MEASW ([22], modified). Petri dishes were further incubated for 4 weeks at 24 °C. Subsequently, cork and wood pieces were transferred to 50 mL tubes containing 20 mL of sterile sea water. Samples were incubated at 24 °C for one month. In parallel, the strains were also plated on Syntetic Nutrient Agar-sea water (SNASW; 1 g KH2PO4, 1 g KNO3, 0.5 g MgSO4 • 7H2O, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, 20 g agar in 1 L of sea water) supplemented with sterile pine needles. Petri dishes were incubated at 24 °C for one month.

Morphological structures were observed, and images captured using an optical microscope (Leica DM4500B, Leica microsystems GmbH, Germany) equipped with a camera (Leica DFC320, Leica microsystems GmbH, Germany). Macro- and microscopic features were compared with the available description of Roussoellaceae and Thyridariaceae [14,15,17,18,20].

2.3. DNA Extraction, PCR Amplification, and Data Assembling

Genomic DNA was extracted from about 100 mg of fresh mycelium grown on MEASW plates. Mycelium was disrupted by the mean of a MM400 tissue lyzer (Retsch GmbH, Haan, Germany) and DNA extracted using a NucleoSpin kit (Macherey Nagel GmbH, Duren, DE, USA) following the manufacturer’s instructions. The quality and quantity of DNA were measured spectrophotometrically (Infinite 200 PRO NanoQuant; TECAN, Switzerland); DNA was stored at −20 °C.

The partial sequences of five genetic markers were amplified by PCR. Primer pairs ITS1/ITS4 [23], LR0R/LR7 [24], NS1/NS4 [23] were used to amplify the internal transcribed spacers, including the 5.8S rDNA gene (nrITS), 28S large ribosomal subunit (nrLSU) and 18S small ribosomal subunit (nrSSU). The translation elongation factor (TEF1α) and RNA polymerase II subunit (RPB2) were amplified by using primer pairs EF1-1018F/EF1-1620R [25] and fRPB2-5F/fRPB2-7R [26].
Amplifications were run in a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) programmed as described in Table 2. Reaction mixtures consisted of 20–40 ng DNA template, 10× PCR Buffer (15 mM MgCl₂, 500 mM KCl, 100 mM Tris-HCl, pH 8.3), 200 µM each dNTP, 1 µM each primer, 2.5 U Taq DNA Polymerase (Qiagen, Chatsworth, CA, USA), in 50 µL final volume. For problematic cases, additional MgCl₂ and/or 2.5% DMSO facilitated the reaction.

Amplicons, together with a GelPilot 1 kb plus DNA Ladder, were visualized on a 1.5% agarose gel stained with 5 mL 100 mL⁻¹ ethidium bromide; PCR products were purified and sequenced at the Macrogen Europe Laboratory (Madrid, Spain). The resulting Applied Biosystem (ABI) chromatograms were inspected, trimmed and assembled to obtain consensus sequences using Seqencer 5.0 (GeneCodes Corporation, Ann Arbor, Michigan, USA http://www.genecodes.com). Newly generated sequences were deposited in GenBank (Table 1).

2.4. Sequence Alignment and Phylogenetic Analysis

A dataset consisting of nrSSU, nrITS, nrLSU, TEF1α and RPB2 was assembled on the basis of BLASTn results and of recent phylogenetic studies focused on Roussolliaceae and Thyridariaceae [18,20]. Reference sequences were retrieved from GenBank (Table 1).

Sequences were aligned using MUSCLE (default conditions for gap openings and gap extension penalties), implemented in MEGA v. 7.0 (Molecular Evolutionary Genetics Analysis), visually inspected and trimmed by TrimAl v. 1.2 (http://trimal.cgenomics.org) to delimit and discard ambiguously aligned regions. Since no incongruence was observed among single-loci phylogenetic trees, alignments were concatenated into a single data matrix with SequenceMatrix [27]. The best evolutionary model under the Akaike Information Criterion (AIC) was determined with jModelTest 2 [28].

Phylogenetic inference was estimated using Maximum Likelihood (ML) and Bayesian Inference (BI) criteria. The ML analysis was generated using RAxML v. 8.1.2 [29] under GTR + I + G evolutionary model and 1000 bootstrap replicates. Support values from bootstrapping runs (MLB) were mapped on the globally best tree using the “−f a” option of RAxML and “−x 12345” as a random seed to invoke the novel rapid bootstrapping algorithm. BI was performed with MrBayes 3.2.2 [30] with the same substitution model (GTR + I + G). The alignment was run for 10 million generations with two independent runs each containing four Markov Chains Monte Carlo (MCMC) and sampling every 100 iterations. The first 25% of generated trees were discarded as “burn-in”. A consensus tree was generated using the “sumt” function of MrBayes and Bayesian posterior probabilities (BPP) were calculated. Consensus trees were visualized in FigTree v. 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree).

Two strains of Occultibambusa bambusae (Occultibambusaceae) were used to root the tree. Due to topological similarity of the two resulting trees, only ML analysis with MLB and BPP values was reported (Figure 1).

DNA diagnostic characters were visually identified by the presence of heterozygous bases. For each locus, aligned sequences of the individual clusters containing new species, were inspected. Nucleotide diversities of the novel species were annotated when occurred (Tables S1–S18).

Sequence alignments and phylogenetic trees were deposited in TreeBASE (http://www.treebase.org, submission number S24773).

Following phylogenetic tree inspection, isolates that clustered in the same group and that derived from the same substrate were subjected to PCR-fingerprinting by using the micro- and mini-satellite primers (GTG) and M13 [31,32] to exclude duplicates from further analysis. DNA fingerprints were visualized with 1.5% agarose gel stained with 5 mL 100 mL⁻¹ ethidium bromide while a GelPilot 1 kb plusDNA Ladder was used as a reference. Images were acquired with a Gel Doc1000 System (Bio-Rad, Hercules, CA, USA) and fingerprints analyzed using Bionumerics v 7.6 (http://www.applied-maths.com).
Table 1. Dataset used for phylogenetic analysis. Genbank sequences including newly generated nrITS, nrLSU, nrSSU, TEF1-α and RPB2 amplicons relative to the novel species of Roussoellaceae and Thyridariaceae, to Parathyridaria robiniae MUT 2452 and MUT 4893 and to Parathyridaria ramulicola MUT 4397.

| Species | Strain Code | Source | nrITS | nrSSU | nrLSU | TEF-1α | RPB2 |
|---------|-------------|--------|-------|-------|-------|--------|------|
| Arthopyrenia salicis Massal | CBS 368.94 | Salix bark | KF443410 | AY538333 | AY538339 | KF443404 | KF443397 |
| Neoroussosella bambusae Liu and Hyde | MFLUCC 11-0124 | Dead branch of Bambusa | KJ74827 | -- | KJ74839 | KJ74848 | KJ74856 |
| N. alishanense Karunarathna, Kuo, Phookamsak and Hyde | AKTW 03 FU31016 | Pennisetum purpureum | MK503816 | MK503828 | MK503822 | MK336181 | MN037756 |
| AKTW 11 FU31018 | Pennisetum purpureum | MK503818 | MK503830 | MK503824 | MK336182 | MN037757 |
| N. entadae Jones and Hyde | MFLUCC 18-0243 | Leucaena sp. | MK347786 | MK347893 | MK348004 | MK360065 | MK434866 |
| N. heveae Senwanna, Phookamsak and Hyde | MFLUCC 17-1983 | Twig of Hevea brasiliensis | MH590693 | -- | MH590689 | -- | -- |
| N. leucaenae Jones and Hyde | MFLUCC 18-1544 | Decaying pod of Leucaena | MK347767 | MK347874 | MK347984 | MK360067 | MK434876 |
| | MFLUCC 17-0927 | Pterocarpus sp. | MK347733 | MK347841 | MK347950 | MK360066 | MK434896 |
| Neoroussosella lignicola sp. nov. | MUT 4904 | P. pavonica | KT699129 | MN556307* | MN556319* | MN605894* | MN605914* |
| | MUT 5008 | P. oceanica leaves | MN556317* | MN556308* | MN556320* | MN605895* | MN605915* |
| | MUT 5373 | P. pavonica | KU314953 | KU314954 | MN556321* | MN605896* | MN605916* |
| Pararoussoella juglandicola | CBS 145037 | -- | MK442607 | -- | MK442543 | MK442699 | MK442671 |
| Crous and Schumach | (Phookamsak, Dai and Hyde) | Bamboo | KU940129 | KU872121 | KU863118 | -- | -- |
| P. mukdahanensis | MFLUCC 11-0201 | Rosa sp. | MG8289391 | MG829154 | MG829048 | MG829224 | -- |
| (Phukhams., Camporesi and Hyde) | Crous | MFLUCC 17-0796 | Dead aerial spines of Rosa canina | MG828922 | MG829138 | MG829032 | -- | -- |
| P. rosarum Jones and Hyde | MFLUCC 15-0052 | Dead branch of bamboo | KJ74828 | -- | KJ74840 | KJ74849 | KJ74857 |
| (Phukhams., Camporesi and Hyde) | Phukhams., Camporesi and Hyde | MFLUCC 10-0556 | Dead branch of bamboo | KY026584 | -- | KY000659 | KY651249 | KY678394 |
| Roussoella chiangraina | MFLUCC 14-0584 | Dead petiole of Elaeis guineensis | MH742329 | -- | MH742326 | -- | -- |
| Phookamsak, Liu and Hyde | MFLUCC 15-0276a | Dead petiole of Elaeis | MH742330 | -- | MH742327 | -- | -- |
| R. doimaesalongensis | MFLUCC 15-0276b | Dead petiole of Elaeis | MH742330 | -- | MH742327 | -- | -- |
| Species                                      | CBS | Origin                                      | GenBank Accessions | Update Status |
|----------------------------------------------|-----|---------------------------------------------|-------------------|---------------|
| R. euonymi Crous and Akulov                  | CBS 143426 | Fallen branches of Euonymus europaeus | MH107915 -- | MH107961 -- MH108007 |
| R. hysterioides (Ces.) Höhn.                 | CBS 546.94 | Phyllostachys | KF443405 -- | KF443381 -- KF443399 -- KF443392 |
| R. intermedia Ju, Rogers and Huhndorf        | CBS 170.96 | Bamboo | KF443407 -- | KF443390 -- KF443382 -- KF443398 -- KF443394 |
| R. japonensis Kaz. Tanaka, Liu and Hyde      | MAFF 239636 | Twigs of Sasa veitchii | KJ474829 -- | AB524480 -- AB524621 -- AB539114 -- AB539101 |
| R. kunmingensis Jiang, Phookamsak and Hyde   | KUMCC 18-0128 | Dead bamboo | MH453491 -- | -- MH453487 -- MH453480 -- MH453484 |
| R. mangrovei Phukhams. and Hyde              | MFLUCC 16-0424 | Dead branches of Rhizophora | MH025951 -- | -- MH023318 -- MH028246 -- MH028250 |
| Roussoella margidorensis sp. nov. R. japonensis Kaz. Tanaka, Liu and Hyde | MUT 5329 | P. pavonica | KU314944 MN556309* MN556322* MN605897* MN605917* |
| Roussoella mediterranea sp. nov.             | MUT 5369 | P. pavonica | KU314947 KU314948 MN556324* MN556399* MN605899* MN605919* |
| R. mexicana Crous and Yáñez-Mor.             | CPC 25355 | Leaf spots of Coffea arabica | KT950848 -- | -- KT950862 -- -- |
| R. neopustulans Dai, Liu and Hyde            | MFLUCC 11-0609 | Bamboo | KJ474833 -- | -- KJ474841 KJ474850 |
| R. nittidula Sacc. and Paol.                 | MFLUCC 12-0003 | Bamboo | KU940130 KU872122 KU863119 -- -- |
| MOSA תת-10-686 | MFLUCC 11-0182 | Bamboo | KJ474835 -- | -- KJ474843 KJ474852 KJ474859 |
| Roussoella padinae sp. nov.                  | MUT 5341 | P. pavonica | KU158153 KU158176 MN556325* MN605900* MN605920* |
| Roussoella padinae sp. nov.                  | MUT 5365 | P. pavonica | KU158170 KU158179 MN556326* MN605901* MN605921* |
| R. pseudohysterioides Dai and Hyde           | MFLUCC 11-0624 | Bamboo | KU940131 KU872123 KU863120 KU940198 -- | |
| R. pustulans (Ellis and Everh.) Ju, Rogers and Huhndorf | K1T709 | Culms of Sasa kurilensis | KJ474830 AB524482 AB524623 AB539116 | AB539103 |
| R. scabrispora (Höh.) Aptroot                 | MFLUCC 11-0624 | Bamboo | KJ474836 -- | -- KJ474844 KJ474853 KJ474860 |
| R. scabrispora (Höh.) Aptroot                 | RSC | Bamboo | KX650566 -- | -- KX650566 KX650537 -- |
| R. siamensis Phookamsak, Liu and Hyde         | MFLUCC 11-0149 | Bamboo | KJ474837 KU872125 KJ474845 KJ474854 KJ474861 |
| R. thailandica Dai, Liu and                  | MFLUCC 11-0621 | Bamboo | KJ474838 -- | -- KJ474846 -- |
| Species                                      | Collection Code | Description                      | Accession Numbers                  |
|----------------------------------------------|-----------------|----------------------------------|-----------------------------------|
| **Hyde**                                     |                 |                                  |                                   |
| R. tuberculata Dai and Hyde                  | MFLUCC 13-0854  | Bamboo                           | KU940132 KU872124 KU863121 KU940199 |
| R. verrucispora Kaz. Tanaka, Liu and Hyde    | CBS 125434      | Sasa kurilensis                  | KJ478432 AB52448 AB524622 AB539115 |
| R. yunnanensis Jiang, Phookamsak and Hyde    | KUMCC 18-0115   | Dead bamboo                      | MH453492 -- MH453488 MH453481 -- |
| **Rousseloellopsis macrospora** (Hino and Katum.) Hino and Katum | MFLUCC 12-0005  | Bamboo                           | KJ739604 KJ739608 KJ474847 KJ474855 KJ474862 |
| Ro. tosaensis (Hino and Katum.) Hino and Katum | KT 1659         | Culms of bamboo                  | -- AB524484 AB524625 AB539117 AB539104 |
| **Thyridariaceae**                           |                 |                                  |                                   |
| Cycasicola goaensis Jones and Hyde           | MFLUCC 17-0754  | Cycas sp.                        | MG828885 MG829112 MG829001 MG829198 -- |
| C. leucaenae Jones and Hyde                  | MFLUCC 17-0914  | Leucaena leucocephala           | MK34772 MK347833 MK347942 MK360046 MK343900 |
| Liua muriformis Phookamsak, Jiang and Hyde   | KUMCC 18-0177   | Dead hanging branches of Lonicera maackii | MK433599 MK433595 MK433598 MK426798 MK426799 |
| **Parathyridaria percutanea** (Ahmed, Stevens, van de Sande and de Hoog) Jaklitsch and Voglmayr | MFLUCC 868.95    | Human                            | KF322118 KF366451 KF366449 KF407987 KF366452 |
| P. ramulicola Jaklitsch, Fourn and Voglmayr  | CBS 128203      | Human                            | KF322117 KF366450 KF366448 KF407988 KF366453 |
| P. robiniae Mapook, Camporesi and Hyde        | CBS 141479      | Twigs of Ribes rubrum            | NR_147657 KX650514 KX650565 KX650536 KX650584 |
| **Parathyridaria flabelliae sp. nov.**        | MFLUCC 14-1119  | Dead branch of Robinia pseudoacacia | KY511142 -- KY511141 KY549682 -- |
| P. oceanica                                  | KC339235        |                                  | MN556311* KF636775 MN605913* MN605933* |
| P. pavonica                                  | KM355998        |                                  | MN56328* MN605904* MN605924* |
| P. pavonica                                  | KM355993        |                                  | MN56328* MN605904* MN605924* |
| Parathyridaria tyrrhenica sp. nov.           | MFLUCC 4859     | F. petiolata                      | KR014355 KT587315 KP671716 MN605909* MN605929* |
| P. petiolata                                  | KR014358        |                                  | KT587317 KP671720 MN605910* MN605930* |
| Parathyridaria tyrrhenica sp. nov.           | MFLUCC 4886     | F. petiolata                      | KR014366 KT587309 KP671740 MN605911* MN605931* |
| P. pavonica                                  | KU314951        |                                  | MN56329* MN605912* MN605932* |
| **Parathyridariella dematiacea** sp. nov. | MUT 4419 | *P. oceanica* rhizomes | KC339245 | MN556313* | KF636786 | MN605905* | MN605925* |
|------------------------------------------|---------|-------------------------|---------|-----------|---------|-----------|-----------|
| MUT 4884                                 | *F. petiolata* | MN556317* | KT587329 | KP671726 | MN605906* | MN605926* |
| MUT 5310                                 | *P. paezonica* | KU255057 | MN556314* | MN556330* | MN605907* | MN605927* |
| MUT 5381                                 | *P. paezonica* | KU314959 | MN556331* | MN605908* | MN605928* |
| **Thyridaria acaciae** (Crous and Wingf.) Jaklitsch and Voglmayr | CBS 138873 | Leaves of *Acacia tortilis* | KP004469 | -- | KP004497 | -- | -- |
| **T. brousso netiae** (Sacc.) Traverso | TB | *Hippocrepis emerus* | KX650567 | -- | KX650567 | KX650538 | KX650585 |
| | TB1 | *Amorpha fruticosa* | KX650568 | KX650515 | KX650568 | KX650539 | KX650586 |
| **Thyridariella mahakoshae** Devadatha, Sarma, Wanas., Hyde and Jones | NFCCI 4215 | Decaying wood *Avicennia marina* | MG020435 | MG020441 | MG020438 | MG023140 | MG020446 |
| **T. mangrovei** Devadatha, Sarma, Hyde, Wanas. and Jones | NFCCI 4213 | Decaying wood *Avicennia marina* | MG020434 | MG020440 | MG020437 | MG020443 | MG020445 |
| | NFCCI 4214 | Decaying wood *Avicennia marina* | MG020436 | MG020442 | MG020439 | MG020444 | MG020447 |

**Occultibambusaceae**

**Occultibambusa bambusae** Dai and Hyde | MFLUCC 11-0394 | Bamboo | KU940124 | -- | KU863113 | KU940194 | KU940171 |
| | MFLUCC 13-0855 | Bamboo | KU940123 | KU872116 | KU863112 | KU940193 | KU940170 |

**Ohleriaceae**

**Ohleria modesta** Fuckel | MGC | Branches of *Chamaecytisus proliferus* | KX650562 | -- | KX650562 | KX650533 | KX650582 |
| | OM | Branches of *Chamaecytisus proliferus* | KX650563 | KX650513 | KX650563 | KX650534 | KX650583 |

**Torulaceae**

**Dendryphion europaeum** Crous and Schumacher | CPC 22943 | *Heracleum sphondylium* | KJ869146 | -- | KJ869203 | -- | -- |
| **Torula herbarum** (Pers.) Link | CBS 111855 | n.a. | KF443409 | KF443391 | KF443386 | KF443403 | KF443396 |
| | CBS 595.96 | | KF443408 | KF443387 | KF443385 | KF443402 | KF443395 |
| **Torula hollandica** Crous | CBS 220.69 | *Delphinium dead stem* | KF443406 | -- | M877717 | -- | -- |

* = newly generated sequences; n.a. = not available
Table 2. Primers and PCR conditions used to amplify specific gene marker.

| Forward and Reverse Primers | Thermocycler Conditions | References |
|-----------------------------|-------------------------|------------|
| ITS ITS1-ITS4               | 95 °C: 5 min, (95 °C: 40 s, 55 °C: 50 s, 72 °C: 50 sec) × 35 cycles; 72 °C: 8 min; 4 °C: ∞ | [23] |
| LSU LR0R-LR7                | 95 °C: 5 min, (95 °C: 1 min, 50 °C: 1 min, 72 °C: 2 min) × 35 cycles; 72 °C: 10 min; 4 °C: ∞ | [24] |
| SSU NS1-NS4                 | 95 °C: 5 min, (95 °C: 1 min, 50 °C: 1 min, 72 °C: 2 min) × 35 cycles; 72 °C: 10 min; 4 °C: ∞ | [23] |
| TEF-1α 1018F/1620R          | 95 °C: 5 min, (95 °C: 1 min, 50 °C: 1 min, 72 °C: 2 min) × 40 cycles, 72 °C: 10 min; 4 °C: ∞ | [25] |
| RPB2 fRPB2-5F/fPB2-7cR      | 94 °C: 3 min, (94 °C: 30 s, 55 °C: 30 s, 72 °C: 1 min) × 40 cycles, 72 °C: 10 min; 4 °C: ∞ | [26] |

3. Results

3.1. Phylogenetic Inference

Preliminary analyses carried out individually with nrITS, nrSSU, nrLSU, TEF1α and RPB2 denoted no incongruence in the topology of the single-locus trees. The combined five-markers dataset—built on the basis of BLASTn results and of recent phylogenetic studies [18,20]—consisted of 81 taxa (including MUT isolates) that represented 16 genera and 56 species (Table 1). A total of 63 sequences (2 nrITS, 8 nrSSU, 13 nrLSU, 20 TEF1α and 20 RPB2) were newly generated while 261 were retrieved from GenBank.

The combined dataset had an aligned length of 3390 characters, of which 1683 were constant, 657 were parsimony-uninformative and 1050 parsimony informative (TL = 218, CI = 0.422018, RI = 0.825243, RC = 0.348267, HI = 0.877952).

Strains MUT 4893 and MUT 2452 were identified as Parathyridaria robiniae, the rest of the strains represented seven new species and one new genus (Figure 1). Parathyridaria tyrrenica sp. nov. (MUT 5371 and MUT 4966) formed a sister clade to Parathyridaria flabelliae sp. nov. (MUT 4859 and MUT 4886) with high statistical support (BYPP = 1.00; MLB = 100%); these two novel species are closely related to P. ramulicola (BYPP = 1.00; MLB = 100%) and clustered with other Parathyridaria species in the Thyridariaceae family. Within this family, four isolates (MUT 5310, MUT 5381, MUT 4419 and MUT 4884) clustered together with the genera Thyridariella, Liua and Cycasicola, and formed a strongly supported monophyletic lineage (BYPP = 1.00; MLB = 100%). Therefore, we have introduced the novel genus Parathyridariella, typified by the new species Parathyridariella dematiacea sp. nov.

The three strains, MUT 4904, MUT 5373 and MUT 5008, represented a novel species Neoroussoella lignicola sp. nov. and formed an independent and robust clade (BYPP = 1.00; MLB = 100%), within the Neoroussoella group in the Roussoellaceae.

Two sister clades within the Roussoella group were represented by the new species Roussoella padinae sp. nov. (MUT 5503, MUT 5341 and MUT 5365) and Roussoella mediterranea sp. nov. (MUT 5306 and MUT 5369). Finally, MUT 5329 Roussoella margidorensis sp. nov. clustered together with R. nitidula, R. pseudohysterioides, R. thailandica and R. tubercolata (BYPP = 0.99; MLB = 71%) but was phylogenetically distant from these species.
Figure 1. Phylogram generated from RAxML analysis based on a combined dataset of nrITS, nrSSU, nrLSU, TEF1α and RPB2 partial sequences. The tree is rooted to Occultibambusa bambusae. Branch numbers indicate BYPP/MLB values; Bar = expected changes per site (0.06).

Nucleotide divergence between each novel species and members of the same clusters were annotated for each locus, when occurred (Tables S1–S18).

3.2. Taxonomy

Parathyridariella gen. nov. V. Prigione, A. Poli, E. Bovio and G.C. Varese
MYCOBANK: MB 832836
Type species. Parathyridariella dematiacea sp. nov.
Etymology. In reference to the phylogenetic proximity to the genus Thyridariella.
Phylogenetic placement. Thyridariaceae, Sordariomycetes, Ascomycota. The genus Parathyridariella gen. nov. clusters together with genera Cycascola, Liua and Thyridariella (Figure 1).
Parathyridariella dematiacea sp. nov. V. Prigione, A. Poli, E. Bovio and G.C. Varese

MYCOBANK: MB 832837

Figure 2

Type. Italy, Tuscany, Mediterranean Sea, Elba Island (LI), Ghiaie ISL, 14–15 m depth, 42°49'04"N, 10°19'20"E, form the green alga Flabellia petiolata, 20 March 2010, R. Mussat-Sartor and N. Nurra, MUT 4884 holotype, living culture permanently preserved in metabolically inactive state by deep-freezing at Mycotheca Universitatis Taurinensis (MUT).

Additional material examined. Italy, Ligury, Mediterranean Sea, Riva Trigoso, Punta Manara (GE), 5–21 m depth, 44°15′08.62″N 9°24′17.64″E, from the seagrass Posidonia oceanica, March 2008, MUT 4419.

Etymology. In reference to the color of the colony on culture media.

Description. Growing actively on Pinus pinaster and Quercus ruber cork. Showing a floccose growth mainly on Pinus pinaster. Hyphae 2.8–4.8 µm wide, septate, hyaline to lightly pigmented. Chlamydospores numerous, mostly in chain, intercalary or solitary, globose to subglobose, from brownish to dark brown, 7–10 × 6–8 µm diameter.

Sexual morph not observed. Asexual morph with differentiated conidiogenesis not observed.

Colony description. Colonies on MEASW attaining 28–34 mm diam after 28 days at 24 °C, mycelium from dark grey/black to dark green, dense with radial grooves and concentric rings, submerged edges; reverse dark green. Brown exudate present above the concentric rings. Growth on OASW reaching 40–54 mm diam at 24 °C and 21–29 mm diam at 15 °C; colonies on PDA attaining 36–49 mm diam and 15.5–22.5 mm diam at 24 °C and 15 °C, respectively.

Figure 2. Parathyridariella dematiacea sp. nov. 28-days-old colony at 21 °C on MEASW (A) and reverse (B); solitary (C) and in chain (D) chlamydospores. Scale bars: 10 µm (C, D).
Parathyridaria tyrrhenica sp. nov. A. Poli, V. Prigione, E. Bovio and G.C. Varese
MYCOBANK: MB 832838

Type. Italy, Tuscany, Mediterranean Sea, Elba Island (LI), Ghiaie ISL, 14–15 m depth, 42°49’04”N, 10°19’20”E, from the brown alga Padina pavonica, March 2010, R. Mussat-Sartor and N. Nurra, MUT 5371 holotype, living culture permanently preserved in metabolically inactive state by deep-freezing at MUT.

Additional material examined. Italy, Tuscany, Mediterranean Sea, Elba Island (LI), Ghiaie ISL, 14–15 m depth, 42°49’04”N, 10°19’20”E, from the green alga Flabellia petiolata, March 2010, R. Mussat-Sartor and N. Nurra, MUT 4966.

Etymology. In reference to Tyrrhenian Sea.

Description. Growing actively on Pinus pinaster wood and Quercus ruber cork. Hyphae 5 µm diameter, septate, hyaline to brownish, sometimes wavy or swollen, forming hyphal strands.

Sexual morph not observed. Asexual morph with differentiated conidiogenesis: not observed.

Colony description. Colonies growing on MEASW, reaching 10 mm diam after 28 days, at 21 °C, mycelium funiculose, yellowish, lightly ochre at the edges; reverse light yellow, lighter at the edges. Growth on OASW reaching 48–50 mm diam at 24 °C and 26–29 mm diam at 15 °C; colonies on PDA attaining 31–46 mm diam and 16–19 mm diam at 24 °C and 15 °C, respectively.

Figure 3. Parathyridaria tyrrhenica sp. nov. 28-days-old colony at 21 °C on MEASW (A) and reverse (B); mycelium (C), black and white arrows indicate hyphal strands and wavy hyphae, respectively. Scale bar: 10 µm.
Parathyridaria flabelliae sp. nov. E. Bovio, A. Poli, V. Prigione and G.C. Varese
MYCOBANK: MB 832839

Type. Italy, Tuscany, Mediterranean Sea, Elba Island (LI), Ghiaie ISL, 14–15 m depth, 42°49′04″N, 10°19′20″E, from the green alga Flabellia petiolata, March 2010, R. Mussat-Sartor and N. Nurra, MUT 4859 holotype, living culture permanently preserved in metabolically inactive state by deep-freezing at MUT.

Additional material examined. Italy, Tuscany, Mediterranean Sea, Elba Island (LI), Ghiaie ISL, 14–15 m depth, 42°49′04″N, 10°19′20″E, from the green alga Flabellia petiolata, March 2010, R. Mussat-Sartor and N. Nurra, MUT 4886.

Etymology. In reference to the original substratum, the green alga Flabellia petiolata.

Description. Growing actively on Pinus pinaster and on Quercus ruber cork. Hyphae 2.6–5 µm wide, septate and hyaline. Chlamydospores numerous, globose or subglobose, from light to dark brown, unicellular (4 × 5 µm diameter) and multicellular (up to four-celled; 8 × 12 µm diameter).

Sexual morph not observed. Asexual morph with differentiated conidiogenesis not observed.

Colony description. Colonies growing on MEASW, reaching 37–44 mm diam after 28 days at 21 °C, funiculose, whitish with submerged edges; reverse brown in the middle, lighter at edges. Growth on OASW reaching 60 mm diam at 24 °C and 33–35 mm diam at 15 °C; colonies on PDA attaining 53–64 mm diam and 23–24 mm diam at 24 °C and 15 °C, respectively.

Figure 4. Parathyridaria flabelliae sp. nov. 28-days-old colony at 21 °C on MEASW (A) and reverse (B); unicellular and multicellular chlamydospores (C). Scale bar: 10 µm.
Neorousoella lignicola sp. nov. A. Poli, E. Bovio, V. Prigione and G.C. Varese

MYCOBANK: MB 832840

Figure 5

Type. Italy, Tuscany, Mediterranean Sea, Elba Island (LI), Margidore ISL, 14–15 m depth, UTM WGS84 42°45'29"N, 10°18'24"E, from the brown alga Padina pavonica, March 2010, R. Mussat-Sartor and N. Nurra, MUT 5373 holotype, living culture permanently preserved in metabolically inactive state by deep-freezing at MUT.

Additional material examined. Italy, Tuscany, Mediterranean Sea, Elba Island (LI), Margidore ISL, 14–15 m depth, UTM WGS84 42°45'29"N, 10°18'24"E, from the brown alga Padina pavonica, March 2010, R. Mussat-Sartor and N. Nurra, MUT 4904.

Italy, Tuscany, Mediterranean Sea, Elba Island (LI), Margidore ISL, 14–15 m depth, UTM WGS84 42°45'29"N, 10°18'24"E, from the seagrass Posidonia oceanica, March 2010, R. Mussat-Sartor and N. Nurra, MUT 5008.

Etymology. In reference to the lignicolous behavior.

Description. Growing efficiently on Pinus pinaster wood. Hyphae 2–4.4 µm wide, septate, hyaline, assuming toruloid aspect when growing into wood vessels and forming chains of two-celled chlamydospores which, at maturity, protrude from the vessels. Chlamydospores 7.4 × 5.2 µm, from light to dark brown, globose or subglobose.

Sexual morph not observed. Asexual morph with differentiated conidiogenesis not observed.

Colony description. Colonies growing on MEASW, reaching 28–29 mm diam after 28 days at 21°C, from grey to dark green, floccose with irregular edges, reverse dark grey. Clear exudate often present. Growth on OASW reaching 27–40 mm diam at 24 °C and 14.5–26 mm diam at 15 °C; colonies on PDA attaining 38–45 mm diam and 19–29 mm diam at 24 °C and 15 °C, respectively.

Figure 5. Neorousoella lignicola sp. nov. 28-days-old colony at 21 °C on MEASW (A) and reverse (B); two-celled chlamydospores inside wood vessels (C). Scale bar: 10 µm.
**Roussoella margidorensis** sp. nov. E. Bovio, V. Prigione, A. Poli and G.C. Varese
MYCOBANK: MB 832841

**Figure 6**

**Type.** Italy, Tuscany, Mediterranean Sea, Elba Island (LI), Margidore ISL, 14–15 m depth, UTM WGS84 42°45'29“N, 10°18'24“E, from the brown alga *Padina pavonica*, March 2010, R. Mussat-Sartor and N. Nurra, MUT 5329 holotype, living culture permanently preserved in metabolically inactive state by deep-freezing at MUT.

**Etymology.** In reference to the area of origin, Margidore.

**Description.** Growing actively on *Pinus pinaster* wood. *Hyphae* approx. 2 µm wide, septate, brownish.

Sexual morph not observed. Asexual morph and differentiated conidiogenesis not observed.

**Colony description.** Colonies growing on MEASW, attaining 33–34 mm diam after 28 days at 21 °C; whitish, lighter to the edge, umbonate in the middle, reverse ochre. Caramel diffusible pigment produced. Growth on OASW reaching 45 mm diam at 24 °C and 27 mm diam at 15 °C; colonies on PDA attaining 45 mm diam and 23 mm diam at 24 °C and 15 °C, respectively.

![Figure 6](image_url)

**Roussoella mediterranea** sp. nov. A. Poli, E. Bovio, V. Prigione, and G.C. Varese
MYCOBANK: MB 832842

**Figure 7**

**Type.** Italy, Tuscany, Mediterranean Sea, Elba Island (LI), Margidore ISL, 14–15 m depth, UTM WGS84 42°45'29“N, 10°18'24“E, from the brown alga *Padina pavonica*, March 2010, R. Mussat-Sartor
and N. Nurra, MUT 5369 holotype, living culture permanently preserved in metabolically inactive state by deep-freezing at MUT.

Additional material examined. Italy, Tuscany, Mediterranean Sea, Elba Island (LI), Margidore ISL, 14–15 m depth, UTM WGS84 42°45′29″N, 10°18′24″E, from the brown alga Padina pavonica, March 2010, R. Mussat-Sartor and N. Nurra, MUT 5306 (identical to MUT 5306 on the basis of micro- and minisatellite analyses)

Etymology. In reference to the geographical origin, Mediterranean Sea.

Description in culture. Growing actively on Pinus pinaster wood and poorly colonizing Quercus ruber cork. Hyphae 2.4 µm wide, septate, dematiaceous. Chlamydospores 4.5 × 5.7 µm, from unicellular to 4-celled; branched chains of light to dark brown chlamydospores often present.

Sexual morph not observed. Asexual morph with differentiated conidiogenesis not observed.

Colony description. Colonies growing on MEASW, reaching 55 mm diam after 28 days at 21 °C, light grey, floccose, with umbonate area in the middle, reverse brown with lighter edges. Dark exudate present. Growth on OASW reaching 67–72 mm diam at 24 °C and 33–38 mm diam at 15 °C; colonies on PDA attaining 69–76 mm diam and 32.5–39 mm diam at 24 °C and 15 °C, respectively.

Figure 7. Roussoella mediterranea sp. nov. 28-days-old colony at 21 °C on MEASW (A) and reverse (B); unicellular and multicellular chlamydospores indicated by a black arrow (C). Scale bar: 10 µm.

Roussoella padinae sp. nov. V. Prigione, E. Bovio, A. Poli and G.C. Varese
MYCOBANK: MB 832843
Figure 8
Type. Italy, Tuscany, Mediterranean Sea, Elba Island (LI), Margidore ISL, 14–15 m depth, UTM WGS84 42°45’29”N, 10°18’24”E, from the brown alga *Padina pavonica*, March 2010, R. Mussat-Sartor and N. Nurra, MUT 5503 holotype, living culture permanently preserved in metabolically inactive state by deep-freezing at MUT.

Additional material examined. Italy, Tuscany, Mediterranean Sea, Elba Island (LI), Margidore ISL, 14–15 m depth, UTM WGS84 42°45’29”N, 10°18’24”E, from the brown alga *Padina pavonica*, March 2010, R. Mussat-Sartor and N. Nurra, MUT 5341 and MUT 5345 (identical to MUT 5503 on the basis of micro- and minisatellite analyses)

Etymology. In reference to the original substratum, *Padina pavonica*.

Description in culture. Growing efficiently on *Quercus ruber* cork and poorly colonizing *Pinus pinaster* wood. Hyphae 3 µm wide, septate, brownish, assuming toruloid aspect when growing into wood vessels and forming chains of two-celled chlamydospores which, at maturity, protrude from the vessels. Chlamydospores 5–7 × 4 µm, from light to dark brown, subglobose, ellipsoidal or cylindrical.

Sexual morph not observed. Asexual morph n with differentiated conidiogenesis not observed.

Colony description. Colonies growing on MEASW, reaching 53 mm diam after 28 days at 21 °C, from grey to dark green, floccose in the middle, with radial grooves, fimbriate edges, reverse brown. Growth on OASW reaching 57.5–65 mm diam at 24 °C and 30–35 mm diam at 15 °C; colonies on PDA attaining 60–69 mm diam and 30–34 mm diam at 24 °C and 15 °C, respectively.

**Figure 8.** *Roussoella padinae* sp. nov. 28-days-old colony at 21 °C on MEASW (A) and reverse (B); toruloid hyphae (C) and two-celled chlamydospores (D) inside wood vessels. Scale bars: 10 µm.
4. Discussion

The description of these new taxa was particularly challenging because neither asexual nor sexual reproductive structures developed in axenic conditions. Therefore, we were unable to describe the range of anatomical variations and diagnostic features among these newly recognized phylogenetic lineages. Indeed, strictly vegetative growth without sporulation is a common feature of many marine fungal strains [10,11,33]. Possibly, these organisms rely on hyphal fragmentation for their dispersal, or alternatively, the differentiation of reproductive structures may be obligatorily dependent on the peculiar environmental conditions under which they live (e.g., wet-dry cycles, high salinity, low temperature, high pressure, etc.). During the study of these fungi, we tried to mimic the saline environment by using different culture media supplemented with natural sea water or sea salts. Although these culture methods were applied to induce sporulation, we observed that only media supplemented with sea water supported a measurable growth of vegetative mycelium (data not shown). The method introduced by Panebianco et al. [22] to induce sporulation by placing wood and cork specimens on the colony surface with their subsequent transfer into sea water, was only partially successful: out of seven species, three (P. dematiacea, P. flabellae, R. mediterranea) developed chlamydospores in the mycelium above the wood surface, two (N. lignicola, R. padinae) gave rise to resting spores inside wood vessels. Most of the strains preferred to colonize P. pinaster wood rather than Q. ruber cork. These structures were interpreted as “chlamydospores” instead of “conidia” for the following reasons: i) They were characterized by a very thick cell wall, a typical feature of resting spores; ii) conidigenous cells were never observed. Additional efforts to force the development of reproductive structures by using SNASW and pine needles, were also unsuccessful.

Both R. padinae and N. lignicola displayed a similar lignicolous behavior, growing and producing chlamydospores inside wooden vessels, although of different size and shape. The ability to form hyphae and to grow inside the wood vessels has been reported for a number of dark septate endophyte fungi in terrestrial environment [34] and, recently, for Posidoniomyces atricolor Vohnik and Řeblová, a marine endophyte that lives in association with the roots of P. oceanica [35]. By definition, endophytes live inside living plant tissues. To induce sporulation, sterilized specimens of dead wood were employed, therefore R. padinae and N. lignicola were inferred to be “lignicolous fungi” rather than “endophytes”. The observation of this growth characteristic in two different genera, may find its reason in an evolutionary adaptation to marine life in association with lignocellulosic matrices. Therefore, we can hypothesize their ecological role as saprobes involved in degrading organic matter.

Notwithstanding the lack of exhaustive descriptions of morphological features, the strongly supported phylogenetic and molecular analysis, conducted with five different genetic markers (nrSSU, nrITS, nrLSU, TEF1α and RPB2) undoubtedly pointed out the differences among these species and their belonging to new taxa. This is also supported by the DNA diagnostic characters identified in the individual loci (Table S1–S18). In particular, the present study introduces four new species of Roussoellaceae and three new species of Thyridariaceae. Indeed, only MUT 2452 and MUT 4893 were ascribable to the previously described P. robindae (Figure 1). In the case of MUT 4884, the holotype of P. dematiacea, a novel genus was proposed since it formed a defined cluster with MUT 5310 and MUT 4419, well separated by the genera Cycasicola, Liua and Thyridariella.

Most of the Roussoellaceae and Thyridariaceae described to date are associated with terrestrial plants, especially bamboo and palm species [15,16]. In fact, only two species, R. mangrovei and R. nitidula have been retrieved from the marine environment (www.marinefungi.org). However, considering the present study, we can infer that these families are well represented in the sea, thus improving our knowledge on the largely unexplored fungal marine biodiversity.

Supplementary Materials: The following are available online at www.mdpi.com/1424-2818/12/4/144/s1, Table S1: The eight variable sites detected in the nrITS region among P. dematiacea and its neighbor species, Table S2: The single variable site detected in the nrLSU region among P. dematiacea and its neighbor species, Table S3: The five variable sites detected in the nrSSU region among P. dematiacea and its neighbor species, Table S4: The six variable sites detected in the TEF1α partial gene among P. dematiacea and its neighbor species, Table S5: The six
variable sites detected in the nrITS region among *P. tyrrenica*, *P. flabelliae* and their neighbor species, Table S6. The eight variable sites detected in the nrLSU region among *P. tyrrenica*, *P. flabelliae* and their neighbor species, Table S7. The eight variable sites detected in the TEF1α partial gene among *P. tyrrenica*, *P. flabelliae* and their neighbor species, Table S8: The 33 variable sites detected in the RPB2 partial gene among *P. tyrrenica*, *P. flabelliae* and their neighbor species, Table S9: The two variable sites detected in nrITS region among R. *mediterranea*, R. *padinae*, and the neighbor species, Table S10: The single variable site detected in nrLSU region among R. *mediterranea*, R. *padinae*, and the neighbor species, Table S11: The six sites detected in the TEF1α partial gene among R. *mediterranea*, R. *padinae* and the neighbor species, Table S12: The six sites detected in the RPB2 partial gene among R. *mediterranea*, R. *padinae* and the neighbor species, Table S13: The eight variable sites detected in the nrITS region among *N. lignicola* and its neighbor species, Table S14: The three variable sites detected in the nrLSU region among *N. lignicola* and its neighbor species, Table S15: The eight variable sites detected in the nrSSU region among *N. lignicola* and its neighbor species, Table S16: The ten sites detected in the TEF1α partial gene among *N. lignicola* and its neighbor species, Table S17: The three variable sites detected in the nrITS region among *R. margidoriensis* and its neighbor species, Table S18: The 29 variable sites detected in the TEF1α partial gene among *R. margidoriensis* and its neighbor species

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**Conflicts of Interest:** The authors declare no conflict of interest.

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