Preliminary phylogenetic and morphological studies in the *Plectania melastoma* lineage (Ascomycota, *Pezizales*)

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Summary: Phylogenetic studies conducted on one collection from Greece and two from Cyprus, the latter morphologically matching *Plectania zugazae* and *Plectania melastoma* respectively, reveal that the delimitation of these two species is still far from clear. The authors choose to keep the two species separate on phylogenetic and microscopic basis only. The presence of *P. zugazae* is confirmed for the first time for Cyprus and Greece and only for the second time worldwide. Useful macro- and microscopic pictures are provided and a tentative key of the accepted *Plectania* species is proposed.

Keywords: ITS, 28S LSU, phylogeny, *Sarcosomataceae*, Mediterranean, Cyprus.

Introduction

According to Carbone *et al.* (2013), the genus *Plectania* Fuckel should be limited to few species, such as the type species *Plectania melastoma* (Sowerby) Fuckel, *P. zugazae* Calonge & A. Garcia, *P. rhytidia* (Berk.) Nannf. & Korf, *P. milleri* Paden & Tylutki and *P. megalocrater* (Malençon & Le Gal) M. Carbone, Agnello & Konstantinidis, even though other species for which no molecular data is yet available could also belong to this genus. In addition, the division into five sections proposed by Korf (1957b), Paden (1983) and Carbone *et al.* (2012) was rejected, since these clades were monophyletic and worth of being considered independent genera.

Two collections from Cyprus, previously identified by the second author as *P. melastoma* and *P. zugazae* respectively, based on their morphological characters, have been subsequently subjected to molecular testing in order to confirm or reject these preliminary identifications and clarify the uncertain taxonomical status of *P. zugazae*, the latter so far known only from the type collection in Spain. Despite recorded from different localities and collected during different seasons, both collections occurred in typically Mediterranean habitats of open, mossy soil in forests of *Pinus brutia* with a *Cistus* spp. understorey, at elevations between 450 and 550 m a.s.l.

In addition, during the revision of the paper, thanks to the courtesy of Vasileios Kaounas, the first author obtained an additional collection from Greece fitting the micro-morphological concept of *P. zugazae* followed in the present study.

P. zugazae was described from Spain and differs from other species mainly by the following combination of characters: cup 5–25 mm in diam., entirely black, sessile, gregarious, with rhizomorphs, asci measuring 250–300 × 16–18 µm, smooth ellipsoid ascospores, 18–22 × 12–14 µm, with granular content, covered by a gelatinous sheath, simple and filiform paraphyses (Calonge *et al.*, 2003). Carbone *et al.* (2013), after sequencing and studying two typical collections (one of them being an isotype), amended the original description of micro-characters and decided to keep this species separate from *P. melastoma* mainly due to a lower Q ratio, derived from more broadly ellipsoid ascospores, as well as a slight difference in spores size, and macroscopically for the lack of the typical orange granules on the external surface, although water mounts have revealed an evident covering of an encrusting pigment very similar to that of *P. melastoma*.

P. melastoma is a well-known species described by many authors both in scientific articles and field books. Macroscopically it is mainly characterized by small ascomata with a black hymenium, contrasting with the margin and external surface, which are covered with orange granules, a feature still visible in dried ancient specimens (see Agnello & Carbone, 2012). From a microscopic point of view, *P. melastoma* is mainly characterized by ellipsoid to subfusoid ascospores, 21.8–25 × 10–12.4 µm, Q= 1.86–2.25, Qm= 2.0, minutely verrucose and sometimes covered by a gelatinous sheath.

The present work aims to update the morphological concept of *P. zugazae* and to ascertain whether this species represents an independent taxon or simply an intraspecific variation of *P. melastoma*.

Material and methods

Morphological study. — The microscopic studies were based on both fresh and dried specimens. Two optical microscopes were used: Olympus CX41 trinocular and a LEICA BM E binocular with plan-achromatic objectives 10×, 40×, 60× and 100× magnifications in oil immersion. The following main reagents were used: Melzer's reagent, Cotton Blue, Congo Red and KOH. Water mounts were used for the observation of the pigmentation and measurements. At least 30 ascospores were measured from each apothecium. Species concepts have been based on the original descriptions and, in some cases, on the type revisions.

DNA extraction, amplification and sequencing. — DNA was extracted and amplified from dried specimens following the methods published before (Alvarado *et al.*, 2010; Alvarado *et al.*, 2012). The primers LR0R and LR5 (Vilgalys & Hester, 1990) were used to amplify and sequence the 28S nuclear large ribosomal region (nrLSU), while ITS1F and ITS4 (White *et al.*, 1990; Gardes & Bruns, 1993) were used to amplify the internal transcribed spacer region. Sequences were visually inspected searching for reading errors in MEGA5.0 (Tamura *et al.*, 2011). Validated sequences were stored in GenBank under the accession numbers listed in *Studied and sequenced collections*.

Phylogenetic analyses. — The sequences obtained were aligned with the closest relatives and their matches obtained with BLAST searches. Most sequences came from earlier works of the present authors (Carbone *et al*., 2013), but also some others produced by Kopcke *et al.* (2002), Peterson *et al.* (2004), Hansen *et al.* (2008) and Gordon (unpub.). Sequences were first aligned in MEGA 5.0 software using its ClustalW application and then corrected manually. The aligned loci were loaded in PAUP* 4.0b 10 (Swofford, 2001) and subjected to MrModeltest 2.3 (Nylander, 2004). The best models were implemented in MrBayes 3.1 (Ronquist & Huelsenbeck, 2003), where a Bayesian analysis was performed (ITS1-5.8S-ITS2-28S nLSU data partitioned, 2 simultaneous runs, 6 chains, temperature set to 0.2, sampling every 100th generation) until convergence parameters were met after about 180,000 generations. Finally a full search for the best-scoring maximum likelihood tree was performed in RAxML (Stamatakis, 2006) using the standard search algorithm (ITS1-5.8S-ITS2-28S nLSU data partitioned, 2000 bootstrap replications). Significance threshold was set above 0.9 for posterior probability (PP). Significance thresholds were above 70% for bootstrap (BP) and 99% for posterior probability (PP).

Studied and sequenced collections

***Plectania zugazae*:** Cyprus, Ayia Paraskevi, ca 550 m a.s.l., on moss, under *Pinus brutia* and *Cistus salvifolius*, *leg*. M. Loizides, 3-III-2010 (TUR-A 199784; Genbank: KM610323, KM610325). Prastio, ca 450 m a.s.l., on moss, under *Cistus* sp. and *Pinus brutia*, *leg*. M. Loizides, 8-III-2012 (TUR-A 199785; Genbank: KM610322, KM610324).

Other studied and cited collections

***Plectania melastoma*:** Italy, Tuscany, Marina di Vecchiano, on *Erica scoparia*, 26.IV.2012, *leg. et det.* M. Carbone & G. Cacialli (TUR-A 195783); Italy, Apulia, Brindisi, Bosco Preti, on *Erica arborea*, 28.IV.2012, *leg. et det.* C. Agnello (TUR-A 195784). ***Plectania zugazae*:** Spain, Valladolid, Valdestillas, among mosses under *Pinus* sp., 8.IV.2001, *leg*. A. Garcia Blanco & J. Mori, *det*. F.D. Calonge (AVM 1467,

Plate 1 – *Plectania zugazae*

a-b: holotype and isotype (photo A. Garcia); c-d: TUR-A199785 (photo M. Loizides); e: TUR-A 199784 (photo M. Loizides); f: *Plectania melastoma* TUR-A 195783 (photo M. Carbone).

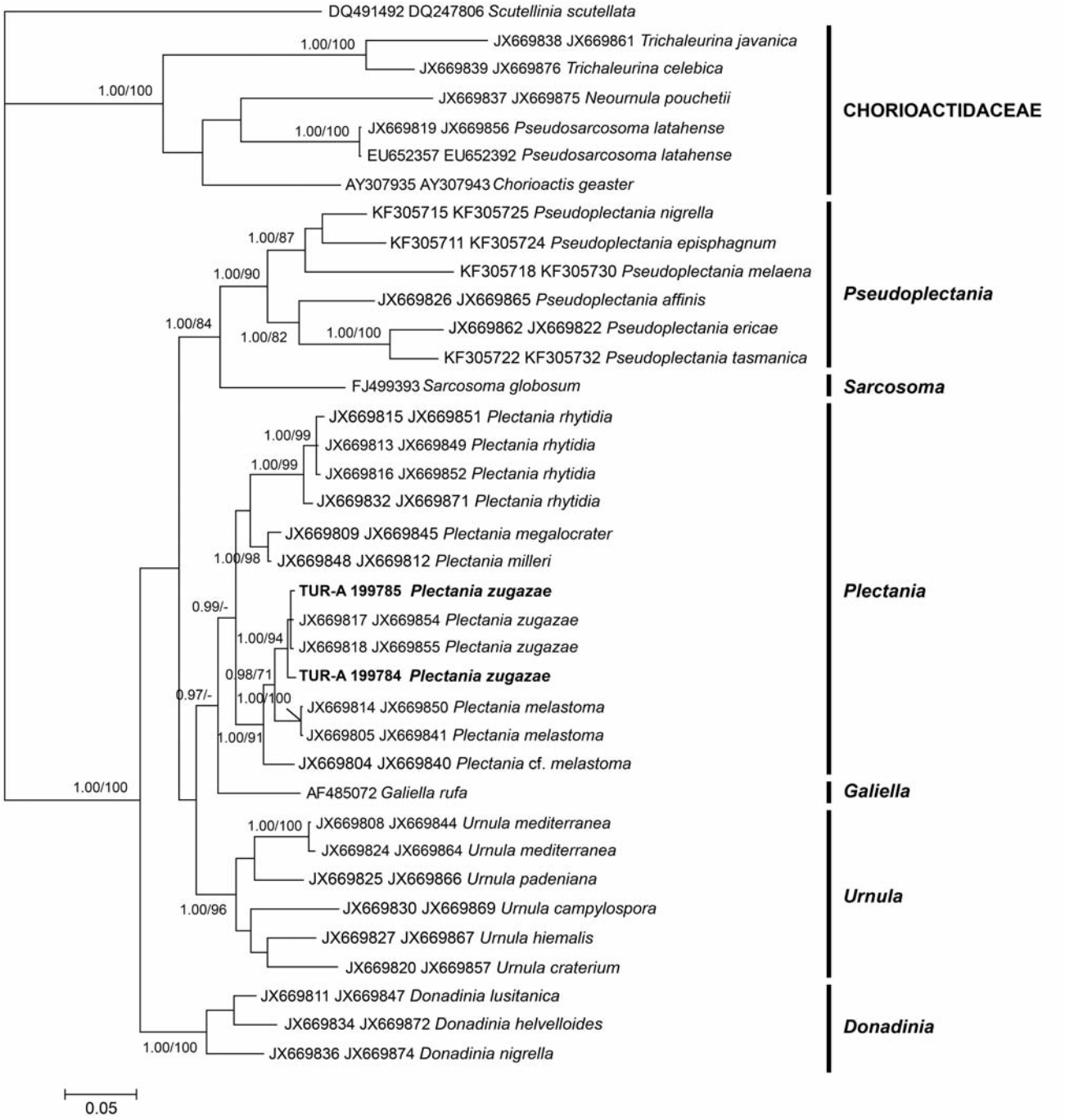

Fig. 1. Consensus phylogram obtained in MrBayes 3.1 after the analysis of an ITS-28S nLSU combined alignment of the genus *Plectania* and other genera in the families *Sarcosomataceae* and *Chorioactidaceae*. Only nodes significantly supported by at least one of the two analyses conducted were annotated with bayesian posterior probabilities and maximum likelihood bootstrap proportions (left to right). Bold characters are employed to highlight the samples analyzed in the present study.

private duplicate of the holotype MA-fungi 53068); Valladolid, Valdestillas, among mosses under *Pinus* sp., 15.IV.2007, *leg*. J. Mori & M. Sanz, *det*. A. Garcia Blanco (AVM 2086). Greece, Attica, Nea Makri, in forest with *Pinus halepensis*, *Cistus monspeliensis* and *Erica arborea*, 06.III.2014, *leg*. V. Kaounas, VK 3271 (duplicate in TUR-A).

Phylogenetic results

Phylogenetic inference derived from combined ITS-28S nLSU analysis produced a tree topology very similar to those already published for the family *Sarcosomataceae* (Carbone *et al.*, 2013; Carbone *et al.*, 2014). *Plectania zugazae* and *P. melastoma* seem to constitute a single monophyletic clade comprising three main lineages: one composed of typical *P. melastoma*, a second integrated by *P. zugazae* and the Cypriot individuals studied in the present work (plus the cited Greek collection, although not shown here), and a third one formed by a single sample, *Plectania* cf. *melastoma* TUR-A 195785. The degree of genetic differentiation seems to be similar to that presented by other species of this genus, such as *P. rhytidia* or *P. milleri*. A very small proportion of intraclade variability was detected in *P. zugazae* clade, where sample ALV3175 displayed several polymorphic sites and a single point mutation. These differences are seemingly not a product of geographic isolation, because these were not present in the other Cypriot sample, ALV3714.

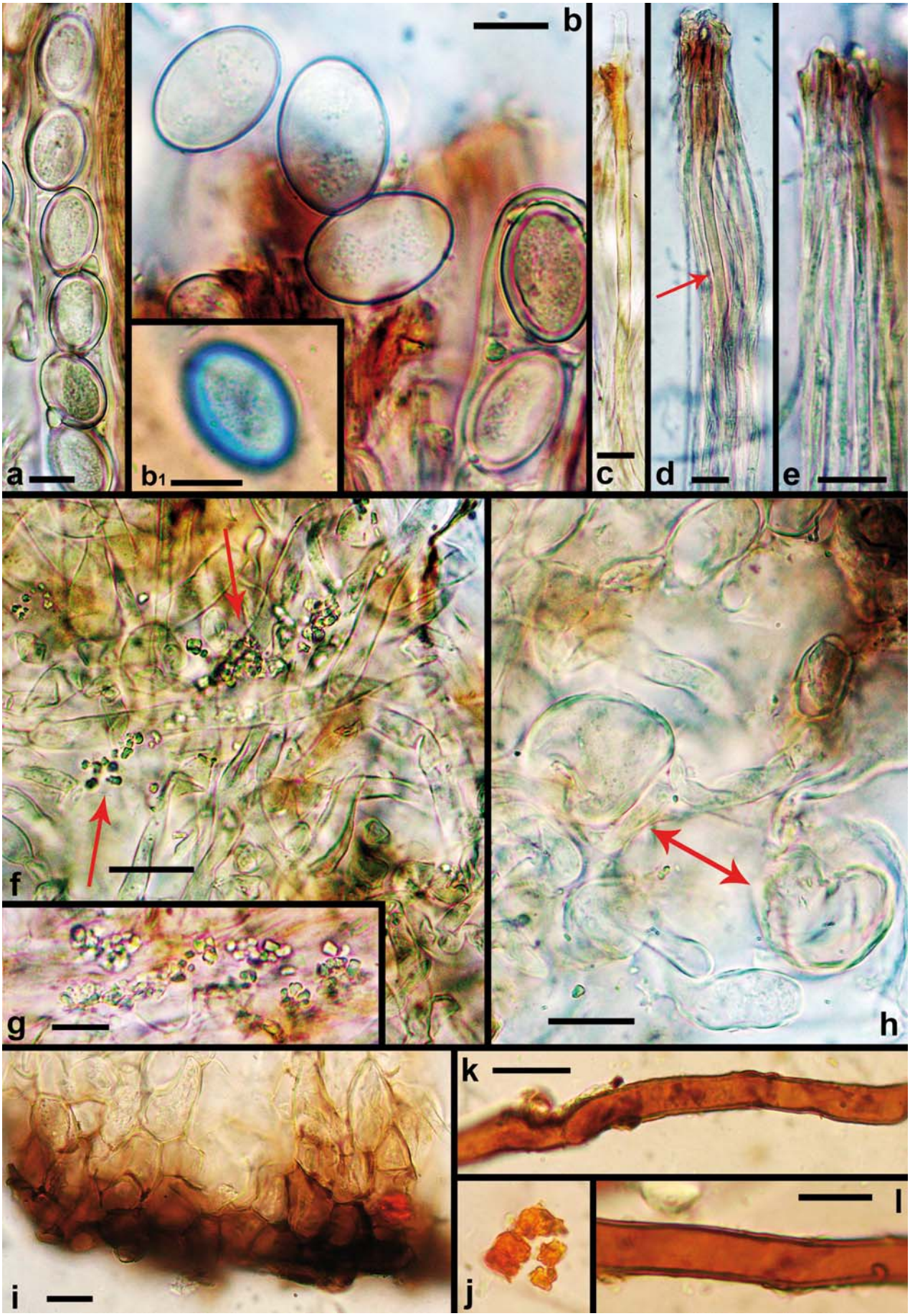

Plate 2 – *Plectania zugazae*

All in water mounts (except b1). a: not fully mature thick-walled ascospores; b: 3 mature thin-walled and 2 immature thick-walled ascospores; b1: spores ornaments; c: hymenial hair; d-e: bundles of paraphyses (red arrow for hymenial hair); f: medullary excipulum (red arrows for crystals); g: crystals; h: globose elements in the transition layer between medullary and ectal excipulum; i: ectal excipulum; j: crystal of the external surface; k-l: external hairs. Photos: M. Carbone.

Taxonomy

Plectania zugazae Calonge & A. Garcia, *Bol. Soc. Micol. Madrid*, 27: 18 (2003).

Original diagnosis

Ascomata sessilia, cupulata deinde discoidea applanata, atra, gregaria, 5-25 *mm diam., cum rhizomorpha. Ectale excipulum bistratosum cum textura globulosa-angularis; stratum externum cum parietes crassiores, incrustate, nigrum, stratum internum cum elementa hyalina et parietes tenuis. Tomentum pilis septatis cum pigmentum nigrum,* 4-10 *µm latis. Medullare excipulum cum hyphis dissolutis, hyalinis. Subhymenium cum textura intricata et hyphis hyalinis, septatis,* 2-4 *µm diam. Asci octospori,* 250-300 × 16-18 *µm, jodo non caerulescentes. Ascosporae ellipsoideae,* 18-22 × 12-14 *µm, laeves, aguttulatae. Paraphyses filiformes,* 2-3 *µm diam.,* 4 *µm ad apices, non ramosae, septatae.*

Description based on the two Cyprus collections

Apothecia nearly spherical at first, at maturity becoming cup-shaped, up to 1 (–1.5) cm in diam. and 0.8 (–1) cm high, attached to the substrate by means of numerous black, threadlike filaments or often with a rooting rhizomorph. **Hymenium** dark sepia-brown to black, mostly smooth. **External surface** more or less concolorous to the hymenium, rough, with often indistinct, pale brownish to orange granules, usually confined to the cup margin, but sometimes also covering the entire external surface. **Flesh** very tough, odorless.

Asci on average 350–375 × 13–16 µm, cylindrical, operculate, inamyloid, eight-spored, with walls up to 1 µm thick and a tapered, flexuous, aporhynchous base. **Paraphyses** not exceeding the length of the asci, 1.8–2 µm wide, cylindrical, septate, sometimes anastomosing, branched below, pale brownish at low magnification. The apex is simple or in few cases slightly lobed. Extracellular, amber-brown, gluey pigment is present in the upper part of dried specimens observed in water mounts. **Hymenial hairs** cylindrical, as long as the paraphyses, 3–3.5 µm wide, with a simple apex, and a single septum at the very base. **Ascospores** ellipsoid, rarely slightly subfusoid, (17.5–) 19–22 (–24) × (12–) 12.5–15 (–15.5) µm, most frequently seen 21–22 × 13.5–14 µm, Q = 1.45–1.6, hyaline, very minutely verrucose (if seen in heated lactic Cotton Blue mounts), without oil drops but filled with a granular content, walls up to 1 µm thick, depending on the stage of maturity, i.e. mature spores thin-walled, less mature spores thick-walled; sometimes a thin gelatinoid sheath has been observed covering the ascospores, especially while still inside the ascus. In young asci, developing ascospores are globose. **Subhymenium** composed by a dense *textura intricata* of cylindrical, frequently septate, hyphae, with thickened, more or less dark brown walls. At low magnifications, it appears uniformly brown. **Medullary excipulum** of *textura intricata* with cylindrical, septate, hyphae, 2–4 µm wide, with walls on average 0.5–0.8 µm thick, immersed in a gelatinous matrix. In the lower part: i) whitish to pale yellowish cubic crystals 2–3 µm long are present among the hyphae or cover their external surface; ii) the hyphae are arranged parallel, resembling a *textura prismatica*, but some elements become inflated or globose, appearing then as a *textura globulosa*. **Ectal excipulum** of *textura angularis* made up of elements up to 20 µm wide, very dark brown due to the colored walls and the presence of an incrusting pigment. **External hairs** cylindrical, septate, 5–6(–7) µm wide, very long, straight, sometimes notched, smooth, originating from the ectal excipulum where they can be encrusted at the very base. They are brown due to an epimembranaceous pigmentation, with walls thickened up to 1 µm. **Amber crystals** few to abundant, present among the hairs and in the outer surface of the ectal excipulum.

Ecology

Usually growing in small groups during late winter and spring, on mossy calcareous ground, in mountainous *Pinus* forests with a *Cistus* understorey.

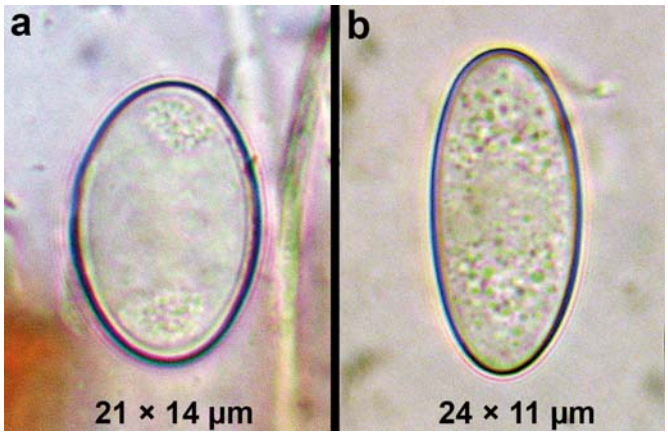

Plate 3. Comparison of ascospores in water mount. a: *Plectania zugazae*, TUR-A 199784; b: *Plectania melastoma*, TUR-A 195783. Photo: M. Carbone

Discussion

Macroscopically there is no doubt that the collection TUR-A199785 (Plate 1: c-d) perfectly matches *Plectania zugazae* (Plate 1: a-b) and, at the same time, it is remarkably different from collection TUR-A 199785 (Plate 1: e). In fact, the latter fits much better the species concept of *Plectania melastoma* due to the prominent orange granules, which are not confined to the margin, but covering the entire external surface (the same could be said for the Greek samples, see Cover). However, the present molecular results suggest that these macroscopic differences do not have a phylogenetic basis.

On the contrary, examination under light microscope of AVM 1467, AVM 2086, both collections from Cyprus and the Greek one, proves they are all identical in anatomical structure, measures and type of pigmentation. We compared these micro-morphological results with what we consider to represent typical *P. melastoma* collections (TUR-A 195783, see Plate 1: f; TUR-A 195784) and noticed that the five *P. zugazae* collections have ascospores 12–14 (–15) µm wide, and a length/width ratio Q = 1.45–1.6 whilst *P. melastoma* has slightly longer ascospores (see Plate 3) but, most crucially, 10–12 µm wide and a higher Q of 1.8–2.2, also in fully matured specimens (as seen in Agnello & Carbone, 2012). The same can be said for the sample *Plectania* cf. *melastoma* (TUR-A 195785) which is macroscopically identical to *P. melastoma*, but has a higher Q and a lower width of the spores. Regarding the remaining microscopic features, our comparison has not revealed noticeable differences, except the presence of numerous, more globose elements in the transition layer between medullary and ectal excipulum of *P. zugazae*. In addition, spore warts are very small, not easy to be highlighted, thus a comparison between *P. zugazae* and *P. melastoma* ascospores surface must be reevaluated with more precise techniques (like SEM microscope).

Combined phylogenetic and morphological results are open to three interpretations of the data: (1) retaining *P. zugazae* as an independent species, although not necessarily macroscopically different from *P. melastoma*; (2) changing the status of *P. zugazae* to an intraspecific level; (3) synonymizing *P. zugazae* with *P. melastoma*. At present, with the data we currently have at our disposal, we have chosen to follow the first approach, which is the most conservative and scientifically correct. Data from future collections should seek

Tentative key of *Plectania* species[1]

1a. Ascospores inequilateral, transversally striated and furrowed on a side, apothecia black, vertically wrinkled on the external surface ..... ***Plectania rhytidia*** (Berk.) Nannf. & Korf (= *P. platensis* (Speg.) Rifai)

Note. The above synonymy is mainly based on Carbone *et al.* (2010) and Carbone *et al.* (2013). *Plectania cyttarioides* (Rehm) Korf, from North Carolina (USA) and *Plectania kohniae* Korf & W.Y. Zhuang, from Azores, belonging to the section *Plicosporae* Korf, are very poor known species in need of molecular validation; the latter is said to differ mainly by its smaller ascospores (Korf & Zhuang, 1991). *Plectania rugosa* (Le Gal) M. Carbone, Baglivo & Agnello is very probably just an overmature or aberrant macrosporic form of *P. rhytidia*.

1b. Mature ascospores very minutely verrucose[2] (Cotton Blue, 100× in oil immersion) **2**

1c. Mature ascospores smooth **3**

2a. Apothecia external surface and margin covered by orange granules, mature ascospores < 12 µm wide ..... ***P. melastoma***

2a. Apothecia external surface covered or not by orange or brownish granules often restricted at the margin, mature ascospores > 12 µm wide ..... ***P. zugazae***

3a. Ascospores 22–31 × (8–) 9–11 µm, dark hyphae running from the external layer into the medullary excipulum, North Western America ..... ***P. milleri*** Paden & Tylutki

3b. Ascospores 20.5–29.5 × 9–13 (15) µm (Le Gal, 1958); 19.4–28 × 7.4–11.8 µm (Carbone *et al.*, 2011b), no dark hyphae running in the flesh, so far known from Morocco and Greece only ..... ***P. megalocrater*** (Malençon & Le Gal) M. Carbone, Agnello & Konstantinidis

Note. Results of Carbone *et al.* (2013) suggested that *P. milleri* and *P. megalocrater* could be considered as conspecific. The *P. milleri* sample analyzed was studied in depth by Carbone *et al.* (2011a), and so was that of *P. megalocrater* by Carbone *et al.* (2011b). All collections clearly match with their respective protologues, and differ from each other in some morphological, ecological and biogeographical features. Until further studies will clarify their status, we prefer to keep them separate.

[1] Only the species accepted in this study are treated. We prefer not to include other species such as *Plectania chilensis* (Mont.) Gamundí, *P. yunnannensis* W.Y. Zhuang and *P. modesta* Otani until their placement will be confirmed by molecular analysis.

[2] We consider mature ascospores those with thin wall; warts are very small and so sometimes not so easily detectable.

to confirm if the different pattern observed in spores width (and Q), could be used as a reliable basis to discriminate the three lineages related to *P. melastoma*, and evaluate to which extent macroscopical features can vary and overlap between the two species.

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Erratum

In the previous issue (Vol. 6, fasc. 5) in the Martin Bemmann's article entitled "The story so far... An Interim Bibliography of Hans-Otto Baral for the Years 1981-2014" one article is missing in the list of publications authored and co-authored by H.-O. Baral:
Helleman S., Lindemann U., Baral H.-O. & Yeates C. 2013. — *Micropeziza filicina* sp. nov. (*Helotiales*), a fern inhabiting species of intermediate generic position, with an emendation of the genus *Micropeziza* Fuckel. *Ascomycete.org*, 5 (4): 129-136.