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Rickiella edulis and its phylogenetic relationships within Sarcoscyphaceae

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Summary

Romero, A. I., G. Robledo, K. F. LoBuglio & D. H. Pfister. 2012. *Rickiella edulis* and its phylogenetic relationships within Sarcoscyphaceae. *Kurtziana* 37 (1): 79-89.

Rickiella edulis is reported from Argentina for the first time and is documented with photographs of fresh specimens and molecular data. Previously the species was known as *R. transiens* (= *Phillipsia transiens*) and was reported from southern Brazil and Paraguay. Phylogenetic analyses based on SSU rDNA and LSU rDNA shows its placement in a monophyletic family, the Sarcoscyphaceae. The relationship of *Rickiella*, *Phillipsia* and *Nanoscypha* however could not be resolved from phylogenetic analyses of the ITS, SSU, and LSU rDNA sequences. The excipular tissue of *Rickiella* is shot through with regularly spaced channels and cavities. Because of this feature, the genus *Rickiella* is recognized as distinct from *Phillipsia*. *Phillipsia* and *Nanoscypha* are morphologically distinct but diversity within *Phillipsia* remains a topic for further research. A new tribe in the Sarcoscyphaceae is proposed to accommodate the genus *Wynnea*.

Key words: Argentina, *Nanoscypha*, Pezizales, *Phillipsia*, Phylogeny

Resumen

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Rickiella edulis se registra por primera vez para la Argentina y se documenta a través de fotografías de materiales frescos y de datos moleculares. Originalmente la especie fue conocida como *R. transiens* (= *Phillipsia transiens*) y registrada para Brasil y Paraguay. Análisis filogenéticos basados en los marcadores SSU y LSU muestran su ubicación en la familia monofilética, Sarcoscyphaceae. Sin embargo, las relaciones entre los géneros *Rickiella*, *Phillipsia* y *Nanoscypha* no se pudieron resolver a partir del análisis filogenético basados en los marcadores de ITS, SSU y LSU rDNA. Las características particulares del excípulo de *Rickiella*, lacunoso y con cavidades, lo diferencian de *Phillipsia. Phillipsia* y *Nanoscypha* son morfológicamente distinguibles pero la diversidad dentro de *Phillipsia* es un tema para futuras investigaciones. Se propone una nueva tribu dentro de Sarcoscyphaceae para acomodar el género *Wynnea*.

Palabras clave: Argentina, *Nanoscypha*, Pezizales, *Phillipsia*, Filogenia.

Introduction

A recent collection of *Rickiella edulis* (Speg.) Pfister from NW Argentina renewed interest in the status of this unusual taxon and in the known distribution of the species. Pfister (1987), in publishing on his discovery of the older name for *R. transiens* Sydow, reviewed the small literature then available regarding the genus and the single species recognized in it. To this review there is nothing new to add. Korf (1983) in an earlier study considered *Rickiella* to be a synonym of *Phillipsia* from which it differs in possessing an excipulum with many regularly spaced lacunae or cavities. None of the phylogenetic studies involving the family Sarcoscyphaceae and related families in the Pezizales have included this species (Hansen and Pfister 2006, Harrington et al. 1999, Perry et al. 2007, Pfister et al. 2008). This recent collection allows us to review the morphology, to consider the distribution of the species, and to sample DNA for phylogenetic study.

The history of the family Sarcoscyphaceae has been reviewed by Harrington et al. (1999). No suitable material of *R. edulis* was available at that time. That study was based on SSU rDNA (Small Subunit Ribosomal DNA) sequences and had limited samples, generally with only a single example for each genus. Harrington et al. (1999) and subsequent studies have demonstrated a monophyletic family Sarcoscyphaceae. Based on Harrington's study, *Nanoscypha* was used as an outgroup species by Hansen et al. (1999) in their studies of *Phillipsia* using ITS rDNA (Internal Transcribed Spacer) sequences.

Using ITS, SSU, and LSU rDNA (Large Subunit Ribosomal DNA) we have returned to investigate the relationships among members of the Sarcoscyphaceae in an attempt to elucidate the placement of *Rickiella* particularly with reference to *Phillipsia,* the genus in which Korf (1983) suggested the type species of *Rickiella* might be placed. We also are able to elaborate on the distribution of *R. edulis*.

Materials and Methods

Herbarium Specimens Used for Morphological Evaluation and DNA Samples

Specimen for DNA extraction: *Rickiella edulis*, collected by Gerardo Robledo in 2007, was sent to D. Pfister by A. I. Romero from BAFC Mycotheca (BAFC #51697). *Specimens examined*: ARGENTINA, **Prov. Salta**, *La Caldera*, Camino de tierra que une Ruta 9 (Camino de Cornisa) con General Güemes, 24º40'20.5''S, 65º22'4.8''W, on dead fallen logs, 1349 m asl, **Robledo 871**, **872**, **873,** 20-II-2007 (CORD) and #51698/99 (BAFC) (acronyms according to Thiers, 2011).

Kompsoscypha phyllogena (Seaver) Pfister was collected by D.J. Lodge and L. Millman in 2009 (without other data) at El Yunque, Baisley Watershed, Puerto Rico (FH #DHP 10-690). Two specimens of *Phillipsia* (FH #113 and #114) were collected in the Dominican Republic (Jardín Botanico Nacional "Dr. Rafael M. Moscoso" Santo Domingo, Republica Dominicana, without data) by S. Cantrell et al. (2002). Specimen #114 was confirmed to be *P. crispata* based primarily on spore morphology and 95% ITS rDNA sequence similarity to *P. crispata* (GenBankAF117355, T. Læssøe AAU-44895a, and GenBank AF117354, T. Læssøe AAU-44801, Hansen et al. 1999). Specimen #113 was confirmed to be *P. carnicolor* based on 97% ITS rDNA sequence similarity to *P. carnicolor* (GenBank AF117353, D. Pfister DHP-7126, Hansen et al. 1999).

DNA Samples: DNA samples of *Rickiella* were obtained from the specimen of *R. edulis* (Robledo 873/BAFC 51697, see above) sent by A.I. Romero. DNA samples of the *Phillipsia* species used in this expanded study were obtained from K. Hansen (Naturhistoriska Riksmuseet Stockhom, Sweden) and were the same genomic DNA samples used in a previous study by Hansen et al. (1999): *Phillipsia carnicolor* D. Pfister DHP-7126, *P. olivacea* Halling-5456, *P. olivacea* T. Læssøe AAU-43162, *P. lutea* G.J. Samuels and P. Searwar NY-4113, and *P. domingensis* D. Pfister DHP-7169. These DNA samples were used in amplification of the LSU and SSU rDNA region. A DNA sample of *Pithya cupressina*, which originated from a previous study by Harrington et al. (1999), was used to amplify the LSU rDNA region.

DNA Isolation, PCR, and Sequencing Techniques

DNA was extracted from the herbarium specimen of *R. edulis* (#51697) and *K. phyllogena* (DHP 10-690) using the Qiagen DNeasy Plant Mini Kit (cat. no. 69104). A 1/10 and 1/100 dilution of the DNA was used for PCR amplification of the ITS, SSU and LSU rDNA regions. The ITS rDNA region was amplified using ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). PCR parameters were as previously described (LoBuglio et al. 1993), using 35 PCR amplification cycles. The SSU was amplified using the NS1, NS2, NS4, NS8 (White et al. 1990) and SL1, SL122, SL344 (Landvik et al. 1996) primers. Amplification of the LSU rDNA region utilized the primers LROR and LR5 (Monclavo et al. 2000). All PCR reactions were done in a Peltier Thermal cycler PTC-200 (MJ Research, Watertown, MA), and used EconoTaq DNA Polymerase (Lucigen, Middleton, WI). PCR amplification, purification, and sequencing techniques were as described in Hansen et al. (2005). Sequencher 4.6 (GeneCodes, Ann Arbor, Michigan) was used to edit the DNA sequences obtained. The DNA sequences determined in this study were deposited in GenBank (*R. edulis* ITS=JQ260808, LSU=JQ260809, SSU=JQ260819; *K. phyllogena* LSU=JQ260810, SSU=JQ260820; *P. carnicolor* DHP-7126 LSU=JQ260811, SSU=JQ260821; *P. carnicolor* #113 LSU=JQ260812, SSU=JQ260822; *P. crispata* #114 LSU=JQ260813, SSU=JQ260823; *P. olivacea* Halling-5456 LSU=JQ260814, SSU=JQ260824; *P. olivacea* AAU-43162 LSU=JQ260815, SSU=JQ260825; *P. lutea* NY-4113 LSU=JQ260816, SSU=JQ260826; *P. domingensis* DHP-7169 LSU=JQ260817, SSU=JQ260827; and *Pithya cupressina* LSU=JQ260818).

DNA Sequence Analyses

Alignment of the DNA sequences was done using Se-Al v 2.0a8 (Rambaut 1996).

As previously described (LoBuglio and Pfister 2010), DNA sequence alignments were analyzed using: MrBayes v3.0b4 (Ronquist and Heulsenbeck 2003) for obtaining Bayesian posterior probabilities (PP); Maximum Parsimony using PAUP 4.0b10 (MP; Swofford 2002); and Maximum-Likelihood with RAxML-HPC2 on Abe through the Cipres Science Gateway (ML; Miller et al. 2009). Branch support for MP and ML analyses was determined by 1000 bootstrap replicates.

The ITS sequence of *Rickiella* obtained in this study was aligned with ITS sequences of the *Phillipsia* and *Nanoscypha* species included in the study by Hansen et al. (1999) (study S403 TreeBASE, http://purl.org/phylo/treebase/phylows/study/TB2:S403). *Sarcoscypha coccinea* (DQ491486) and *Sarcoscypha austriaca* (U66012) were included in this analysis as outgroup species. The hypervariable ITS1 region (as described by Hansen et al. 1999) was aligned with multiple gaps. Parsimony analyses were carried out with these gapped positions either included or excluded, and followed a phylogenetic search protocol using Maximum Parsimony as outlined by Hansen et al. (1999).

The DNA sequences determined in this study (*Rickiella*, *Kompsoscypha*, *Phillipsia* species, and *Pithya*), were included in the SSU and LSU phylogenetic analysis along with the following sequences from GenBank (SSU and LSU respectively): *Nanoscypha tetraspora* AF006314+DQ220374, *Pseudopithyella minuscula* AF006317+AY544658, *Sarcoscypha coccinea* AY544691+AY544647, *Microstoma floccosum* AF006313+DQ220370, *Cookeina tricholoma* AF006311+AY945860, *Pithya cupressina* AF006316, *Chorioactis geaster* AF104340+AY307944, *Wynnea* sp./*americana* AF006319+AY945848, *Wolfina aurantiopsis* AF104664+AY945859, *Desmazierella acicola* AF104341+AY945854, *Neournula pouchetii* AF104666+AY307940, *Sarcosoma latahense* FJ176806+FJ176860, *Urnula craterium* AF104347.1+AY945851, and *Galiella rufa* AF004948+AY945850. The outgroup taxa were: *G. rufa,* and *U. craterium.*

A third data set, which combined the ITS, LSU, and SSU rDNA data, was constructed and analyzed. This data set included 12 taxa: *P. domingensis* DHP-7169*, P. lutea* NY-4113, *P. olivacea* Halling-5456, *P. olivacea* AAU-43162, *P. crispata* #114, *P. crispata* AAU-44895a, *P. carnicolor* #113, *P. carnicolor* DHP-7126, *Nanoscypha tetraspora, R. edulis,* and *Pithya cupressina* and *Sarcoscypha coccinea* as the two outgroup species.

Hypothesis Testing

Approximately Unbiased (AU) tests (Shimodaira 2002; Ruhfel et al. 2008; Mathews et al. 2010) were conducted with the SSU and LSU rDNA data set using the R (http://www.r-project.org/) package, Scaleboot, to statistically evaluate alternative phylogenetic hypotheses on the evolution of *Rickiella*, *Phillipsia* and *Nanoscypha*. The three hypothesis tree topologies tested were: 1) All species of *Phillipsia* are monophyletic; 2) *Nanoscypha* and *Phillipsia* species are monophyletic; and 3) *Rickiella* and *Phillipsia* species are monophyletic. Constraint trees were first drawn in MacClade 4.05 (Maddison and Maddison 2004) to enforce the above mentioned tree topologies and then tested against the best ML tree.

Results

The ITS1F-ITS4 sequence of *Rickiella* was 613 bp long. It is alignable with the ITS of *Phillipsia* species from Hansen et al. (1999), but shows a region of 80 unique base pairs (bp) at approximately 69 bp in ITS1. In the study by Hansen et al. (1999), a hypervariable region among the ITS sequences of *Phillipsia* and *Nanoscypha* species at this same ITS1 region was described. Parsimony analysis of *Rickiella*, *Phillipsia*, and *Nanoscypha* ITS sequences (with *S. coccinea* and *S. austriaca* as the outgroup) showed that both *Rickiella* and *Nanoscypha* were unresolved within a highly supported (100%) *Phillipsia* clade (results not shown). Trees were identical whether gapped positions (present in the ITS1 hypervariable region) were included or excluded from the analyses as was previously found (Hansen et al. 1999).

The combined SSU and LSU alignment of the 24 taxa in Figure 1 included 2662 bp, none of which were excluded in the phylogenetic analyses. MP and ML bootstrap support and PP values for the family Sarcoscyphaceae was 100%. This was the case when the data set was analyzed 3 times, each using 3 different outgroups: 1) *Urnula craterium* and *Galiella rufa*; 2) *Neournula pouchetii, Desmazierella acicola, Sarcosoma latahense, Chorioactis geaster*, and *Wolfina aurantiopsis*; and 3) no outgroup. Tree topologies from Bayesian and ML analyses did not conflict with the parsimony tree presented in Figure 1, with respect to relationships within the Sarcoscyphaceae among statistically supported branches (≥ 60% MP, 70% ML, and 90% PP). As shown in Figure 1, Bayesian, MP, and ML phylogenies supported a monophyletic clade comprised of *Rickiella, Nanoscypha* and the five species of *Phillipsia* examined (MP had low support, 66%, compared to ML bootstrap, 90%, and Bayesian PP values, 100%). Within this clade the five species of *Phillipsia*, *Rickiella*, and *Nanoscypha* collapses to a polytomy. Inclusion of the ITS data, in the LSU, and SSU data set (as described in materials and methods) did not improve the phylogenetic resolution among the *Phillipsia*, *Rickiella*, and *Nanoscypha* species (data not shown). As described above for the LSU-SSU phylogeny presented in Figure 1, the ITS, LSU, and SSU data also supported a monophyletic clade (with 100% and 97% support, MP and ML Bootstrap respectively) comprised of *Rickiella, Nanoscypha* and the five species of *Phillipsia* examined. Within this clade there was no bootstrap support among the species of *Phillipsia*, *Rickiella*, and *Nanoscypha*. Thus, the relationship among the three genera, *Rickiella*, *Phillipsia*, and *Nanoscypha*, was not resolved from phylogenetic analyses of rDNA sequence data.

The AU test (Table 1) rejected hypothesis tree #1, which forced all species of *Phillipsia* to be a monophyletic group, at a 5% significance level. The second hypothesis which forced *Nanoscypha* and *Phillipsia* species to be monophyletic, thus excluding *R. edulis*, could be marginally rejected with a variance of +/- 0.41 at a 5% significance level. Hypothesis tree #3, which forced *Phillipsia* species and *R. edulis* to be monophyletic, was not rejected by the AU test.

Discussion

In the present study a clade is identified that includes species that have been referred to as the genera *Phillipsia, Nanoscypha* and *Rickiella* (Fig. 1). This group, which has reasonable support (Fig. 1), is characterized by moderate to large ascomata, and generally ellipsoid to alantoid spores which are often asymmetrical or flattened on one side. Ascospores are smooth or marked with longitudinal ribs. Within this clade, relationships among the taxa could not be resolved indicating that more data is needed to clarify how the *Rickiella, Phillipsia* and *Nanoscypha* lineages are related. AU tests support the lack of monophyly of the genus *Phillipsia* (Table 1). Clearly species of *Phillipsia* need additional taxonomic attention. The ability to reject tree #2, which excludes *Rickiella* from a monophyletic clade with *Phillipsia* and *Nanoscypha* species, but not reject tree #3, which considers *Rickiella* and *Phillipsia* monophyletic and excludes *Nanoscypha*, suggests that *Rickiella*, but not *Nanoscypha*, may be embedded within a group of *Phillipsia* species (Table 1). It is interesting to note that

Table 1.
Results of hypothesis testing as determined by Approximately Unbiased (AU, Shimodaira 2002) tests. P-values presented are corrected by Alkaike weights where values greater than 5% (with astericks) indicate tree toologies that are not significantly different from the best tree.

Tree Topology	Likelihood	P-value	Variance	Tree Rejected
Best Tree	-9047.003734	67.5	0.52	
1. Phillipsia Monophyletic	-9056.886057	3.65	0.41	Yes
2. Phillipsia + Nanoscypha Monophyletic	-9056.054433	5.27	0.41	Yes
3. Phillipsia + Rickiella Monophyletic	-9047.333917	51.93*	0.37	No

Rickiella and *Nanoscypha* each had unique regions in ITS1 which could not be aligned with each other or with any of the unique regions in this ITS1 hypervariable region described for *Phillipsia* species by Hansen et al. (1999).

On molecular grounds, as far as studied, there is no justification for accepting or rejecting the genera *Rickiella* and *Nanoscypha* but other characters must be considered in determining generic boundaries. There is considerable morphological variation within the clade. Pigments vary from yellow, pink, scarlet, purple to dark greenish. The construction and cell arrangement of the excipular tissues also prove to be diverse. *Phillipsia* species and *R. edulis* have an outer excipulum composed of a generally thin prosenchymatous layer as seen in median section. *Nanoscypha* species have an outer layer that is composed of angular to globose cells. *Rickiella edulis* is the most distinct morphologically in its unique regularly lacunose excipular tissue (Fig. 2). This feature is unknown in other members of the clade. Given these morphological differences we recognize *Rickiella* as a genus distinct from *Phillipsia*. Anamorphic states might prove helpful. Some *Phillipsia* species, *Nanoscypha tetraspora* and several other taxa in the Sarcoscyphaceae produce anamorphic states that are placed in the form genus *Molliardomyces* (Paden 1984, Pfister 1973) but we do not have information on an anamorphic state in *Rickiella.*

Species of *Phillipsia* are diverse; a constellation of species or species complexes center on *P. domingensis*. These fungi produce large ascomata, up to 10 cm diam, have thick flesh, and have spores with longitudinal ridges. ITS sequence data (Hansen et al. 1999) showed little variation within the *P. domingensis* group and all isolates within this group shared an identical 32 bp in the ITS1 hypervariable region (Hansen et al. 1999). Two taxa, *P. carnicolor* and *P. crispata*, differ from the P. *domingensis* group in their smaller ascomatal size and spore ornamentation. *P. crispata* and *P. carnicolor* each have a unique DNA sequence in the ITS1 hypervariable region (Hansen et al 1999). These species produce apothecia up to 2.5 cm diam and have ascospores that are smooth or have very fine longitudinal striate. *Phillipsia olivacea* may be morphologically distinct as well. In this species ascomata are large (up to about 3.5 cm diam) and have dark green hymenial pigments and smooth or indistinctly striate ascospores that often are nearly alantoid. Furthermore, *P. olivacea* has a 38 bp sequence in the ITS1 hypervariable region that can be aligned with *P. carnicolor* but is distinct from the unique DNA sequences (found at this ITS1 position) in the other species in this clade. Both Rifai (1968) and Moravec (1997) noted some of these morphological distinctions within the genus *Phillipsia* and suggested that subgeneric or generic recognition of them might be

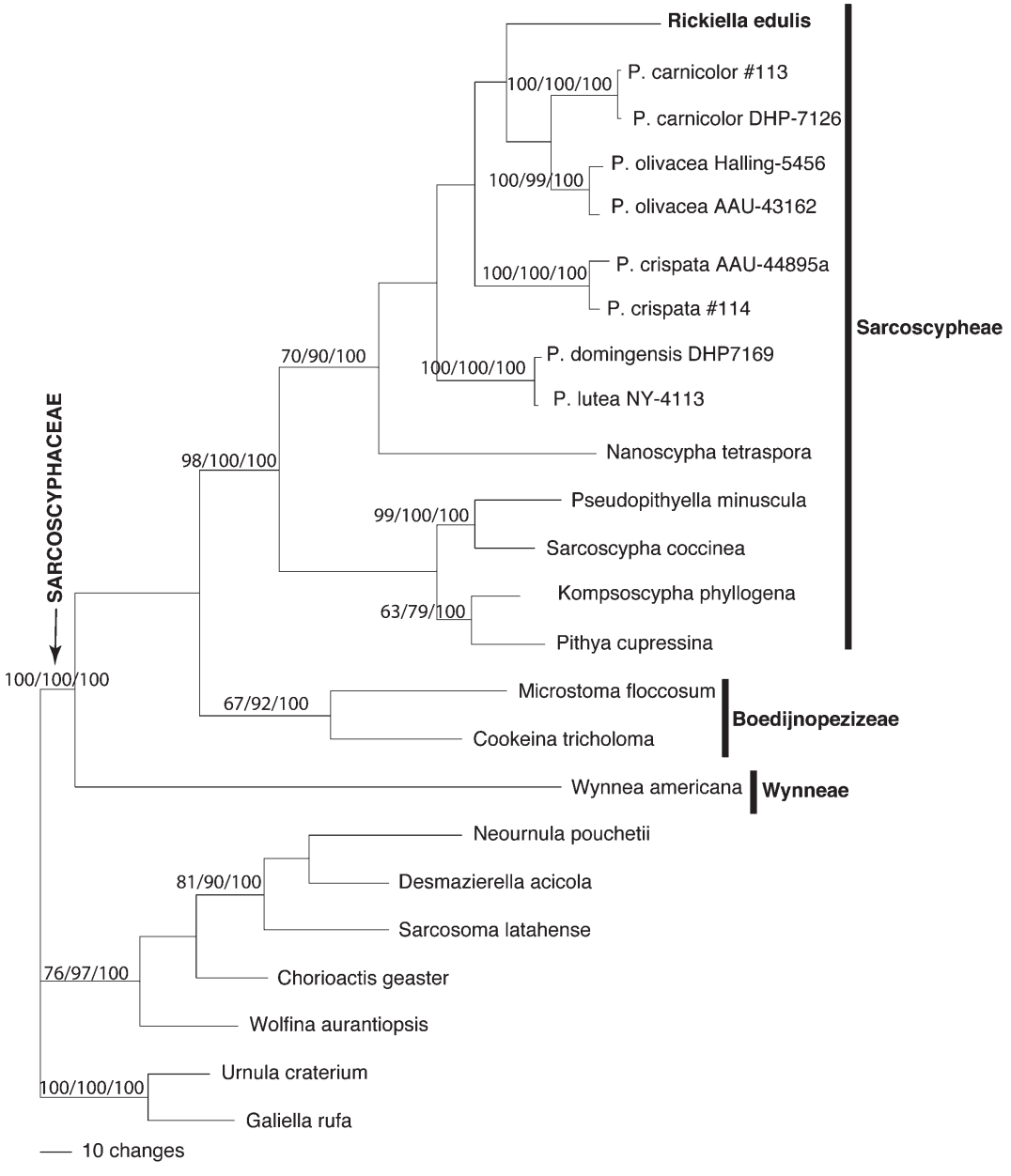

Fig. 1. One of 3 most parsimonious trees (tree length of 1015) based on LSU and SSU rDNA sequence data (2662 bp). Parsimony and Maximum-Likelihood Bootstrap values below 60%, and Bayesian posterior probabilities below 90% are not displayed. The outgroup taxa were, *G. rufa,* and *U. craterium*. The vertical line indicates tribes in the *Sarcoscyphaceae: Sarcoscypheae, Boedijnopezizeae* and *Wynneae*.

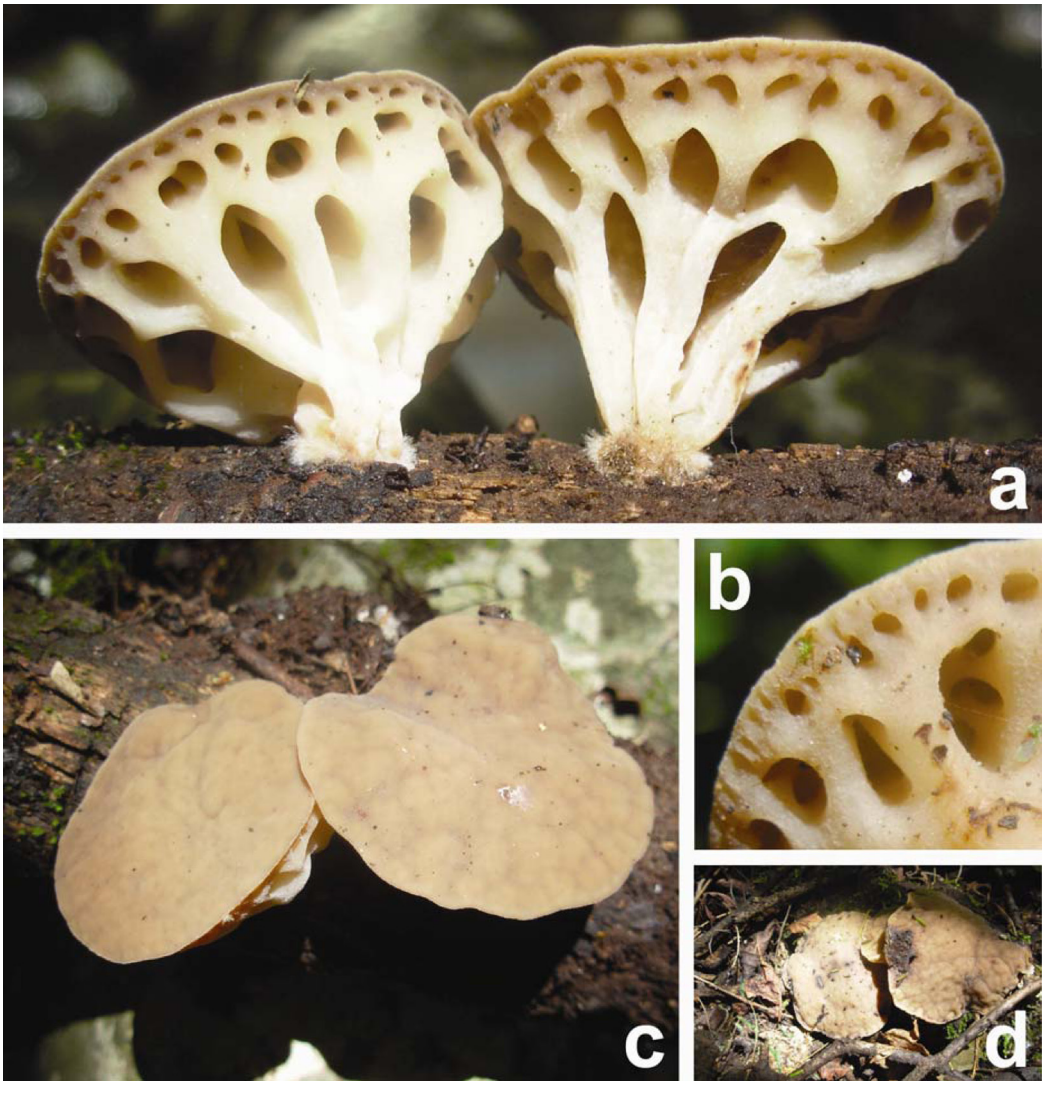

Fig. 2. Macroscopic characters of fresh specimens of *Rickiella edulis* a) lateral view, b) detail of the lacunose excipular tissue, c) upper view and d) habit of the species as could be founded in the field. Photo credits G. Robledo.

appropriate. This view is supported given the DNA sequence differences between species in the ITS1 hypervariable region.

Given the lack of support with the markers we have thus far sampled we are hesitant to break apart *Phillipsia* but at the same time we are confident in the morphological characters that we have outlined here to support the recognition of both *Nanoscypha* and *Rickiella*. Since its introduction *Nanoscypha* has been accepted by all workers who point to the morphologically distinct excipular construction in this species as a critical character.

In this study the monophyly of the Sarcoscyphaceae is confirmed and *Wynnea*, which has been placed both in this family and in the Sarcosomataceae, is shown to fall within this well circumscribed family. Our molecular data show three groups (Fig. 1). Two of these groups correspond to the tribes Sarcoscypheae

and Boedinopezizeae, as discussed and proposed originally by Korf (1970). *Wynnea* falls outside both of these tribes and deserves its own tribe. We here provide a diagnosis for a new tribe in the family Sarcoscyphaceae to accommodate *Wynnea* alone.

Wynneae Pfister, **tribe nov.**

MycoBank no. 564069

Ascis ut in familia. Ascomis proceris, spathulatis, quarum aliquae observor glomeratae dense in stipite communi, generaliter surgente a sclerotio sepulto. Ascosporis cum cristis longitudinalibus notoriis CB-.

Type genus: Wynnea Berk. & M. A. Curtis, J. Linn. Soc. Bot. 9: 424. 1867.

For literature on this genus see Pfister (1979) and Zhuang (2004).

***Rickiella* ecology and distribution**

Collections of *Rickiella edulis* are known only from three areas in South America. The lack of collections is striking given the particular morphology of the ascomata. Other than the collection from northwestern Argentina, the species is known from a few examples collected between 1880 and 1922 in Guarapí, Paraguay, Paraguarí Department, the type locality of *Peziza edulis* Speg., and Rio Grande do Sul state in southeastern Brazil, the type locality of *R. transiens* (Pfister 1987). Traditional biogeographical summaries place these three areas in three different phytogeographic provinces/regions (Fig.3) within the Neotropical Region (Cabrera and Willink 1973, Morrone 2001). The locality in Paraguay corresponds to Gran Chaco; localities in Rio Grande do Sul correspond to Atlantic Rain Forest/Paranaense Forest; and the northeastern Argentina site corresponds to Yungas Mountain Rain Forests. These three phytogeographic regions show different floristic elements of different origins and, even though the three areas are more or less at similar latitudes, there are different climatic characteristics and altitudes. For example, the area in Argentina where *R. edulis* was collected has snow at least once a year. In recent biogeographic contributions a new phytogeographic region, the Dry Seasonal Neotropical Forests (DSNF), has been proposed based on distributions of particular tree species and tree species assemblages (Prado 2000, Pennington et al. 2000). *Rickiella edulis* collections come from the DSNF (Fig.3). In this scenario, it could be supposed that either *R. edulis* is potentially a widely spread species that is extremely rare or that it has been overlooked, perhaps because these areas have not been sufficiently sampled. We know nothing about its requirements for ascomata production; ascomata may occur only rarely, during a brief season or may be particularly ephemeral. In northwestern Argentina, the species was found growing on dead fallen logs, but the type specimen was said to occur "*sur la terre*." No particular reference to ecology was reported for collections from southern east Brazil. Collections from Argentina seem to occur on soil (Fig. 2d) but they grew on fallen logs, some of which were partly buried. It is probably the case that the type collection was growing in this way and was misinterpreted as growing on soil. Being a wood-inhabiting species, like nearly all member of the Sarcoscyphaceae, it is plausible that *R. edulis* follows the distribution of some woody plant of the DSNF. At this juncture such a suggestion is speculative given how little information is at hand. Fungal distribution patterns have received little attention and such distinctive fungi as *R. edulis* might be helpful in taking an integrated approach to plant and fungal distributions.

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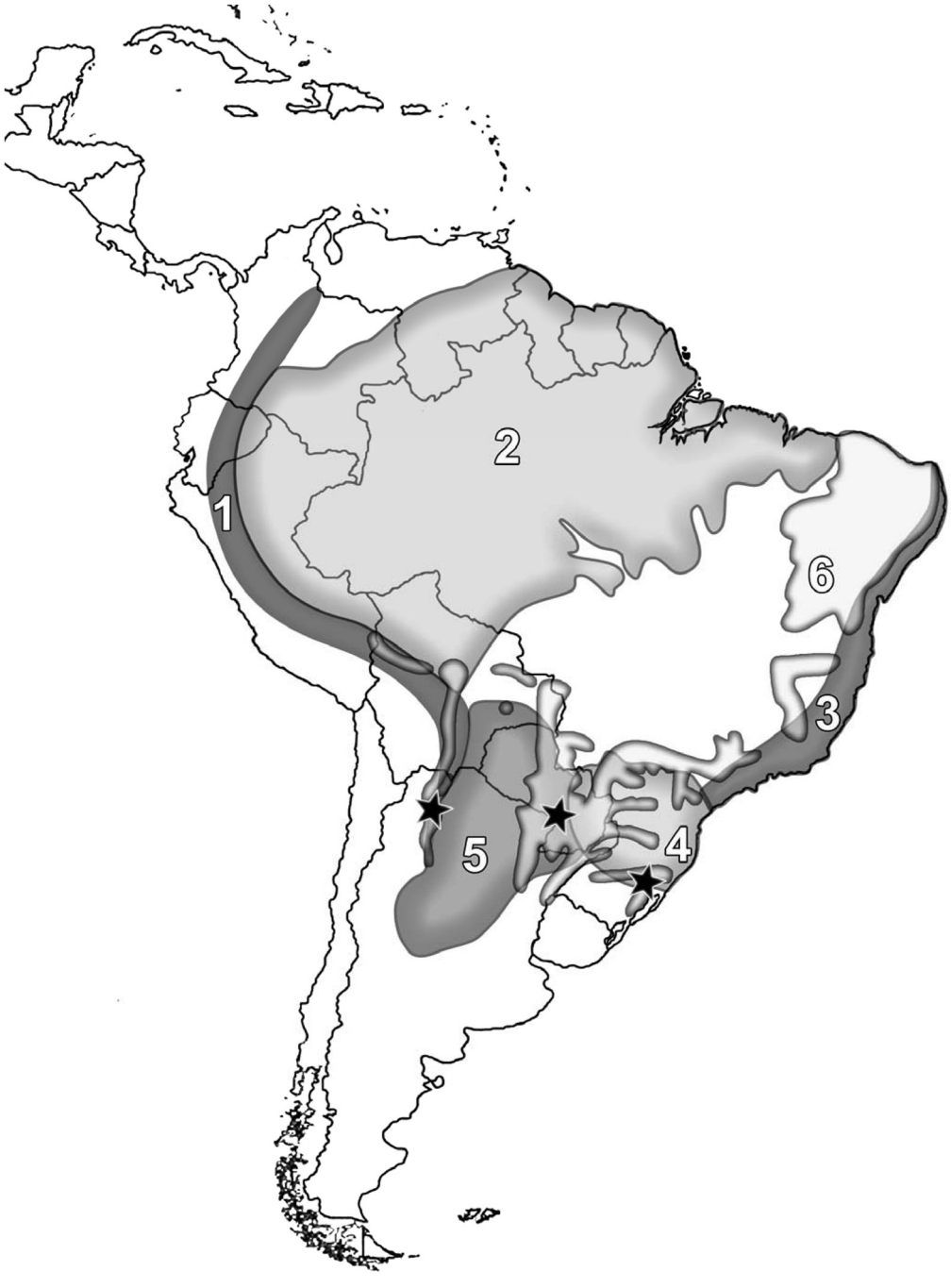

Fig. 3. Known distribution of *Rickiella edulis* (★black stars). Phytogeographic regions in South America are numbered as follows: 1) Yungas Mountain rain forests, 2) Amazonian forest, 3) Atlantic rain forest, 4) Paranaense forests, 5) Gran Chaco and 6) Dry seasonal neotropical forests.

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