Auriculibuller fuscus gen. nov., sp. nov. and *Bullera japonica* sp. nov., novel taxa in the Tremellales

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Seven phylloplane yeast strains that were collected in the Arrábida Natural Park, Portugal, and identified preliminarily as *Bullera alba*, the anamorphic stage of *Bulleromyces albus*, were investigated. In contrast to *Bulleromyces albus*, these isolates produced a brownish pigment when grown on potato dextrose agar. The pigment caused darkening of the cultures and diffused into the culture medium. Mating studies revealed that the Arrábida isolates did not react with the different mating types of *Bulleromyces albus*, but were sexually compatible with them and produced mycelium with clamp connections, haustoria and transversally septate basidia that ejected the basidiospores. Various taxonomic criteria that were evaluated during the present study and comparison with other sexual taxa of the Tremellales indicated that this teleomorph should be classified in a novel genus. Therefore, *Auriculibuller fuscus* gen. nov., sp. nov. (type strain, PYCC 5690^T = CBS 9648^T) is proposed. In addition, during the course of this investigation, a member of a novel *Bullera* species, *Bullera japonica* sp. nov. (type strain, PYCC 4534^T = CBS 2013^T), was found among collection isolates that were identified formerly as *Bullera alba*. In molecular phylogenetic analysis of the D1/D2 domains of the 26S rDNA and the internal transcribed spacer region, the two taxa were found to be closely related, but distinct at the species level.

INTRODUCTION

The phenotypic circumscription of the basidiomycetous anamorphic genus *Bullera* Derx is based on the production of rotationally symmetrical ballistoconidia, absence of bud-forming stalks, white to cream or yellowish cultures, inositol assimilation, production of starch-like compounds (a few exceptions are known for the two latter tests), CoQ10 as the major ubiquinone system and the presence of xylose in whole-cell hydrolysates (Boekhout & Nakase, 1998). Presently, the genus *Bullera* comprises approximately 30 species that belong to the Tremellales, based on various molecular phylogenetic analyses (Fell *et al.*, 2000; Nakase, 2000; Bai *et al.*, 2001; Scorzetti *et al.*, 2002). Until now, two teleomorphic genera, *Bulleromyces* Boekhout and Fonseca (Boekhout *et al.*, 1991) and *Bulleribasidium* Sampaio, Weiss and Bauer (Sampaio *et al.*, 2002) have had

their anamorphic counterparts in *Bullera*. However, no teleomorph is known for most *Bullera* species. Implementation of molecular phylogenetic analyses based on DNA sequence data has emphasized the need to integrate mitospore taxa, such as *Bullera*, into the classification system that was originally built exclusively for sexual fungi.

Ballistoconidia-producing yeasts are found frequently in the phylloplane. Forceful liberation of propagules seems to constitute an ecological advantage for colonization and dissemination in the plant leaf environment (Nakase, 2000). A 2 year survey (1997–1998) of the phylloplane yeast communities that are found on selected plants from two study sites in the Arrábida Natural Park, Portugal, was carried out by Inácio *et al.* (2002). This natural park encompasses different microclimatic areas with specific formations of typically Mediterranean vegetation. Among a vast majority of isolates of basidiomycetous affinity, seven yeast strains were identified preliminarily as *Bullera alba* Hanna, the anamorphic stage of *Bulleromyces albus* Boekhout and Á. Fonseca. In contrast to *Bulleromyces albus*, these isolates produced a brownish pigment when

Abbreviations: ITS, internal transcribed spacer; MCMC, Markov chain Monte Carlo.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are shown in Table 1.

grown on potato dextrose agar. The pigment caused darkening of the cultures and diffused into the culture medium. Subsequent mating studies revealed that the new isolates did not react with the different mating types of *Bulleromyces albus*, but were sexually compatible with each other and produced clamped mycelium with haustoria, basidia and basidiospores in culture. Several taxonomic criteria that have been evaluated during the present study indicate that these Arrábida isolates should be accommodated in a novel genus, rather than in any existing genus of the Tremellales. Moreover, during the course of this investigation, a novel *Bullera* species was found among collection isolates that were identified previously as *Bullera alba*.

METHODS

Two methods were used for isolation: one was based on the plating of leaf washings, as described by Inácio *et al.* (2002), and the other on the spore-fall method. Leaves were collected from different plants (see Table 1) in the Arrábida Natural Park [for further details, see Inácio *et al.* (2002)]. A list of cultures of the novel taxa described in this study is shown in Table 1. For microscopy, cultures were grown on MYP agar [malt extract, 0.7% (w/v); yeast extract, 0.05% (w/v); soytone peptone, 0.25% (w/v); and agar, 1.5% (w/v)] at room temperature (20–23 °C) and examined with an Olympus BX50 microscope by using phase-contrast optics. For determination of sexual compatibility, pairs of 2–4-day-old cultures were mixed on MYP agar, incubated at room temperature and examined for production of mycelium and clamp connections after 2 weeks.

Physiological and biochemical characterization followed the methods described by Yarrow (1998). Additional assimilation tests were performed by using aldaric acids and aromatic compounds as described by Fonseca (1992) and Sampaio (1999), respectively.

For determination of the extent of DNA relatedness, total genomic DNA was extracted and purified by using the procedures described by Sampaio *et al.* (2001). For DNA–DNA reassociation experiments,

a Gilford Response UV-VIS spectrophotometer and its thermal programming software were used and the methods of Kurtzman *et al.* (1980) were followed.

DNA sequence analysis, DNA extraction, PCR amplification, purification and cycle sequencing followed the protocol of Fell *et al.* (2000). Alignments were made with MegAlign (DNASTar) and corrected visually. To estimate phylogenetic relationships, we applied the Bayesian Markov chain Monte Carlo (MCMC) method of phylogenetic inference (Larget & Simon, 1999), as implemented in the computer program MRBAYES (Huelsenbeck & Ronquist, 2001). This method allows estimation of the *a posteriori* probability that groups of taxa are monophyletic, given the DNA alignment (i.e. the probability that corresponding bipartitions of the species set are present in the true unrooted tree, including the given species). Four incrementally heated simultaneous Monte Carlo Markov chains were run over 1 000 000 generations by using the general time-reversible model of DNA substitution with Γ -distributed substitution rates (GTR + G), random starting trees and default starting parameters of the DNA substitution model. Trees were sampled every 100 generations, resulting in an overall sampling of 10 000 trees. From those trees that were sampled after the process had reached a stationary stage, a consensus tree was computed to obtain estimates for *a posteriori* probabilities. This Bayesian approach to phylogenetic analysis was repeated several times, always using random starting trees and default starting values for the model parameters to test the reproducibility of the results.

RESULTS AND DISCUSSION

Latin diagnosis of *Auriculibuller* Sampaio *et* Fonseca gen. nov.

Fungus dimorphus. *Basidiomata nulla*. *Basidia plerumque transversalitre, interdum longitudinalitre septata, fibulata, in fasciculis parvis an binatim disposita*. *Basidiosporae globosae, a sterigmatibus eiciuntur, gemmis vel repetitione germinant*. *Hyphae hyalinae, fibulatae, haustoriis tremelloideis*. *Status anamorphosium in genus Bulleram pertinet*. *Typus generis: Auriculibuller fuscus in opere ipso descriptus*.

Table 1. Source, sexuality and GenBank accession numbers of cultures of the novel taxa described in this study

Species*	Isolation source	Sexuality†	GenBank no.	
			D1/D2	ITS
<i>Auriculibuller fuscus</i> :				
PYCC 5690 ^T =A2AVS4 ^T	<i>Acer monspessulanum</i> , Arrábida, Portugal	MT A1	AF444762	AF444668
PYCC 5691=A2AVS5	<i>Acer monspessulanum</i> , Arrábida, Portugal	MT A2	AF444763	AF444669
PYCC 5739=A2QVSI1	<i>Quercus faginea</i> , Arrábida, Portugal	MT A2	AF444764	AF444670
PYCC 5740=A1CVV1	<i>Cistus albidus</i> , Arrábida, Portugal	MT A2	AF444761	AF444667
A2AVS6	<i>Acer monspessulanum</i> Arrábida, Portugal	MT A1		
2AVF3	<i>Acer monspessulanum</i> , Arrábida, Portugal	MT A2		
A5QSV2	<i>Quercus faginea</i> , Arrábida, Portugal	MT A2		
<i>Bullera japonica</i> PYCC 4534 ^T =CBS 2013 ^T	<i>Zelkova</i> sp., Japan	ANA	AF444760	AF444666

*CBS, Centraalbureau voor Schimmelcultures, Yeast Division, Utrecht, the Netherlands; PYCC, Portuguese Yeast Culture Collection, FCT-UNL, Portugal.
†ANA, Anamorphic; MT, mating type.

Description of *Auriculibuller Sampaio and Fonseca gen. nov.*

Auriculibuller (Au.ri.cu.li.bul'ler. L. fem. n. *auricula* ear-lobe, referring to the auricularioid nature of the basidia; N.L. n. *Bullera* referring to the anamorphic yeast genus *Bullera*; N.L. masc. n. *Auriculibuller* auricularioid yeast that is similar to the genus *Bullera*).

Dimorphic. Basidiocarps are absent. Basidia are typically transversally septate or occasionally longitudinally septate, arranged in small clusters or in pairs and clamped. Basidiospores are globose, rotationally symmetrical, produced at the apex of tubular sterigmata and forcibly discharged; germination occurs by budding or repetition. Hyphae are hyaline and clamped with tremelloid haustoria. Anamorphic stage produces ballistoconidia and budding yeast cells and is classified in the genus *Bullera*. The type species is *Auriculibuller fuscus*.

Latin diagnosis of *Auriculibuller fuscus Sampaio, Inácio, Fonseca et Fell sp. nov.*

*Cultura in striis post septem dies crenea, fuscans aetate progrediente. Post unum mensem cultura fusca, mucilaginea, superficie laevi, nitida. In statu unicellulari cellulae ovoideae, (3) 4–5 × 5–8 µm. Sterigmata 1–2 × 7–30 µm, interdum usque ad 60 µm longa. Ballistoconidia globosa (4–5.5 µm) ad subglobosa (4.5–5 × 6–7 µm). Mycelium hyphis fibulatis, 1.5–2 µm diametro, haustoriis tremelloideis. Basidia cylindracea, transversaliter septata, bicellulata. Basidiosporae globosae ad subglobosae, 5–7 µm diametro, parietibus tenuibus, eiciuntur a sterigmatibus (1–2 × 8–15 µm), gemmis vel repetitione germinant. Characteres biochemici physiologicique *Auriculibulleri fusci* in tabula 2 describuntur.*

Description of *Auriculibuller fuscus Sampaio, Inácio, Fonseca and Fell sp. nov.*

Auriculibuller fuscus (fus'cus. L. masc. adj. *fuscus* dark-coloured, referring to the greyish-brown darkening of the cultures that occurs upon ageing).

Streak culture after 1 week is cream-coloured; becomes darker upon ageing. After 1 month, cultures are greyish-brown, mucilaginous, smooth-surfaced and glossy. Yeast cells are ovoid after 4 days on MYP agar, measuring (3) 4–5 × 5–8 µm. Sterigmata are 1–2 × 7–30 µm or occasionally up to 60 µm in length. Ballistoconidia are globose (4–5.5 µm) to subglobose (4.5–5 × 6–7 µm) (Fig. 1). Hyphae are 1.5–2 µm in diameter with clamp connections and haustoria, which are formed after crossing of sexually compatible strains (Fig. 2). Haustorial cells are globose to slightly elongated (Fig. 2). Basidia initials are at first globose and then become cylindrical (Fig. 3); they are arranged in small clusters or isolated (Fig. 3). Mature basidia are two-celled, normally transversally septate and cylindrical, measuring 5–7 × 20–30 µm. Basidiospores are globose to slightly subglobose (5–7 µm in diameter), thin-walled, ejected from sterigmata that measure 1–2 × 8–15 µm

Fig. 1. Line drawings of yeast cells (Y), ballistoconidiogenic cells and ballistoconidia (B indicates released ballistoconidia) of *Auriculibuller fuscus* (PYCC 5690^T and PYCC 5739) observed on MYP agar after 4–7 days. Bar, 10 µm.

and germinate by budding or repetition (Fig. 3). Biochemical and physiological properties of *A. fuscus* are given in Table 2 and its phylogenetic placement is shown in Fig. 5.

The type strain is PYCC 5690^T = CBS 9648^T. A complementary mating reference strain is PYCC 5739 = CBS 9650. The seven cultures of *A. fuscus* that are listed in Table 1 were isolated in 1997 and 1998 from leaves of different Mediterranean trees and shrubs that were collected at the

Fig. 2. Line drawings of the sexual stage of *Auriculibuller fuscus* (PYCC 5690^T × PYCC 5739) observed on MYP agar after 10–15 days. Yeast cells with appressoria-like structures (upper left part), conjugation and early stages of mycelium formation (upper right part) and hyphae with clamp connections and haustoria (lower part). Bar, 10 µm.

Fig. 3. Line drawings of the basidial stage of *Auriculibuller fuscus* (PYCC 5690^T × PYCC 5691) and comparison with *Bulleromyces albus*. (a) Basidia of *A. fuscus* in different developmental stages: non-septate globose, non-septate cylindrical, septate and septate with sterigmata; note one basidiospore between the two sterigmata on the lower right part. (b) Basidia of *Bulleromyces albus* (PYCC 4539 × PYCC 4560); note the different basidial shape and type of septation and the sessile mode of basidiospore formation. Bar, 10 µm.

Arrábida Natural Park, Portugal. All cultures were isolated by using the spore-fall method, except for strain 2AVF3, which was isolated by using the leaf-washing method. Sexual compatibility and identical microsatellite-primed PCR fingerprints with primer M13 supported conspecificity of those isolates (data not shown). Sexual compatibility seems to be biallelic, as crossing experiments suggest the presence of two mating types.

A. fuscus is able to complete its life cycle on culture media. When sexually compatible strains are mixed on MYP agar,

Table 2. Physiological and biochemical characteristics of *Auriculibuller fuscus* and *Bullera japonica*

Taxa: 1, *Auriculibuller fuscus*; 2, *Bullera japonica*. Characteristics are scored as: +, growth; –, no growth; D, delayed growth; V, variable growth. Both taxa were positive for assimilation of the carbon compounds D-glucose, D-galactose, D-glucosamine, D-ribose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, sucrose, maltose, α,α-trehalose, methyl α-D-glucoside, cellobiose, salicin, melibiose, lactose, raffinose, melezitose, soluble starch, erythritol, ribitol, xylitol, D-glucitol, D-mannitol, inositol, glucono-δ-lactone, D-gluconic acid, D-glucuronic acid, succinic acid, citric acid, L-malic acid, saccharic acid, mucic acid and gallic acid and the nitrogen compounds ethylamine, L-lysine and cadaverine. Both taxa were positive for growth with 0.01% cycloheximide, growth at 25 °C, formation of starch-like compounds, splitting of arbutin, hydrolysis of urea and the diazonium blue B reaction. Both taxa were negative for fermentation of D-glucose, assimilation of the carbon compounds inulin, D-tartaric acid, methanol, ethanol, vanillic acid, ferulic acid, veratric acid, *p*-hydroxybenzoic acid, *m*-hydroxybenzoic acid, protocatechuic acid, catechol, salicylic acid, gentisic acid and phenol and the nitrogen compounds potassium nitrate, sodium nitrite, creatine and creatinine. Both taxa were negative for growth in vitamin-free medium, growth with 0.1% cycloheximide and growth at 35 °C (these data are available in tabular form at http://www.crem.fct.unl.pt/dimorphic_basidiomycetes/Databases/databases.htm).

Characteristic	1	2
Carbon compounds:		
L-Sorbose	–	D
Glycerol	V	D
Galactitol	V	+
DL-Lactic acid	V	+
L-Tartaric acid	V	+
D-Tartaric acid	V	+
Growth at 30 °C	V	+

cells with appressorium-like structures develop (Fig. 2). Conjugation between two cells is difficult to observe, due to the twisting and ramifications of the conjugating hyphae (Fig. 2). One-week-old crossings show conspicuous clamped mycelium with haustoria that are able to attach to hyphae of deuteromycetous culture contaminants, which suggests that *A. fuscus* is capable of mycoparasitism in nature. Basidia initials are formed after 6–8 weeks at room temperature. Transfer of agar blocks of this age to 2% water agar stimulated basidial formation. Mature basidia (septated, with sterigmata and basidiospores) could be detected approximately 1 week after transfer to water agar.

Latin diagnosis of *Bullera japonica* Sampaio, Fonseca et Fell sp. nov.

Cultura in striis post septem dies cremea, fuscans aetate progrediente. Post unum mensem cultura fusca, mucilaginea, superficie laevi, nitida. Cellulae ovoideae ad cylindraceae,

(1.5) 2–3 × 5–10 (12) μm . Sterigmata 1–2 × 8–16 μm . *Ballistoconidia globosa* (5–6 μm diametro) ad subglobosa (4–5 × 5–6 μm). *Characteres biochemici physiologici Bullerae japonicae in tabula 2 describuntur.*

Description of *Bullera japonica* Sampaio, Fonseca and Fell sp. nov.

Bullera japonica [ja.po'ni.ca. N.L. adj. *japonica* referring to the origin (Japan) of the single culture of this species that is presently known].

Streak culture after 1 week is cream-coloured; becomes darker upon ageing. After 1 month, cultures are greyish-brown, mucilaginous, smooth-surfaced and glossy. Yeast cells are ovoid to cylindrical after 4 days on MYP agar, measuring (1.5) 2–3 × 5–10 (12) μm . Sterigmata are 1–2 × 8–16 μm ; ballistoconidia are globose (5–6 μm) to subglobose (4–5 × 5–6 μm) (Fig. 4). Biochemical and physiological properties of *Bullera japonica* are presented in Table 2 and its phylogenetic placement is shown in Fig. 5.

The type strain of *Bullera japonica* (PYCC 4534^T=CBS 2013^T) was isolated by K. Tubaki in Japan from *Zelkova* sp.

The phylogenetic placement of *A. fuscus* and *Bullera japonica* was inferred by using the D1/D2 domains of the 26S rDNA and the Bayesian MCMC method of phylogenetic inference (Fig. 5). As expected, both taxa belong to the Tremellales. Sequence data indicated that the two novel taxa described in this report are related most closely to one another. Their D1/D2 sequences differed by three nucleotide substitutions and one single-base indel. The four strains of *A. fuscus* that were sequenced (see Table 1) have identical sequences; therefore, only the sequence of the type strain was used in molecular analyses. In the analysis shown in Fig. 5, the pair *A. fuscus*–*Bullera japonica* has, as its closest teleomorphic relative, *Papiliotrema bandonii* Sampaio, Gadanho, Weiss and Bauer. Both *Auriculibuller* and *Papiliotrema* have auricularioid basidia. In order to study in more detail the relationship between

A. fuscus and *Bullera japonica*, the complete internal transcribed spacer (ITS) region (ITS1 + 5.8S rDNA + ITS2) of the two taxa was investigated (the phylogenetic tree is available at http://www.crem.fct.unl.pt/dimorphic_basidiomycetes/Databases/databases.htm). Again, the four strains of *A. fuscus* showed identical sequences that differed from that of *Bullera japonica* by seven substitutions and one single-base indel. In ITS analysis, *P. bandonii* was the closest relative of *A. fuscus* and *Bullera japonica*. Nuclear DNA–DNA reassociation experiments between two strains of *A. fuscus* (PYCC 5690^T and PYCC 5739) yielded high relatedness values (92–93 %), whereas each of these strains produced intermediate values in reassociation experiments with *Bullera japonica* (52–58 % relatedness). Moreover, crossings of *Bullera japonica* with the two mating types of *A. fuscus* invariably gave negative results. Physiological and biochemical characterization of the two novel taxa, presented in Table 2, showed that, except for the assimilation of L-sorbose, *A. fuscus* and *Bullera japonica* are indistinguishable. As indicated in Table 2, a few tests gave variable results for *A. fuscus*.

The two novel taxa share a peculiar cultural characteristic: when grown on potato dextrose agar, they produce a brownish pigment that darkens the cultures and diffuses into the agar. This trait is absent in *Bulleromyces albus* and motivated a deeper analysis of the pigment-producing isolates.

The genus *Auriculibuller*, described in the present report, is the third genus to accommodate the sexual stages of the mitosporic ballistoconidial genus *Bullera*. The other two genera are *Bulleromyces* and the recently described genus *Bulleribasidium* (Sampaio *et al.*, 2002). Despite similar basidial morphologies, *Auriculibuller* and *Bulleribasidium* are clearly distinct, based on the molecular phylogenetic hypothesis presented in Fig. 5. From an evolutionary point of view, *Auriculibuller* and *Bulleromyces* seem to share a recent common ancestor. The phylogenetic placement of these two genera correlates with the morphological comparison that is also presented in Fig. 5. Whereas *A. fuscus*, *P. bandonii* and *Tremella exigua* Desm. have clavate to cylindrical basidia with obliquely to transverse septa, *Bulleromyces albus* and three *Tremella* species of the Indecorata group *sensu* Chen 1998, namely *Tremella indecorata* Sommerf. Fr., *Tremella moriformis* (Fr.) Smith ex Berk. and *Tremella nivalis* C.-J. Chen, have subglobose to pyriform, longitudinally or obliquely cruciate-septate basidia. In Fig. 5, the clade for which basidial types are presented contains two well-supported monophyletic groups. One includes the species with a tremelloid basidial plan and the other includes *Auriculibuller* and *Papiliotrema*, which have clavate basidia with obliquely to transverse septation. This topology was also observed in maximum-parsimony analysis (data not shown), but the bootstrap values were generally lower than the *a posteriori* probability values of MCMC analyses. As can be observed in the illustrations that are included in Fig. 5, *T. exigua* shows

Fig. 4. Line drawings of yeast cells (Y), ballistoconidiogenic cells and ballistoconidia (B indicates released ballistoconidia) of *Bullera japonica* (PYCC 4534^T) observed on MYP agar after 4–7 days. Bar, 10 μm .

Fig. 5. Phylogenetic tree of *Auriculibuller fuscus*, *Bullera japonica* and related taxa of the Tremellales, inferred by using Bayesian MCMC analysis of an alignment of the D1/D2 region of 26S rDNA. Numbers on branches are estimates for a posteriori probabilities, i.e. probabilities that the respective groups are monophyletic given the alignment. The topology was rooted with *Filobasidium floriforme*, *Filobasidium uniguttulatum* and *Filobasidium capsuligenum*. Names in bold type correspond to organisms discussed in the comparison of basidial types and/or to novel taxa described in this report. GenBank accession numbers of sequences are indicated after strain numbers. Schematic illustrations of basidial types are inserted for *Auriculibuller*, *Bulleromyces*, *Papiliotrema* [redrawn from Sampaio *et al.* (2002)] and selected *Tremella* species [redrawn from Chen (1998)]; the basidial types of *Tremella moriformis* and *Tremella indecorata* are basically similar to that of *Tremella nivalis* and are not shown].

an intermediate basidial morphology. In the various MCMC analyses that were carried out during this study, the position of this species was found to vary and never received high statistical support. In the ITS tree (see Supplementary Figure, available at http://www.crem.fct.unl.pt/dimorphic_basidiomycetes/Databases/databases.htm), species with *Tremella*-type basidia were again well-separated from *Auriculibuller* and *Papiliotrema*, the latter being placed in the vicinity of the novel genus that is described in this report. The possible conspecificity between *A. fuscus* and *Bullera japonica*, which is suggested by pigment production, nutritional similarity and D1/D2 sequence data, was ruled out, based on greater sequence divergence of ITS data, absence of sexual compatibility and lack of high DNA–DNA reassociation values.

The unravelling of novel sexual taxa and mitosporic species, such as those described here, is essential to expand our assessment of the diversity of the Tremellales and allied taxa of the Tremellomycetidae. Accumulation of more data on the Tremellales by means of multi-disciplinary approaches will set the basis for a more accurate perspective on the evolutionary history of these organisms and will consequently contribute to improved classification proposals. Another issue in the systematics of the Tremellales is the genus concept for this group. The polyphyletic nature of *Tremella*, as depicted in Fig. 5, suggests that the tremelloid basidial type, the key character for the genus, needs to be combined with other markers for adequate generic differentiation. Molecular studies have also indicated that asexual genera, such as *Bullera* and *Cryptococcus* Vuillemin, are polyphyletic (Fell *et al.*, 2000). On the other hand, the same approaches have validated the generic concept of *Fibulobasidium* Bandoni and fostered the segregation of species from heterogeneous assemblages into natural groups, such as *Dioszegia* Zsolt emend. Takashima, Deak and Nakase (Takashima *et al.*, 2001). The proposal of *Auriculibuller* in this report follows this trend.

ACKNOWLEDGEMENTS

J. I. and M. G. were supported by grants PRAXIS/XXI/BD/19833 and SFRH/BD/1170/2000, respectively, and J. W. F. was supported by the USA National Science Foundation, Division of Environmental Biology (DEB 0206521). We thank Michael Weiß (University of Tübingen, Germany) for preparing the Latin diagnoses and for advice concerning the MCMC phylogenetic analyses.

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