ACTA MYCOLOGICA
Vol. 48 (1): 113–122
2013
DOI: 10.5586/am.2013.013

Battarrea phalloides in Macedonia: genetic variability, distribution and ecology

MARÍA P. MARTÍN[1], KATERINA RUSEVSKA[2], MARGARITA DUEÑAS[1] and MITKO KARADELEV[2]

[1]Departamento de Micología, Real Jardín Botánico-CSIC,
Plaza de Murillo 2, S-28014 Madrid, maripaz@rjb.csic.es
[2]Ss. Cyrl and Methodius University, Faculty of Natural Science and Mathematics
Institute of Biology Gazi Babad, bb, P.O. Box 162, MKD-1000 Skopje

Martín M.P, Rusevska K., Dueñas M., Karadelev M.: *Battarrea phalloides in Macedonia: genetic variability, distribution and ecology*. Acta Mycol. 48 (1): 113–122, 2013.

Morphological and molecular analyses of *Battarrea phalloides* from Macedonia were done. While *B. phalloides* specimens shown three kind of spore ornamentation, each one related to a clade in the phylogenetic ITS nrDNA tree; all specimens from Macedonia shown spores with anastomosing truncate ridges and very low variability of the ITS nrDNA sequences. The low genetic variability of these specimens, could be because of genetic drift.

Key words: Gasteromycetes, Basidiomycota, DNA barcoding, spore ornamentation, taxonomy, biodiversity hotspot

INTRODUCTION

Two species of the genus *Battarrea* Pers. (Basidiomicota, Agaricales) have been cited in Europe: *B. phalloides* (Dickson) Pers. and *B. stevenii* (Linbosch.) Fr. According to Fries (1832), the slender habit allowing to separate *B. phalloides* from *B. stevenii*. Hollós (1904) after examining specimens all over the world was the first to clearly defend the idea of a single polymorphic species. However, Maublanc and Malençon (1930), Cunningham (1944), Moravec (1958), Pegler et al. (1995), and Calonge (1998) emphasive the presence of a gelatinose volva in *B. phalloides* as the main character to distinguish this species from *B. stevenii*.

Martín and Johannesson (2000) analyzed 35 basidiomes from different geographical areas (Austria, Burundi, France, Hungary, Italy, Mexico and Spain) with regard to basidiome size, presence or absence of gelatinose hyphae in the volve, spore size and spore ornamentation; as well as, the internal transcribed spacer regions of the nuclear ribosomal DNA sequences (ITS nrDNA) and concluded that *B. phalloides*

specimens did not cluster together in a monophyletic clade, they appear in three different lineages mixed with *B. stevenii* specimens. The only feature related with each lineage was the spore ornamentation (under SEM): a) spores with anastomosing truncate ridges, b) spores finely verrucose and c) spores finely reticulate. Although not typus collections were analysed, Martín and Johannesson (2000)'s study support the idea of Hollós (1904): *B. phalloides* and *B. stevenii* belong to a single taxon.

Jeffries and McLain (2004) analyzed the collection (78 specimens) of *B. phalloides* and *B. stevenii* from the herbarium of the Kew Royal Botanic Gardens, and fresh specimens collected in Suffolk (UK), from the type locality of *B. phalloides*. Based on morphological and molecular analyses on single-strand conformation polymorphism (SSCP), as well as sequence comparison of the ITS nrDNA, these authors confirmed the results of Martín and Johannesson (2000): *B. phalloides* and *B. stevenii* are conspecific.

Battarrea phalloides [=*B. stevenii*] develops on dry, sandy localities, and it has a broad area of distribution; it is known from Africa, Asia, Australia, Europe and North and South America (Cunningham 1944; Bottomley 1948; Moreno et al. 1995; Watling et al. 1995; Calonge 1998; Martín, Johannesson 2000). Notwithstanding, the fact that it has wide distribution, it occurs only locally, namely individually or in a population of few specimens. In Europe it is a rare species, e.g. in Hungary and France is part of Natura 2000 (the network to assure the long-term survival of Europe's most valuable and threatened species and habitats). Moreover, *B. phalloides* is red listed in Armenia, Austria, the Czech Republic, France, Germany, Hungary, Poland, Spain, and the United Kingdom, protected by law in the United Kingdom and critically endangered in Germany.

In Macedonia, the first data for *B. phalloides* are from the monastery of St. Jovan Bigorski, in river Radika valley, a hornbeam forest, at 700 meters altitude, from 1924 (Lindtner 1931-1932); it is not found again. Another locality is Golem Grad Island (Prespa Lake), where the population grows in Greek juniper forest (Karadelev 2000). In the National Collection of Fungi there is a collection from the area of Lake Dojran from 1988 (under *B. stevenii*), and its habitat now is under strong anthropogenic pressure. The species is incorporated in the Preliminary Red List of Fungi of Macedonia, and it is categorized in the group of species existing only on threatened or rare habitats (Karadelev 2000).

Since information about the genetic diversity of an organism is fundamental to establish Biodiversity Action Plans (English Nature 2002); the main purpose of this work was to study the variability of the ITS nrDNA sequences from *B. phalloides* specimens collected in Macedonia, as well as to give more information about the distribution and ecology of this species.

MATERIAL AND METHODS

Taxon sampling and morphological studies. Fifty-five specimens were collected during 1987 to 2008 in 2 localities in Macedonia (Fig. 1) and are deposited in the Macedonian Collection of Fungi (MCF) at Mycological Laboratory, Institute of Biology, Faculty of Natural Sciences and Mathematics. Dried specimens were used for light

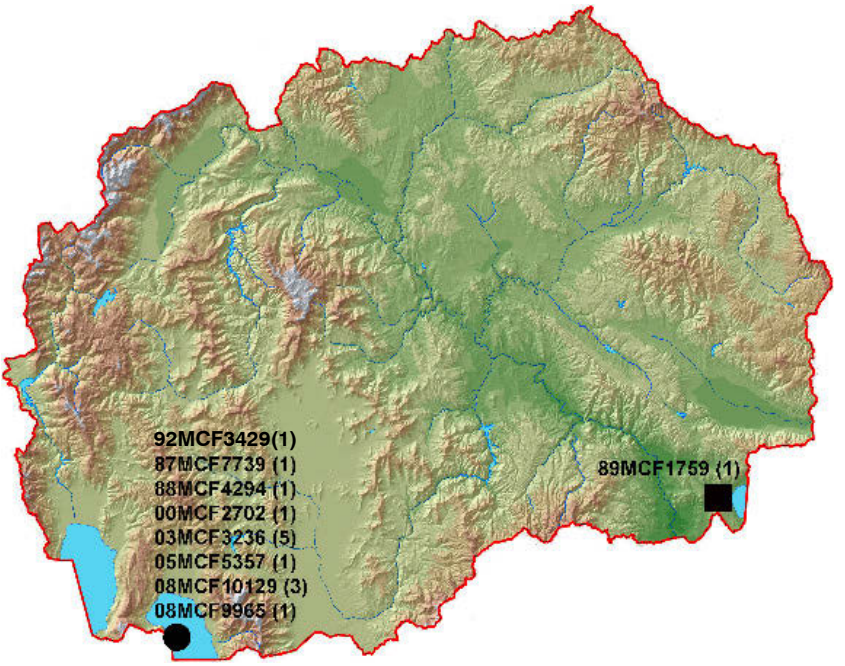

Fig. 1. Geographical positions of collections examined in this study. Herbarium MCF numbers of collections from each locality is included. The number of basidiomes in indicated between parenthesis. ● Island Golem Grad; ■ area of Lake Dojran.

microscope and SEM (scanning electron microscope) studies. Measurements and photographs were made from microscopic sections mounted in 5% KOH and examined at magnifications up to 1000X with an LW Scientific microscope. SEM was carried out after coating dried glebal samples in gold with Balzers SCD 004 sputter coater with a Hitachi S-3000N SEM.

DNA extraction and sequencing. Genomic DNA was extracted from dried basidiomes with a DNeasy Plant Mini Kit (QIAGEN, Ililden, Germany) following instructions of the manufacturers. Lysis buffer incubation was done overnight at 55°C. The DNA was isolated and processed during March 2010. To identify and prevent DNA contamination, the "reagent blank" (all reagents used during sample processing but lacking sample) control was included (Sundquist, Bessetti 2005). The primer pair ITS1F and ITS4 was used to obtain amplifications of both ITS regions, including the 5.8S of the ribosomal RNA gene cluster and small flanking parts of SSU and LSU genes (White et al. 1990; Gardes, Bruns 1993).

Individual reactions to a final volume of 25 µl were carried out using illustra™PuReTaq™Ready-To-Go™ PCR Beads (GR Healthcare, Buckinghamshire, UK) with a 10-pmol μl^{-1} primer concentration following the thermal cycling conditions used in Martín and Winka (2000). Negative controls lacking fungal DNA were run for each experiment to check for contamination. Results of amplifications were assayed from 5 µl aliquots by gel electrophoresis of 2% Pronadisa D-1 Agarose (Lab. Conda, Spain). Before sequencing the amplification products were cleaned with QIAquick gel PCR

purification kit (QAGEN, Valencia, California). When more than 20 ng/µl were obtained both strands were sequenced separately with primers mentioned above at MACROGEN, INC (Korea).

Sequencher 4.2 (Gene Codes, Ann Arbor, Michigan, USA) was used to obtain the consensus sequence from the two strands of the ITS nrDNA of each isolate. BLAST searches with MEGABLAST option were used to compare the sequences obtained against the sequences in the National Center of Biotechnology Information (NCBI) nucleotide databases (Altschul et al. 1997). The new consensus sequences have been lodged in the EMLB-EBI and UNITE databases.

Alignment and phylogenetic analyses. The ITS nrDNA sequences obtained were aligned separately using Se-Al v2.0a11 Carbon (Rambaut, 2002) for multiple sequences. Sequences obtained in this study were compared with homologous sequences mainly from Martín and Johannesson (2000), and Jeffries and McLain (2004). Where ambiguities in the alignment occurred, the alignment, generating the fewest potentially informative characters, was chosen. Alignment gaps were marked "–", unresolved nucleotides and unknown sequences were indicated with "N".

The polarity of characters was assessed with outgroup comparison, using *Tulostoma beccarianum* (AF097752) and *Chlamydopus meyenianus* (AF097756) as outgroup.

A maximum parsimony analysis (MP) was carried out; minimum length Fitch trees were constructed using heuristic searches with tree-bisection-reconnection (TBR) branch swapping, collapsing branches if maximum length was zero and with the MulTrees option on in PAUP*4.0b10 (Swofford 2003). Gaps were treated as missing data. Nonparametric bootstrap support (Felsenstein 1985) for each clade, based on 10,000 replicates using the fast-step option, was tested. The consistency index, CI (Kluge, Farris 1969), retention index, RI (Farris 1989), and rescaled consistency index, RC (Farris 1989), were obtained.

A second analysis was done by Bayesian approach (Larget, Simon 1999; Huelsenbeck et al. 2001) using MrBayes 3.1 (Ronquist, Huelsenbeck 2003). The analysis was performed assuming the general time reversible model (Rodríguez et al., 1990) including estimation of invariant sites and assuming a HKY + I model as suggested by hierarchical likelihood ratio test in MrModeltest 2.3 (Nylander 2004). According to Rodríguez et al. (1990) only reversible models allow the calculation of the substitution rates. Two independent and simultaneous analyses starting from different random trees were run for 2.000.000 generations with four parallel chains and trees and model scores saved every 100th generation. The default priors in MrBayes were used in the analysis. Every 1.000th generation tree from the two runs was sampled to measure the similarities between them and to determine the level of convergence of the two runs. The potential scale reduction factor (PSRF) was used as a convergence diagnostic and the first 25% of the trees were discarded as burn-in before stationary was reached. Both the 50% majority-rule consensus tree and the posterior probability of the nodes were calculated from the remaining trees with MrBayes. Phylogenetic trees were drawn using TreeView (Page 1996).

RESULTS

During 21 years of survey, 15 *Battarrea* specimens were collected and they are stored at the MCF Herbarium with the numbers 87MCF7739, 88MCF4294, 89MCF1759, 92MCF3429, 00MCF2702, 03MCF3236, 05MCF5357, 08MCF9965 and 08MCF10129 were analyzed by molecular approach. In Figure 1, the collection and the number of basidiomes to each locality is indicated. Only in collection 89MCF1759, specimen with robust basidioma, up to 35 cm high, was located (Fig. 2 morphotype or habit "*B. stevenii*"), the rest of specimens show slender habit (Fig. 3). Under SEM, the spore ornamentation of all Macedonian specimens appears with anastomosing truncate ridges (Figs 4 and 5).

The ITS region was successfully amplified and sequenced from dried basidiomes using ITS1F/ITS4 primer pair, except from collection 92MCF3429. The total length of the *Battarrea* sequences, including the ITS1, 5.8S and ITS2 genes and small flanking regions of SSU and LSU genes, was 630 base pairs (bp). In general, after purification of the amplified product, if the DNA concentration was greater than to 20 ng/µl, the sample was sequenced directly. Among the complete sequences, the nucleotides differences were 2 bp in ITS1 region due to 1 transition and 1 transversion, and

Fig. 2. Basidiomes of *Battarrea phalloides,* collection 89MCF1759, robust "*B. stevenii*" habit. Scale bar = 15 cm.

Fig. 3. Basidiomes of *Battarrea phalloides,* collection 00MCF2702, slender "*B. phalloides*" habit. Scale bar = 10 cm.

11 in ITS2 region due to 4 deletions, 3 transitions and 4 transversions. The eight new sequences obtained in this study have been submitted to the international database (EMBL) with numbers HF913778-HF913785.

The new sequences of *Battarrea* were aligned with 26 sequences from GenBank. The ITS nrDNA dataset contained 34 taxa and 710 aligned characters with a variable region of 66 base pairs (excluded in the phylogenetic analyses). Out of the total 810 positions, 533 were constant, 123 variable parsimony-uninformative and 154 parsimony informative. In the phylogenetic analysis under heuristic search, 100 most parsimonious trees (MPTs) were obtained (tree length = 375 steps long, consistency index CI=0.8453, CI excluding uninformative characters = 0.7603, retention index RI=0.9430, rescaled consistency index RC=0.9972). The 50% majority-rule tree of the Bayesian analysis (Fig. 6) has similar topology that the parsimony strict consensus tree (data not shown); three main clades are resolved. The clades are indicated as A, B and C, according to the spore ornamentation mentioned in Martín and Johannesson (2000): clade A with anastomosing truncate ridges, clade B finely verrucose and clade C finely reticulate.

All sequences from Macedonia specimens group together in clade A with sequences from Israel, Hungary, Kenya and Spain specimens. This clade is highly supported (bs> 97%; pp= 1.0). From clade B, two strongly supported subclades (bs=100%; pp=1.0) are resolved, one (B1) constituted by collections from Spain (including a specimen from Balearic Island) and Hungary, and another (B2) which includes specimens from Burundi, Israel and Cyprus, as well as the specimen from the typus locality of *B. phalloides* in Great Britain. In clade C, all sequences belong to specimens from Spain (including one from Canaria Islands) and Kenya.

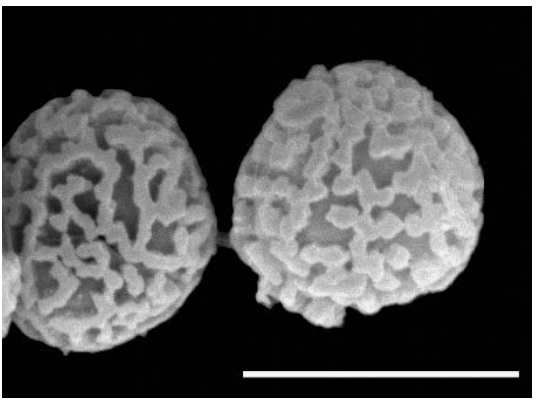

Fig. 4. *Battarrea phalloides.* SEM images of basidiospore ornamentation, collection 89MCF1759 with robust "*B. stevenii*" habit. Scale bar = 5 μm.

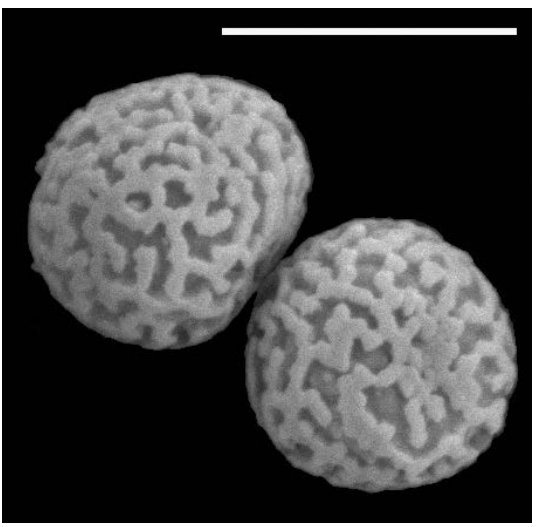

Fig. 5. *Battarrea phalloides.* SEM images of basidiospore ornamentation, collection 08MCF9965 with slender "*B. phalloides*" habit. Scale bar = 5 μm.

DISCUSSION

Although Macedonia is part of the Mediterranean basin biodiversity hotspot, the third richest biodiversity hotspot in the world, the *Battarrea* specimens shown very low genetic variability, comparing with other countries, such as Spain.

Battarrea collections from Macedonia originate from two localities, Island Golem Grad and vicinity of Lake Dojran. Both of them are situated in the south part of country, first in south-west and the second in south-east.

Island Golem Grad (also known as a Snake Island) is non-populated area of 18 hectares located in Lake Prspa, at about 1000 m altitude, a few kilometres from Greek and Albanian territory. The entire island is encircled by 20 to 30 meter high rocks. The Greek or Crimean juniper (*Juniperus excelsa* M. Bieb.) is the dominant forest species on the island, and it forms a well-developed and old forest type. The prevailing forest community on the island is *Biaro tenuifoliae-Juniperetum excelsae*, commonly known as Greek juniper forest. Several rare plant species in Macedonia (*Biarum tenuifolium*

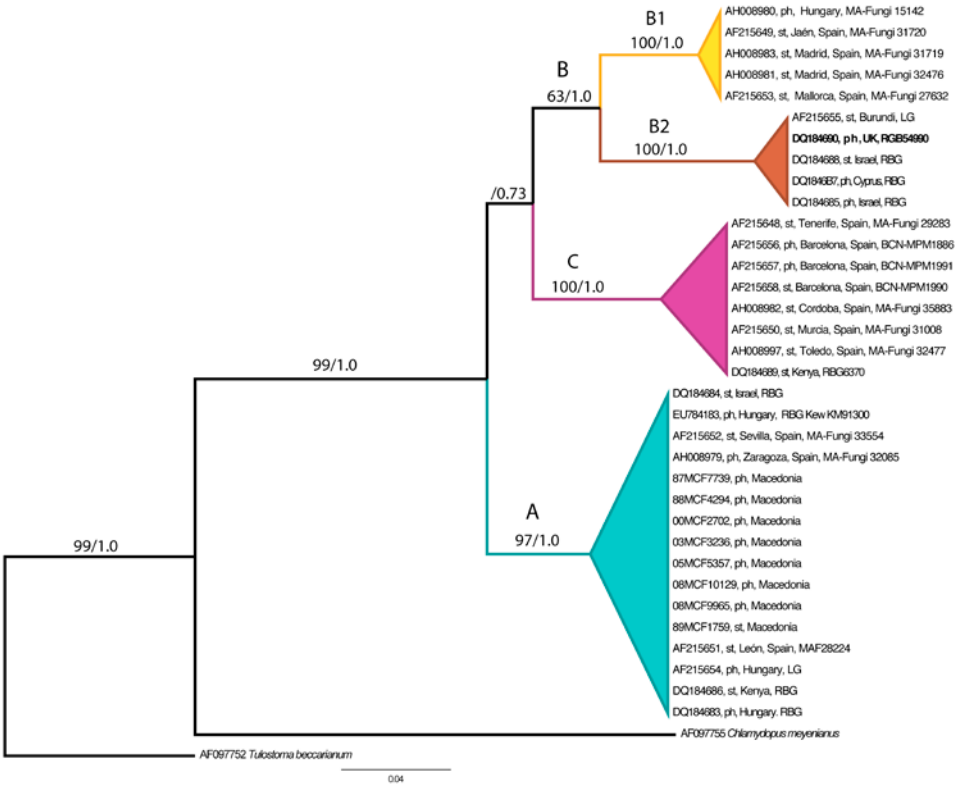

Fig. 6. The 50% majority-rule consensus tree of Bayesian analysis inferred from ITS nrDNA sequences of *Battarrea phalloides* specimens from Macedonia (indicates with the MCF herbarium number), as sequences retrieved from the GenBank. Bootstrap and posterior probabilities values are indicated on the branches. The morphotypes or habits "*B. phalloides*" and "*B. stevenii*" are indicated as "ph" and "st" after the herbarium MCF number or the sequence accession number from the GenBank. In black the sequence of a specimen from the type locality of *B. phalloides*. Names clades (A, B and C) related to the spore ornamentation mentioned in Martín and Johannesson (2000).

(L.) Schott., *Asterolinon linum-stellatum* (L.) Duby, *Celtis glabrata* Stev, *Lilium candidum* L.) have been found only in the Greek juniper forest on Golem Grad Island. The distribution area of Greek juniper reaches from Iran and Lebanon through Asia Minor, Crimea up to the Balkan Peninsula, and Macedonia lies in its eastern and northern borders of distribution. Nowadays, Golem Grad is the only locality where *Battarrea* is found in Macedonia, because in the other not more basidiomes are found recently; the assumptions are that there are more than a hundred specimens growing in a small patch on the island. This fungus grows in Greek juniper forest on sandy soil with a large concentration of guano from cormorants, whose bird colony inhabits and nests on the island. These unique conditions seem very favourable for development of the species, which is the reason why it occurs in a relatively high number.

Unlike the island Golem Grad the area of Lake Dojran, as a populated area has completely different ecological characters. The precise locality of *Battarrea*

collection is the tourist camp Achikot which lies at 140 m altitude. This locality belongs to the sub-mediterranean climate zone. It is represented mainly by the Mediterranean pseudomacchia forest with domination of *Quercus coccifera* L. Among with *Phillyrea media* L., *Quercus pubescens* Willd. and *Carpinus orientalis* Mill. Only one sample was collected from this locality which grows on sandy soil, near the lake, under planted willow tree. The data from this locality is not confirmed after its first finding.

Concerning above mentioned ecological features of two localities of *Battarrea* in Macedonia it could be concluded that macroscopic characteristic (size of the basidioma) are in correlation with the ecology of the habitat, as is mentioned in Maublanc and Malençon (1930) that slender "*B. phalloides*" habit is confined to northern, cooler and humid regions (like Island Golem Grad in our case), and the robust "*B. stevenii*" habit to the southern, subtropical, hot and dry regions (like area of Lake Dojran in presented study).

To conclude, the low genetic variability of *B. phalloides* specimens from Macedonia could be because of genetic drift, but more specimens and other molecular methods should be analyzed.

Acknowledgments. This research received support from the SYNTHESYS Project http://www.synthesys.info/ which is financed by European Community Research Infrastructure Action under the FP7 "Capacities" Program.

REFERENCES

Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389-3402.

Bottomley A.M. 1948. Gasteromycetes of South Africa. Bothalia 4: 473-810.

Calonge F.D. 1998. Gasteromycetes, I. Lycoperdales, Nidulariales, Phallales, Sclerodermatales, Tulostomatales. Flora Mycologica Iberica. 3. J. Cramer, 271 pp.

Cunningham G.H. 1944. The Gasteromycetes of Australia and New Zealand. New Zealand, Dunedin.

English Nature 2002. Project synopsis, Biodiversity Programme Research Project-Sandy stilt puffball *Battarrea phalloides*. Colchester: English Nature.

Farris J.S. 1989. The retention index and the rescaled consistency index. Cladistics 5: 417-419.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783-793.

Fries N. 1832. *Battarrea stevenii* (Lib.) Fries. Syst. Mycol. 3: 7.

Gardes M., Bruns T.D. 1993. ITS Primers with enhanced specificity for Basidomycetes—applications to the identification of mycorrhizae and rusts. Mol. Ecol. 1: 113-118.

Hollós L. 1904. *Die Gasteromyceten Ungarns*. 278. XXIV. Tab. Leipzig (part of series: Gasteromycetes Hungariae, cum tabulus 31).

Huelsenbeck J.P., Ronquist F., Nielsen R., Bollback J.P. 2001. Bayesian inference of phylogeny and its impact on evolutionary biology. Science 294: 2310-2314.

Jeffries P., McLain L. 2004. Synonymy between *Battarrea phalloides* and *B. stevenii*. English Nature Research Reports. Report Number 625.

Karadelev M. 2000. Preliminary Red List of Macrofungi in the Republic of Macedonia. European Council of Conservation of Fungi, Newsletter 10: 7-11.

Kluge A.G., Farris J.S. 1969. Quantitative phyletics and the evolution of anurans. Systematic Zoology 18: 1-32.

Larget B., Simon D.L. 1999. Markov chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. Mol. Biol. Evol 16: 750-759.

Lindtner V. 1931–1932. *Battarrea phalloides* (Dicks.) Pers. aus Südserbien. Extrait du Bulletin de l'Institut et du Jardin Botaniques de l'Université de Beograd, 2 (1/2): 104-105.

Martín M.P., Johannesson H. 2000. *Battarrea phalloides* and *B. stevenii,* insight into a longstanding taxonomic puzzle. Mycotaxon 76: 67-75.

Martín M.P., Winka K. 2000. Alternative methods of extracting and amplifying DNA from lichens. Lichenologist 32: 189-196. http://dx.doi.org/10.1006%2Flich.1999.0254

Maublanc A., Malençon G. 1930. Rechercher sur le *Battarraea guicciardiana* Ces. Bull. Soc. Mycol. Fr. 46: 43-73.

Moravec Z. 1958. Tulostomataceae. (In:) A. Pilàt (ed.). Flora ČSR. Praha 585-626 pp.

Moreno G., Altés A., Ochoa C., Wright J.E. 1995. Contribution to the study of the Tulostomataceae in Baja California, Mexico. I. Mycologia 87: 96–120. http://dx.doi.org/10.2307%2F3760953

Nylander J.A.A. 2004. MrModeltest v2. Uppsala, Sweden: Evolutionary Biology Center, Uppsala University. Program distributed by author.

Page R.D.H. 1996. TreeView: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosciences 2: 357-358.

Pegler D.N., Laessoe T., Spooner B.M. 1995. Bristish puffballs, Earthstars and stinkhorns, an account of the British gasteroid fungi. Kew: Royal Botanic Gardens.

Rambaut A. 2002. Se-Al: sequences alignment editor v2.0a11. Edinburgh: Institute of Evolutionary Biology, University of Edinburgh. http://tree.bio.ed.ac.uk/software/

Rodríguez F., Oliver J.F., Martin A., Medina J.R. 1990. The general stochastic model of nucleotide substitution. J. Theor. Biol. 142: 485-501.

Ronquist F., Huelsenbeck J.P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixedmodels. Bioinformatics 19: 1572-1574. http://dx.doi.org/10.1093%2Fbioinformatics%2Fbtg180

Sundquist T., Bessetti J. 2005. Identifying and preventing DNA contamination in a DNA-Typing Laboratory. Profiles in DNA. September: 11-13.

Swofford D.L. 2003. PAUP*4.0: phylogenetic analysis using parsimony (*and other methods). Sunderland, Massachusetts: Sinauer Associates.

Watling R., Gücin F., Isiloglu M. 1995. *Battarraea phalloides* – its history, biology and extention to its distribution. Nova Hedwigia 60 (1/2): 13-18.

White T.J., Bruns T., Lee S., Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. (In:) M.A. Innes, D.H. Gelfand, J.J Sninsky, T.J White (eds). PCR protocols: a guide to methods and applications. San Diego, California: Academic Press: 315-322.