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Phylogenetic position of *Battarrea japonica* (Kawam.) Otani

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Abstract Based on parsimony analyses of partial sequences of ribosomal DNA, we suggest that the fungus *Battarrea japonica* should be placed in the Ascomycota, Eurotiales, rather than in the Basidiomycota, Tulostomatales, as previously supposed. Additionally, sequence data suggest that *B. japonica* is closely related to *Pseudotulostoma volvata*, a taxon recently described as a member of the Elaphomycetaceae.

Key words : *Battarrea japonica*, Eurotiales, phylogeny, *Pseudotulostoma*

Introduction

Battarrea japonica (Kawam.) Otani, known in Japanese as "Kobo-fude," was first described as *Dictyocephalos japonicus* Kawamura by Kawamura (1954) , and was later transferred to the genus *Battarrea* Pers. by Otani (1960). This species had historically been placed in the Basidiomycota, Tulostomatales, however, because Otani (1960) could not observe its basidia at the time of his studies, he was unable to accurately determine its taxonomic position. Asai *et al.* (2004) recently conducted detailed observations of *B. japonica* including its immature fruit-bodies, and showed that the fungus is characterized by its stalked fruit-body, exposed apical gleba, and woody volvate base, and particularly in its unopened fruit-bodies, which have asci, a thick-walled peridium, and ornamented ascospores. These characteristics suggest that *B. japonica* belongs to Ascomycota, however, its phylogenetic position at the ordinal level is uncertain. Indeed, stalked ascomata are also found in several taxa in the Eurotiales and Onygenales, and the development of unopened ascomata is similar to that found in the hypogeous taxa of the Pezizales or Eurotiales. Thus, the following four hypotheses were considered: *B. japonica* is placed 1) in the Tulostomatales, Basidiomycota; 2) in the Pezizales, Ascomycota; 3) in the Eurotiales, Ascomycota; or 4) in the Onygenales, Ascomycota.

In the present study, we aimed to clarify the phylogenetic position of *B. japonica* and tested the above-mentioned hypotheses. We also discuss the taxonomic treatment of *B. japonica*.

Materials and Methods

Samples

Two samples of *B. japonica* were used in the present study. The first was collected by M. Taniguchi on 26 September, 2003 in Miyakawa, Mie Prefecture, and the second by H. Sato and T. Nara in Kawauchi, Fukushima Prefecture on 27 September, 2003. The samples included both mature and immature fruit-bodies. We accept the evidence of Asai *et al.* (2004) that asci are present in the fruit-bodies of *B. japonica*. Examples of the samples used in this study were deposited in the herbarium of the National Science Museum (TNS-F11151, TNS-F11152).

DNA extraction

For the extraction of DNA, core tissues of fructifications were scooped out and ground to a fine powder under liquid nitrogen. Their DNA

was then extracted with a DNeasy Plant Mini Kit (Qiagen, Inc., Valencia, CA, USA) following the instructions provided by the manufacturer. The small subunit ribosomal DNA (SSU rDNA) was amplified with the primer pairs NS1, NS4, NS5, NS6, NS7 and NS8 (White *et al.*, 1990). The 5′ terminal end of the large subunit (LSU) rDNA gene containing D1/D2 regions was amplified with the primer pairs NL1 and NL4 (O'Donnell, 1993). The components for the polymerase chain reaction (PCR) were used per the manufacturer's instructions for KOD-Plus DNA Polymerase (Toyobo Co., Ltd., Osaka, Japan). The PCR program was 2 min at 94°C (initial denaturation); 30 cycles of 15 s at 94°C, 30 s at 52 to 54°C, and 1 min at 68°C. PCR products were visualized on an electrophoresis gel after etidium bromide staining, and target bands were purified with a DNeasy Kit and used directly for sequencing with a Big Dye Terminator Cycle Sequencing FS Ready Reaction Kit (ver. 3.0) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The standard conditions recommended by the vendor were used for the sequencing. The obtained sequences have been deposited in the DNA Data Bank of Japan (DDBJ) under the following numbers: AB161193 to AB161196.

DNA analysis

The sequences of *B. japonica* were analyzed against sequences deposited in the GenBank database. Taxon sampling was carried out for those species hypothetically related to *B. japonica*. Data sets including the new sequences were prepared for both LSU rDNA and SSU rDNA, and were aligned using ClustalX, version 1.81 (Thompson *et al.*, 1997) in order to clarify the phylogenetic position of *B. japonica*. The obtained sequence alignments were manually adjusted using the program BioEdit, version 5.0.9 (Hall, 1999), and were analyzed using the program PAUP*4.0 beta 10 (Swofford, 2003). A parsimony analysis was also carried out using a heuristic search with the random stepwise addition and tree-bisection-reconnection (TBR) options of the program. Gaps were treated as missing data. All characters were equally weighted. Bootstrap values (1000 replicates) were also calculated.

Results and Discussion

Approximately 560 bp were amplified with NL1 and NL4 for LSU rDNA partial sequencing. Overall, the aligned dataset (579 bp) for the LSU rDNA D1/D2 region included 36 sequences, including the sequence data produced in the present study. Parsimony analysis of the LSU-D1/D2 region clearly indicates that *B. japonica* should be placed in the Ascomycota rather than in the Basidiomycota (bootstrap value 93%). Our analysis additionally suggests that this fungus has evolutionary origins within the Eurotiomycetes (Fig. 1, bootstrap value 96%). Furthermore, the fungus was found not to be related to other hypogeous genera in the Pezizales (*Tuber* F. H. Wigg. or *Terfezia* (Tul. et C. Tul.) Tul. et C. Tul.) and the hypotheses that *B. japonica* is related to the species Tulostomatales or Pezizales were therefore rejected. Out of a total of 579 characters, 247 characters were constant, 70 variable characters were parsimony-uninformative and 262 were informative. A heuristic search found 12 most parsimonious trees. The tree length was 1089, The Consistency Index (CI) was 0.5051, the Homoplasy Index (HI) was 0.4949, and the Retention Index (RI) was 0.7121.

A total of approximately 1800 bp were amplified with the primers NS1, NS4, NS5, NS6, NS7 and NS8 for SSU in rDNA. Overall, the aligned dataset of 1840 bp included 34 sequences, including the sequence data produced in this study. Parsimony analysis of SSU rDNA found that *B. japonica* should be placed in the Eurotiales (bootstrap value 88%), and that it is most closely related to *Pseudotulostoma volvata* (Fig. 2) (bootstrap value 96%) and was never evolutionarily related to other fungi with stalked ascomata in the Eurotiomycetes, *Onygena equina* (Willd.) Pers., *Penicilliopsis clavariiformis* Solms-Laubach or *Trichocoma paradoxa* Junghuhn (Fig. 2). Out of

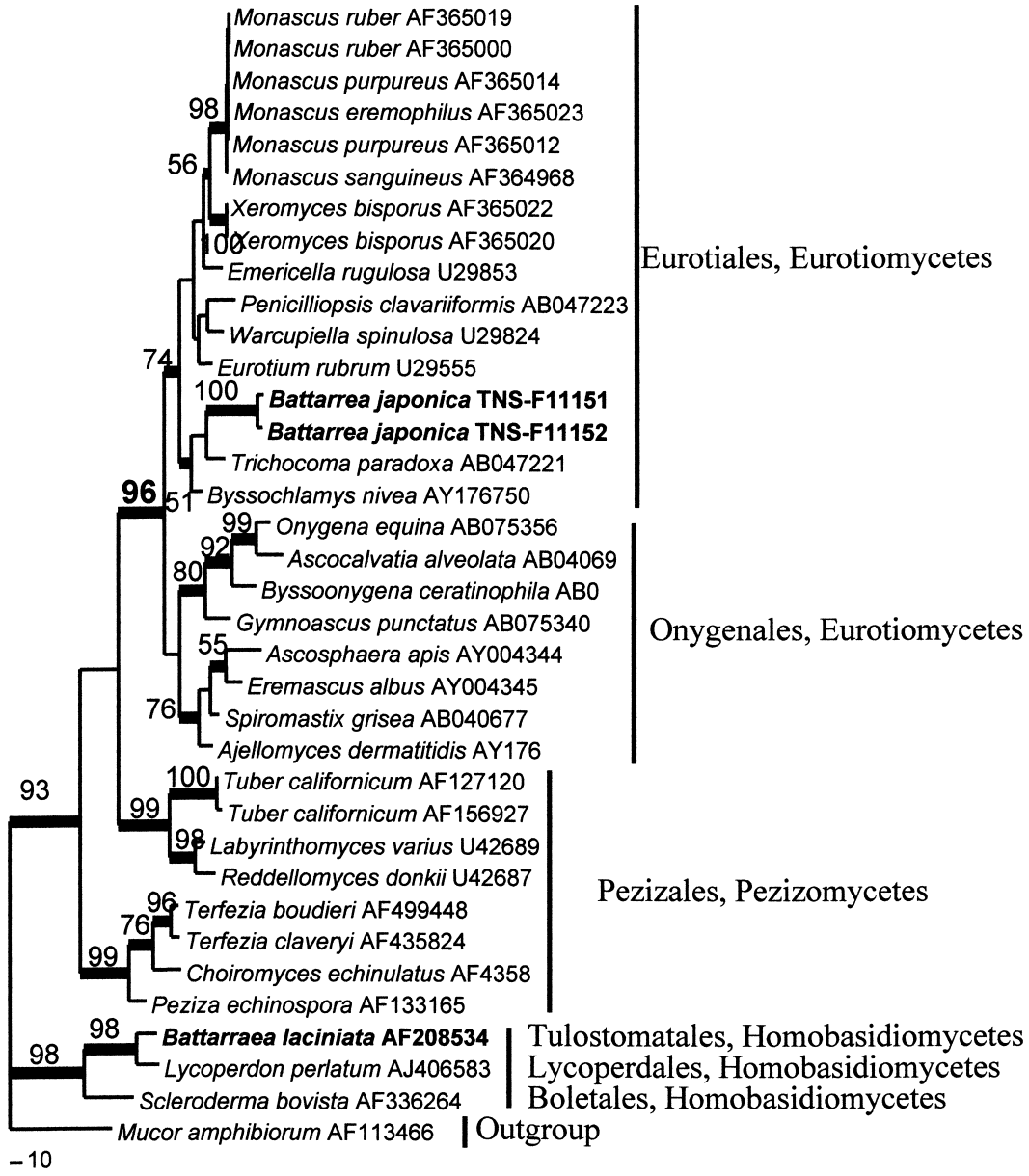

Fig. 1. One of the 12 most parsimonious trees of 36 fungal taxa hypothetically related to *Battarrea japonica* based on 579 characters, including gaps, of the 5′ terminal of LSU rDNA including the D1/D2 region. Out of a total of 579 characters, 247 characters were constant, 70 variable characters were parsimony-uninformative and 262 were informative. A heuristic search found 12 most parsimonious trees. The tree length was 1089, the Consistency Index (CI) was 0.5051, the Homoplasy Index (HI) was 0.4949 and the Retention Index (RI) was 0.7121.

a total of 1840 characters, 1564 characters were constant, 126 variable characters were parsimony-uninformative and 150 were informative. A heuristic search found 59 most parsimonious trees. The tree length was 508, CI was 0.6260, HI was 0.3740 and RI was 0.6979.

Thus, the evidence is conclusive that *B. japonica* cannot be treated as a member of *Battarrea*,

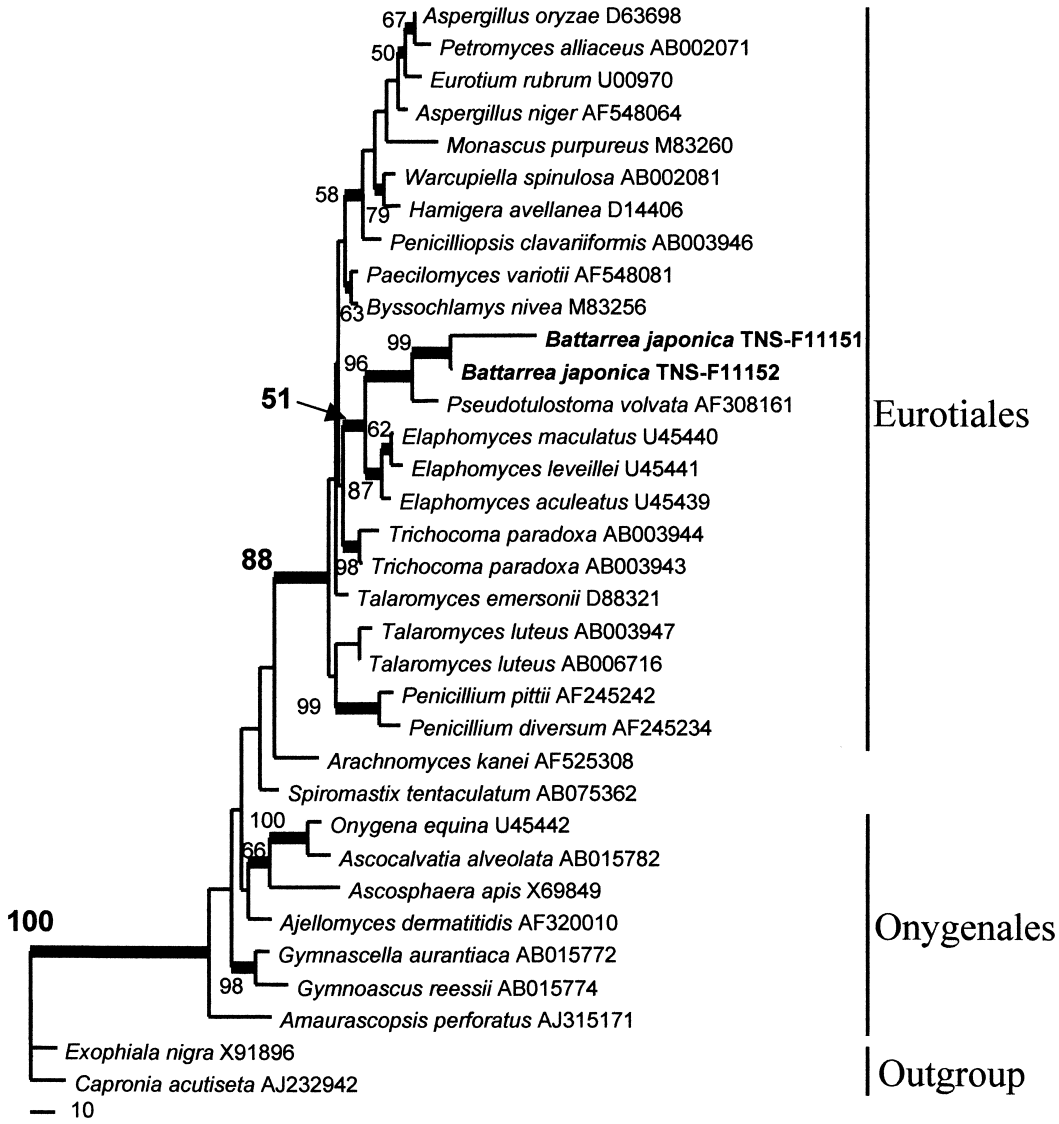

Fig. 2. One of the 59 most parsimonious trees of 34 fungal taxa mainly belonging to Eurotiomycetes based on 1840 characters, including gaps, of SSU rDNA operons. Out of a total of 1840 characters, 1564 characters were constant, 126 variable characters were parsimony-uninformative and 150 were informative. A heuristic search found 59 most parsimonious trees. The tree length was 508, CI was 0.6260, HI was 0.3740 and RI was 0.6979.

Battarreaceae, Tulostomatales, Basidiomycota; molecular data from the present study show that this species should be placed in the Ascomycota, Eurotiales, and that *Pseudotulostoma volvata* O. K. Mill. *et* T. Henkel in particular may be closely related. Asai *et al.* (2004) compared the morphology of *B. japonica* to the description of the genus *Pseudotulostoma*, and found that the characteristics of *B. japonica* are well fitted to the original description of this genus; nevertheless, *B. japonica* and *P. volvata* can be clearly distinguished from each other by their stipe length (*B. japonica*: 85–145 mm, *P. volvata*: 35–71 mm).

The genus *Pseudotulostoma* O. K. Mill. *et* T. Henkel was recently established based on the monotypic species, *P. volvata*, described by

Miller *et al.* (2001), who propose that *P. volvata* be placed in the Elaphomycetaceae. However, our analysis did not find in *B. japonica* the robust independent cluster typical of the Elaphomycetaceae including *P. volvata* (bootstrap value 51%, Fig. 2). Miller *et al.* (2001) also report that Elaphomycetaceous linkage including *P. volvata* was supported with a relatively low bootstrap value (58%), but emphasize the fact that the unopened fruit-bodies of *P. volvata* are highly similar to those of *Elaphomyces*. At present, we accept their treatment of the Elaphomycetaceae with the caveat that more detailed molecular study may require reevaluation of our concept of the Elaphomycetaceae.

Pseudotulostoma volvata is placed in the Ascomycota despite the fact that its asci are not observed. Asai *et al.* (2004) report that the asci of *B. japonica* can be found only when its fruit-bodies are immature. The species *Elaphomyces*, which is related to *Pseudotulostoma*, also has evanescent asci. Evanescent asci are thus considered to be a common development among the Elaphomycetaceous genera. Additionally, the fact that the unopened ascomata of *B. japonica* is highly similar to the fruit-body found in the genus *Elaphomyces* suggests that this species, which we believe should be treated as *Pseudotulostoma*, may also exist as a species of *Elaphomyces*.

Species of the genus *Elaphomyces* related to *Pseudotulostoma* are well known to form ectomycorrhiza with trees (Trappe, 1962; Miller and Miller, 1984). Miller *et al.* (2001) suggest that *P. volvata* may be also ectomycorrhizal because of the apparent restriction of the species to stands dominated by ectomycorrhizal *Dicymbe* Benth. trees and its placement in the ectotrophic Elaphomycetaceae. Although the nutritional habitat of *B. japonica* is of uncertain statute, because of the phylogenetic relatedness of *B. japonica* to *P. volvata*, we may find its mycorrhizal status with further study.

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