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Note

Based on DNA sequences of ITS and *rpb2*, *Amylostereum orientale* is reported for the first time in Japan

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ABSTRACT

Amylostereum laevigatum has been reported from Japan growing on *Cryptomeria japonica* and *Chamaecyparis obtusa*. Using morphological and molecular data (internal transcribed spacer (ITS) nrDNA and *rpb2* markers), we demonstrate that some specimens under *A. laevigatum* from Japan belong to *A. orientale*, a species described from China. *Amylostereum orientale* differs from *A. laevigatum* mainly in having smaller basidiospores. This is the first citation of *A. orientale* in Japan.

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Amylostereum Boidin (Russulales, Basidiomycota) is a genus of corticioid fungi mainly growing on conifers, with resupinate to reflexed basidiomes, and characterized by lamprocystidia and narrowly cylindrical to ellipsoid, smooth and amyloid basidiospores (Eriksson and Ryvarden 1973; Boidin and Lanquetin 1984; Telleria and Melo 1995). According to MycoBank (<http://www.mycobank.org>), five species are included in this genus: *A. areolatum* (Fr.) Boidin, *A. chailletii* (Pers.) Boidin (the type species), *A. ferreum* (Berk. & M.A. Curtis) Boidin & Lanq., *A. laevigatum* (Fr.) Boidin, and *A. sacratum* (G. Cunn.)

Burds. (= *Gloeopeniophorella sacrata* (G. Cunn.) Hjorstam & Ryvarden); and recently, He and Li (2013) described a new species from China, *A. orientale* S.H. He & Hai J. Li. As mentioned in Slippers et al. (2003), for many years, *A. areolatum* and *A. chailletii* were known to establish obligate mutualistic relationships with woodwasps of the genus *Sirex*; Tabata and Abe (1997) showed that specimens of *A. laevigatum* from Japan establish this relationship with the woodwasps *Urocera antennatus* Marlatt and *U. japonicus* Smith, effectively inoculating new wood (Slippers et al. 2003).

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Amylostereum laevigatum has been reported in Japan (Tabata and Abe 1997) growing on *Cryptomeria japonica* D. Don and *Chamaecyparis obtusa* Sieb. & Zucc. (Tabata et al. 2000). This species was originally described from Sweden (Fries 1828), where it is widespread (Eriksson and Ryvarden 1973; Legon 2005). It has been reported from Macaronesia: Madeira (Telleria et al. 2008), and the Azores islands (Dennis et al. 1977; Telleria et al. 2009a,b); as well as from North America (Ginns and Lefebvre 1993), and China (Maekawa et al. 2002).

Based on analysis of the internal transcribed spacer (ITS) region of nrDNA, the barcode for fungi (Schoch et al. 2012), as well as on the manganese-dependent peroxidase gene, Tabata et al. (2000) showed that specimens of *A. laevigatum* from Japan and *A. laevigatum* from France grouped separately; also, they reported differences in the spore size and in their hosts. Tabata et al. (2000), without providing spore dimensions, noted that specimens from Japan present smaller basidiospores than those from France. In the description of *A. orientale*, He and Li (2013) reported the spore size of this species as smaller than *A. laevigatum* from Europe, but they did not include specimens from Japan to confirm the presence of *A. orientale* in that country.

Therefore, the aim of this research was to clarify the identity of *Amylostereum laevigatum* from Japan using a barcoding approach (Schoch et al. 2012), and to provide an accurate revision of the morphological characters.

Specimens examined: JAPAN, Ochidani Park, Tottori City, Tottori Pref., on unidentified wood, 2 Jul 2008, leg. H. Suhara, TUMH61905 (TUMH); JAPAN, Nankan-machi, Tamana-gun, Kumamoto Pref., on *Cryptomeria japonica*, Jun 2004, leg. N. Maekawa, TUMH61916 (TUMH).

Cultures examined: JAPAN, Ochidani Park, 2 Jul 2008, leg. H. Suhara, TUFC11625 (isolated from TUMH61905; Tottori University); JAPAN, Nankan-machi, Jun 2004, leg. N. Maekawa, TUFC12106 (isolated from TUMH61916; Tottori University); JAPAN, without locality, cultures B1361 and B1362 isolated from *Urococcus japonicus* mycelium (Iowa State University); JAPAN, Motoyama, culture B1368 isolated from basidiospores obtained from basidiome growing on *Cryptomeria japonica*; JAPAN, B1369 from *Chamaecyparis obtusa* (Iowa State University). [Data from B1361, B1362, and B1369 as indicated in Tabata et al. (2000), and provided by T. Harrington. Data from B1368 as indicated in Tabata and Abe (1999)].

The genomic DNA from cultures B1361, B1362, B1368, B1369 was extracted using the FTA® Indicating Micro Cards WB120211 (Whatman, Maidstone, England), following Telleria et al. (2014). The ITS nrDNA and *rpb2* markers were amplified using Illustra™ PureTaq™ Ready-To-Go™ PCR Beads (GE Healthcare, Buckinghamshire, UK). The ITS nrDNA was amplified with the primer pair ITS1/ITS4 (White et al. 1990; Liu et al. 1999), following thermal cycling conditions described in Martín and Winka (2000). To amplify and sequence ribosomal polymerase two, subunit two (*rpb2*), regions 5–7, nested-PCR was performed with the following combination of primers: the primer pair RPB2-5F/RPB2-7.1R (Liu et al. 1999; Matheny et al. 2007) was used in the first amplification, and the pair RPB2-6F/RPB2-7.2R (Matheny et al. 2007) in the second one, using 1 µL of the first amplification as target DNA. The PCR products were purified using the QIAquick Gel Extraction (Qiagen, Hilden, Germany) as indicated by the manufacturer.

All these protocols were carried out at the Real Jardín Botánico, RJB-CSIC (Madrid, Spain). Purified amplicons were sent to Macrogen (Belgium/South Korea) for sequencing.

From cultures TUFC12106 and TUFC11625, the genomic DNA was isolated following Hosaka and Castellano (2008). The ITS nrDNA and *rpb2* markers were amplified using dNTPs, KOD FX Neo (TOYOBO Co., Ltd., Osaka, Japan) and PCR Buffer for KOD FX Neo. The ITS nrDNA was amplified using the primer pair ITS5/ITS4 (White et al. 1990), following a PCR program with an initial denaturation at 94 °C (1 min) was followed by 35 cycles of 94 °C (15 s), 55 °C (30 s) and 72 °C (1 min), and final extension at 72 °C (5 min). The *rpb2* was amplified as indicated above. The PCR products were purified using the MonoFas DNA Purification Kit (GL Sciences, Tokyo, Japan), and sequenced with ABI 3130 DNA sequencer (Applied Biosystems Inc., California, USA) in Tottori University.

Consensus sequences were assembled using Geneious Pro v9 (<http://www.geneious.com>; Kearse et al. 2012). Prior to the alignments, sequences generated in the present research were compared with homologous sequences in EMBL/GenBank/DDBJ (Cochrane et al. 2011) using the BLASTn algorithm (Altschul et al. 1997) to check for contamination. Two alignments, one to each marker, were generated including the new sequences and selected sequences from *Amylostereum* species from EMBL/GenBank/DDBJ. In both alignments, *Echinodontium tinctorium* (Ellis & Everh.) Ellis & Everh. sequences were included as outgroup, since in Tabata et al. (2000), the ITS sequence of this species, as well as other *Echinodontium* species closely matched the ITS sequence of *Amylostereum*.

Three analyses were performed for each alignment. First, a maximum parsimony (MP) analysis using the program PAUP 4.0a147 (Swofford 2003), as indicated in Telleria et al. (2013), with a default setting to stop the analyses at 100 trees. Exhaustive searches were conducted, and gaps treated as missing data. Tree scores, including tree length, consistency index (CI), homoplasy index (HI), and retention index (RI) were calculated from each exhaustive search; non-parametric searches (Felsenstein 1985) were used to calculate branch support (bootstrap, MPbs), performing 10,000 replicates using the fast-step option. Second, a maximum likelihood (ML) analysis was performed using the RAXML (Stamatakis 2014) in the CIPRES portal (CIPRES Science Gateway v.3.3; Miller et al. 2010), assuming a GTR model (default model); nonparametric bootstrap (MLbs) support (Felsenstein 1985) for each clade, based on 1000 replicates was tested. Finally, a Bayesian analysis (Larget and Simon 1999; Huelsenbeck et al. 2001) was performed using the software MrBayes v.3.2. (Ronquist et al. 2012), and assuming the general time reversible model (Rodríguez et al. 1990), including estimation of invariant sites and assuming a discrete gamma distribution with six categories (GTR+I+G) selected by PAUP*4.0b10; the 50% majority-rule consensus tree and the posterior probability (pp) of the nodes were calculated from the remaining trees with MrBayes. Moreover, to assess whether a barcoding gap exists (Hebert et al. 2004), Kimura-2-parameter (K2P) pair-wise ITS distances were calculated using the software PAUP 4.0a147. On the basis of the widely used threshold for barcoding studies, a barcoding gap exists if the average interspecific difference is 10 times the average intraspecific difference (Hebert et al. 2004).

The 12 new consensus sequences have been lodged in the EMBL/GenBank/DDBJ (Accession Numbers included in Figs. 1, 2). A matrix of 534 aligned nucleotide positions was produced for the ITS (459 characters are constant, 31 parsimony informative and 44 parsimony-uninformative). The 100 most parsimonious trees gave a length of 93 steps, CI = 0.8710, RI = 0.9481, and RC = 0.8257. The topology of the parsimony strict consensus tree (not shown) was similar to the ML (not shown) and the 50% majority rule Bayesian tree (Fig. 1): sequences of *A. laevigatum* from Japan appeared separated from sequences of *A. laevigatum* from Europe. The sequences from Japan grouped in the *A. orientale* clade along with the *A. orientale* sequences from He and Li (2013) with very high support (MPbs = 100%, MLbs = 98%, pp = 1.0). For the *rpb2* gene, an alignment with 702 characters (541 characters are constant, 113 parsimony-informative and 48 parsimony uninformative) was produced. The 100 most parsimonious trees gave a length of 246 steps, CI = 0.7510, RI = 0.9402, and RC = 0.7061. The *rpb2* topologies obtained with each analysis were identical to each other; the 50% majority rule strict Bayesian tree is shown in Fig. 2. Sequences of *A. laevigatum* from Japan form a clade of their own, clearly separated from *A. laevigatum* from Europe. The alignments and trees were deposited in TreeBASE (Submission ID S20149).

On the other hand, the K2P distances matrix (Table 1) indicates that the maximum genetic distance between ITS sequences of any two isolates of *A. laevigatum* from Japan is 0.00196, and between any two isolates of *A. laevigatum* from Europe is 0.00241; however, the large genetic distance between *A. laevigatum* from Japan and all the specimens of *A.*

laevigatum from Europe (minimum 0.01894) confirms that they belong to different species. Moreover, the low value between *A. laevigatum* from Japan and *A. orientale* (maximum 0.00590), demonstrates that collections studied from Japan correspond to the species *A. orientale*. In *Amylostereum*, a barcoding gap exists in the ITS marker since the interspecific values are ten times higher than intraspecific values (Table 1), allowing identification to the species level.

The revision of morphological features of TUMH61905 and TUMH61916 was performed following Telleria and Melo (1995) and He and Li (2013). A short description of these collections is provided.

Basidiomes resupinate, adnate and coriaceous; hymenium and context brownish with numerous lamprocystidia; hyphal system monomitic, with generative hyphae with clamps, thin-walled; lamprocystidia thick-walled, brownish, 22–26 × 5–6 µm; young cystidia subulate, hyaline, thin-walled, smooth; basidia subclavate to clavate, with a basal clamp connection and four sterigmata, 26 × 5–6 µm; basidiospores ellipsoid, hyaline, thin-walled, amyloid, 6.5 × 3.5–4.5 µm (very few observed).

As detected in Tabata et al. (2000), our molecular and morphological analyses clearly separate Japanese *A. laevigatum* from European *A. laevigatum*. The position in the ITS nrDNA tree, as well as the genetic K2P distance values obtained show that Japanese collections studied belong to *A. orientale*, instead of *A. laevigatum*. Although no *rpb2* sequences from *A. orientale* from China are included in our analyses, the position of the Japanese collections in the *rpb2* tree, forming a group of its own separated from *A. laevigatum*, support our

Fig. 1 – Phylogenetic tree obtained from Bayesian inference of ITS sequences of *Amylostereum* spp., including *Echinodontium tinctorium* as outgroup. The host trees and localities are shown next to the accession numbers. Parsimony bootstrap support (MPbs), likelihood bootstrap support (MLbs) and posterior probability (pp) indicated above branches. New sequences obtained in this work are marked in bold face, and culture numbers are provided.

Fig. 2 – Phylogenetic tree obtained from Bayesian inference of *rpb2* sequences of *Amylostereum* spp. including *Echinodontium tinctorium* as outgroup. The host trees and localities are shown next to the accession numbers. Parsimony bootstrap support (MPbs), likelihood bootstrap support (MLbs) and posterior probability (pp) indicated above branches. New sequences obtained in this work are marked in bold face, and culture numbers are provided.

Table 1 – Matrix of pairwise intra- and interspecific Kimura-2 Parameter (K2P) distance among *Amylostereum* spp. ITS nrDNA sequences. The gray shading indicates the interspecific K2P among *A. orientale* and *A. laevigatum*, from Japan and Europe.

	1	2	3	4	5	6
1 <i>Amylostereum orientale</i>	0					
2 <i>Amylostereum laevigatum</i> , Japan	0.00590	0.00196				
3 <i>Amylostereum laevigatum</i> , Europe	0.01881	0.01894	0.00241			
4 <i>Amylostereum chaillatii</i>	0.02189	0.01990	0.00991	0.00412		
5 <i>Amylostereum ferreum</i>	0.02556	0.02360	0.01173	0.01381	0	
6 <i>Amylostereum areolatum</i>	0.03338	0.03530	0.02547	0.03124	0.02738	0.00590

conclusion. These are the first confirmed records of *A. orientale* in Japan.

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