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FUNGAL MICROBIOLOGY

Greenhouse Seedlings of *Alnus* Showed Low Host Intrageneric Specificity and a Strong Preference for Some *Tomentella* Ectomycorrhizal Associates

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Abstract Ectomycorrhizal (ECM) fungal associates of *Alnus* are relatively few in comparison with those associated with other tree hosts. The composition of ECM assemblages associated with *Alnus* seems to change very little across the Northern Hemisphere. However, *Alnus*-associated ECM assemblages from the Western United States, Mexico, and Argentina tend to differ from those in eastern North America and Europe, presumably due to their different biogeographic histories. *Alnus glutinosa* is a northern European species subjected to diverse environmental conditions. To address intrageneric host preference within two distantly related *Alnus* species (*Alnus acuminata* and *A. glutinosa*), we tested the ECM colonization on seedlings of both species inoculated with natural soil from *A. acuminata* forests. Two tomentelloid ECM fungi from *A. acuminata* natural soils were determined from the anatomotyping and molecular analysis. Both species colonized *A. glutinosa* seedlings and presented similar relative abundances. Additional soil sequence data from *A. acuminata* sites suggest that a variety of tomentelloid taxa occur, including several unidentified *Tomentella* lineages. Maximum-likelihood and Bayesian inference analyses based on internal transcribed spacer (ITS) sequences from various locations do not reflect associations of taxa based on their biogeographic origin, and clades are in general constituted by sequences from diverse regions, including South America, Mexico, USA, and Europe. Results illustrate the probable role of specific tomentelloid fungi in the early colonization of seedlings in *A. acuminata* forests as well as their importance in the structure of the ECM propagule community at the sites.

Keywords Yungas forests · Ectomycorrhizal specificity · Ion Torrent · Tomentelloid taxa

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Introduction

In Argentina, *Alnus acuminata* Kunth is known as "aliso del cerro" and grows in the Yungas at the northwestern corner of the country. Their roots host a tripartite symbiosis in which arbuscular mycorrhizal (AM) fungi, ectomycorrhizal (ECM) fungi, and actinorhizas are involved [1–5]. In this type of synergistic associations, the ECM fungi are known to stimulate the bacterial colonization of roots as well as providing mineral nutrition [6], in particular enhancing organic phosphorous acquirement in addition to the nitrogen fixation that occurs in the presence of *Frankia* [7, 8]. More specifically, the higher P acquisition ability of *Alnus*-associated ECM fungi is suggested to be the result of a greater plant demand of phosphorous, given the continuous N provision by *Frankia* [8]. Throughout this highly effective symbiosis, *Alnus* spp. are able to restore and improve the fertility conditions of mountainous lands subject to erosive process and to colonize nutrient depauperate substrates, improving soil quality and facilitating the plant succession [9, 10]. At the early stages of colonization, the ECM fungal propagule abundance is a prime determinant of early stages of community development and might affect long-term dominance [11, 12].

It is known that *Alnus* associates with fewer ECM fungi than most other ECM tree hosts [13–16], although their specialization is stronger due to restricted receptivity [11]. In addition, the co-occurrence of nitrogen-fixing *Frankia* bacteria strongly modifies soil nitrogen concentrations [17] and lowers the pH levels affecting the associated ECM assemblage [18–20]. The composition of *Alnus*-associated ECM communities seems to change very little across the Northern Hemisphere [21], and they seem to display little intrageneric specificity within *Alnus* [22, 5]. The majority of *Alnus* species are widespread either in Asia, Europe, and North America, while *A. acuminata* inhabits Central and South America. The arrival of *Alnus* into the Americas likely occurred from Asia throughout the Bering Sea land bridge, with existing fossil records from Oregon confirming this hypothesis [23]. Therefore, it is supposed that *A. acuminata* is the most recent descendent of those species that initially entered from Asia, probably reaching northern South America in the mid-Pleistocene, ca. 1 million years ago as suggested by the fossil evidence [24]. In addition, phylogenetic studies have demonstrated that the Latin American species (*A. acuminata* and *A. jorullensis*) are more closely related to western North American species (*A. rhombifolia* and *A. oblongifolia*) than species that occur in both western and eastern North America [25, 26].

Alnus-associated ECM assemblages from the Western United States, Mexico, and Argentina are similar but presumed to have dissimilarities with those associated to eastern North America and European *Alnus* species [21], possibly due to their different biogeographic histories [25]. However, more recently, Põlme et al. [16], focusing on contrasting biogeographic patterns, suggest that the overall community in South America and eastern North America is more similar than the ECM community associated to *Alnus* spp. in western North America. The eastern *Alnus* species migrated from Europe probably through the North Atlantic land bridge >30 MA [24]. In addition to contrasting biogeographic patterns, different diversity patterns of some ECM fungal groups, such as ascomycetes and the basidiomycete genus *Alnicola*, between these two biogeographic *Alnus* lineages have been described [21]. However, Pritsch et al. [27] comparing some of the *Alnus*-associated ECM taxa between the European and Argentinian assemblages found high similarities among species of *Tomentella* and *Lactarius*. The overall data indicate a relatively uniform array of ECM species, with some level of host preference [16, 21], globally distributed and highly adapted to *Alnus* species [22, 11]. However, novel ECM species are expected based on previous rarefied accumulation curve analysis of *Alnus*-associated ECM fungi [16] from a biogeographic study at global scale. In this study, the less frequent groups exhibited substantial differences in distribution by hosts and regions, and most rare taxa exhibited a restricted geographical range. Intrageneric phylogenetic relations among *Alnus* spp. were defined as the cause of a large part of the ECM fungal community structure within *Alnus* at the global scale.

In order to address intrageneric host preference in *Alnus*, we tested ECM colonization on seedlings of *A. acuminata* and *A. glutinosa* inoculated with natural soil from *A. acuminata* forests under greenhouse conditions. *A. glutinosa* is a north-central European species [28] and thus has evolved under different environmental conditions than *A. acuminata* which is native to subtropical and tropical cloud forests in Central and South America. Colonization of *Frankia* was also evaluated. In addition, we further characterized the ECM community present at the *A. acuminata* sites based on deep DNA sequencing of soil samples. We predict that most ECM fungi from *A. acuminata* natural soils will colonize *A. glutinosa* seedlings, but the relative abundance of ECM species will differ between hosts if considering their biogeographic origin and differential preference towards fungal symbionts occurring in their own natural soil's ECM propagule community.

Materials and Methods

Soil and Seed Collection

A. acuminata soil samples and seeds were collected in June 2010 in northwestern Argentina, in Catamarca and Tucumán Provinces. Soils were obtained from five locations under pure *A. acuminata* forests from the Parque Nacional Campo de Los Alisos (27° 43,184′ S, 65° 54,186′ W; 27° 42,371′ S, 65° 54,705′ W; 27° 19,829′ S, 65° 55,941′ W; 27° 19,754′ S, 65° 55,231′ W; 27° 20,892′ S, 65° 57,782′ W) between 1262 and 1890 masl. At each site, two composite soil samples of approx. 2 kg were collected with a spade under the trees upon removal of the litter layer. Soil samples were stored at 4 °C for a period of 2 weeks until the greenhouse experiments were conducted. A fraction of the pooled samples were also subjected to standard soil chemical analyses. Soil physicochemical characteristics were as follows: pH (H_2O)=5.8; electrical conductivity (EC)=1.13 mmhos/cm; organic matter=7.2 %; carbon=4.2 %; nitrogen=0.39 %; C/N=10.7; NO_3= 673 mg kg^{-1}; and available P=20.09 mg kg^{-1}. Seeds of *A. glutinosa* were provided by the Laboratorio de Botánica, Departamento de Biología Vegetal y Ecología, Universidad del País Vasco/EHU, Spain. Seeds were stored at 4 °C until used.

Experimental Design

The greenhouse experiment involved natural soil (inoculum) from *A. acuminata* sites and two *Alnus* species treatments (*A. acuminata* and *A. glutinosa*), with 12 replicates for each species and 12 replicate controls per treatment. Seeds were

surface sterilized with 10 % sodium hypochlorite (NaOCl) for 5 min and then thoroughly rinsed with deionized water. Seeds were sown in sterilized Petri dishes on a humid absorbent paper to promote germination inside culture chambers. Two-week-old seedlings were placed individually in plastic containers (capacity of 350 ml and approx. 450 g of soil). Substrate was prepared by thoroughly mixing the natural soil from the 10 samples and sterilized vermiculite (1:2). For controls, the natural soil was steam sterilized three times (120 °C for 1 h with 24 h at room temperature between the 3 cycles), stabilized for 2 weeks, and then mixed in equal proportions with sterilized vermiculite (60 min at a pressure of 2 atm). To prevent cross contamination between pots, a thin layer of autoclaved sand was added at the top of each plastic container. Temperature in the greenhouse was between 20 and 30 °C (14-h photoperiod; 10-h natural light supplemented during the winter time with 4-h artificial light). Plants were watered daily, and fertilizer was not added. After 6 months (from July to December 2010), plants were uprooted and shoots and roots were separated. The aerial portion and roots were used to estimate plant growth parameters, for this purpose and to analyze *Frankia* and ECM fungi colonization; roots were gently washed with water to remove adhering particles.

Measurements

The plant growth parameters length and dry weight of root and shoot were measured. *Frankia* nodules were extracted, and their dry weight was obtained. The percentage of ECM colonization was calculated as the number of ECM root tips divided by the total number of root tips [29]. The percentage of colonization by each ECM morphotype was calculated for each sample by dividing the number of root tips colonized by each ECM morphotype by the total number of root tips and multiplying by 100 [30]. The ECM root tips were extracted carefully from soil samples and sorted into morphotypes according to their morphological and anatomical features using a Wild M5A stereomicroscope at ×10–40 magnification. Criteria for sorting ECM morphotypes included color, mantle layers, branching pattern, emanating hyphae, presence of rhizomorphs and cystidia, following Agerer's methodology [31, 32]. Presence of Hartig net was confirmed in all morphotypes.

Molecular Identification of ECM Root Samples

Clusters of ECM root tips belonging mostly to one individual morphotype from each seedling were inserted into 1.5-ml microtubes containing 500 μl 2 % cetyltrimethylammonium bromide (CTAB) DNA extraction buffer (100 mM Tris–HCl (pH 8.0), 1.4 M NaCl, and 20 mM EDTA) and stored at −20 °C. One to five root tips from each morphotype per soil core were subjected to DNA extraction using the CTAB chloroform method [33]. The internal transcribed spacer (ITS) region, including the 5.8S ribosomal DNA (rDNA) locus, was amplified via PCR with ITS1-f and ITS4 as well as ITS1-f and ITS4-b primer pairs [34]. PCR reactions were performed in 50-μl reaction tubes with 1.1× Reddy Mix™ PCR Master Mix (2.5 mM $MgCl_2$) (ABgene®; Thermo Fisher Scientific, Inc., UK) according to the manufacturer's instructions. Cycling conditions consisted of 2 min of activation at 94 °C, followed by 35 cycles for 45 s at 94 °C, 30 s at 50 °C, and 60 s, +1 s/cycle, at 72 °C, and a 10-min final extension at 72 °C. PCR products were checked for positive amplification on 1 % agarose gels, and the amplified products were sent to Macrogen Inc. (Seoul, South Korea) for purification and sequencing using the BigDye™ Terminator kit and run on ABI 3730xl. ECM voucher material has been deposited at CORD herbarium.

Diversity of ECM Taxa in the Sampled *A. acuminata* Sites

In order to characterize the ECM taxa diversity at the sampling sites, a soil sampling was carried out at the same locations for subsequent deep sequencing of fungal communities as follows. Sixty soil cores, each ca. 4 cm in diameter and 10–15 cm long and taken more than 2 m from each other, were pooled for a composite sample. Genomic DNA was extracted from 1 g of dry soil using NucleoSpin® Soil kit (Macherey-Nagel Gmbh & Co., Düren, Germany), according to the manufacturer's protocol. The ITS2 region (ca. 250 bp) of the nuclear ribosomal rDNA repeat was PCR amplified as described in Geml et al. [35]. Two hundred fifty microliters of the sample was used for emulsion PCR according to the Ion PGM™ 200 Xpress™ Template Kit manual and sequenced using an Ion Torrent Personal Genome Machine (PGM; Life Technologies, Guilford, CT, USA) at the Naturalis Biodiversity Center.

The initial cleanup of the sequence data was carried out as described in Geml et al. [35]. The resulting 611,493 quality-filtered sequences served as input for operational taxonomic unit (OTU) clustering. Although there is no universal cutoff value for species delimitation in fungi due to a substantial variability in nucleotide substitution rates and ages of species across fungal lineages, it has been shown that 2–3 % ITS sequence divergence usually represents different species in many basidiomycete lineages [36], and a 97 % sequence similarity cutoff value tends to provide a conservative, yet reasonably accurate estimate of total species diversity in fungal communities [37–39]. Therefore, we clustered the quality-filtered sequences into OTUs based on 97 % sequence similarity using OTUpipe [40], while removing 223,468 putatively chimeric sequences. We compared representative sequences of the OTUs using USEARCH [41] against the latest release of quality-checked UNITE+INSD fungal ITS sequence database containing both identified and unidentified sequences, many of which are assigned to Species Hypothesis groups as defined by Kõljalg et al. [42]. OTUs that did not have at least 80 % similarity over at least 150 bp to any fungal

sequence in INSD were excluded from further analyses. Finally, 77 OTUs belonging to the /amanita, /hebeloma-alnicola, /clavulina, /inocybe, /cortinarius, /paxillus-gyrodon, /russula-lactarius, and /tomentella-thelephora lineage were recovered (data not shown). For this study, 16 OTUs belonging to the /tomentella-thelephora lineage and with at least sequences of 200 bp in length were selected and incorporated into the multiple sequence alignment described below as well as the most similar sequences from the UNITE database. Sequences of OTUs included in the alignment have been submitted to GenBank (Table 1). ECM root sequences and soil OTU sequences were identified based on their phylogenetic placement and assigned to ECM fungal lineages according to Tedersoo et al. [43]. Whenever available, we used the Species Hypothesis (SH) numbers for species identification [42], which is assigned for the taxa discovered in clustering on different similarity thresholds (97–99 %). This term was created with the purpose of improved accuracy and ease of comparison among studies.

Phylogenetic Analyses

ITS sequence chromatograms of ECM root sequences were visually revised and manually corrected where necessary using BioEdit 7.0.5.3 [44]. The sequences of *Tomentella* spp. generated for this study from the alder root tips DNA have been deposited into GenBank (Table 2). Additionally, 16 ITS2 sequences generated from soil samples as described above were incorporated into the alignment (Table 1). Sequences generated in this study were combined into a data set with additional closely related public sequences obtained throughout Basic Local Alignment Search Tool (BLAST) searches in GenBank and UNITE data sets.

A total of 65 sequences including the out-group (*Tomentellopsis* sp.) were used for analyses. We constructed the multiple sequence alignment using MUSCLE [45]. Phylogenetic analyses were performed using the maximum-likelihood (ML) method and Bayesian inference (BI). ML analyses were conducted in PhyML 3.0 [46] under the TPM1+I+G model of DNA substitution, previously determined as the best-fit model through the AICc and BIC as implemented in jModelTest 2.0 [47]. ML analyses were conducted using an estimated proportion of invariable sites, gamma distribution parameter, and transition/transversion ratio, and the best option of tree topology search. Bootstrap analyses were run with 300 replicates to assess the support of the branches. BI analyses were conducted in MrBayes 3.2.2 [48] with four incrementally heated simultaneous Monte Carlo Markov chains over 10 million generations under GTR+G+I model of DNA substitution. Random trees were used as the starting point, and the sample frequency occurred once every 1000 generations, resulting in 10,000 sampled trees. With those trees sampled after the process had reached stationary, a majority rule consensus tree was computed to estimate the posterior probabilities.

Data Analyses

Analysis of variance (ANOVA) using the InfoStat statistical package [49] was used to examine the relationships between the response variables (plant growth, ECM colonization, individual ECM morphotype colonization and their relative

Table 1 GenBank accession numbers, number of reads and size of the "tomentelloid" sequences (OTUs obtained from the soil analyses at the *Alnus acuminata* sites) included in the alignment and their best BLAST parameters

GenBank	OTU #	Reads #	bp	Best BLAST-identified match		
				Specimen	Accession #	% identity
KJ140268	OTU_255	3	222	*T. testaceogilva*	UDB002979	95.2
KJ140269	OTU_330	9528	352	*T. testaceogilva*	UDB002979	99.7
KJ140270	OTU_334	1958	351	*T. ellisii*	UDB002982	99.7
KJ140271	OTU_394	1467	321	*Thelephora* sp.	DQ195591	100
KJ140272	OTU_509	2079	351	*Thelephora alnii*	UDB003353	98.8
KJ140273	OTU_703	28	345	*T. ellisii*	UDB002982	94.2
KJ140274	OTU_708	298	222	*T. cinereoumbrina*	UDB003298	89.9
KJ140275	OTU_990	7	253	*Tomentella* sp.	UDB002929	99.2
KJ140276	OTU_1034	478	261	*Tomentella fuscocinerea*	UDB018524	92.3
KJ140277	OTU_1412	8	246	*T. testaceogilva*	UDB002979	99.5
KJ140278	OTU_2000	5	251	*T. testaceogilva*	UDB002979	98.3
KJ140279	OTU_2124	8	225	*T. testaceogilva*	UDB002979	98.1
KJ140280	OTU_2796	106	297	*T. testaceogilva*	UDB002979	99.3
KJ140281	OTU_2983	21	351	*T. ellisii*	UDB002982	96.4
KJ140282	OTU_3040	252	277	*T. testaceogilva*	UDB002979	99.6
KJ140283	OTU_3115	106	260	*T. testaceogilva*	UDB002979	99.6

Table 2 GenBank and UNITE accession numbers of the sequences from voucher collections and environmental sources used in the phylogenetic analysis. When available, the Species Hypothesis (SH) numbers are given for the corresponding sequence as published by Kõljalg et al. [42]

Collection	Herbarium number	Origin	ITS accession No.	SH
ECM sp. 1 (*Tomentella* sp.)	EN238 (CORD)	Argentina	KC782503	–
ECM sp. 2 (*Tomentella* sp.)	EN243 (CORD)	Argentina	KC782508	–
Tomentella atramentaria	TAA149211	Russia	AF272904	–
Tomentella botryoides	TAAM149614	Russia	UDB000257	202530.06FU
Tomentella bryophila	TAA164410	Estonia	AF272908	–
Tomentella castanea	TL-6886	Denmark	UDB000120	195957.06FU
T. cf. *ellisii*	AB10 (CORD)	Argentina	DQ195592	222911.06FU
T. cf. *sublilacina*	AB06 (CORD)	Argentina	DQ195590	195954.06FU
Tomentella coerulea	TU100487	Australia	UDB016683	203126.06FU
T. coerulea	TU108828	Estonia	UDB000958	219962.06FU
T. ellisii	–	Argentina	UDB002982	222911.06FU
T. fuscocinerea	TAAM149918	Estonia	UDB000240	219977.06FU
Tomentella galzinii	TAAM166821	Estonia	UDB000260	219966.06FU
Tomentella lapida	TU108555	Estonia	UDB000273	203170.06FU
Tomentella lateritia	TU100385	Australia	UDB016705	202764.06FU
T. lateritia	TAAM167067	Estonia	UDB000267	202551.06FU
Tomentella lilacinogrisea	TU108886	Estonia	UDB000953	202620.06FU
Tomentella pilosa	TAAM152428	Estonia	UDB000241	195965.06FU
Tomentella punicea	TAAM158081	Estonia	UDB000271	202489.06FU
Tomentella stuposa	TAA159498	Estonia	AF272902	–
Tomentella subtestacea	MC01-546	Denmark	UDB000034	219871.06FU
Tomentella terrestris	TAAM159557	Estonia	UDB000221	195959.06FU
T. testaceogilva	TU100932	Argentina	UDB002972	195954.06FU
T. testaceogilva	–	Argentina	UDB002979	195954.06FU
T. testaceogilva	–	Argentina	UDB002978	195954.06FU
Tomentella umbrinospora	TAAM149462	Estonia	UDB000233	202530.06FU
Thelephora albomarginata	TU100195	Estonia	UDB003349	195954.06FU
Thelephora alnii	TU114333	Estonia	UDB003353	195955.06FU
Tomentella sp.	AB08 (CORD)	Argentina	DQ195591	202496.06FU
Tomentella sp.	–	Estonia	UDB002929	219858.06FU
Tomentella sp. 3 clone 3492	–	Mexico	HQ271384	213382.06FU
Tomentella sp. 4 clone 4394	–	Mexico	HQ271385	195955.06FU
Tomentellopsis sp.	TSHY1	Finland	AJ410784	199523.06FU
Uncultured basidiomycete	–	Greece	FM866364	–
Uncultured ECM fungus	–	Ecuador	HE979082	195954.06FU
Uncultured ECM fungus	–	Ecuador	HE979087	202496.06FU
Uncultured ECM fungus	–	Ecuador	HE979536	202496.06FU
Uncultured ECM fungus	–	France	JX989964	195955.06FU
Uncultured ECM fungus	–	Eastern United States	HE978942	195954.06FU
Uncultured ECM fungus	–	Ecuador	HE979543	195954.06FU
Uncultured fungus	–	Eastern United States	GU174338	213401.06FU
Uncultured *Thelephoraceae*	–	Puerto Rico	JX548277	205648.06FU
Uncultured *Tomentella* clone 1 A1-2	–	Western United States	JX198510	195955.06FU
Uncultured *Tomentella* clone 1 A8-10	–	Western United States	JX198519	222911.06FU
Uncultured *Tomentella* clone 2 B1-10	–	Western United States	JX198524	222919.06FU
Uncultured *Tomentella* clone 2 B3-9	–	Western United States	JX198514	220146.06FU
Uncultured *Tomentella* clone 3 B10-2	–	Western United States	JX198522	202450.06FU

Table 2 (continued)

Collection	Herbarium number	Origin	ITS accession No.	SH
Uncultured *Tomentella* clone 4 A4-4	–	Western United States	JX198508	195954.06FU
Uncultured *Tomentella* clone B7-8	–	Western United States	JX198539	202460.06FU

abundance, number of *Frankia* nodule lobes, and dry weight of nodules) and host trees. Before analysis, number of *Frankia* nodules lobes per seedling and dry weight, ECM colonization, individual ECM morphotype colonization, and relative abundance were transformed to rank to accomplish the normality and homogeneity criterion and analyzed statistically by ANOVA, the equivalent to the nonparametric analyses [50]. All differences among means were evaluated using Tukey's test.

Results

Colonization and Growth Parameters

ECM colonization was achieved in both species treatments inoculated with natural soils. Only two ECM morphotypes (ECM sp. 1 and ECM sp. 2) were identified showing well-developed mantle and a Hartig net (Figs. 1 and 2) and later determined as *Tomentella* spp. Poorly developed mantles and senescent morphotypes were initially separated as different; however, posterior DNA analysis showed that they belonged to the same identified *Tomentella* taxa. Both *Alnus* species presented high ECM colonization percentages, with 86.6 % for *A. acuminata* and 74.7 % for *A. glutinosa* (Table 3). No ECM root tips were registered in the sterilized controls.

Plant growth parameters (shoot height, shoot dry weight, and *Frankia* nodules) showed significant differences among control treatments, but no differences were observed among host species (Table 3), at exception of the root dry weight that was significantly higher in *A. glutinosa* than in *A. acuminata* ($P<0.00001$). Root length was similar between non-sterilized *A. glutinosa* seedlings and sterilized controls, but differed between non-sterilized *A. acuminata* seedlings and sterilized controls.

Colonization of ECM sp. 2 in *A. acuminata* was significantly higher than that in *A. glutinosa* (Table 3). Both ECM morphotypes presented similar relative abundances in *A. glutinosa*, although ECM sp. 2 presented a higher relative abundance in *A. acuminata* ($P<0.0002$) (Fig. 3). *Frankia* nodule dry weight values significantly differed between soil treatments, but not between *Alnus* species (Table 3).

Molecular Analysis and Phylogenetic Reconstruction

The ITS region of the two ECM morphotypes separated by their anatomical features were amplified with both the ITS1-f/ITS4 and ITS1-f/ITS4-b primer pairs. Seedlings were colonized by two dominant morphotypes, both of which belonged to the /tomentella-thelephora lineage. Sequences varied between 608 and 776 bp. The exception was a single sequence that matched an unclassified *Corticiaceae* specimen (accession number EF538420.1) with 99 % identity. The lack of an associated morphotype for this sequence in addition to poor definition at the taxonomic level obtained from the BLAST search precludes us from considering it as an ECM symbiont.

In the phylogenetic reconstruction, both analyses, the ML and BI, yielded congruent tree topologies (Fig. 4). In both cases, the two tomentelloid ECM taxa appear in different clades, but closely related to other *Tomentella* species known to be associated with *Alnus*. ECM sp. 1 presented a sequence similarity of 99 % matching *Tomentella* cf. *ellisii* (DQ195592) and formed a distinct clade together with an additional *T. ellisii* (UDB002982) sequence (94 % sequence similarity). These sequences, along with some of the environmental sequences generated in this study, formed a well-defined clade in accordance to the Species Hypothesis number (SH222911.06FU).

ECM sp. 2 presented a sequence similarity of 100 % matching *Tomentella* cf. *sublilacina* (DQ195590) and 99 % to *Tomentella testaceogilva* (UDB002972) and additional *T. testaceogilva* sequences from Argentina (Fig. 4). It is worth mentioning that the sequence corresponding to *T.* cf. *sublilacina* (DQ195590) was originally named by Pritsch et al. [27] based on 99.8 % similarity with *T. sublilacina* (UDB002972). The later sequence then was reassigned to *T. testaceogilva* in the database probably due to a misidentification of the voucher material. At the same time, this group of sequences formed the second well-supported clade, along with additional environmental sequences sharing the same SH number (SH195954.06FU).

Sequences of tomentelloid OTUs generated from the habitat soil samples represented an array of taxa distributed throughout the tree topology. The majority of them had closely related sequences in the UNITE database, while some (OTUs 703, 708, 1034) appear to be different from formerly sequenced species (Table 1). Of the OTUs matching formerly sequenced taxa, several were identified to species. In particular, OTUs 334 and 2983 belonged to the *T. ellisii* clade (SH2229111.06FU) that also included ECM sp. 1, while OTUs 255, 330, 1412, 2000, 2124, 2796, 3040, and 3115 had identical or nearly identical sequences to *T. testaceogilva*

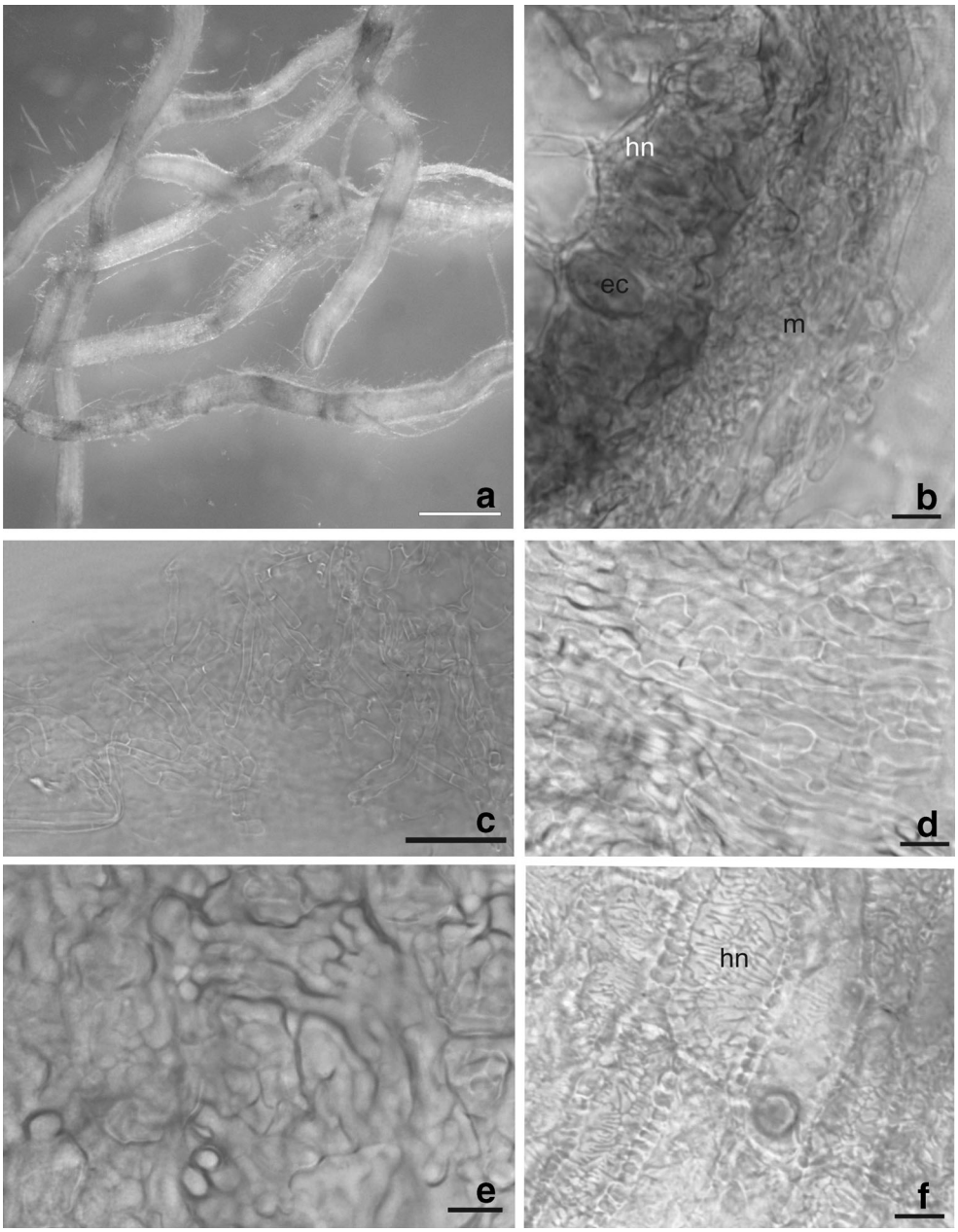


Fig. 1 **a** Light micrographs of ECM sp. 1 (*Tomentella* sp.) on *Alnus glutinosa*. **b** Cross section showing the mantle layers (*m*), Hartig net (*hn*), and epidermal cells (*ep*). **c** Outer mantle layer plectenchymatous. **d** Middle mantle layer plectenchymatous. **e** Inner mantle layer pseudoparenchymatous. **f** Longitudinal section of the mantle showing the Hartig net (*hn*). *Bars*: **a** 0.5 mm; **c** 50 μm; **b**, **d**–**f** 10 μm

(SH195954.06FU) to which ECM sp. 2 likely belong as well. Identified tomentelloid taxa only recovered from the habitat soil samples but not from the root samples included OTU 509, with 99.7 % match to *Thelephora alnii* (SH195955.06FU), and OTUs 394 and 990, both matching unidentified *Tomentella* spp. (SH202496.06FU and SH219858.06FU, respectively), from studies on ECM communities associated with *Alnus* spp. Table 1 summarizes information such as accession numbers, number of reads, and size of the "tomentelloid" OTU sequences obtained from the soil analyses at the *A. acuminata* sites as well as their best BLAST parameters.

Most clades depicted in Fig. 4 do not reflect an association of taxa based on biogeographic origin. On the contrary, each main clade characterized by unique SH numbers (representing species complexes or most likely the same species) comprised sequences from diverse regions, including Argentina or other South American locations, Western United States, Mexico, Eastern United States, and Europe, except the clade represented by the SH202496.06FU which provisionally includes only sequences from South America.

Discussion

Both *Alnus* species presented high ECM colonization percentages growing under soils collected from native *A. acuminata*

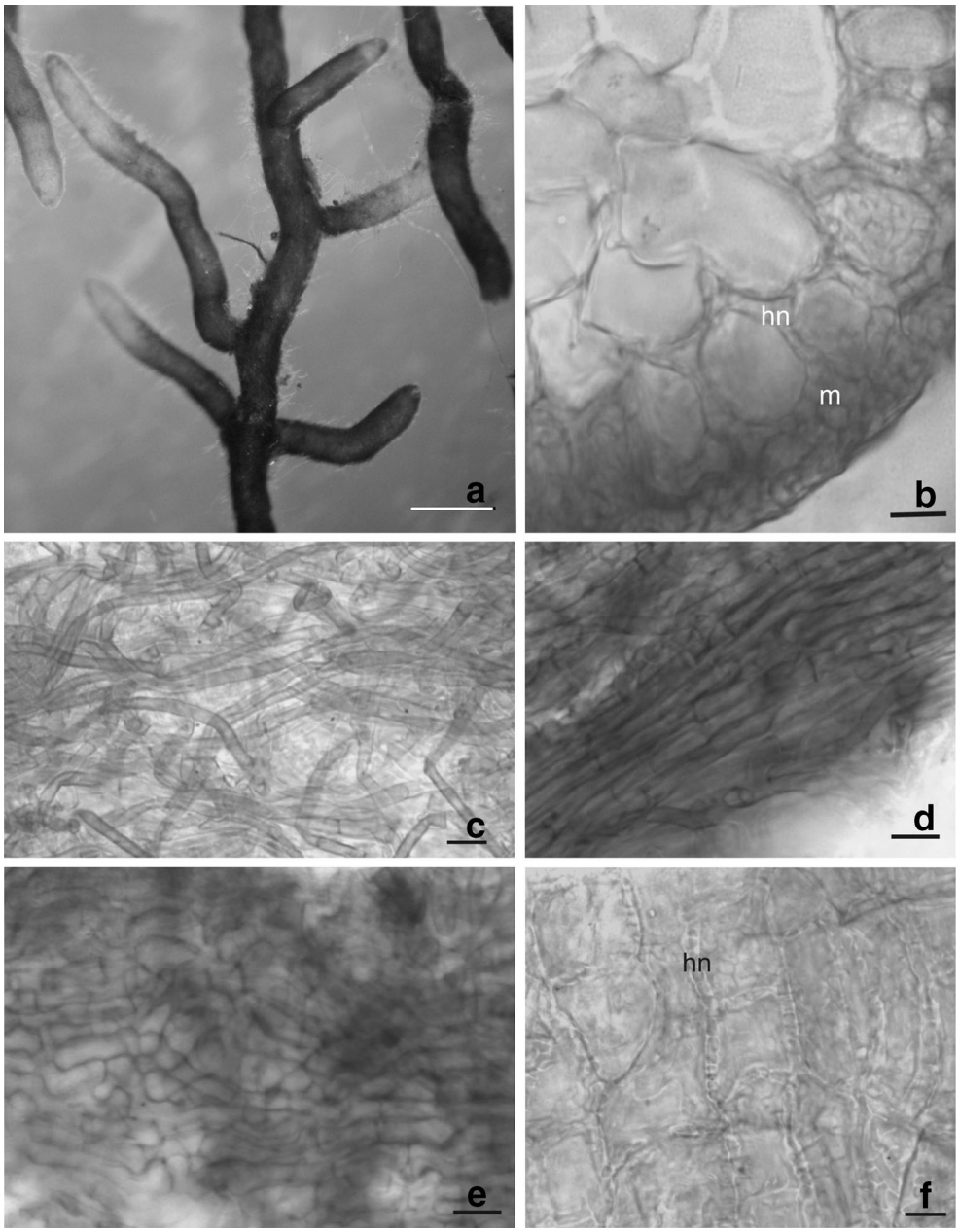


Fig. 2 **a** Light micrographs of ECM sp. 2 (*Tomentella* sp.) on *Alnus glutinosa*. **b** Cross section showing the mantle layers (*m*) and Hartig net (*hn*). **c** Outer mantle layer plectenchymatous. **d** Middle mantle layer plectenchymatous. **e** Inner mantle layer plectenchymatous to pseudoparenchymatous. **f** Longitudinal section of the mantle showing the Hartig net (*hn*). *Bars*: **a** 0.5 mm; **b**–**f** 10 μm

Table 3 Growth of *Alnus acuminata* and *A. glutinosa* (Ag) inoculated with natural soils of *A. acuminata* and control treatments (control *A. acuminata* and *A. glutinosa*)

Treatments	Plant variables					ECM variables		
	Shoot height (cm)	Shoot dry weight (g)	Root length (cm)	Root dry weight (g)	Nodules dry weight (g)	ECM %	ECM % sp. 1	ECM % sp. 2
Control *A. glutinosa*	17.07±2.34b	0.77±0.20b	21.92±3.61a	0.84±0.39b	0.001±0.003b	0	0	0
A. glutinosa	26.33±4.28a	2.06±0.43a	22.75±1.36a	1.49±0.36a	0.05±0.02a	74.77±13.10b	46.83±27.57aa	53.16±27.57aa
Control *A. acuminata*	12.17±3.32c	0.43±0.28b	16.75±2.67b	0.43±0.28c	0.002±0.01b	0	0	0
A. acuminata	28.17±1.74a	2.23±0.43a	20.71±2.13a	0.92±0.26b	0.05±0.01a	86.60±10.99a	27.75±32.88aa	72.24±34.30ab

Mean and standard error of 12 samples followed by different letters indicate significant differences ($P<0.05$) in each column as determined by Tukey's HSD test. Additionally, for the ECM % sp. 1 and sp. 2, different letters indicate significant differences ($P<0.05$) within the same host

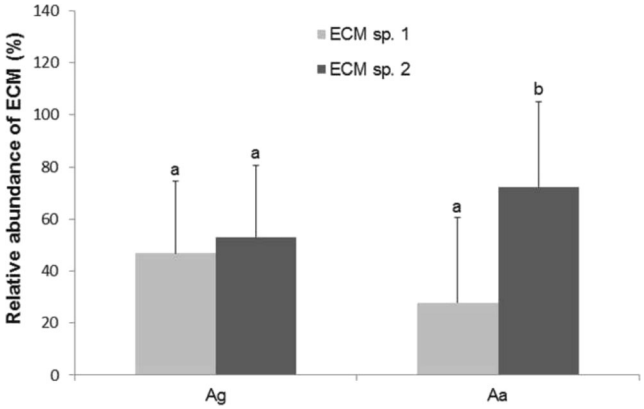


Fig. 3 Relative abundance of ECM morphotypes present in *Alnus glutinosa* (*Ag*) and *Alnus acuminata* (*Aa*) seedlings from the greenhouse experiment. *Different letters* indicate significant differences between ectomycorrhizal types for each host according to Tukey's post hoc test at $P<0.05$ ($n=12$ replicates)

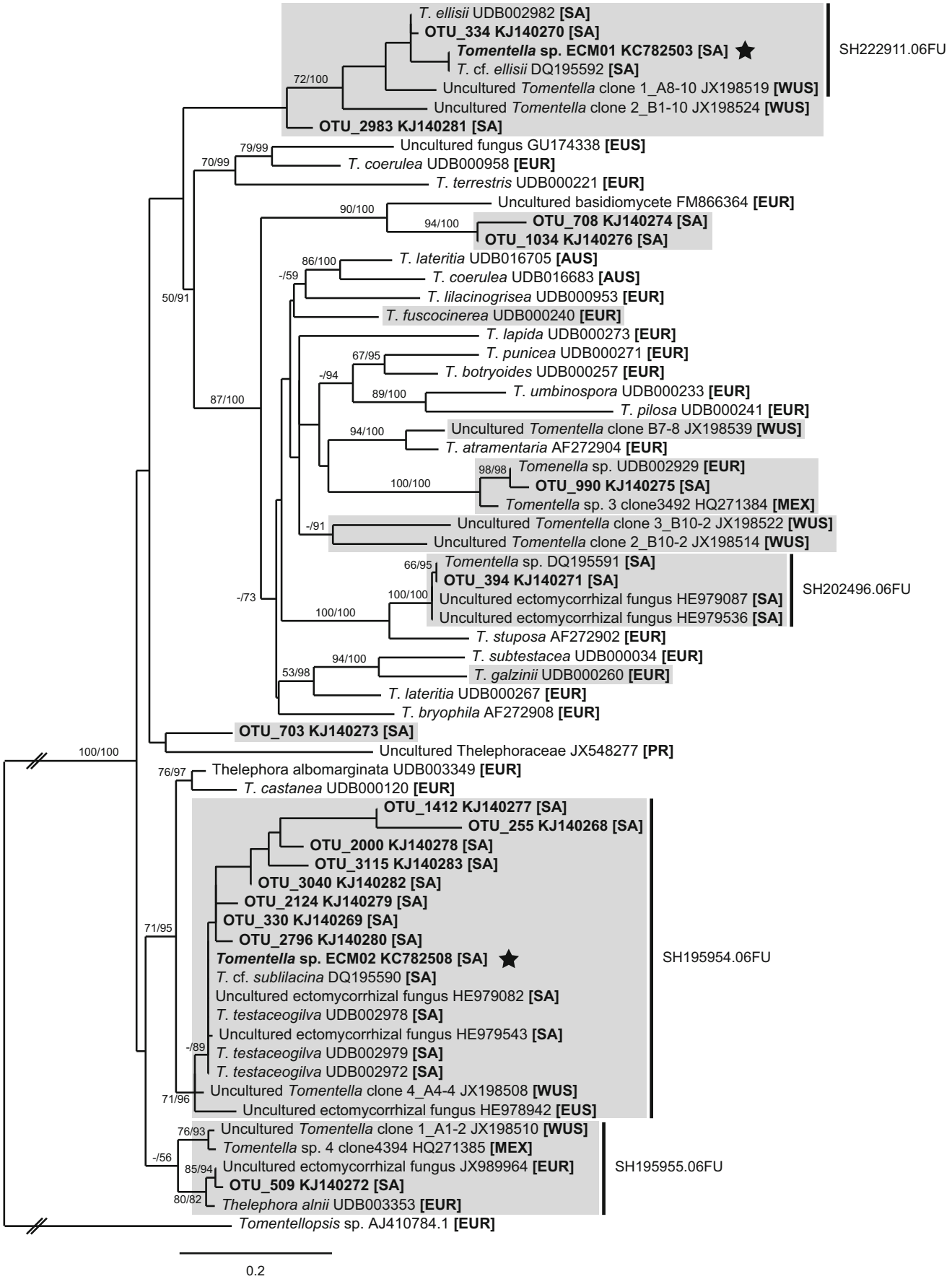
forests; however, *A. acuminata* showed the highest colonization value. In addition, both *Alnus* species had higher growing parameter values in the non-sterilized soil treatments, compared with sterilized controls, thus indicating a high affinity for ectomycorrhizal and actinorhizal association. *Frankia* colonization was abundant in both hosts. Few studies [51, 52] have concluded that some *Frankia* strains present a wide range of suitable hosts and are capable of surviving as inocula in the soil. Results on *Alnus* suggest that *Frankia* strains are promiscuous in their infection as previously observed in *A. acuminata* [1, 5] and that actinorhizal plants can be infected by *Frankia* strains present in foreign soils [53, 54].

Two tomentelloid ECM taxa colonized both *A. acuminata* and *A. glutinosa* seedlings in the greenhouse experiment and dominated the root systems after 6 months of culture. Previous greenhouse experiments have shown *Tomentella* as well as *Alnicola* species as dominant symbionts on *A. rhombifolia* [55] in the Western United States. It is known that the harvesting time can influence the ECM-associated species, because a single early harvest may miss many minor types that have not yet developed, and a late harvest might show only the dominant types [56]. *A. glutinosa* seedlings were receptive to native *Tomentella* species from *A. acuminata* forests. Even though DNA sequences generated from soil samples included other ECM genera as well (e.g., *Alnicola*, *Amanita*, *Alpova*, *Clavulina*, *Cortinarius*, *Inocybe*, *Lactarius*, *Russula*), these types were not registered on the seedlings at the harvest time. Previous studies indicated that *Tomentella* is the most diverse and abundant ECM genus associated with *Alnus* in Mexico [21], which seem to be also true for *A. acuminata* in Argentina, which is also supported by the diversity of tomentelloid OTUs generated from the habitat soil samples. The placement of both tomentelloid taxa in two clades clustered with other *Tomentella* spp. associated with alder in the phylogenetic analysis (Fig. 4) provides additional support to previous phylogenetic reconstructions of *Tomentella* species in association with *Alnus* [42, 21, 16].

Therefore, our results confirm former reports of strikingly high sequence similarity among various dominant alder-associated ECM fungi across distant geographic areas [3, 4, 21, 16, 27, 15] as well as the existence of a number of unidentified endemic taxa suggested to occur at local scales [57, 16]. Results also provide further support to the hypothesis of recent co-migration of these ECM fungi with *Alnus* from the Northern Hemisphere [21].

Soil sequence data suggest that a variety of tomentelloid taxa occur at the native *A. acuminata* sites, including several unidentified *Tomentella* lineages that may or may not represent undescribed species. A sequence count of these OTUs was variable, as shown in Table 1. It is worth to note that while in sequencing data with very high coverage, the reliability of OTUs with a low number of sequences (e.g., <5) may be questioned, and read count cannot be used as a single measure of how reliable the OTU sequence is. For example, OTU 990 is a rather rare OTU with just seven sequences; nonetheless, it is the only one in that lineage and is highly similar to a formerly published sequence. Similarly, other OTUs represent taxa not previously reported from Argentina, such as OTU 509 that is closely related to *T. alnii* (SH195955.06FU) and other two *Tomentella* species represented by OTUs 990 and 394 (SH219858.06FU and 202496.06FU, respectively). Thus, several tomentelloid taxa remain to be characterized for *A. acuminata* at the regional scale. Indeed, previous studies have indicated that further sampling along the *Alnus* distribution range would reveal additional undiscovered taxa [16]. Our observations suggest that several *Tomentella* species and their propagules are readily available in *A. acuminata* forest soils. Mycobiont propagule abundance is considered as a prime determinant of early stages of community development and might affect long-term dominance [11].

Tomentella species from Argentina sites colonized *A. acuminata* and *A. glutinosa* seedlings. This pattern is in accordance with previous studies that indicate low host specificity at the intrageneric level in *Alnus* [21, 22, 11]. In addition, other studies indicated that the /tomentella-thelephora lineage is the most species rich of the *Alnus*-associated fungi at the global scale [16]. Indeed, *Tomentella* is a widespread ECM fungus that sporulates in the organic soil horizon and is an important component of ECM communities worldwide, including arctic tundra [58], boreal forest [59, 60], and tropical and subtropical rain forest habitats [61, 62]. It has also been proved to be abundant in mature temperate forest stands [63] and described as typical early colonizers usually dominant in the spore banks of the post-disturbance ECM fungal propagule community [64, 65, 63] facilitated by invertebrate dispersal [66]. Data showed that tomentelloid fungi can be of considerable importance in ECM communities linking the


T. ellisii UDB002982 [SA]
OTU_334 KJ140270 [SA]
Tomentella sp. ECM01 KC782503 [SA]
T. cf. ellisii DQ195592 [SA]
Uncultured Tomentella clone 1_A8-10 JX198519 [WUS]
Uncultured Tomentella clone 2_B1-10 JX198524 [WUS]
OTU_2983 KJ140281 [SA]
SH222911.06FU
72/100
Uncultured fungus GU174338 [EUS]
79/99
T. coerulea UDB000958 [EUR]
70/99
T. terrestris UDB000221 [EUR]
Uncultured basidiomycete FM866364 [EUR]
90/100
OTU_708 KJ140274 [SA]
94/100
OTU_1034 KJ140276 [SA]
T. lateritia UDB016705 [AUS]
86/100
T. coerulea UDB016683 [AUS]
-/59
T. lilacinogrisea UDB000953 [EUR]
50/91
T. fuscocinerea UDB000240 [EUR]
T. lapida UDB000273 [EUR]
T. punicea UDB000271 [EUR]
67/95
T. botryoides UDB000257 [EUR]
-/94
T. umbinospora UDB000233 [EUR]
89/100
T. pilosa UDB000241 [EUR]
87/100
Uncultured Tomentella clone B7-8 JX198539 [WUS]
94/100
T. atramentaria AF272904 [EUR]
Tomenella sp. UDB002929 [EUR]
98/98
OTU_990 KJ140275 [SA]
100/100
Tomentella sp. 3 clone3492 HQ271384 [MEX]
Uncultured Tomentella clone 3_B10-2 JX198522 [WUS]
-/91
Uncultured Tomentella clone 2_B10-2 JX198514 [WUS]
Tomentella sp. DQ195591 [SA]
66/95
OTU_394 KJ140271 [SA]
100/100
Uncultured ectomycorrhizal fungus HE979087 [SA]
Uncultured ectomycorrhizal fungus HE979536 [SA]
SH202496.06FU
-/73
100/100
T. stuposa AF272902 [EUR]
T. subtestacea UDB000034 [EUR]
94/100
T. galzinii UDB000260 [EUR]
53/98
T. lateritia UDB000267 [EUR]
T. bryophila AF272908 [EUR]
OTU_703 KJ140273 [SA]
Uncultured Thelephoraceae JX548277 [PR]
100/100
Thelephora albomarginata UDB003349 [EUR]
76/97
T. castanea UDB000120 [EUR]
OTU_1412 KJ140277 [SA]
OTU_255 KJ140268 [SA]
OTU_2000 KJ140278 [SA]
OTU_3115 KJ140283 [SA]
OTU_3040 KJ140282 [SA]
OTU_2124 KJ140279 [SA]
OTU_330 KJ140269 [SA]
OTU_2796 KJ140280 [SA]
71/95
Tomentella sp. ECM02 KC782508 [SA]
T. cf. sublilacina DQ195590 [SA]
Uncultured ectomycorrhizal fungus HE979082 [SA]
T. testaceogilva UDB002978 [SA]
Uncultured ectomycorrhizal fungus HE979543 [SA]
SH195954.06FU
-/89
T. testaceogilva UDB002979 [SA]
T. testaceogilva UDB002972 [SA]
Uncultured Tomentella clone 4_A4-4 JX198508 [WUS]
71/96
Uncultured ectomycorrhizal fungus HE978942 [EUS]
Uncultured Tomentella clone 1_A1-2 JX198510 [WUS]
76/93
Tomentella sp. 4 clone4394 HQ271385 [MEX]
-/56
Uncultured ectomycorrhizal fungus JX989964 [EUR]
85/94
OTU_509 KJ140272 [SA]
SH195955.06FU
80/82
Thelephora alnii UDB003353 [EUR]
Tomentellopsis sp. AJ410784.1 [EUR]
0.2

◀ **Fig. 4** Maximum-likelihood phylogram showing the placement of the two ECM morphotypes found among *Tomentella*/*Thelephora* species and environmental DNA sequences. Bootstrap values (*BS*) and Bayesian posterior probabilities (*BPP*) (as percentages), both >50 %, are shown *near the nodes*. *Alnus*-associated taxa are designated in *gray boxes*. Taxa names in *bold* correspond to sequences generated for this study. *Star* indicates the placement of ECM1 and ECM2. *OTU* operational taxonomic unit generated from eDNA, *SA* South America, *MEX* Mexico, *PR* Puerto Rico, *EUS* Eastern United States, *WUS* Western United States, *EUR* Europe

decomposition of wood process with the germination and early growth of seedlings which commonly become established on decaying logs and branches [67]. In addition to its ECM capacity, some species have been probed to establish specific myco-heterotrophic associations with terrestrial orchids over a broad geographic area in North America [68].

Results from the greenhouse experiment illustrate the probable role of specific tomentelloid fungi in the early colonization of seedlings in *A. acuminata* forests as well as their importance in the structure of the propagule community of ECM fungi at the sites. It was also demonstrated that some *Tomentella* species lack host preference when considering two *Alnus* species from different biogeographic origins and subjected to diverse environmental conditions.

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