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ORIGINAL PAPER

Ectomycorrhizal fungi in Mexican *Alnus* forests support the host co-migration hypothesis and continental-scale patterns in phylogeography

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Abstract To examine the geographic patterns in *Alnus*-associated ectomycorrhizal (ECM) fungal assemblages and determine how they may relate to host plant biogeography, we studied ECM assemblages associated with two *Alnus* species (*Alnus acuminata* and *Alnus jorullensis*) in montane Mexico and compared them with *Alnus*-associated ECM assemblages located elsewhere in the Americas. ECM root samples were collected from four sites in Mexico (two per host species), identified with ITS and LSU rRNA gene sequences, and assessed using both taxon- (richness, diversity, evenness indices) and sequence divergence-based (UniFrac clustering and significance) analyses. Only 23 ECM taxa were encountered. *Clavulina*, an ECM lineage never before reported with *Alnus*, contained the dominant taxon overall. ECM assemblage structure varied between hosts, but UniFrac significance tests indicated that both associated with similar ECM lineage diversity. There was a strikingly high sequence similarity among a diverse array of the ECM taxa in Mexico and those in *Alnus* forests in Argentina, the United States, and Europe. The Mexican and United States assemblages had greater overlap than those present in Argentina, supporting the host–ECM fungi co-migration hypothesis from a common north temperate origin. Our results indicate that *Alnus*-associated ECM assemblages have clear patterns in richness and composition across a wide range of geographic locations. Additional data from boreal western North America as well as the eastern United States and Canada will be particularly informative in further understanding the co-biogeographic patterns of *Alnus* and ECM fungi in the Americas.

Keywords *Alnus* · Americas · Biogeography · Ectomycorrhiza · Fungi · ITS · LSU · Mexico

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Introduction

The evolutionary and ecological significance of mycorrhizal symbioses has been widely recognized (Selosse and Le Tacon 1998; Van der Heijden and Sanders 2002), particularly as the ability to identify and quantify mycorrhizal fungi in natural environments has improved (Horton and Bruns 2001; Peay et al. 2008). To date, however, there has been a strong geographic bias in the study of these symbioses, especially for ectomycorrhizal (ECM) fungi (Bruns and Kennedy 2009). The vast majority of what is known about ECM fungi comes from studies in north temperate and boreal regions, despite the fact that tropical and south temperate regions harbor a high diversity of ECM hosts (Smith and Read 2008). Due to this discrepancy, a major aim of recent ECM research has been to determine the extent to which patterns identified in northern regions are truly global in scope (Dickie and Moyersoen 2008).

The ECM host genus *Alnus* Mill. (Betulaceae) has a widespread distribution, with the majority of the ~30

species occurring in temperate latitudes (Benson and Dawson 2007). Although taxonomists have debated the geographical origin of this genus, multiple authors have agreed it was most likely in Asia (Chen and Li 2004 and references therein). From that region, it appears that ancestors of western north American species (e.g. *Alnus incana* L., *Alnus rubra* Bong., *Alnus rhombifolia* Nutt.) migrated across the Bering Sea land bridge, with *Alnus* fossil records being present in central Oregon since the middle Eocene (Crane 1989). The two *Alnus* species with ranges in central and south America, *Alnus acuminata* Kunth and *Alnus jorullensis* Kunth, are hypothesized to be further descendants of those western north American colonists (Furlow 1979). This relationship is supported by fossil evidence (Crane 1989) as well as phylogenetic studies of the genus, which show *A. acuminata* and *A. jorullensis* as sister taxa nested within the circumpolar *A. incana* complex that includes western north American species such as *A. rubra* (Chen and Li 2004; Navarro et al. 2003).

Because the historical spread of *Alnus* in the Americas is well documented, this genus provides an excellent opportunity to examine how the structure of ECM assemblages may be influenced by host plant migration and biogeography. Similar comparisons have been made for individual ECM species (e.g., Murat et al. 2004; Geml et al. 2008) and ECM genera (e.g., Wu et al. 2000; den Bakker et al. 2004; Matheny et al. 2009; Bonito et al. 2010), but analyses of the entire ECM assemblage are less common. A notable exception is the long-term study of neotropical montane *Quercus*-associated ECM assemblages by Halling, Mueller, and colleagues (http://www.nybg.org/bsci/res/hall). Halling (2001) summarized part of their work by stating that: "[a]lthough we are not the first to note affinities of montane Neotropical Agaricales with taxa in the North Temperate zone (see Moser & Horak, 1975), we do suggest co-migration of mycorrhizal communities; that is, obligate ectomycorrhizal fungal genera have migrated from the North Temperate zone with their associated phanerogams, specifically alder [*Alnus*] and oak [*Quercus*], both of which had a North Temperate origin." While there have been reports of individual ECM lineages in montane neotropical *Alnus* forests that support this hypothesis (Halling 1989, 1997; Moser 2001), no systematic surveys of entire ECM assemblages in these habitats have yet been presented.

Currently, two detailed studies of the belowground ECM assemblages associated with *Alnus* species in the Americas have been conducted. Becerra et al. (2005) examined the ECM assemblages on *A. acuminata* at one site in northwestern Argentina. The assemblage was dominated by multiple *Tomentella* species, with representatives of the genera *Cortinarius*, *Lactarius*, and *Russula* also being present. More recently, Kennedy and Hill (2010) examined the ECM assemblages associated with *A. rubra* at four sites in the northwestern United States. The latter study found a similar dominance of *Tomentella* species and a phylogenetic analysis of ITS rRNA gene sequences indicated that one *Tomentella* taxon present in both studies (*Tomentella* sp. 1) likely represented the same species (99% similarity, 660/663 bases). The study of Kennedy and Hill (2010) also found representatives of the genera *Cortinarius* and *Lactarius* but additionally documented an abundance of ECM roots colonized by two other genera, *Alnicola* and *Alpova*, and recorded a greater number of species overall. The observed differences may be explained by multiple factors (e.g., sampling effort, environmental conditions, specific molecular identification method) but may also reflect differences in host biogeography, as the south American sites were colonized by *A. acuminata* more recently than *A. rubra* in western north America. To examine this hypothesis as well as others about the factors affecting *Alnus*-associated ECM distributions in the Americas, data from additional locations are clearly needed.

In this study, we sampled the ECM assemblages associated with two *Alnus* species, *A. acuminata* and *A. jorullensis*, in the montane forests of central Mexico. This study is the first to examine the ECM assemblages associated with *A. acuminata* at the northern end of its distribution as well as compare the assemblages present on *A. jorullensis* to those of other host species. We also present the phylogenetic analyses of four ECM genera with *Alnus*-associated taxa in the United States, Mexico, and Argentina to examine patterns of sequence similarity at the continental scale. Based on the relatively high genus-level overlap between the north and south American *Alnus*-associated ECM assemblages as well as those sampled in Europe (Pritsch et al. 1997; Tedersoo et al. 2009), we hypothesized that the Mexican assemblages would have similar composition and richness to those present in other regions.

Materials and methods

Study location

ECM samples were collected from four montane sites in Mexico ("Electronic supplementary materials, S1"). Two of the sites were located in Parque Nacional La Malinche, Tlaxcala, in mature forests co-dominated by *A. jorullensis* and *Pinus montezumae*. At Malinche site 1 (19°16.07 N, 98° 02.13 W, elevation=3,283 m), *A. jorullensis* and *P. montezumae* were both present in the canopy; at Malinche site 2 (19°11.29 N, 97°58.97 W, 2,929 m), *A. jorullensis* and *P. montezumae* dominated the sub-canopy and canopy, respectively. These sites were separated by 10 km. The only additional ECM host at those sites was a *Salix* species, which was present in much lower abundance than both

Alnus and *Pinus*. The other two sites were located at the Acatlan volcano, Veracruz. The sites, designated Naolinco 1 (19°40.49 N, 96°51.12 W, 1,816 m) and Naolinco 2 (19°40.98 N, 96°51.48 W, 1,880 m), were separated by 1 km and located on opposite sides of the outer rim of the volcano. Both were exclusively dominated by *A. acuminata*, with no other ECM hosts present. The *A. acuminata* individuals at the Naolinco sites were estimated to be ~20–25 years old based on height and basal diameter (C. Alvarez, personal communication). Climate information for each site is provided in Table 1.

Field sampling design

The design of ECM root tip sampling varied slightly among sites. At both the Malinche sites and at Naolinco 1, two parallel transects separated by >10 m were established and all *Alnus* trees separated by >2 m along each transect were numbered (~20 trees per transect). A total of 20 trees were then randomly selected for ECM root tip sampling. At each tree, root samples were obtained in the immediate vicinity of the trunk (<1 m) from the top 20 cm of soil. Because the *Alnus* root distribution was patchy, multiple individual root samples and associated soil around each tree were pooled into a single ~2,500-cm^3 sample. At the two Malinche sites, a concerted effort was made to collect roots with *Frankia* nodules or directly connected to *Alnus* trees due to the co-occurrence of *P. montezumae* (see results regarding variable success rates). No *Salix* individuals were located near the sampled *Alnus* trees. The Naolinco 2 site was smaller in total area than the other sites, so a similar transect-based randomization was not feasible. Therefore, 20 *A. acuminata* trees separated by >2 m were haphazardly selected for the same type of ECM sampling (see Krebs (1999) for differences between random and haphazard sampling). All samples were collected in July 2010 and stored on ice for a maximum of 72 h prior to laboratory processing.

ECM root tip processing

In the laboratory, roots from each sample were removed from the soil and gently rinsed in non-distilled water. They were then stored in water at 8°C for 24 to 96 h before microscopic examination. For each sample, all roots were examined under a ×10 dissecting microscope and ECM root tips were distinguished from non-ECM root tips based on standard morphological characteristics (e.g., swelling, color, branching morphology, emanating hyphae). Tips from all ECM putative morphotypes present in each sample were removed in approximate proportion to their abundance. From that pool, which varied in size across samples from 0 to >50 tips (typically around 40), eight ECM root tips were randomly selected (or as many present if less than that), placed into CTAB buffer (2% CTAB, 0.1 M Tris pH 8.0, 1 M NaCl, 0.02 M EDTA, 1% PVPP), and stored at −20°C.

ECM molecular protocols

Total genomic DNA from ECM root tips was extracted using the REDExtract-N-Amp Plant PCR kit (Sigma-Aldrich, St. Louis, MO, USA). Individual root tips were added to 10 μl of extraction solution and heated at 65°C for 10 min, followed by 95°C for 10 min. After incubation, 30 μl of dilution solution was added and DNA extracts were stored at −20°C. PCR reactions of the rDNA internal transcribed spacer (ITS) region were conducted using the primer pair ITS1F and ITS4 under conditions previously described (Gardes and Bruns 1993; Kennedy and Hill 2010). For each sample with dual amplification, which represented 9.6% (54/562) of all root tips, both the ITS1F/ITS4B and ITS1F/ITS4A primer combinations were subsequently applied to obtain single amplicons wherever possible. A ~800-bp fragment of rDNA large subunit (LSU) gene was also amplified using the fungal specific primer pair LROR_F and LR5-F under conditions described by Amend et al. (2010). All PCR products were cleaned by combining 0.75 μl of ExoSAP IT (USB Corp., Cleveland, OH, USA), 1.25 μl of PCR-grade water, and 7.5 μl of PCR product. The combination was heated to 37°C for 45 min, followed by 80°C for 15 min. DNA sequencing was performed on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at the Genetics Core Facility at the University of Arizona, USA. Sequence chromatograms were visually confirmed and

Table 1 Annual and July-specific temperature and precipitation data for the four Mexican field sites. Data are calculated from the USDA Forest Service current climate model (http://forest.moscowfsl.wsu.edu/climate/). Temperature units are in degrees Celcius and precipitation is in millimeters

Site	Temperature (mean, max, min)	Precipitation (total)	July temperature (mean, max, min)	July precipitation (total)
Malinche 1	9.6, 18.6, 0.6	928	9.9, 15.2, 4.6	154
Malinche 2	11.9, 21.6 , 1.7	620	12.3, 18.1, 6.3	90
Naolinco 1	16.0, 23.5, 8.5	2,414	16.6, 21.4, 12.0	414
Naolinco 2	15.6, 23.1, 8.2	2,413	16.2, 21.0, 11.6	413

manually corrected where necessary, and contiguous sequences were assembled using Sequencher 4.8 (Gene Codes Corp., Ann Arbor, MI, USA).

ECM taxa identification

Sequences from ECM root tips were grouped across all samples into operational taxonomic units (OTUs) at ≥97% and ≥99% similarity for the ITS and LSU regions, respectively. Those thresholds have been previously shown to distinguish most ECM taxa at the species level (Peay et al. 2008; Porter et al. 2008). Sequences of each OTU (hereafter referred to as taxon) were compared with those in the NCBI and UNITE databases ("Electronic supplementary materials," S2 and S3) and names were designated based upon the taxonomic level supported from the database queries. There was very high overlap in the OTU designations from the two gene regions (95% (394/416) of the identified EM root tips were designated identically); however, in 22 cases they differed. These were all either intra-family (*Hymenogastraceae* and *Hebeloma* (ITS) vs. *Alnicola* (LSU)) or intra-generic (*Tomentella* and *Sebacina*) differences. For the samples with different ITS and LSU intra-family designations, the taxonomic affiliation was left at the family level. For the intra-generic *Tomentella* and *Sebacina* differences, the ITS classification was used due to greater genetic discrimination with this region. Only samples with confirmed host identification (see below) and from ECM lineages identified by Tedersoo et al. (2010) were included in the final analyses.

ECM host identification

Due to the co-dominance of *P. montezumae* at the Malinche sites, additional analyses of ECM root tips were conducted to confirm host root identity. For all root tips with ECM taxa that were not shared with the *A. acuminata* sites, as well as all singletons from all four sites, the plant plastid *trn*L cpDNA region was amplified using the *trn*L_c-*trn*L_d primer pair (Taberlet et al. 1991). The PCR cycling conditions for those reactions were as follows: (1) 94°C for 3 min, (2) 94°C for 45 s, (3) 55°C for 45 s, (4) 72°C for 30 s, (5) back to step two 30 times, and (6) 72°C for 10 min. Size differences among amplicons were determined on 2% agarose gels, with *Pinus* and *Alnus* leaf amplicons present on each gel for reference. A representative sequence from each ECM taxon (including those present on *Pinus*) was submitted to GenBank under the accession numbers HQ271351–HQ271432.

Soil chemical analyses

Soils from five samples at each site were analyzed independently for pH, cation exchange capacity (CEC), available phosphorus (Olsen method), percent carbon, and percent nitrogen at the Instituto de Geologia, Universidad Nacional Autonoma de Mexico. The soils came from the same samples from which ECM root tips were analyzed.

Statistical and phylogenetic analyses

EstimateS (Colwell 2005) was used to estimate ECM richness at each site. The Chao2 estimator was chosen to facilitate comparison with previous *Alnus* ECM studies (Tedersoo et al. 2009; Kennedy and Hill 2010) and computed based on 500 randomizations of sample order without replacement. The same program was also used to calculate for Simpson's index of diversity and evenness. To examine the potential effect of host species on *Alnus* ECM assemblage structure, divergence-based analyses were conducted (Lozupone and Knight 2008). Both weighted and unweighted UniFrac analyses were carried out in Fast UniFrac (Hamady et al. 2010). For these analyses, LSU ECM sequence data were first aligned in MUSCLE (Edgar 2004) and then a maximum likelihood (ML) tree was generated in MEGA 4 (Tamura et al. 2007) using a K2 model of nucleotide substitution and 500 bootstrap replications. Jackknife sample clustering and UniFrac tests of significance were carried out under the default settings. Because local abundances of ECM root tips can be directly influenced by fungi themselves (Van der Heijden and Kuyper 2003; Pickles et al. 2010), all of the taxon- and divergence-based analyses were based on the number of root samples, not the number of ECM root tips.

Phylogenetic analyses of the genera *Tomentella*, *Lactarius*, and *Cortinarius* were conducted using the same ML methods as described in detail in Kennedy and Hill (2010). For the genus *Inocybe*, a LSU alignment was obtained from Matheny et al. (2009) and trimmed to include 30 taxa most related to those sampled in this study. All samples were re-aligned using MUSCLE and a ML tree was generated in PhyML 3.0 (Guindon and Gascuel 2003) on the Montpellier bioinformatics platform (http://www.atgc-montpellier.fr/phyml/) using the following settings: substitution model TN93, proportion of invariable sites fixed, gamma parameter estimated, number of substitution categories=4, starting tree BIONJ, type of tree improvement NNI, optimized topology and branch lengths, and approximate likelihood ratio test (aLRT) scores.

Soil parameters were compared among sites using a series of one-way fixed-factor ANOVAs. Prior to running those analyses, pH, cation exchange, and phosphorus data were log-transformed to reduce variance heterogeneity. Significant differences of means were determined using post-hoc Tukey HSD tests. Analyses were conducted in JMP v4 (SAS Inc, Cary, NC, USA) and considered significant at $P \leq 0.05$.

Results

ECM root tips were present in 79 of the 80 samples collected. Seventy-four percent (416/562) of all root tips sampled were successfully sequenced with at least one primer pair and identified as ECM taxa. Success varied somewhat between gene regions, with ITS and LSU having 70% (390/562) vs. 63% (353/562) ECM identifications, respectively. Amplification of the *trn*L plant plastid revealed that 60% (32/53) of the identified ECM root tips from Malinche 1 and 9% (10/114) from Malinche 2 belonged to *P. montezumae*, not *A. jorullensis*. As a result, only 11 of the samples at Malinche 2 contained *Alnus* ECM root tips, while the other three sites were each represented by 20 samples. All amplified ECM singletons from the Naolinco sites were confirmed to be present on *A. acuminata* roots (data not shown).

A total of only 23 ECM taxa were observed across the four sites (Fig. 1; Table 2). Chao2 taxon richness estimates indicated that the vast majority of ECM taxa were captured at all sites except Naolinco 1, where eight of the 16 taxa were encountered in a single soil sample (Table 2). The dominant taxon overall, *Clavulina* sp. 1, was present in 35% (25/71) of all samples analyzed and represented an ECM lineage never previously reported on *Alnus*. While *Clavulina* sp. 1 was the most abundant taxon on *A. acuminata*, *Cortinarius* sp. 1 had the highest abundance on *A. jorullensis* (Fig. 1). Other ECM taxa present on both

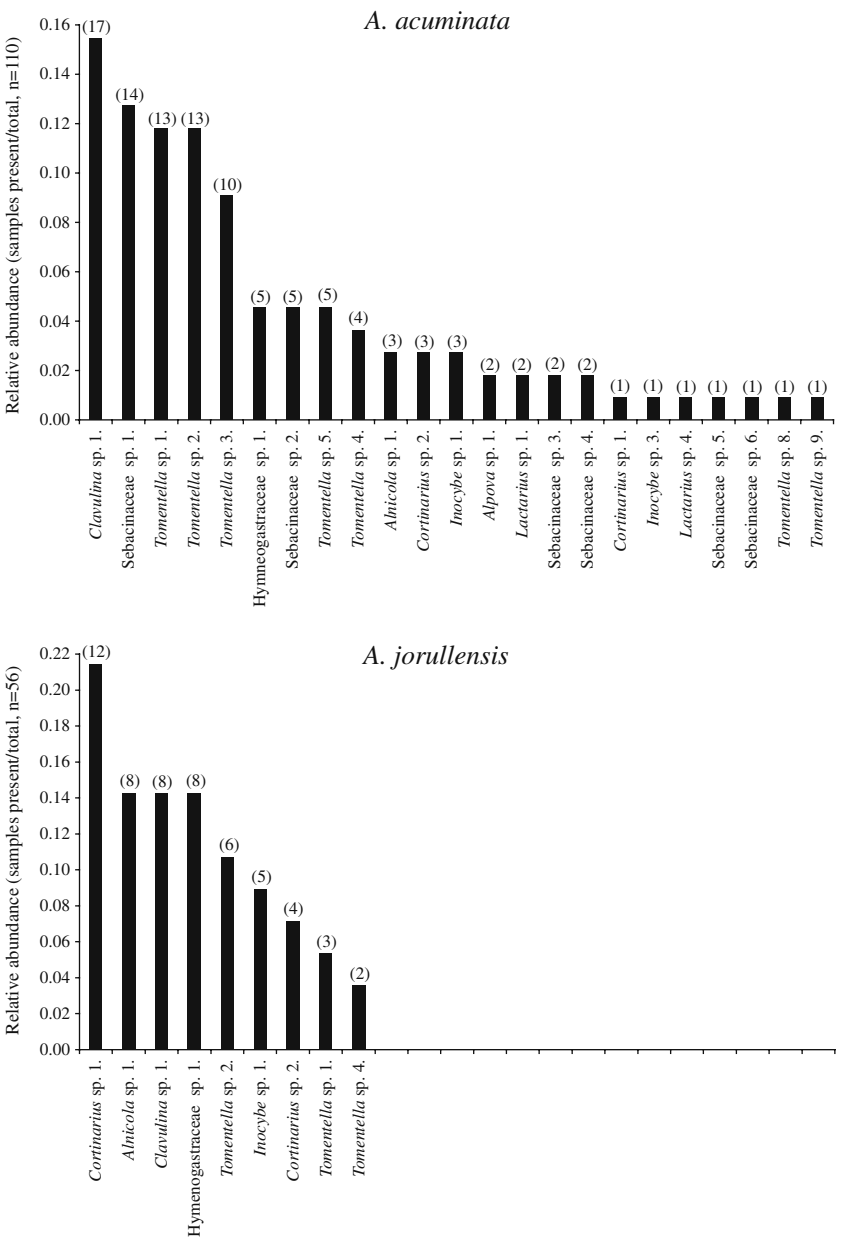

Fig. 1 Rank–abundance curves for the ectomycorrhizal fungal assemblages associated with *A. acuminata* and *A. jorullensis* in Mexico. *Values in parentheses above each bar* represent the total number of samples in which that taxon was encountered on each host. Note that the non-sequential numbering of taxa in the genera *Inocybe*, *Lactarius*, and *Tomentella* reflects the fact that other congeneric taxa were sampled on *P. montezumae*

Table 2 Ectomycorrhizal fungal taxa richness, diversity, and evenness associated with *A. jorullensis* and *A. acuminata* in Mexican montane forests. Samples represent the number of samples in which *Alnus* EMF root tips were present. Taxa estimated are based on Chao 2 estimates, while diversity and evenness are both based on Simpson estimates. All estimates were calculated in the program EstimateS (Colwell 2005)

Host	*A. jorullensis*		*A. acuminata*		Total
Site	Malinche 1	Malinche 2	Naolinco 1	Naolinco 2	
Samples	20	11	20	20	71
Taxa observed	9	5	16	19	23
Taxa estimated	9	5.9	22.7	20.4	24.6
Taxa diversity	8.6	3.9	10.9	13.2	14.1
Taxa evenness	0.76	0.72	0.68	0.69	0.61
Singletons	1	2	8	5	16
Doubletons	1	0	3	5	9

hosts with relatively high abundance (>10 samples total) included multiple *Tomentella* taxa (*Tomentella* sp. 1 and 2), *Alnicola* sp. 1, and Hymenogastraceae sp. 1. Taxa in the family Sebacinaceae, which also have not been previously reported in association with *Alnus*, represented a significant component of the richness of the ECM assemblages sampled (six taxa total) but were only present on *A. acuminata* (Fig. 1).

ECM diversity was higher at both Naolinco (*A. acuminata*) sites than at the two Malinche (*A. jorullensis*) sites (Table 2). This appeared to be due mainly to differences in taxon richness, as evenness was relatively similar among sites (Fig. 1; Table 2). Coleman rarefaction curves indicated that, even when all four sites were rarefied to 11 samples, ECM richness was still significantly higher in the Naolinco sites compared to the Malinche sites (Fig. 2). The structure (i.e., richness, composition, and abundance) of the ECM assemblages also varied across sites. UniFrac jacknife clustering showed that the two Naolinco sites were more similar in structure to each other than to those located at Malinche and vice versa ("Electronic supplementary materials, S4"). This divergence was most apparent in the higher abundance of *Cortinarius* and *Alnicola* taxa at the Malinche sites and the higher and exclusive abundance of *Tomentella* and *Sebacinaceae* taxa at the Naolinco sites, respectively (Fig. 1). Despite these differences, UniFrac tests of significance revealed no differences in ECM lineage representation among sites ($P>0.05$). Soil pH, available phosphorus, percent carbon, and percent nitrogen additionally also varied by site (Table 3). Of these variables, only available phosphorus varied consistently between host sites, with the Malinche (*A. jorullensis*) sites having approximately 70-fold greater phosphorus concentrations than those at Naolinco (*A. acuminata*).

BLAST searches and phylogenetic analyses indicated that many of the *Alnus*-associated ECM taxa sampled in

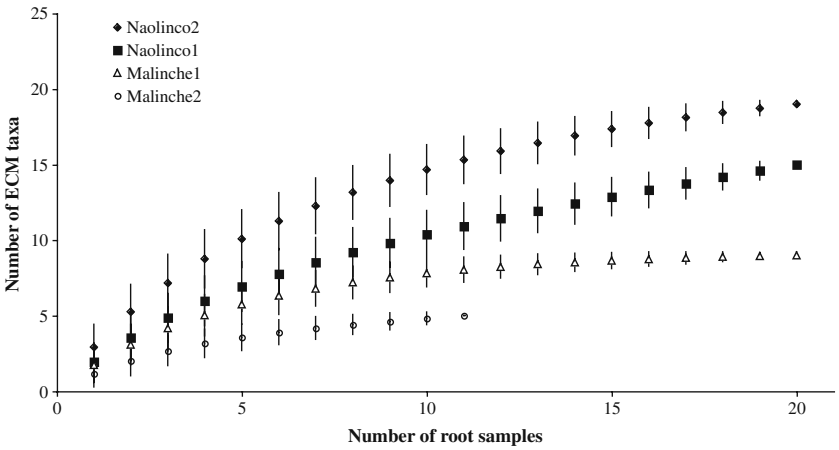

Fig. 2 Coleman rarefaction curves of ECM taxa richness at the four study sites. Means±1 s.d. were calculated in EstimateS (Colwell 2005). Similar differences in richness among sites were also obtained from rarefaction curves calculated in the program EcoSim (http://www.garyentsminger.com/ecosim/index.htm). Rarefying all sites to the lowest total number of ECM occurrences ($n=21$), the mean taxa number and 95% confidence intervals (CI) per site were: Malinche2 mean=5, 95% CI low=5, 95% CI high=5; Malinche1 mean=7.99, 95% CI low=6, 95% CI high=9; Naolinco1 mean=10.83, 95% CI low=8, 95% CI high=14; Naolinco2 mean=11.48, 95% CI low=9, 95% CI high=14

Table 3 Soil chemical characteristics at the four Mexican field sites. Five soil samples from each site were independently analyzed at the Instituto de Geologia, Universidad Nacional Autonoma de Mexico. The characteristics at each site are given as mean (± 1 s.d.). Means not followed by the same letter were significantly different in post-hoc Tukey tests following one-way fixed-factor ANOVAs

Site	Host	pH (H_2O 1:2.5)	CEC (uS/cm)	P (mg/kg)	C (%)	N (%)
Malinche1	*A. jorullensis*	5.76 (0.18) a, b	194.6 (145.4) a	52.2 (25.8) b	5.92 (1.83) a	0.38 (0.13) a
Malinche2	*A. jorullensis*	5.90 (0.41) b	158.5 (53.5) a	86.6 (22.1) b	4.40 (0.96) a	0.32 (0.08) a
Naolinco1	*A. acuminata*	5.34 (0.11) a	301.1 (39.7) a	1.18 (0.53) a	6.22 (1.02) a	0.52 (0.04) a
Naolinco2	*A. acuminata*	5.30 (0.17) a	274.9 (46.6) a	0.76 (0.13) a	13.46 (2.45) b	1.14 (0.17) b

Mexico were very closely related to those present in other *Alnus* forests ("Electronic supplementary materials, S2"). Within the genus *Tomentella*, for example, the closest related taxon to each of the five most abundant Mexican *Tomentella* taxa came from studies of *Alnus*-associated ECM in either Argentina or the United States and not Mexico (Fig. 3). In all of those cases, sequence similarity was ≥97% ("Electronic supplementary materials, S5"), the cutoff typically used to group ITS sequences at the species level. Matching patterns of high sequence similarity (≥97%) between Mexican taxa and north or south American *Alnus*-associated ECM were also observed with representatives of the genera *Cortinarius*, *Inocybe*, and *Lactarius* (Fig. 3). In addition, many of the ECM taxa sampled in American *Alnus* forests showed high similarity to *Alnus*-associated taxa from Europe. The relatively high aLRT scores in these analyses indicate that the ML trees represented accurate reconstructions of phylogenetic history.

Discussion

The *Alnus*-associated ECM assemblages in Mexican forests shared many characteristics with those present elsewhere in the Americas. Overall, taxa richness on both *A. jorullensis* and *A. acuminata* was relatively low, which supports all previous studies of *Alnus*-associated ECM assemblages showing that they typically have much lower richness than those of other hosts (Kennedy and Hill 2010 and references therein). Like other American *Alnus*-associated ECM assemblages sampled to date, the ECM assemblages in Mexico were only composed of basidiomycete ECM lineages. This contrasts with the *Alnus*-associated ECM assemblages in Europe, which contain a number of ECM ascomycete lineages (Tedersoo et al. 2009). Also similar to the other two American assemblages, *Tomentella* was the most diverse ECM genus in Mexico and present in relatively high abundance, particularly on *A. acuminata*. At the same time, the Mexican *Alnus*-associated ECM assemblages had some unique features. Both the overall dominance of a *Clavulina* taxon and the high richness of ECM taxa in the Sebacinaceae represented globally novel ECM associations for *Alnus*. While neither of these ECM lineages has previously been documented in *Alnus* forests, they have been reported as abundant in *Quercus*-dominated Mexican montane forests (Morris et al. 2009), suggesting that the environmental conditions present in Mexico may be particularly conducive to their growth. In addition, the high abundance of *Cortinarius* sp. 1 on *A. jorullensis* was unexpected given that *Cortinarius* species consistently represent a sub-dominant role in *Alnus*-associated ECM assemblages in the United States (Kennedy and Hill 2010), Argentina (Beccera et al. 2005), and Europe (Pritsch et al. 1997; Tedersoo et al. 2009).

Comparing *Alnus*-associated ECM assemblages across the Americas, the ECM assemblages in Mexico had greater overlap with those present in the United States on *A. rubra* than those in Argentina on *A. acuminata*. This was evidenced by the shared *Cortinarius* clades, a greater number of common *Tomentella* clades, and the presence of both *Alnicola* and *Alpova* ECM lineages in Mexico and the United States but not in Argentina. The high general similarity between the United States and Mexican *Alnus*-associated ECM assemblages lends clear support to the hypothesis of Halling (2001) that there has been a co-migration of *Alnus* hosts and ECM fungi from north temperate regions into neotropical montane habitats. While the overlap between the United States and Mexico was higher than that with Argentina, the similarity between the latter two assemblages also supports a continual expansion of this host genus from north to south America. Mexican and Argentinean assemblages both shared *Tomentella*, *Lactarius*, and *Inocybe* clades with very high sequence similarity. In addition, the higher ECM taxa richness on *A. acuminata* in the older part of its range (i.e., Mexico) is also suggestive of a north-to-south expansion of this species. Additional data from boreal western North America as well as the eastern United States and Canada will be particularly informative in further examining *Alnus*–ECM fungi co-biogeographic patterns in the Americas. We hypothesize that the western boreal *Alnus*-associated ECM assemblages will have considerable overlap with those in the western United States, central, and south America due to their common biogeographic history (Chen and Li 2004). In

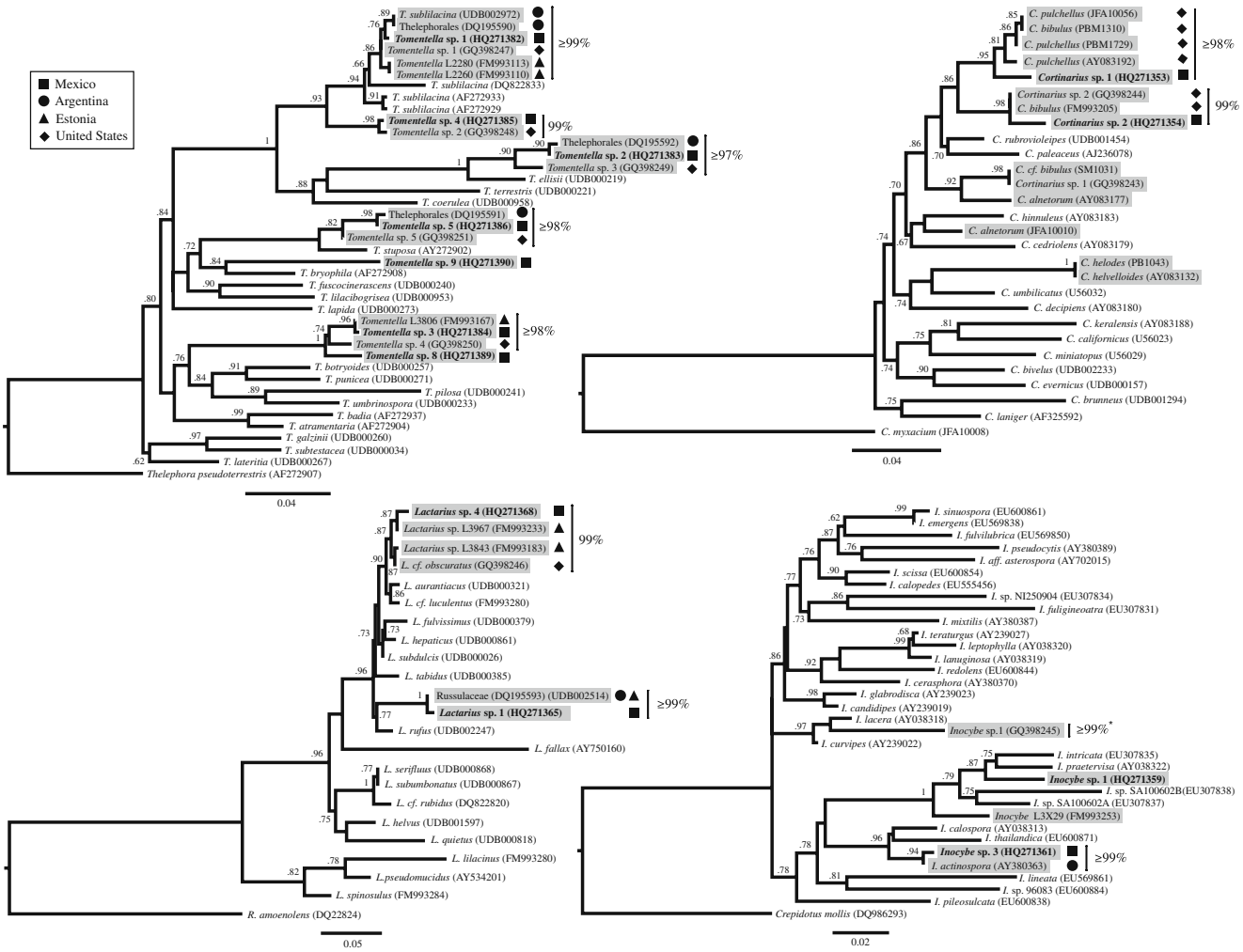

Fig. 3 Phylogenetic reconstructions of taxa in the ECM genera *Tomentella*, *Cortinarius*, *Lactarius*, and *Inocybe* based on rDNA ITS or LSU sequences. Nodes are labeled with aLRT scores from the maximum likelihood analysis above 0.60. Taxa are labeled with species name or unique identifier and GenBank or UNITE number in *parentheses*. *Alnus*-associated taxa are designated in *gray boxes*, with the Mexican *Alnus*-associated taxa in *bold*. *Symbols* next to selected *Alnus*-associated taxa indicate the geographic area from which they were obtained. The percentage values for the selected groups represent pair-wise comparisons between all group members (specific values for each comparison are provided in "Electronic supplementary materials, S5"). *Thelephora pseudoterrestris*, *Cortinarius myxacium* (sensu J. Ammirati), *Russula amoenolens*, and *Crepidotus mollis* were designated as outgroups. Note that many of the species epithets used in the *Inocybe* analysis are provisional; see Matheny et al. (2009) for full details about the *Inocybe* nomenclature used. *Asterisk*, *Inocybe* sp. 1 (GQ398245), which was sampled in *A. rubra* forests in the United States by Kennedy and Hill (2010), shares 99% sequence similarity with another *Inocybe* taxon, provisionally named *I. crucifera* Moreau and Matheny ad. int., collected under *Alnus alnobetula* subsp. *suaveolens* in Corsica, Europe (P-A. Moreau and P. Matheny, personal communication)

contrast, *Alnus*-associated ECM assemblages from eastern north America, which are present on host species that colonized the continent via the North Atlantic land bridge from Europe (Chen and Li 2004), may likely have greater similarity to European *Alnus*-associated ECM assemblages (e.g., presence of ascomycetes, a greater diversity of *Alnicola* taxa).

Our phylogenetic analyses indicate that many ECM taxa present in *Alnus* forests in both the western United States and Argentina were also present in Mexico. Because our data are only based on root tip sequences and do not include any fruit body comparisons or laboratory mating tests, we are not able to say that geographically disparate samples with high sequence similarity are in fact the same ECM species. It is clear, however, that there are many common *Alnus*-associated ECM clades present throughout the Americas. These results correspond well with the recent study of Pritsch et al. (2010), who compared some of the *Alnus*-associated ECM taxa in Argentina with those in Europe and found very high sequence similarity among taxa in the genera *Tomentella* and *Lactarius*. The high global similarity among many *Alnus*-associated ECM taxa is a striking pattern that contrasts with the more regional distribution patterns observed in many ECM species (Wu et

al. 2000; Murat et al. 2004; Geml et al. 2008) and subgenera (Matheny et al. 2009; Bonito et al. 2010). The reasons for this pattern are not clear, but we have previously hypothesized that it may be due to the co-presence of nitrogen-fixing *Frankia* bacteria (Kennedy and Hill 2010). These bacteria strongly alter soil nitrogen concentrations (Bormann et al. 1994) in ways that may differentially affect ECM taxa (Lilleskov et al. 2002; Toljander et al. 2006) and/or favor the presence of those with greater enzymatic capacities for nutrients aside from nitrogen. Although our current data cannot address this hypothesis directly, we have experiments about the effects of *Frankia* on *Alnus*-associated ECM assemblages in progress.

There was considerable overlap in the dominant ECM taxa associated with *A. acuminata* and *A. jorullensis*, with the former species also hosting a number of additional low-abundance taxa. The differential abundance of shared ECM taxa on the two hosts could indicate some level of host preference, a pattern well documented both within (Morris et al. 2008; Morris et al. 2009) and among (Kennedy et al. 2003; Ishida et al. 2007; Smith et al. 2009) other ECM host genera. The lack of significance in the UniFrac tests, however, indicated that intra-generic host specificity is low. A lack of intra-generic host specificity was also experimentally demonstrated by Molina (1981). Given the confounded design of this study (i.e., host species is co-correlated with differences in distance, elevation, climate, soil phosphorus, and tree age), no clear conclusions can be drawn about which factors were most responsible for the differential abundance of ECM taxa across sites. Interestingly, the low ECM richness observed on *A. jorullensis* does not reflect the ECM capacity of the Malinche sites. From the 42 *P. montezumae* ECM root tips that we sampled at those sites, 24 taxa were identified as belonging to a diverse array of genera ("Electronic supplementary materials, S3"). This much higher richness in fewer ECM root tips reiterates the distinctive nature of *Alnus*-associated ECM assemblage richness compared to other hosts. Despite a clear intermingling of root systems, there was no overlap in the ECM assemblages on *Alnus* and *Pinus* at the Malinche sites. This result may reflect an absence of *Alnus*-associated ECM ascomycetes, which Tedersoo et al. (2009) found were the only ECM taxa that co-occurred on *Alnus* and other hosts.

In summary, we found that the *Alnus*-associated ECM assemblages in Mexico contained novel host associations but also shared many features with those located in other regions of the Americas. The continental-scale similarity in taxon richness and composition indicates that *Alnus* ECM assemblages have clear patterns across a wide range of geographic locations. Greater sampling of *Alnus*-associated ECM assemblages in boreal western north America and throughout eastern North America, both above and below the ground, will help in further investigating the role of host plant migration and biogeography in ECM assemblage structure. In addition, a better understanding of the effects of *Frankia* bacteria on *Alnus* ECM assemblages may also reveal much about the latter's exclusive nature.

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