Ticodendron incognitum and *Neea pittieri* associated ectomycorrhizal fungi in Neotropical mountain forest

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Abstract

Mycorrhizal associations have crucial importance in the ecology of the majority of terrestrial plants. However, information about mycorrhizal associations of multiple exotic plant taxa remains scant. In order to verify the recently described *Ticodenrum incognitum* (Ticodendraceae) ectomycorrhizal status I performed root sampling of 15 individuals in Alberto Manuel Brenes Nature Reserve, Costa Rica. For a better description of the local ectomycorrhizal species pool and for estimating potential host preference I sampled adjacent *Neea pittieri* individuals for putative ectomycorrhizal status. Results confirmed that *Ticodenrum incognitum* forms ectomycorrhiza with tomentella-thelephora and boletus lineages. In spite of overall scarce ectomycorrhizal associations, comparison of ectomycorrhizal communities of adjacent host species showed clear host preferences.

Key words – ectomycorrhizal – host abundance theory – host preference – Ticodendraceae

Introduction

Understanding the mycorrhizal root traits of plants is critical in understanding ecosystem processes from landscape to global scale, but information about the mycorrhizal status is scant for multiple plant groups. The majority of the mycorrhizal studies were conducted in temperate regions and tropical taxa remain often understudied as compared to their temperate relatives. In spite of "latitudinal bias" the evidence suggests that arbuscular mycorrhiza is the predominant mycorrhizal type in tropical forests (St John & Uhl 1983, Béreau & Garbaye 1994, Onguene & Kuyper 2001, St John 1980, McGuire 2007). Nevertheless, ectomycorrhizal (EcM) associations have been recognized in all major tropical regions involving a high variety of obligately mutualistic fungal and host taxa with independent evolutionary origins (Becker 1983, Connell & Lowman 1989, Hart et al. 1989, Henkel 2003). In the Neotropics, EcM associations of *Pakaraimaea* (Dipterocarpaceae), *Quercus* (Fagaceae), *Coccoloba* (Polygonaceae), *Aldina* (Fabaceae subfamily Papilionoideae), *Dicymbe* (Fabaceae subfamily Caesalpinioideae), *Gnetum* (Gnetaceae), *Neea* (Nyctaginaceae), *Guapira* (Nyctaginaceae), *Oreomunnea* (Juglandaceae) have been characterized to various degrees, and exhibit a high variation in diversity and host preference (Henkel et al. 2002, Henkel 2003, Morris et al. 2009, Smith et al. 2011,2013, Tedersoo et al. 2010, Moyersoen 2006, Corrales et al. 2016, Séne et al. 2015, Põlme et al. 2017).

Recently described *Ticodendron incognitum* Gómez-Laur. and L.D. Gómez (Gómez-Laurito

& Gómez 1989) from Mesoamerica Atlantic slope forests is the only extant species in Ticodendraceae family (Fagales). In spite of its wide presence in wet mid-elevation forests, these habitats were hard to access until recently and this species were overlooked by the scientific community due to its "ordinary" alder appearance (Hammel & Burger 1991). Initially the species was described from Costa Rica, but further research expanded its distribution to southern Mexico and Panama. Fossil records from the Eocene suggest that this family was widely distributed in North America and Europe (Manchester 2011). As Fagales comprises predominantly EcM hosting taxa it has been proposed that the EcM habit is ancestral to the whole group (Tedersoo & Brundett 2017). The EcM habit are characteristic to the majority of Betulaceae and Casuarinaceae taxa which are sister groups to Ticodendraceae (Larson-Johnson 2016). Confirmation of *T. incognitum* EcM status would indicate that these families might have had common EcM ancestor, especially when considering temperate history of Ticodendraceae. In spite of these evolutionary implications of the possible EcM habit in *T. incognitum,* there is no documented record of the mycorrhizal status of this species. The only published study about *T. incognitum* rhizoplane described the new endophytic genus *Ticogloea* (Weber et al. 1994). To the best of my knowledge, *T. incognitum* mycorrhizal associations have not been clearly verified, although A. Corrales reported from Panama the non-ectomycorrhizal status (pers. comm.).

In order to ascertain *Ticodendron incognito* mycorrhizal status I performed root sampling in Costa Rica cloud forest, applying a combination of anatomical and morphological evidence. For better characterization of the local EcM fungal species pool and to asses possible host preference, I collected root samples from neighboring *Neea pittieri* (Nyctaginaceae) individuals with putative EcM status (Tedersoo et al. 2010). Based on its phylogenetic position I hypothesized that *Ticodendron incognitum* forms ectomycorrhizal associations.

Material and methods

Sampling design

Sampling was performed in Alberto Manuel Brenes Nature Reserve (10.211015, -84.596265), Costa Rica. The mountainous sampling area was covered by pristine neotropical forest situating between 900 and 1200 meters above sea level. The area receives 4000mm rainfall annually. *Neea* and *Ticodendron* trees were scarce and scattered around ca 4km^2 area. *Ticodendron* trees were large, often reaching the canopy whereas *Neea pittieri* individuals were small trees in the understory reaching up to 3m in height. Host trees were determined with the help of the local expert Dr. Victor Madrigal Elizondo. *Ticodendron incognitum* individuals were determined based on tree stems and leafs using binoculars. All together 34 root samples were collected, including 15 individuals of *Ticodendrum incognitum* and 19 individuals of *Neea pittieri* across several square kilometers. In order to collect fine roots from each tree individual and ascertain host identity, main root was traced from the tree stem.

Soil samples were placed into plastic bags and processed within 24 hours after collection. Roots were carefully cleaned under tap water and placed into large Petri dishes filled with water. Only vital tree roots were processed. Ectomycorrhizal morphotypes were distinguished under stereomicroscope based on color and roughness of mantle, presence of emanating hyphae and rhizomorphs. If available, at least two EcM root tips from each morphotype per soil sample were stored in CTAB buffer (1% cetyltrimethylammonium bromide, 100 mM Tris–HCL (pH 8.0), 1.4 M NaCl, 20 mM ethylenediaminetetraacetic acid) for molecular analyses.

Molecular and statistical analyses

DNA was extracted from EcM root tips using Thermo Scientific Phire Plant Direct PCR Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. In the course of the study, PCR was performed by use of 5x HOT FIREPol Blend Master Mix Ready to Load (Solis BioDyne, Tartu, Estonia). In EcM root tips, fungal rDNA Internal Transcribed Spacer (ITS) region was amplified with a forward primer ITSOF (5`-acttggtcatttagaggaagt-3`) in

combination with reverse primer LR0B(5′-acccgctgaacttaagc-3′). In case of PCR failure we combined ITSOF with ITS2 (5′-gctgcgttcttcatcgatgc-3′) and 58SF (5`- atgcatcgatgaagaacgc-3`) with LR0B in order to amplify a shorter fragment of fungal DNA. PCR reactions were run under the following conditions: 95 °C for 15 min; five cycles of 42 °C for 30 s, 72 °C for 60 s and 92 °C for 45 s; 35 cycles of 65 °C for 30 s, 72 °C for 60 s and 92 °C for 45 s, followed by a final cycle of 65 °C for 30 s and 72 °C for 10 min for all primer combinations. PCR products were separated by electrophoresis through a 1.5% agarose gel in $0.5 \times$ TBE buffer (45 mM Tris Base, 45 mM Boric Acid, 1 mM EDTA (pH 8.0)), visualized under UV light and purified using Exo-Sap enzymes (Sigma, St. Louis, MO, USA).

Sequencing of fungal DNA was performed with primers ITS5 (5`-ggaagtaaaagtcgtaacaagg - 3') and ITS4 (5′- tcctccgcttattgatatgc -3′). Sequences were assembled, checked, trimmed and manually corrected in Sequencher 4.10.1 software (GeneCodes Corp., Ann Arbor, MI, USA). Sequences were confirmed to belong to EcM fungal lineages (cf. Tedersoo et al. 2010) by use of BLASTn searches against the International Sequence Databases (INSD) or UNITE (Abarenkov et al. 2010).

In order to illustrate the presence of fungal mantle in *T. incognitum* root tips, 8µm cross sections were cut with a TBS Minotome Plus OM2563 microtome. Cross sections of roots were photographed with Nikon Eclipse 80i at 400-600x magnification (Fig. 1).

To estimate the relative importance of host effect to EcM community I applied multivariate analysis of variance as implemented in the adonis function of the Vegan package of R (Oksanen et al. 2007). For relevant illustration I constructed a nonmetric multidimensional scaling (NMDS) plot in ecodist package of R (Goslee & Urban 2007).
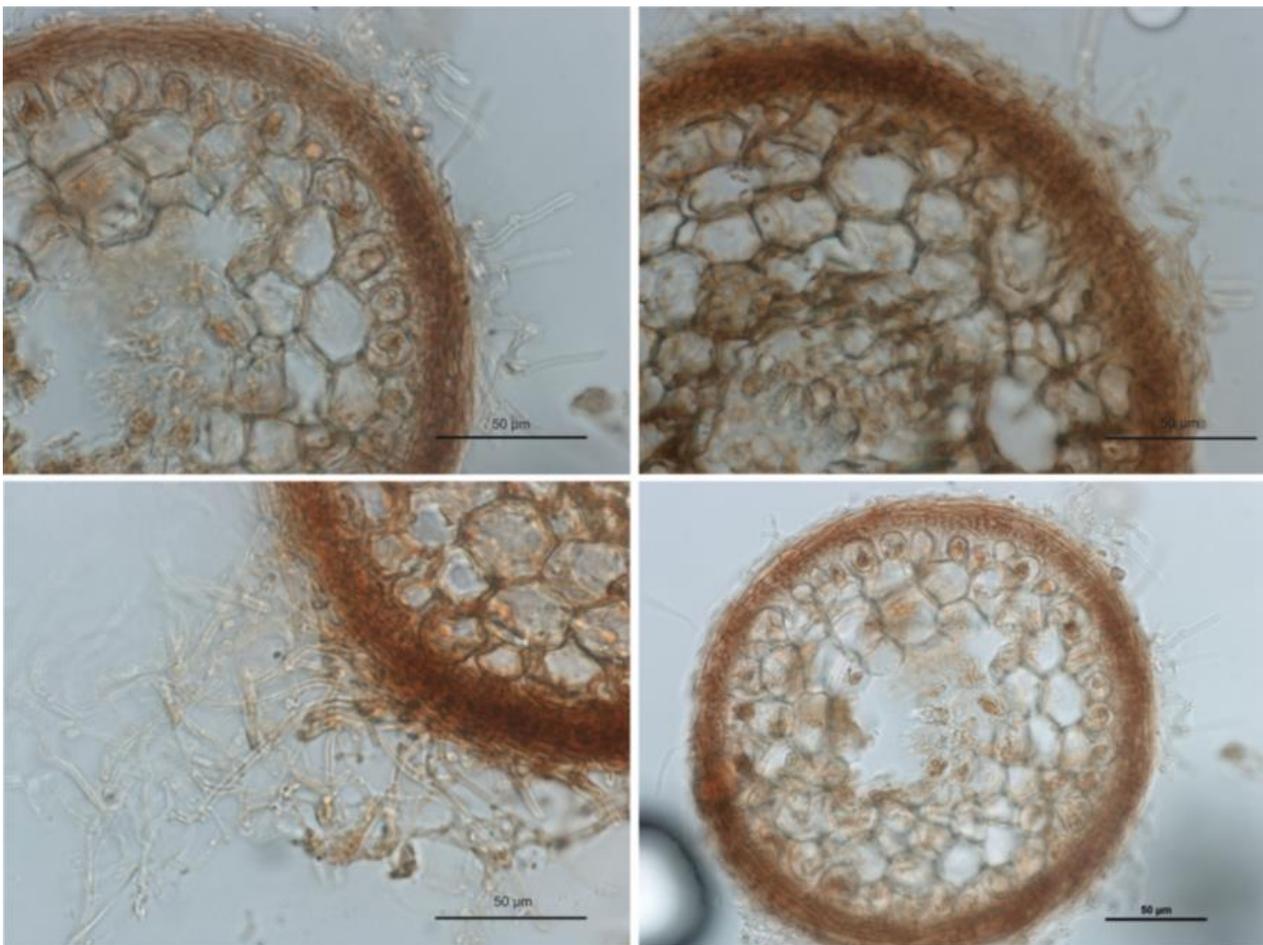

Fig. 1 – Light micrographs of *Ticodendron incognitum* root tip cross sections from UDB023273 (Boletales). Fungal mantle and hyphae are visible. Scale Bars: 50 µm.

Results

In the course of the study 81 EcM root tips were extracted. Thirty-eight EcM root tips were associated with *Neea pittieri* and 43 with *Ticodendron incognitum*. After DNA extraction, PCR and sequencing, 30 (78.9%) of sequences associated with *N. pittieri* and 16 (37.2%) associated with *T. incognitum* yielded high quality EcM fungal sequences.

Based on 97% similarity threshold we distinguished 14 EcM OTUs (Table 1). Ten OTUs were associated with *Neea pittieri* and five OTUs with *Ticodendron incognitum*. Most OTU rich ectomycorrhizal lineage was /tomentella-thelephora containing eight OTUs. Other lineages were represented with the following number of OTUs: /boletus (four), /pisolithus-scleroderma (one) and /inocybe (one). Latter two lineages were associated only with *Neea pittieri*. Only one fungal OTU was shared between two hosts. Accordingly, host effect was evident when tested with permutational anova (R=0.203; F=5.63; P=0.001; Fig. 2). Four OTUs were found only once in the dataset (i.e. singletones), three of them were associated with *N. pittieri* and one with *Ticodendron incognitum*. Sequences from 11 OTUs clustered into Species Hypothesis (SH) with 97% similarity threshold (Table 1). Five of SH-s were exclusively associated with *N. pittieri*. None of the SH associated with *T. incognitum* was exclusive. *T. incognitum* shared fungal SH-s with *Oreomunnea mexicana* root symbiont and with *Tomentella* fruitbodies collected beneath *Neea* and *Coccoloba* communities from Central America. The latter SH was shared also with *Neea pittieri*. *Neea pittieri* shared SH from /tomentella –thelephora lineage with *Guapira macrocarpa* from Mexico and /boletus SH with *Pinus taeda* from North Carolina.
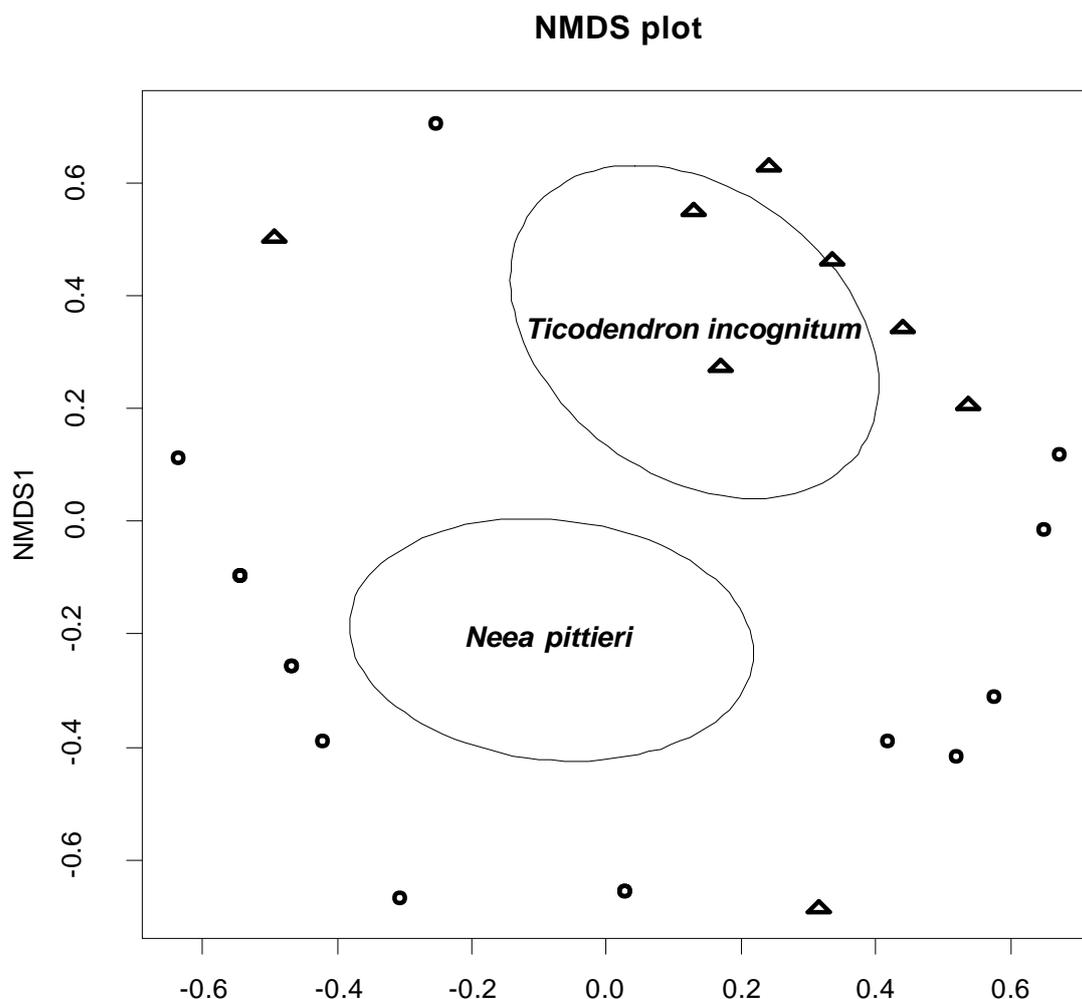
NMDS plot

Fig. 2 – Nonmetric multidimensional scaling ordination plot with 95% confidence intervals demonstrating the *Ticodendron incognitum* (triangles) and *Neea pittieri* (circles) associated EcM fungal communities.

Table 1 Distribution of fungal species hypothesis and OTUs at 97% cutoff level, associating with *Ticodendron incognitum* and *Neea pittieri* individuals in Costa Rica forest. Asterisks denote species hypothesis that are exclusively associated with *Neea pittieri*.

EcM lineage	/tomentella-thelephora	/tomentella-thelephora	/tomentella-thelephora	/tomentella-thelephora	/tomentella-thelephora	/tomentella-thelephora	/tomentella-thelephora	/tomentella-thelephora
No. of sequences in SH	3	9	11	3	2	3	2	2
Species hypothesis	SH1190450.08*	SH1140548.08*	SH1140532.08	SH1140724.08*	SH1140853.08*	SH1140726.08*	SH1140821.08	SH1233043.08
Neea pittieri	UDB023243							
Neea pittieri		UDB023245			UDB023246 UDB023247			
Neea pittieri	UDB023250							
Neea pittieri	UDB023251							
Neea pittieri								
Neea pittieri		UDB023253 UDB023262				UDB023254		
Neea pittieri			UDB023255					
Neea pittieri				UDB023258 UDB023259				
Neea pittieri		UDB023260				UDB023261		
Neea pittieri						UDB023262		
Neea pittieri			UDB023263					
Neea pittieri		UDB023264 UDB023265						
Neea pittieri				UDB023266				
Neea pittieri		UDB023267				UDB23268		
Neea pittieri		UDB023269 UDB023270						
Neea pittieri					UDB023271		UDB023272	
Ticodendron incognitum			UDB023274					
Ticodendron incognitum								
Ticodendron incognitum			UDB023276					
Ticodendron incognitum								
Ticodendron incognitum								
Ticodendron incognitum								
Ticodendron incognitum								
Ticodendron incognitum								UDB023282

Table 1 Continued.

EcM lineage	/boletus	/boletus	/boletus	/boletus	/pisolithus-scleroderma	/inocybe
No. of sequences in SH	4	2				2
Species hypothesis	SH1169225.08	SH1179911.08				SH1213002.08
Neea pittieri	UDB023244					
Neea pittieri						
Neea pittieri						UDB23248
Neea pittieri						
Neea pittieri					UDB23249	
Neea pittieri						
Neea pittieri						UDB23256 UDB023257
Neea pittieri						
Neea pittieri						
Neea pittieri						
Neea pittieri						
Neea pittieri						
Neea pittieri						
Neea pittieri						
Neea pittieri						
Ticodendron incognitum		UDB023273		UDB033271		
Ticodendron incognitum				UDB033272		
Ticodendron incognitum		UDB023275				
Ticodendron incognitum		UDB023277	UDB023278			
Ticodendron incognitum			UDB023271	UDB033273		
Ticodendron incognitum			UDB023280	UDB033275		
Ticodendron incognitum		UDB023281		UDB033276		
Ticodendron incognitum						

Discussion

To the best of my knowledge this is the first study assessing *Ticodendron incognitum* ectomycorrhizal status. Examination of *T. incognitum* roots confirmed the scarce association with EcM fungi that belonged mainly to /tomentella-thelephora and /boletus lineages, which are commonly ectomycorrhizal with Fagales host trees in temperate and montane tropical regions (Smith et al. 2007, Corrales et al. 2016). Previous studies have shown high variation of EcM diversity of neotropical communities which are sometimes comparable with temperate regions (Tedersoo et al. 2010, Smith et al. 2011, Henkel et al. 2012, Amadou et al. 2014, Séne et al. 2015, Corrales et al. 2016, Põlme et al. 2017). Although the presence of EcM root tips were scarce, suggesting low ecological importance, the overall low diversity and richness of EcM community in current study is partly attributable to limited sampling effort, rendering direct comparison between studies unreliable.

In spite of limited sampling effort, I found evidence of host preference of adjacent but phylogenetically distant hosts. *Neea pittieri* and *Ticodendron incognitum* harbored distinct fungal communities and shared only one fungal OTU. Current findings corroborate with Tedersoo et al. (2010) who reported low diversity and high host preference for dispersed, low density hosts from Polygonaceae and Nyctaginaceae in Ecuador. These authors hypothesized that isolated EcM host plants function as discrete habitat islands with limited photosynthetic resources and inter-connectivity. Accordingly, studies from EcM dominated communities in neotropics report high EcM fungal diversity, suggesting that prevalence of suitable hosts enhances dispersal and resource availability being major driver of EcM fungal diversity (Smith et al. 2011, 2013, Corrales et al. 2016). The contrasts in observed host preference between studies could be partly ascribed to phylogenetic proximities of examined host species – Smith et al. (2011) sampled hosts from the same family, while Tedersoo et al. (2010) and present study addressed hosts from different families and orders, respectively. However, latter study by Smith et al. (2013) examining *Pakaraimaea dipterocarpacea* and *Dicymbe jenmanii* from different subclasses in nearby savanna ecosystem, documented moderate host effect suggesting its conditional nature.

Few non-exclusive EcM SHs associated with *Ticodendron incognitum* and *Neea pittieri* were distributed mainly in Central America, with one exception from North America. One of the SH associated with *Ticodendron incognitum* was shared with *Oreomunnea mexicana* (Juglandaceae) and mutual EcM symbiont of *Ticodendron incognitum* and *Neea pittieri* falls in the same SH with *Tomentella* fruitbodies found under mixed community of *Coccoloba* spp. and *Neea* spp. from Mexico. The overall high ratio of novel SH-s implicate that EcM communities in these regions remain understudied.

In spite of low sampling effort current results suggest that EcM association of *Ticodendron incognitum* are very moderate. It has been proposed that various taxa among Fagales have reduced or lost their ancestral capacity to form EcM, which has often been attributed to development of actinorrhizal symbiosis or shift to herbaceous life forms in other orders (Tedersoo & Brundett 2017). Generalization about EcM biogeography predicts its lower ecological importance in tropical regions which has been attributed mainly to rapid turnover of organic matter and weak soil stratification (Tedersoo et al. 2012). I propose that inconsistent EcM root colonization of *T. incognitum* is a result of reduction of EcM habit with expansion to mid-elevation tropical habitats in Mesoamerica, compared to its temperate ancestors from Tertiary when many extant EcM groups of plants and fungi evolved (Hammel & Burger 1991, Zanne et al. 2014).

In conclusion, this study confirms my hypothesis about concordance of the *Ticodendron incognitum* phylogenetic placement and its EcM habit. The low diversity and scarce EcM associations of *T. incognitum* and *Neea pittieri* corroborates host abundance theory (Tedersoo et al. 2010). However, additional sampling of adjacent populations would enable to make more confident generalizations about putative low ecological importance of EcM habit of these species in Central American forests.

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