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Ecology of vertical life in harsh environments: The case of mycorrhizal symbiosis with secular cliff climbing trees (*Juniperus phoenicea* L.)

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ABSTRACT

The Phoenician juniper tree (*Juniperus phoenicea* L.) is emblematic of Mediterranean ecosystems. Secular trees from a relict population are present in the cliffs of the Ardèche gorges (protected natural reserve). This population shows a remarkable adaptability to major physical, nutrient and water availability constraints. The basis of its adaptation to hostile environmental conditions is poorly understood. The aim of the study is to decipher the diversity and structure of the Phoenician juniper mycorrhizal community in order to highlight mycorrhizal characteristics related to the particular ecology of this relict population. We hypothesized that a long-term co-evolution between arbuscular mycorrhizal (AM) fungi and the plant partner, emphasized by a geographic isolation, may have selected a specific mycorrhizal community playing a key role in *J. phoenicea* adaptation. 454-sequencing of AM fungal community from Phoenician juniper secular trees revealed a complex community, notably composed of *Rhizophagus* and poorly-affiliated *Glomeraceae* clades. The AM fungal community characterized was mainly related to those found in arid and semi-arid habitats, strengthening the ecological specificity of the Phoenician juniper AM fungal communities to harsh environmental conditions.

1. Short note

Juniperus phoenicea L. (family *Cupressaceae*) is a small (8–12 m tall) gynodioic tree native to the Mediterranean basin, mainly distributed in its western part. It is a light-demanding tree resisting to dry climates and characterized by high pioneer properties (Quézel and Médail, 2003). Populations of *J. phoenicea* subsp. *turbinata* are characteristic of the coastal areas while *J. phoenicea* subsp. *phoenicea* are growing up to 2000 m (e.g. on the Atlas mountain slopes). The subsp. *phoenicea* shows a low level of geographic differentiation and a conservation of local characters by isolation from other subspecies (Boratynski et al., 2009).

In France, populations of *J. phoenicea* subsp. *phoenicea* stretch to the nature reserve of the Ardèche gorges (Mathaux et al., 2016), in the south of Rhône-Alpes. This reserve is characterized by a 200-m

deep canyon composed of urgonian limestones and an extensive plateau mainly covered by typical Mediterranean forest. Old Phoenician juniper trees found on the large walls of the limestone cliffs have adapted their growth to the topological and physico-chemical constraints (Mathaux et al., 2016). This ecosystem, functioning for millennium – trees have been dated to be thousand-year-old (Mathaux et al., 2016) – is probably among the last native ones in France, representing a possible relict population (geographic and physical isolation, confined distribution). Its natural regeneration is reaching a critical threshold due to erosion and to the attested warming in the Rhône-Alpes region (Météo-France Centre-Est, 2011). Phoenician juniper trees establish a symbiosis with arbuscular mycorrhizal (AM) fungi (Boullard, 1986), a key parameter in ecosystem functioning (Smith and Read, 2008). Characterizing Phoenician juniper mycorrhizal diversity may represent a major insight to better understand the biological functioning of this particular ecosystem.

Three secular Phoenician trees (J1, J2, and J3; Fig. 1) located on the reserve canyon cliffs (Gaud, Saint-Remèze, France; Fig. A1) were

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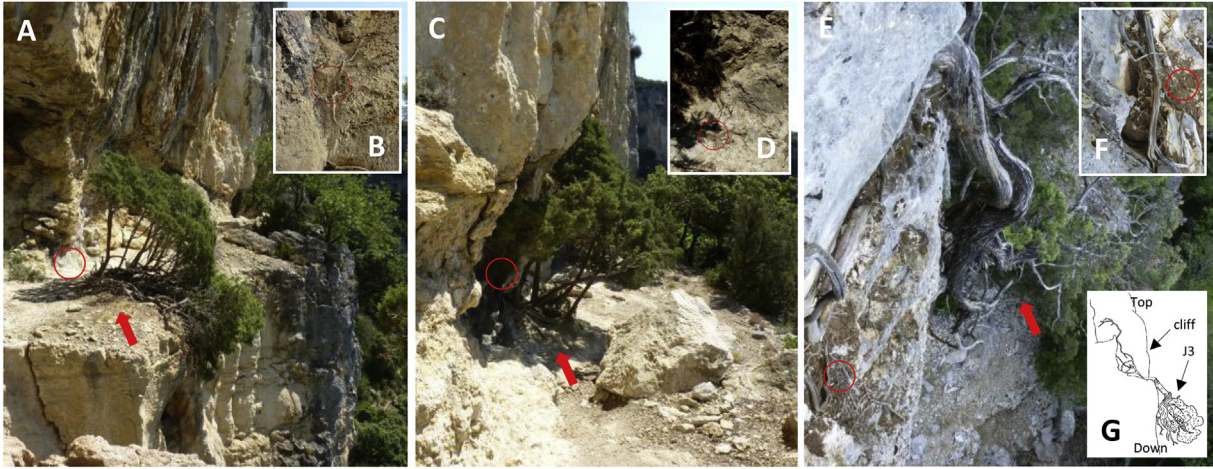


Fig. 1. Photos of selected *J. phoenicea* trees in the protected nature reserve of the Ardèche gorges (Gaud, Saint-Remèze, France). Phoenician juniper trees (A) J1, (C) J2 and (E) J3 are indicated with red arrows. Red circles indicate root sampling area for each tree, visualized more precisely in (B), (D), (F) for J1, J2 and J3, respectively. (G) Schema of the general morphology of J3 growing downwards on the cliff. The trees J1 (44°21'57"N – 4°27'06"E, 268 m altitude) and J2 (J2, 44°21'56"N 4°27'08"E, 268 m altitude) are distant from 25 m and are both located on a 2-m wide rocky shelf (A and C) used as pathway by feral goats. The tree J3 (44°21'44"N, 4°26'58"E, 190 m altitude) presents an original morphology since it grows down directly along the cliff (G). The juniper roots were shown to penetrate in rock crevices partially filled by soil deposits (B, D and E). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reached using rock-climbing equipment. The selected trees are hundred-years-old (141, 128 and 216 years old for tree J1, J2, and J3, respectively), and are characterized by their extremely slow radial growth rates (0.37, 0.21 and 0.25 mm per year, respectively) (Appendix). Only three trees could be sampled because of the very low accessibility of root systems (rock crevices) and tree instability.

A 18S-based 454-pyrosequencing approach was applied to characterize the Phoenician juniper AM fungal community. The 18S rRNA gene as well as the primer set were selected because of their reliability to assess AM fungal diversity in environmental samples (Öpik et al., 2013; Van Geel et al., 2014). 454 data-processing (for more details, see Appendix file) lead to 4647 high quality sequences from approximately 10,000 raw sequences. All the analyses were performed both with and without singletons because it has been shown that half of the singletons may represent true target taxa (Brown et al., 2015). Finally, the sequence number between environmental samples was rarefied to improve statistical robustness.

The AM fungal community was composed of 77 molecular operational taxonomic units (MOTUs) (42 without singletons), based on a 97% sequence similarity. A high diversity coverage (>97%) was reached, with less than 10 MOTUs not retrieved (Boneh estimates) (Table 1), demonstrating a suitable sequencing effort for

comparison of community richness, diversity and composition between environmental samples (Lemos et al., 2011). The AM fungal diversity and evenness among trees tended to be different, contrary to community richness (Table 1).

Approximately 80% of sequences were shared between at least two trees. Differences in the AM fungal community were only due to low abundant MOTUs membership (Fig. A2). On the other hand, the genetic structure of the AM fungal community was significantly different among trees ($P < 0.001$; Unifrac tests on genetic distances; Table A1). Phylogeny-based approaches are demonstrated as powerful approaches compared to similarity-based approaches to reliably analyse microbial community in environment (Senés-Guerrero and Schüßler, 2016). The differences observed in the AM fungal community structures among the juniper trees might be related to abiotic factors. The soil deposits surrounding the juniper roots showed variations for several chemical parameters (CaCO₃ content, SOC and SOM; Table A2) between J3 and the two other trees. Heterogeneity of physical and nutritive constraints and water availability are known as potential drivers of mycorrhizal communities (Tedersoo et al., 2003; Johnson et al., 2010; Jumpponen et al., 2010). The variation in abiotic factors were postulated as the main factor driving both plant and AM fungal communities in

Table 1
Richness and diversity indexes.

Juniper tree ^a	Number of MOTUs	Chao (lci; hci) ^b	Shannon (lci; hci) ^b	Inverse simpson (lci; hci) ^b	Simpson evenness	Shannon evenness	Coverage (%) ^c	Boneh estimation
J1	42	70	1.55	3.10	0.07	0.41	97.69	7.58
		(52; 121)	(1.46; 1.64)	(2.93; 3.28)				
J1(- s)	21	21	1.41	2.96	0.14	0.46	99.79	3.29
		(21; 24)	(1.34; 1.49)	(2.82; 3.13)				
J2	26	61	0.96	1.59	0.06	0.30	98.49	4.37
		(36; 152)	(0.87; 1.06)	(1.50; 1.69)				
J2(- s)	21	21	0.90	1.56	0.07	0.29	99.95	0
		(21; 21)	(0.85; 0.96)	(1.50; 1.63)				
J3	20	48	0.51	1.22	0.06	0.17	98.89	3.26
		(27; 133)	(0.43; 0.60)	(1.18; 1.27)				
J3(- s)	13	14	0.45	1.20	0.09	0.18	99.75	1.34
		(13; 24)	(0.39; 0.52)	(1.16; 1.24)				

^a J1, J2, J3 indicate the juniper trees 1, 2 and 3, respectively. (- s) indicates the data without the singletons. All data correspond to normalized data based on random subsamplings.

^b lci and hci indicate the lower and higher 95% confidence intervals, respectively.

^c Good's coverage: sum of probabilities of observed classes calculated as $(1 - (n/N))$, where n is the number of singleton sequences and N is the total number of sequences.

successionally stable plant populations (Habitat hypothesis; Zobel and Öpik, 2014), which is the case of the Phoenician juniper population investigated. However, the impact of abiotic factors cannot be robustly evaluated in the current study due to the limited sampling and 454-data treatment intrinsic biases (Gihring et al., 2012).

The phylogenetic assignment of MOTUs (Fig. 2) revealed a predominance of *Glomeraceae* (*Rhizophagus*, 47% of sequences; *Sclerocystis*, 0.03%; *Septoglosum*, 0.27%), in accordance with previous results on *Juniperus procera* (Wubet et al., 2006). The majority of MOTUs (52% of sequences) had uncertain affiliation regarding reference strains but constituted nine well-resolved clades (Glo-a to Glo-i; Table A3) related to known virtual taxa (Öpik et al., 2014). The most abundant MOTU (1382 sequences), shared between all trees (Fig. A1) was affiliated to a cluster composed of *Rhizophagus intraradices*/*Rhizophagus proliiferus*, distinct from the cluster defined by well-known *Rhizophagus irregularis*. The worldwide distribution of *Rhizophagus* suggests a lack of specialization. However, some species present a rapidly evolving genotypic diversity (Angelard et al., 2014) having to differential effects on plant growth (Koch et al., 2006).

The need to develop AM fungal diversity analyses at the community and population levels appears crucial to reliably evaluate AM fungal ecology and adaptation to environmental changes. The originality of the AM fungal community associated to the Phoenician juniper population was assessed using an ecosystem-related approach crossing taxonomic assignment, genetic structure and host origin data, as described in Appendix. Forty four percent of MOTUs (44% of sequences) were closely related to AM fungal sequences from desert and xeric shrublands (Biome) (Blast analysis;

Table 2

Comparison of AMF genetic structures from the relict Phoenician juniper population and different shrubland habitats corresponding to different biomes.

Comparison with limestone cliffs ^a	UWscore ^b	P-value ^c
<i>Tropical dry broadleaf forest biome-related habitats</i>		
Dry afro-montane forest	0.91,667	0.0170,093
Seasonal tropical forest	0.97,744	0.00100,921
<i>Tropical shrubland biome-related habitat</i>		
Arid montane shrubland	0.913,445	0.0650,088
<i>Subtropical dry broadleaf forest biome-related habitat</i>		
Dry eucalyptus forest	0.923,016	0.00400,924
<i>Subtropical shrubland biome-related habitats</i>		
Fynbos	0.931,909	0.105,009
Semi-arid mediterranean shrubland	0.931,909	0.0660,085
<i>Desert and xeric shrubland biome-related habitats</i>		
Desert	0.918,605	0.044,092
Dolomitic vegetation	0.880,386	0.358,008
Gypsophilous vegetation	0.914,079	0.0790,092

^a "Limestone cliffs" represents the habitat of AM fungal MOTUs from Phoenician juniper trees J1, J2, J3. The biome and habitat references were defined in MAARJAM database.

^b "UWscore" indicate the score obtained with the unweighted Unifrac tests.

^c Statistical tests were done at the habitat level ($P < 0.05$).

Table A4). The genetic structure of AM fungal community from the Ardèche Gorges limestone cliffs showed significant similarity with AM fungal communities from arid and semi-arid habitats characterized by low water availability (unweighted unifrac test; Table 2 and Table A5). For example, the similarity with the dolomitic habitat may reflect the comparable abiotic and physical constraints between both habitats (drought due to rainwater percolation and high level of calcium carbonate). Only habitats in Tropical and

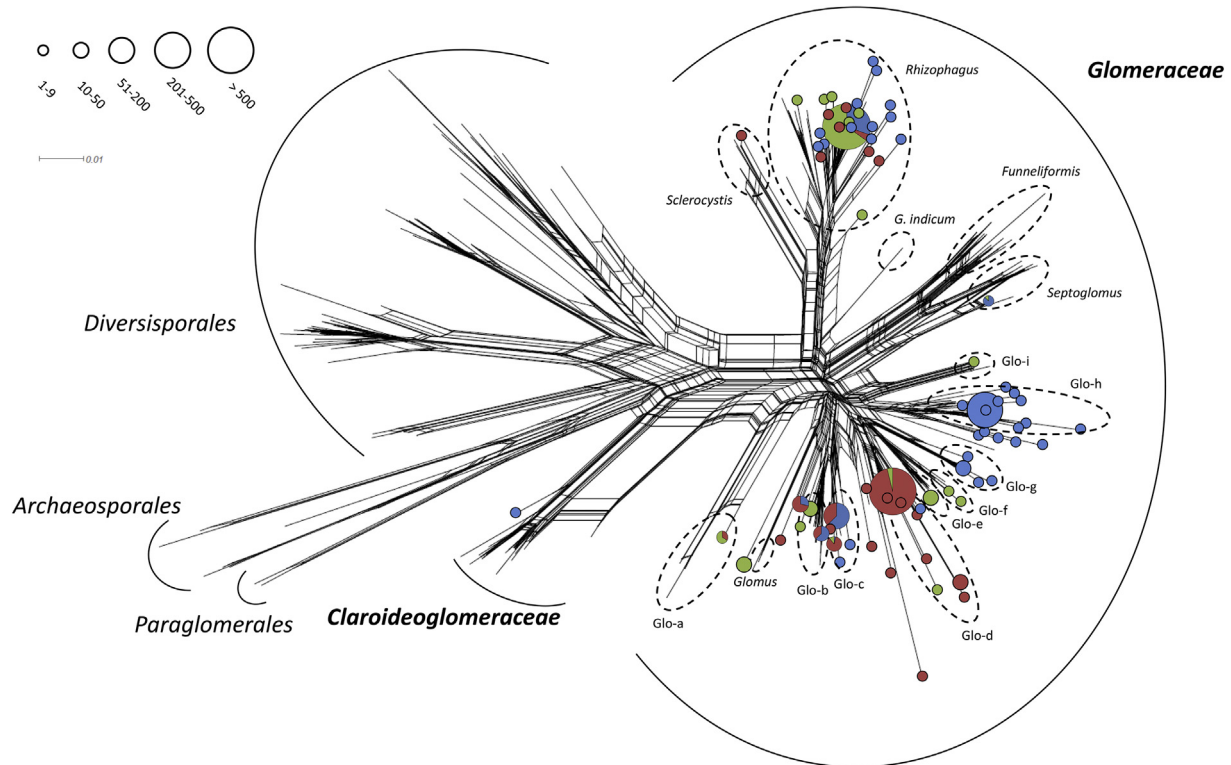


Fig. 2. Network phylogenetic analysis of AM fungal 18S sequences from the Phoenician juniper trees (J1, J2, J3). All sequences were clustered in MOTUs (97% similarity). 18S sequences from public database used as references are from Krüger et al. (2012) and MAARJAM database (Öpik et al., 2013). The circles indicate the presence of MOTUs and the size is indicative of the sequence number in MOTUs (see scale at the top-right corner). Blue color corresponds to sequences from J1, red from J2 and green from J3. Glo-a to Glo-i correspond to *Glomeraceae* clades with uncertain position in *Glomus* « sensu lato » closely related to one or several fungal virtual taxa (MAARJAM database) (see Table A3 for details). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

subtropical dry broadleaf forest biomes showed significant differences. Overall, the results highlighted the specific status of the Phoenician juniper AM fungal community regarding harsh environmental conditions, and support the habitat preference of AM fungal taxa detailed in Helgason and Fitter (2009), rather than the plant host preference.

The current study constitutes a preliminary exploration of AM fungal community associated to a relict Phoenician juniper population, growing in extreme conditions on limestone cliffs. Unraveling the complexity of mycorrhizal symbiosis associated to relict plant populations represents a major insight towards a better understanding of microbial life in harsh ecosystems, and the impacts of long-term environmental changes on plant-fungal interactions (Woolbright et al., 2014). Climate-induced die off in juniper woodlands has been shown to strongly affect soil microbial functioning (Lloret et al., 2015), but many gaps still exist in our knowledge of plant-fungal symbiosis responses to climate changes (Johnson et al., 2013; Kivlin et al., 2013). A more extensive exploration of Phoenician juniper tree ecotypes and other juniper relict populations (El-Bana et al., 2015) may allow the determination of an “island” effect (Peay et al., 2007) of the relict Phoenician juniper on AM fungal community and improve our knowledge about the laws that rule mycorrhizal symbiosis during long-term co-evolution.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jaridenv.2016.07.008>.

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