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Do Sebacinales commonly associate with plant roots as endophytes?

Marc-André SELOSSE[a,*], *Marie-Pierre DUBOIS*[a], *Nadir ALVAREZ*[b]

[a] *Centre d'Ecologie Fonctionnelle et Evolutive (CNRS, UMR 5175), Equipe co-évolution, 1919 Route de Mende, 34293 Montpellier Cedex 5, France*

[b] *Institute of Biology, University of Neuchâtel, 11 rue Emile-Argand, 2000 Neuchâtel, Switzerland*

Sebacinales are basal Hymenomycetes with diverse mycorrhizal abilities, ranging from ectomycorrhizae to ericoid and orchid mycorrhizae. Several previous PCR or isolation works raised the possibility that Sebacinales are endophytes in plant roots. We tested this hypothesis in an isolation-independent approach by using specific PCR primers for ribosomal DNA of Sebacinales on AM mycorrhizal or non-mycorrhizal roots. Thirty-nine plant species were sampled on a Caribbean and two European sites (3 repetition per species and site), covering 25 families in monocots and eudicots. PCR signals were obtained from 40 samples (28.9 %) from 27 species (69.2 %) and all sites. Whenever sequencing was successful, a sequence belonging to Sebacinales was recovered. A phylogenetic approach revealed that 13 of them belonged to clade B (encompassing ericoid and orchid mycorrhizal species) and 4 to clade A (usually encompassing only ectomycorrhizal species). These data suggest that Sebacinales may be endophytic in many angiosperm roots, and that this condition is plesiomorphic in Sebacinales. They bridge the gap between physiological studies, inoculating Sebacinales (*Piriformospora indica* or *Sebacina vermifera*) on diverse plants and molecular ecology, hitherto restricting Sebacinales to mycorrhizal interactions. Structural and functional aspects of the interaction deserve further studies.

Keywords: Endophytes, *Piriformospora indica*, Root fungi, Sebacinales, Tropical fungi

Introduction

In the twentieth century, Sebacinales were considered as a saprotrophic taxon (Wells 1994), mycorrhizal on orchid (e.g. Warcup 1988). Since then, molecular methods not only unravelled their diversity and phylogenetic position as basal Hymenomycetes (Weiß *et al.* 2004; Matheny *et al.* 2007), but also their unexpectedly diverse associations with various plants. Sebacinales were confirmed as associates of several orchids (e.g. Selosse *et al.* 2002a; Suárez *et al.* 2008). They were demonstrated to form ectomycorrhizae on tree roots (Selosse *et al.* 2002a, b; Urban *et al.* 2003) and to colonize thalli of liverworts (Kottke *et al.* 2003). They were shown to be endomycorrhizal on Ericaceae (Berch *et al.* 2002; Allen *et al.* 2003; Setaro *et al.* 2006), worldwide and on many species (Selosse *et al.* 2007). The *ca.* 500 Sebacinales entries currently deposited in GenBank are nearly all from environmental root samples, demonstrating the fascinating diversity and commonness of this taxon, mainly as a mycorrhizal partner of plants. Yet, the diversity of interaction with plants' roots may still be overlooked.

Two cultivable strains of Sebacinales are commonly used as root inoculants on various plant hosts. *Piriformospora indica*

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[*] Corresponding author. Tel.: +33 (0)4 67 61 32 31; fax: +33 (0)4 67 41 21 38.
E-mail addresses: ma.selosse@wanadoo.fr (M.-A. Selosse), marie-pierre.dubois@cefe.cnrs.fr (M.-P. Dubois), nadir.alvarez@unine.ch (N. Alvarez)

improves plant growth and/or stress tolerance (Varma *et al.* 2001), such as on the non-mycorrhizal *Arabidopsis thaliana* (Peškan-Berghöfer *et al.* 2004) and *Nicotiana tabacum* (Barazani *et al.* 2005), but also on Fabaceae and Rhamnaceae (Varma *et al.* 2001), Asteraceae and Solanaceae (Rai *et al.* 2001), Geraniaceae and Euphorbiaceae (Druege *et al.* 2007), as well as Poaceae (Waller *et al.* 2005; Baltrushat *et al.* 2008). There is evidence that *P. indica* enhances apoptosis of host plant cells that it colonizes, but this occurs in living roots. Another *Sebacina vermifera* strain, originally isolated from orchids by Warcup (1988), also colonizes roots, increasing plant growth and sensitivity to herbivores (Barazani *et al.* 2005, 2007), perhaps by impairing ethylene production (Barazani *et al.* 2007). Does this reflect associations allowed by the reduced microbial diversity and artificial lab conditions, but that would never exist *in natura*, or is it the tip of an overlooked ecological niche of Sebacinales? (Selosse *et al.* 2007).

These Sebacinales colonize roots as endophytes, *i.e.* organisms that for all or part of their life cycle grow within living plant tissues, causing an unapparent infection, and especially do not form mycorrhizae, nor cause any obvious disease symptoms (following the definition by Wilson 1995). Intriguingly, a series of classical works reported that strains looking like *S. vermifera* could be root endophytes: some strains were isolated from *Trifolium* spp. pot cultures and plants collected in the field, simply by incubating washed roots on agar plates (Williams 1985; Milligan & Williams 1987; Williams & Thilo 1989); earlier, Peyronel (1923) reported very similar observations and claimed that such fungi, that we feel of possible affinities to Sebacinales, were frequent on plant roots. Recently, Neubert *et al.* (2006) cloned *S. vermifera*-related sequences from *Phragmites australis* tissues. Most of these putative endophytes, such as *P. indica*, are related to *S. vermifera* according to morphological (Williams 1985; Milligan & Williams 1987) or molecular criteria (Weiß *et al.* 2004).

To test whether some Sebacinales commonly occur as root endophytes, a simple PCR assay was performed on three stands from tropical and temperate regions. Since many Sebacinales are uncultivable (Berch *et al.* 2002; Allen *et al.* 2003; Weiß *et al.* 2004), a DNA-based approach was preferred. We investigated the frequency, diversity and phylogenetic affinities of Sebacinales on surface-sterilized roots from various non-mycorrhizal and arbuscular mycorrhizal (AM) plant species.

Materials and methods

Root sampling

Root systems were sampled in 2004 and 2006 from three sites in the Northern hemisphere, *i.e.* two in France (Europe) and one in La Guadeloupe (a tropical Caribbean island; Table 1). In every case, a non-pastured field near a managed forest, rich in herbaceous plant species, was investigated, and most common species were harvested. Seven plant species were common to both European sites (Table 1). For each species, root systems were obtained from three different individuals situated more than 1 m away from each other. They were washed carefully under a dissection microscope within 1 h after harvesting, in order to remove soil and larger and old or damaged roots: only healthy-looking, fine young roots, with no superficial hyphal colonization, were kept for analysis. They were then surface-sterilized using a solution of sodium hypochloride (2 % v/v) and Tween 80 (=polysorbate 80, a non-ionic emulsifier – 5 % w/v) for 10 s and rinsed three times in sterile distilled water. Then, a 0.5 g (fresh weight) sub-sample, containing several roots, was frozen at −80 °C. A subsample of the roots was stained by Trypan blue following the method of Koske & Gemma (1989) to check for AM colonization: all species proved to be AM, with exception of the two *Cardamine* species (Brassicaceae) and *Rumex crispus* (Polygonaceae; not shown).

Molecular analysis

DNA of all roots was extracted as in Selosse *et al.* 2007. Primers ITS3seb (3′-TGAGTGTCATTGTAATCTCAC-′, specific for Sebacinales) and TW13 (3′-GGTCCGTGTTTCAAGACG-′, universal for fungi) were used for amplification of the fungal Intergenic Transcribed Spacer (ITS) plus the 5′ part of the 28S ribosomal DNA (rDNA), following Selosse *et al.* (2007). Amplicons were sequenced from both strands as in Selosse *et al.* (2002a) and these that were too diluted for direct sequencing were cloned as in Julou *et al.* (2005; two attempts per samples) and a minimum of seven clones was sequenced from both strands. Sequencher 4.5 for MacOsX (Gene Codes, Ann Arbor, USA) was used to assemble complementary strands or clones. To check for auto-contamination, sequences were compared to all the fungal sequences obtained in our lab since 2004: no identical sequences were discovered (not shown).

Phylogenetic analyses

Thirty-eight ITS accessions from taxa within Sebacinales and five ITS accessions from two outgroup taxa (*Geastrum saccatum* and *Auricularia auricula-judae*) were included (see Fig 1), together with the recovered sequences (using consensus for successful clones). Alignment was performed using ClustalX (Thompson *et al.* 1997). Maximum Parsimony (MP) analyses were performed using parsimony ratchet (Nixon 1999) as implemented in PAUPrat (http://users.iab.uaf.edu/∼derek_sikes/software2.htm) in order to produce a majority-rule consensus tree. Non-parametric bootstrap analyses were performed using PAUP* version 4.0b10 (Swofford 2002) (1000 replicates, TBR branch-swapping, simple sequence addition, MULTREES, 10 trees per replicate). Model selection was assessed using MrAIC v.1.4.3 (Nylander 2004). Maximum Likelihood (ML) analyses were performed using RAxML version 7.0.0 (Stamatakis 2006) with a 1000 rapid bootstrap. Bayesian inference (BI) analysis was performed in MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001). Two simultaneous MCMCs were run for 1 000 000 generations, saving a tree every 100 generations. Due to burn-in, 4000 sample points were discarded until stationarity was established among the chains. The remaining trees were used to construct a 50 % majority-rule consensus tree and to calculate Bayesian posterior probabilities (BPP). Convergence diagnostic among the two chains was determined by computing the Potential scale reduction factor (Gelman & Rubin 1992) for each parameter. Tracer 1.3 (http://evolve.zoo.ox.ac.uk) was used to assess the effective sample size of the parameters sampled from the MCMC.

Table 1 – A summary of investigated root systems at the three study sites, with frequency of successful amplification using primers ITS3seb and TW13 as well as GenBank accession numbers of sequenced PCR products

Host species[a]	Host families[b]	PCR amplification signal		Successful sequencing
		Strong	Weak[c]	
Cavanière at Grands Fonds (La Guadeloupe, 16°14′25″N, 61°23′29″W, elevation 83 m asl), January 2006				
Axonopus compressus	Poaceae		1	0/3
Blechum brownei	Acanthaceae			0/3
Cissus verticilata	Vitaceae	1 (EU909164)	1	1/3
Heliotropium indicum	Boraginaceae			0/3
Hyptis verticillata[d]	Lamiaceae		1	0/3
Lippia nodiflora	Verbenaceae			0/3
Mimosa pudica	Mimosaceae		1	0/3
Pilea microphylla	Urticaceae		1	0/3
Pilea nummularifolia	Urticaceae	1 (EU909166)	1 (EU909165)[e]	2/3
Piper dilatatum	Piperaceae			0/3
Ruellia tuberosa	Acanthaceae			0/3
Senna obtusifolia	Cesalpinaceae		1	0/3
Sida rhombifolia	Malvaceae		2	0/3
Solanum americanum	Solanaceae	1 (EU909167)	1	1/3
Spermacoce assurgens	Rubiaceae		1 (EU909168)[e]	1/3
Wedelia trilobata	Asteraceae		1	0/3
Total Cavanière		3	12	5/48
Port-Guen at Belle-Isle-en-Mer (Britany, 47°19′52″N, 3°09′01″E, elevation 18 m asl), April 2004				
Allium triquetrum	Liliaceae	1 (EU909169)		1/3
Bellis perennis	Asteraceae			0/3
Cardamine hirsuta	Brassicaceae		3 (EU909170)	1/3
Galium aparine	Rubiaceae			0/3
Geranium robertianum	Geraniaceae	1 (EU909171)		1/3
Hedera helix	Araliaceae			0/3
Lamium purpureum	Lamiaceae			0/3
Myosotis sylvatica	Boraginaceae	1 (EU909172)		1/3
Rumex crispus	Polygonaceae			0/3
Urtica dioica	Urticaceae		1	0/3
Veronica persica	Veronicaceae		1	0/3
Vicia sepium	Fabaceae			0/3
Total Port Guen		3	5	4/36
Arboretum at Nogent-sur-Vernisson (Central France, 47°50′51″N, 2°45′17″E, elevation 152 m asl), May 2004				
Arum maculatum	Araceae	1 (EU909173)		1/3
Bellis perennis	Asteraceae		2 (EU909174)	1/3
Cardamine pratensis	Brassicaceae	1 (EU909175)	2 (EU909176)[e]	2/3
Cyclamen hederifolium[d]	Primulaceae		2	0/3
Geranium robertianum	Geraniaceae			0/3
Glechoma hederacea	Lamiaceae			0/3
Hedera helix	Araliaceae		1	0/3
Lamium purpureum	Lamiaceae			0/3
Myosotis sylvatica	Boraginaceae	1 (EU909177)		1/3
Potentilla fragariastrum	Rosaceae			0/3
Primula acaulis	Primulaceae	1 (EU909180)	1	1/3
Pulmonaria officinalis	Boraginaceae			0/3
Rumex crispus	Polygonaceae		1	0/3
Thymus serpyllum	Lamiaceae			0/3
Urtica dioica	Urticaceae			0/3
Veronica chamaedrys	Veronicaceae			0/3
Vicia sepium	Fabaceae		3 (EU909178)[e]	1/3
Viola reichenbachiana	Violaceae		1 (EU909179)[e]	1/3
Total Arboretum		4	13	8/54
Total out of 39 different plant species		10	30	17/138

a Names of species and genera sampled on the two European sites simultaneously are underlined; all species except *Rumex cripus* and the two *Cardamine* species were checked to be AM mycorrhizal.

b Plant orders covered: monocots: Alismatales, Liliales, Poales; eudicots: Apiales, Asterales, Brassicales, Ericales, Fabales, Gentianales, Lamiales, Malvales, Malpighiales, Polygonales, Rosales, Solanales, Vitales. Orders for which a Sebacinales sequence was obtained are underlined.

c Less than 10 ng μL^{-1} of amplified DNA.

d Naturalized at this site.

e Sequence obtained after cloning from a weak PCR product, as a consensus from 7 clones (otherwise, all sequences are from direct sequencing of PCR products).

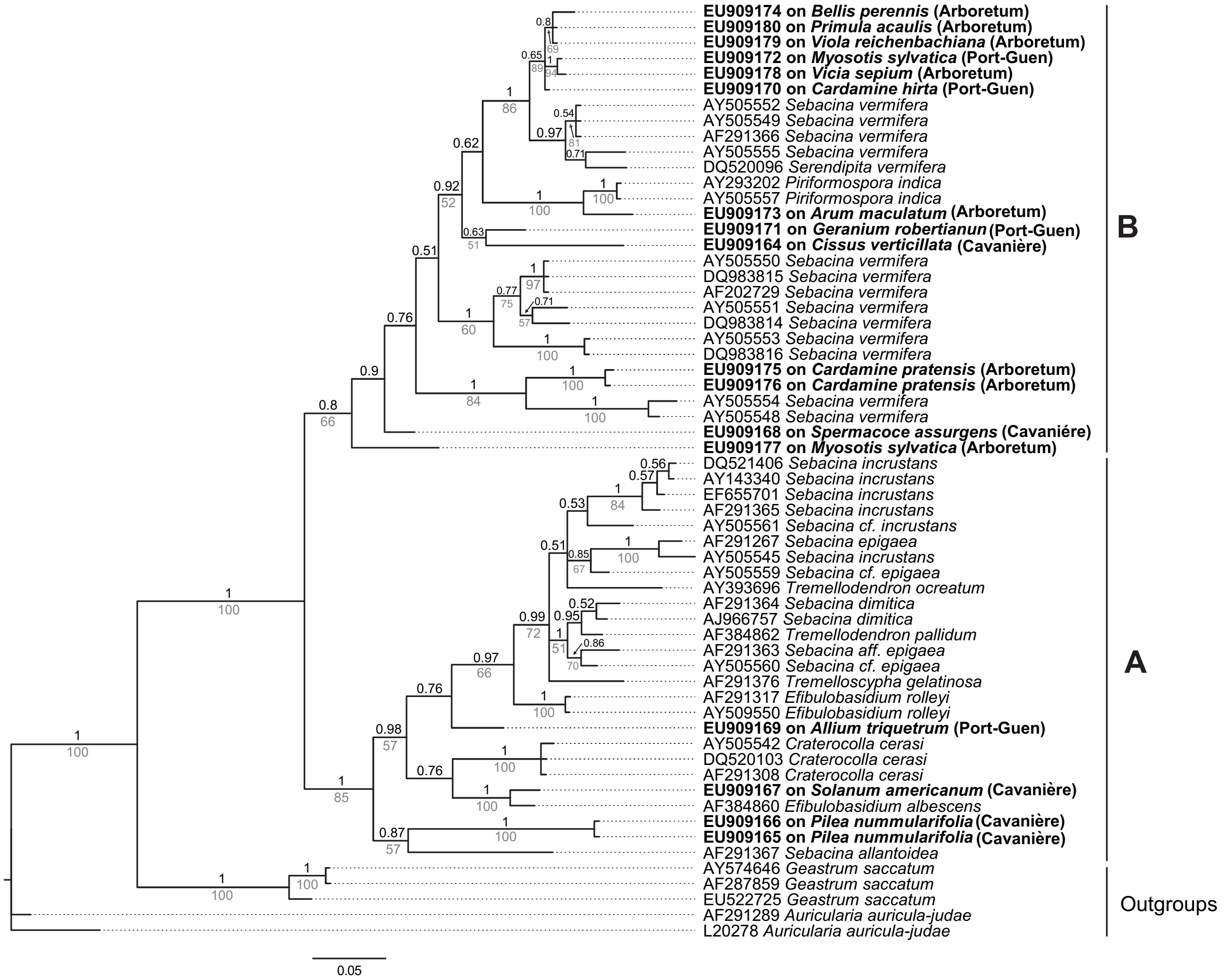

Fig 1 – Phylogenetic affinities of the 17 Sebacinales detected in roots systems. The fifty-percent majority-rule consensus-tree results from a Bayesian Inference analysis, based on the ITS and 5′ part of the 28S rDNA. Numbers at nodes indicate the BPP values (in black) and the corresponding bootstrap supports (>50) obtained in the ML analysis (in grey). Vertical lines highlight clades A and B, according to Weiß *et al.* (2004).

Congruence among topologies obtained using the three different criteria (MP, ML and BI) was evaluated pairwise by computing Explicitly Agree distances (Estabrook *et al.* 1985) using Darwin 5 (Perrier *et al.* 2003).

Results

PCR detection of Sebacinales

A total of 39 plant species from 25 families were investigated from the three sites (some species were sampled from two sites; Table 1). Using primers specific for Sebacinales rDNA, an amplicon was obtained from 40 out of the 138 (28.9 %) investigated root systems. Therefore, 27 out the 39 (69.2 %) investigated plant species and 21 out of the 25 families (84.0 %) provided a PCR signal. Seven plant species were investigated from the two European sites: among them, *Lamium purpureum* never gave any PCR signal, *Myosotis sylvatica* gave a positive signal on both sites, and all five other species gave a PCR signal on one of the two sites only. Success in the PCR amplification of the three replicates was as follows: three positive PCRs among all replicates were obtained for 3 species; two positive PCRs among three for 7 species; one positive PCR among three for 17 species. The fraction of plant species for which root samples produced an amplicon did not vary among sites (Fisher's Exact Test (FET): $P = 0.559 \pm 0.0006$; see Raymond & Rousset 1995). However, three quarters of the PCR signals consisted in weak bands after gel staining (*i.e.* $<10\ \text{ng}\ \mu\text{L}^{-1}$ of amplified DNA; Table 1).

Detection of Sebacinales by sequencing

All the ten strong amplicons were directly sequenced, as well as two of the weak amplicons (Table 1). Cloning attempts were successful for only five weak amplicons. Blast analyses showed that the 17 recovered sequences were all from Sebacinales. No additional fungi were found in cloning procedures, suggesting that sequencing problems resulted of concentration of weak amplicons, but not presence of additional fungal sequences. A sequence at least was recovered from 14 out of the 39 investigated plant species (35.9 %) and 13 out of the 25 families (52.0 %). We recovered two sequences for three species only. *Myosotis sylvatica* showed divergent sequences at the two European sites (40 mismatches among 567 alignable positions, see below). In contrast, when sequences were obtained from two conspecific plants at the same site, they were identical (for *Pilea nummularifolia* at Cavanière) or very similar (a single mismatch among 567 alignable positions for *Cardamine pratensis* at Arboretum). The rate of successful sequencing was not significantly different among sites (FET: $P = 0.851 \pm 0.0003$). Interestingly, sequences were clean and never showed evidence of dual sequences in our root samples. Only three sequences had a position with two overlapping bases (a single possible heterozygote in EU909167, EU909168 and EU909174).

Phylogenetic analysis

The alignment length of the ITS + 28S region was 567 bp and comprised 208 variable characters, among which 167 were parsimony-informative. Under the MP criterion, the heuristic search resulted into 173 equally most parsimonious trees of 651 steps (consistency index: 0.435). The best-fit model was GTR + G and topologies produced during ML and BI searches resulted respectively in tree-lengths of 1.10 and 2.74 (standard deviation [sd] = 0.02) with alpha parameters equal to 0.240 and 0.257 (sd = 0.0003) and minus-log-likelihoods of −3996.69 and −4096.74 (sd < 1.00). For each estimated parameter the effective sample size was higher than 100 and the potential scale reduction factor ranged between 1 and 1.01, attesting the convergence of the two chains. Topologies produced using the three different criteria (MP, ML and BI) were highly similar, with Explicitly Agree distances being lower than 0.08 whatever pair of trees was considered (*i.e.* 0.061 between MP and ML, 0.080 between MP and BI and 0.019 between ML and BI). Since all topologies were extremely congruent, only the tree resulting from the BI analysis is shown, with node supports indicated with both BPP and bootstrap values (>50) from the ML heuristic search (Fig 1). The slight incongruence between MP and probabilistic trees is explained by the position of EU909169 (from *Allium triquetrum*), which branches as a sister taxa of a clade comprising *Craterocolla cerasi* in the MP topology (Bootstrap support <50).

We recovered the two well-supported clades (namely A and B) from previous studies (Weiß *et al.* 2004; Selosse *et al.* 2007). Globally, supports were strong with 68 % of the nodes supported by BPP > 0.8. Four environmental samples clustered into clade A and 13 clustered into clade B. Three of the five taxa sampled in the tropical site are found within clade A, whereas 11 of the 12 taxa sampled in the two temperate sites are found within clade B. However, a FET does not support a trend for biogeographical signal in the phylogenetic hypothesis ($P = 0.053 \pm 0.0002$). Expectedly, samples obtained from the same plant species in the same site clustered together (*i.e.* Sebacinales on *Cardamine pratensis* and *Pilea nummularifolia*). However, when obtained from different plant species and/or different sites, sequences diverged among them, and with the accessions included in the analysis (i.e. tree-distances between a root sample and its closest relative were usually larger than 0.03). This trend for phylogenetic isolation is challenged by one exception: six European sequences form a distinct monophyletic group within clade B (namely, from *Bellis perennis*, *Primula acaulis*, *Viola reichenbachiana* and *Vicia sepium*, *Cardamine hirsuta* and *Myosotis sylvatica*), with moderate support (BPP = 0.65).

Discussion

Sebacinales as root endophytes?

Sebacinales were detected by PCR in *ca.* 29 % of the root systems, and 69 % of the plant species investigated; most investigated species did not systematically reveal Sebacinales and only 3 species consistently produced PCR signals (Table 1). Although various factors may affect PCR results, this suggested a sporadic, non-obligatory association on the plant side. On the fungus side, few taxa were hithertoo found to occur so frequently on plants' roots.

In all, 17 sequences from Sebacinales were recovered. This well corroborates the previous isolation of Sebacinales from AM plants (Williams 1985; Milligan & Williams 1987; Williams & Thilo 1989), such as the well-known endophyte *Piriformospora indica* (Varma *et al.* 2001). Interestingly, a sequence from *Arum maculatum* clustered with *P. indica* with strong support (Fig 1). Sebacinales were thus likely present in AM roots, but also in non-mycorrhizal roots. In Brassicaceae, positive results were obtained from *Cardamine* spp., congruently with the successful inoculations of *P. indica* on *Arabidopsis thaliana* (Peškan-Berghöfer *et al.* 2004). Presence on non-mycorrhizal roots of Polygonaceae is less obvious, since *Rumex crispus* roots only produced a single weak, non-sequencable PCR signal. Hosts belonged to phylogenetically diverse lineages in monocots and eudicots (see orders in Table 1), and this, again, is congruent with the diversity of hosts reported for *P. indica* (see Introduction). However, larger samplings will be necessary to conclude on specificity or preferences, if any, on both sides.

Did our amplicons result of spores or rhizoplan contamination, in spite of surface sterilization? Direct observation of hyphae of Sebacinales in roots based on ultrastructural features such as dolipores (Selosse *et al.* 2002b; Setaro *et al.* 2006; Selosse *et al.* 2007) could reject this hypothesis. However, repartition of Sebacinales in roots is unlikely to be dense or uniform (Peyronel 1923), and this may challenge investigations by electron microscopy. We failed to reveal typical Sebacinales dolipores by transmission electron microscopy from roots providing positive PCR signals (not shown), suggesting that colonization is sparse and not dense. In the future, ITS sequences may provide useful, specific probes for fluorescent *in situ* hybridization (FISH), allowing the screening of large portions of root tissues, but adapting this method to roots will likely require some time. Their limited abundance in roots makes them unlikely to be major nutrient providers for plants, but does not exclude that they can interfere with plant physiology. The high colonization levels observed *in vitro* for *P. indica* and *Sebacina vermifera* strains may result of the absence of competition with other root colonizers, and thus not reflect *in natura* colonization.

Concerning other evidences for root colonization, *in vitro* isolation of Sebacinales from roots would not distinguish between strains arising from spores and strains issuing form hyphae living endophytically. In spite of surface colonization, our protocol may even not fully discard all rhizoplan colonizers. *In vitro* inoculations would be another way of demonstrating root–Sebacinales interactions: indeed, several papers already reported successful inoculations of Sebacinales (e.g. Barazani *et al.* 2005, 2007; Deshmukh *et al.* 2006), using *P. indica* and *S. vermifera* strains. We therefore consider evidences from *in vitro* inoculations as already published, at least for clade B species. Because Sebacinales were successfully inoculated to, or isolated from, various plants in other works, we favour the idea that our cultivation-independent approach detected endophytic fungi. Interestingly, the presence was restricted to roots (no amplicon was obtained from aerial parts, data not shown), so that the word 'endorhizic' (root endophytic, *i.e.* not mycorrhizal – Wilson 1995) best applies for these Sebacinales. In other words, Sebacinales belong to type IV endophytes sensu Rodriguez *et al.* (2009). Whether some endorhizic Sebacinales can also be mycorrhizal deserve further studies.

Diversity of root endophytic Sebacinales

Sebacinales are divided into two clades (Weiß *et al.* 2004), *i.e.* clade A whose species form ectomycorrhizae (and, at the same time, endomycorrhizae on heterotrophic orchids; Selosse *et al.* 2002a, b) and clade B, whose species are either endomycorrhizal on autotrophic orchids and Ericaceae, or endophytic to liverworts (Kottke *et al.* 2003; Selosse *et al.* 2007). Up to now, all putative endophytes clustered in clade B, including *Piriformospora indica* and *Sebacina vermifera* strains (Setaro *et al.* 2006; Selosse *et al.* 2007), and so did 13 of the 17 sequences recovered. Unexpectedly, four putatively endophytic sequences were from clade A (Fig 1). Whether ectomycorrhizal strains themselves can also behave as endophytes deserves further analysis; however, since we cannot grow *in vitro* species of clade A up to now (Weiß *et al.* 2004), re-synthesis experiments are hitherto not allowed. Ectomycorrhizal trees are frequent around the Port-Guen site, and thus clade-A species are present on this site. The presence of clade-A species is more surprising at Cavanière, where no known ectomycorrhizal plant species occur (M.-A. Selosse & J. Fournet, pers. obs.). Little is known on interactions between tropical plant and fungi, and new associations were recently reported from the tropics, including new ectomycorrhizal plant taxa (Ducousso *et al.* 2008). Ectomycorrhizal associations are unlikely on our plants, since no superficial hyphae covered the roots. We may thus face here a new endophytic niche for clade-A species, joining to the growing number of functional and taxonomic differences between tropical and temperate plant-fungi interactions.

Our sampling is too limited to reveal any biogeographic pattern, or ascertain a higher frequency of clade-A endophytism in the tropics. Only a larger sampling could test for this, and confirm the frequency of the monophyletic clade grouping six sequences from temperate sites (Fig 1). However, the occurrence of putatively endophytes in clades A and B, especially in basal positions, suggests that endorhizic abilities are plesiomorphic in Sebacinales. By contrast, true mycorrhizal abilities are more derived: clade A taxa form ectomycorrhizae while some taxa in clade B evolved orchid or ericoid mycorrhizae (Selosse *et al.* 2007). If endorhizic ability was further supported by morphological analyses in clades A and B species, it could be plesiomorphic and have pre-adapted Sebacinales to developing mycorrhizal associations. This would simply mean a transition to a denser colonization of roots, including a morphogenesis more coordinated with the host. Evolution of mycorrhizal symbiosis *via* endorhizic stages may even be more widespread in Asco- and Basidiomycetes, for which mycorrhizal clades repeatedly evolved from saprotrophic ancestors (Hibbett & Matheny, 2009): in this evolution, endorhizic life may have been a frequent intermediary step.

Each sample produced a single sequence, although it encompassed 10–30 root fragments from the same plant. Similarly, very close sequences occurred in two individuals from the same site and plant species (*Cardamine pratensis* at Arboretum and *Pilea nummularifolia* at Cavanière). Such an exclusive colonization at the root level was also reported by direct sequencing in Ericaceae roots (Selosse *et al.* 2007). It can either reflect a loose colonization (so that a single colonization event is observed in each sample) or a dense colonization by individual(s) having the same sequence. What the different sequences reflect – different species or different individuals within the same species – remains unclear. As already stated (Selosse *et al.* 2007), the use of more loci and phylogenetic species circumscription would clarify species number and origin of the sequence diversity in Sebacinales.

Outline and perspectives

Our data bridge the gap between physiological studies, making use of Sebacinales inoculated on plant roots (e.g. Barazani *et al.* 2005, 2007), and molecular ecology data hitherto restricting Sebacinales to ectomycorrhizal, ericoid or orchid mycorrhizal roots. Conciliating these two approaches, we provide evidences that Sebacinales are endorhizic in many root systems *in natura*, and that this may be a plesiomorphic feature among Sebacinales. More work is needed to assess their diversity and exact colonization pattern, as well as the biogeographical and host determinants of this diversity. Precise pictures of the interaction also deserve further studies, e.g. by TEM and FISH methods, as well as the origin of the tremendous diversity of rDNA sequences (intraspecific polymorphism or large species diversity). Having limited evidence that some individuals cover several root systems, the possibility that several plants associate with the same genetic individual that cross soil is an intriguing possibility, reported for other endorhizic fungi such as *Phialocephala* spp. (Sieber & Grünig 2006): soil is thus the next step in the study of Sebacinales diversity.

Beyond a contribution to the emerging diversity of endophytic fungi, the question of the functional impacts of endorhizic Sebacinales in ecosystems and plant physiology remains open. After first reports focusing on outstandingly positive effect on host, a continuum ranging from positive to negative outcomes may await discovery, as for other endophytes: indeed, reduction of herbivore resistance by Sebacinales was already documented (Barazani *et al.* 2005). Endorhizic Sebacinales may reveal diverse mutualistic and/or parasitic stories that will be testable after isolation of some strains.

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