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Fungal diversity in adult date palm (*Phoenix dactylifera* L.) revealed by culture-dependent and culture-independent approaches*

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Abstract: Endophytic flora plays a vital role in the colonization and survival of host plants, especially in harsh environments, such as arid regions. This flora may, however, contain pathogenic species responsible for various troublesome host diseases. The present study is aimed at investigating the diversity of both cultivable and non-cultivable endophytic fungal floras in the internal tissues (roots and leaves) of Tunisian date palm trees (*Phoenix dactylifera*). Accordingly, 13 isolates from both root and leaf samples, exhibiting distinct colony morphology, were selected from potato dextrose agar (PDA) medium and identified by a sequence match search wherein their 18S–28S internal transcribed spacer (ITS) sequences were compared to those available in public databases. These findings revealed that the cultivable root and leaf isolates fell into two groups, namely Nectriaceae and Pleosporaceae. Additionally, total DNA from palm roots and leaves was further extracted and ITS fragments were amplified. Restriction fragment length polymorphism (RFLP) analysis of the ITS from 200 fungal clones (leaves: 100; roots: 100) using *Hae*III restriction enzyme revealed 13 distinct patterns that were further sequenced and led to the identification of *Alternaria*, *Cladosporium*, *Davidiella* (*Cladosporium* teleomorph), *Pythium*, *Curvularia*, and uncharacterized fungal endophytes. Both approaches confirmed that while the roots were predominantly colonized by *Fusaria* (members of the Nectriaceae family), the leaves were essentially colonized by *Alternaria* (members of the Pleosporaceae family). Overall, the findings of the present study constitute, to the authors' knowledge, the first extensive report on the diversity of endophytic fungal flora associated with date palm trees (*P. dactylifera*).

Key words: Date palm tree (*Phoenix dactylifera*), Endophytic cultivable fungi, rDNA internal transcribed spacer (ITS), Phylogenetic analysis, Total DNA diversity analysis

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1 Introduction

The date palm (*Phoenix dactylifera*) is a multipurpose plant species typically grown in the arid and semi-arid regions of the world and is globally valued for its nutritional and health-promoting fruit. Current estimates indicate that Tunisia is the world's tenth largest producer and first African exporter of date palms in value. It has an average annual production of over 100000 t, of which more than 30% are exported. The Deglet Nour variety dominates, accounting for about 60% of the total production. Constituting about

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13% of all Tunisian exports, date palm cultivation is a major agricultural and socio-economic activity in Tunisia.

The cultivation of date palms is, however, often hampered by several constraints, particularly its susceptibility to a wide range of damaging diseases caused by different pathogens living in the same environment. In fact, date palms have been cultivated with in vitro conditions for several years (Drira and Benbadis, 1985). Further knowledge about the fungal flora that may naturally be associated with healthy palm trees is, however, necessary to (1) improve yield, (2) facilitate the occupation of the ecological "niche" and avoid the random colonization of young plantlets by potential fungal pathogens, and (3) help further plantlet acclimation to arid soil conditions. In general, at least part of the endophytic flora of a plant, namely the fungal flora, can be cultivated. If the cultivatable fungi have a reasonable potential of young plantlet colonization, they could be easily used as inoculum for in vitro plantlet producing or, later, in plant nurseries.

However, to the authors' knowledge, no systematic study has so far been performed on the total endophytic flora associated with date palms. Accordingly, the present study was undertaken to extensively assess both the cultivable and non-cultivable fungal floras of this tree. In fact, a number of recent studies have shown that both approaches could bring varying, yet complementary, results. The introduction of molecular methods characterizing fungal flora led to new insights on microbial diversity (Hugenholtz *et al.*, 1998). The literature presents various methods for the characterization of fungal flora. These include the determination of the growth parameters (Steinberg *et al.*, 1997a), analysis of the diversity of β-tubulin sequences (Watanabe *et al.*, 2011), ribosomal genes sequencing (Kodsueb *et al.*, 2006; Crous *et al.*, 2007; Schroers *et al.*, 2009), intergenic 18S–28S internal transcribed spacer (ITS) sequencing (Camara *et al.*, 2002; Braun *et al.*, 2003; Pryor and Bigelow, 2003; Kwasna *et al.*, 2006; Manamgoda *et al.*, 2012), multilocus analysis (Zhang *et al.*, 2009a), and single sequence repeat (SSR) genotyping (Wang *et al.*, 2010). Further methods include terminal restriction fragment length polymorphism (T-RFLP) (Dickie and FitzJohn, 2007), amplified ribosomal DNA restriction analysis (ARDRA) (Sutthisa *et al.*, 2010), as well as ribosomal intergenic spacer analysis (RISA) (Sigler and Zeyer, 2002). Alternatively, denaturing gradient gel electrophoresis (DGGE) (Vallaeys *et al.*, 1997; Ampe *et al.*, 1999; Coppola *et al.*, 2001), temperature gradient gel electrophoresis (TGGE) (Felske *et al.*, 1999), temporal temperature gel electrophoresis (TTGE) (Sonjak *et al.*, 2009), and single-strand conformation polymorphism (SSCP) (Zumstein *et al.*, 2000; Feurer *et al.*, 2004) can be used in molecular analyses. The present work, which aimed to identify and characterize the endophytic flora associated with date palms, opted for the application of the ITS sequencing technique. In fact, sequence data provide valuable grounds for comparison with similar data available in public databases and comparable data in the literature, with the potential to provide interesting geographically related information on plant species. These data can also give indications on the GRAS (generally recognized as safe) status of members of this endophytic flora that could constitute part of developed inocula. The assessment of the GRAS status (for both the plant host and the final consumer) of potential endophytic plant growth promoting fungi (PGPF) is, in fact, essential before developing industrial applications to counteract target plant pathogens. Among endophytic fungi, PGPF species are common inhabitants of a wide range of plant species and can be found in the cells (Petrini, 1991), intercellular space, or vascular systems (Lee *et al.*, 2009) of the plant. However, some endophytic fungal species have been reported to be pathogenic to the plant. It is, therefore, important to characterize endophytic flora while keeping in mind their functional role. Wilson (1995) provided a working definition of the term "endophyte" by analyzing the different levels of endophytic association and stated that "endophytes are fungi which, for all or part of their life cycle, invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues but cause no symptoms of the disease". From a functional point of view, endophytic fungi are divided into two groups, non-pathogenic fungi that promote growth or suppress fungal diseases (Backman and Sikora, 2008) and pathogenic fungi that cause disease in plants (Strobel and Daisy, 2003).

In addition to comprising species that are pathogenic to the plant, endophytes also include fungi that have diverse positive effects on their hosts. The latter include Balansia (Ascomycetes) (Bacon *et al.*,

1977; 1979), though reported elsewhere to be implicated in toxicosis of cattle and humans (Groger, 1972). Other PGPF fungi have been described to protect their hosts against insect pests, pathogens, and even domestic herbivores (Yan *et al.*, 2011). These include *Aspergillus flavus* and *Penicillium sublateritium* (Webber, 1981), which live and feed on the host plant, and in turn, produce functional metabolites that enhance its fitness and resistance against stresses (Rana *et al.*, 1997). Last but not least, a number of species, including selected *Fusarium* sp. are reported to act as antagonists against plant pathogens. Accordingly, PGPF have commonly been used in practice as inocula to improve the growth of plants and suppress pathogens (Steinberg *et al.*, 1997b; Cao *et al.*, 2002; Alabouvette *et al.*, 2009).

Considering the important opportunities that the decipherment of currently undeciphered fungal flora might bring to the field, the present study was undertaken to explore the fungal flora associated with date palms (*P. dactylifera*). In particular and for practical purposes, special focus was given to the cultivable fraction of date palm endophytes, because this fraction could be further used for the development of inocula to promote the health and growth of date palms and to conserve production potential and agro-ecosystem sustainability of Tunisian oases. The non-cultivable fraction of date palm endophytes was also investigated using molecular methods, to provide a more complete overview of the diversity of this mostly undeciphered flora. Accordingly, the diversities of the cultivable and non-cultivable endophytic populations were analyzed in both the roots and leaves of healthy Tunisian date palm trees (*P. dactylifera*), with special focus on the characterization of fungi with potential PGPF effects. Combined ITS-polymerase chain reaction (PCR), random cloning, and phylogenetic analysis were employed to explore and compare the endophytic diversity between the roots and leaves of the same plant.

2 Materials and methods

2.1 Sampling of date palm

Oasis sampling site: the date palm tree samples employed in the present study were from an old cultivar of the "Deglet Nour" variety situated in the Jouali oasis, near Tozeur (Southwest Tunisia), on the Algero-Tunisian border. This date palm field is located in the major date palm production area of Tunisia, with a surface area of 4719 km^2. The sampled date palm trees were approximately 50 years old, with an average height of 7 to 8 m. For the sake of a systematic overview of date palm endophytes, both leaf and root samples were collected from a healthy tree during summer. Healthy mature leaves whose size ranged between 3.5 and 4.5 m were collected from the central crown of the tree (the most active crown). Healthy roots having an average diameter of (0.3± 0.08) cm were gathered from soil surface layers ranging between 0 and 20 cm. All root and leaf samples were collected from a single date palm tree.

Soil texture: the soil at the oases of Tozeur presents a typically carbonated sandy loam texture. The water pH of the oasis is 8.1, total $CaCO_3$ is 119 g/kg, organic matter is 12.08 g/kg, nitrogen is 0.56 g/kg, carbon is 7.03 g/kg, K_2O is 0.18 g/kg, CaO is 49.1 g/kg, MgO is 0.51 g/kg, Na_2O is 0.53 g/kg, and P_2O_5 is 0.31 g/kg (Namsi *et al.*, 2007).

2.2 Isolation of endophytic fungi

Endophytic fungi were isolated from the internal tissues of roots and leaves of a healthy date palm tree. The leaves and roots were washed in running tap water for 30 min. Sixteen adjacent 1 mm×2 mm segments were cut from each washed root and leaf. The samples were surface-sterilized by sequential washes in 90% ethanol for 10 min, 2.5% sodium hypochlorite for 2 min, and 80% ethanol for 3 min. They were then rinsed five times with sterile distilled water and allowed to surface-dry under sterile conditions. In fact, surface-sterilization has previously been reported to present an effective method for the removal of yeasts, fast-growing Zygomycetes, and other epiphyllous organisms from endophyte cultures (Schulz *et al.*, 1993; Arnold *et al.*, 2000). The leaf and root segments were placed on Petri dishes containing potato dextrose agar (PDA) (Collins and Lyne, 1984) and incubated at 28 °C for up to 20 d for the selective isolation of fungal endophyte. Morphologically distinct fungal colonies were re-isolated by sub-culture on appropriate media. Collected spores were stored at −20 °C in 20% sterile glycerol until used for further analysis and molecular identification assays.

2.3 Isolation of genomic DNA from isolates of endophytic fungi

Genomic DNA was extracted from isolates of endophytic fungi using the Wizard SV genomic DNA purification system kit (Promega, France) following the manufacturer's instruction. DNA quality and concentration were assessed with NanoDrop (Spectrophotometer ND-1000, France) in accordance with the manufacturer's instruction.

2.4 Total DNA extraction from leaf and root samples

A slightly modified version of the protocol described by Dellaporta *et al.* (1983) was employed for DNA extraction. In brief, plant tissues were pulverized with liquid nitrogen (−196 °C) to obtain a fine powder and then transferred to an organic solvent-resistant test tube. A warm 2-mercaptoethanol (2-ME)/cetyl trimethyl ammonium bromide (CTAB) extraction solution was added to the pulverized tissues with mixing. The tissues were incubated for up to 60 min at 65 °C with occasional mixing. An equal volume of 24:1 chloroform/isoamyl alcohol was added to the lysate, which was centrifuged for 5 min at 7500×*g* (or 10000 r/min in microcentrifuge for smaller samples) at 4 °C, until lysis was complete. The top (aqueous) phase was recovered. One-tenth volume of CTAB/NaCl solution heated to 65 °C was added to the recovered aqueous phase, which was followed by extraction with an equal volume of chloroform/isoamyl alcohol. The top (aqueous) phase was recovered after centrifugation. Then, one volume CTAB precipitation solution was added and centrifuged for 5 min at 500×*g* (or 2700 r/min in microcentrifuge) at 4 °C. The supernatant was recovered while the pellets were resuspended in a high-salt TE buffer (0.5 to 1.0 ml/g starting material). The nucleic acids were precipitated by adding a 0.6 volume of isopropanol and then centrifuged for 15 min at 7500×*g* and 4 °C. The pellets were washed with 80% ethanol, dried, and resuspended in a minimal volume of TE buffer (0.1 to 0.5 ml/g starting material).

2.5 PCR amplification and sequencing of ITS rRNA genes from both cultivable endophytic fungi and clones

Fungal ITS rDNA fragments were amplified from the DNA extracted from strains or, alternatively, total date palm samples. PCR amplification was performed using universal fungal ITS rRNA gene-specific oligonucleotide primers ITS1 (5′-TCCG TAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTC CGCTTATTGATATGC-3′) (White *et al.*, 1990) with a thermal cycler (M.J. Research, Biometra, Stratagene) in a volume of 25 μl. The DNA sample was mixed with a polymerase reaction buffer, 25 mmol/L $MgCl_2$, 10 mmol/L dNTPs, the above primers (20 μmol/L), and 5 U/μl Taq polymerase (Promega, France). The thermocycling conditions consisted of an initial denaturation step at 94 °C for 2 min, followed by 35 amplification cycles at 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 2 min, and a final polymerization step at 72 °C for 7 min. PCR products were electrophoresed in a 1.5% agarose gel (15 g/L) and visualized by ethidium bromide staining.

2.6 Cloning and sequencing of ITS rRNA gene amplified from the total DNA extracted from the leaf and root samples

The PCR products amplified from the total DNA extracted from the leaf and root samples using the universal external primers ITS1 and ITS4 were immediately ligated into the pCR4-TOPO vector (Invitogen, Carlsbad, CA, USA). Recombinant pCR4-TOPO plasmids were used to transform *Escherichia coli* TOPO10 One Shot competent cells as specified by the manufacturer (Invitrogen, France). For each DNA sample, 100 transformed clones were randomly picked for plasmid extraction (Bimboim and Doly, 1979). Plasmids were then amplified using the primers M13Forward (M13F) and M13Reverse (M13R) (Invitrogen, France) according to the manufacturer's instructions. The fragments obtained were then digested with *Hae*III (Promega, USA) (Dwivedi *et al.*, 2012). Clones were grouped according to RFLP banding patterns and further sequenced using primers M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′). Sequencing was performed on a 3130x1 genetic analyzer (Life Technologies Corporation, Tokyo, Japan) using the Big Dye Terminator v3.1 cycle sequencing kit (Life Technologies, USA) according to the manufacturer's recommendations.

2.7 Molecular analysis of the DNA and PCR products from cultivable isolates

The ITS PCR products of the DNA extracted

from cultivable isolates were purified with Zymoclean™ Gel DNA purification kit to remove primer in excess following the manufacturer's instruction, and were then sequenced using the forward and reverse primers used for ITS PCR amplification, respectively.

2.8 Sequence assembly and analysis

Sequences were subjected to a preliminary basic local alignment search tool (BLAST) analysis (Altschul *et al.*, 1990) in the National Center for Biotechnology Information (NCBI) databases, and sequence matches were used to determine their identities. Multiple sequence alignment was then carried out using ClustalW (Thompson *et al.*, 1994) at the European Bioinformatics Institute Website (http://www.ebi.ac.uk/clustalw/). Phylogenetic analyses were performed using MEGA5 (Tamura *et al.*, 2007) using reference sequences presented in Table 1. For accuracy reasons, sequences from collection strains were preferred, when available, to infer phylogeny. Alternatively, ITS sequences from clone libraries were used. Both anamorphs and corresponding teleomorphic affiliations were used for tree labeling according to data available in databases. Obvious corrections and annotation updates were made. A phylogenetic tree was constructed by maximum parsimony (MP) analysis using the Kimura two-parameter distance method. The robustness of the inferred tree was evaluated by bootstrap (100 replications).

Table 1 Reference sequences used in the phylogenetic analysis and deposited GenBank accession numbers for this study

Strain	Accession number	Reference	Strain	Accession number	Reference
Alternaria bokurai	EU520199	Pryor and Bigelow, 2003	*Ochrocladosporium elatum*	EU040233	Crous *et al*., 2007
Alternaria alternate	AF347031	Pryor and Bigelow, 2003	*Leptospora rubella*	JQ936327	Crous *et al*., 2009
Alternaria japonica	AF229474	Pryor and Bigelow, 2003	*Phoma herbarum*	JQ282910	Zhang *et al*., 2009a
Alternaria macrospora	AF229469	Pryor and Bigelow, 2003	*Fusarium equiseti*	AB587000	Watanabe *et al*., 2011
Alternaria radicina	AF229472	Pryor and Bigelow, 2003	*Fusarium incarnatum*	AB586988	Watanabe *et al*., 2011
Alternaria arborescens	AF347033	Pryor and Bigelow, 2003	*Fusarium oxysporum*	AB586994	Watanabe *et al*., 2011
Alternaria solani	AF229475	Pryor and Bigelow, 2003	*Fusarium phyllophilum*	AB587006	Watanabe *et al*., 2011
Alternaria infectoria	AF229480	Pryor and Bigelow, 2003	*Pythium inflatum*	JQ898463	Tambong *et al*., 2006
Embellisia proteae	AY278842	Pryor and Bigelow, 2003	*Pythium inflatum*	AY598626	Lévesque and de Cock, 2004
Pleospora herbarum	KC009768	Zhang *et al*., 2009a	*Pythium catenulatum*	AY598675	Lévesque and de Cock, 2004
Pleospora herbarum	JX397964	Zhang *et al*., 2009a	Uncultured root fungus	EU144681	Porras-Alfaro *et al*., 2008
Pleospora tarda	AF229481	Zhang *et al*., 2009a	*Fusarium* sp.	JX391934	This study
Davidiella tassiana	AF393705	Kwasna *et al*., 2006	*Fusarium equiseti*	JX391935	This study
Davidiella macrospora	AF297231	Kwasna *et al*., 2006	*Alternaria bokurai*	JX391938	This study
Davidiella dianthi	AF393698	Kwasna *et al*., 2006	*Alternaria bokurai*	JX391937	This study
Fungal endophyte	HM537053	Zhang *et al*., 2013	*Curvularia* sp.	JX391936	This study
Cladosporium australiense	HM147999	Braun *et al*., 2003	*Alternaria* sp.	JX391940	This study
Cladosporium oxysporum	AF393720	Braun *et al*., 2003	*Alternaria* sp.	JX391950	This study
Cladosporium herbarum	AJ244227	Braun *et al*., 2003	*Curvularia* sp.	JX391943	This study
Cochliobolus microlaenae	JN601032	Dimuthu *et al.*, 2012	*Cladosporium* sp.	JX391945	This study
Curvularia coicis	AF081447	Manamgoda *et al*., 2012	*Cladosporium herbarum*	JX391946	This study
Curvularia gladioli	AF071337	Manamgoda *et al*., 2012	*Pythium* sp.	JX391949	This study
Curvularia gudauskasii	AF071338	Manamgoda *et al*., 2012	*Curvularia* sp.	JX391944	This study
Preussia africana	JQ031265	Zhang *et al*., 2012	Fungal endophyte	JX391947	This study
Preussia australis	AY510413	Arenal *et al*., 2005	*Alternaria alternata*	JX391941	This study
Preussia sp.	HQ607926	Rodrigues *et al*., 2011	*Alternaria* sp.	JX391942	This study
Massarina corticola	JX421713	Zhang *et al*., 2009b	Fungal endophyte	JX391948	This study
Corynespora cassiicola	FJ852657	Dixon *et al*., 2009	*Alternaria* sp.	JX391939	This study
Uncultured Pleosporales	JQ247356	Rodrigues *et al*., 2013	*Alternaria* sp.	JX391951	This study

2.9 Nucleotide sequence accession numbers

The nucleotide sequence data reported here were deposited in the GenBank under the accession numbers: R2 (JX391934), R3 (JX391935), Fc1s (JX391936), Fc3s (JX391937), Fc4s (JX391938), 2C2 (JX391939), 1C2 (JX391940), 2G5 (JX391941), 2F4 (JX391942), 1C12 (JX391943), 2B6 (JX391944), 1E1 (JX391945), 1G10 (JX391946), 2E6 (JX391947), 2H7 (JX391948), 2A3 (JX391949), 1C5 (JX391950), 2B8 (JX391951).

3 Results

3.1 Morphological and molecular analyses of the cultivable endophytic fungi from mature palm trees

The emergence of diverse colony morphotypes was observed on the PDA agar media. A total of 13 fungal colonies presenting distinct morphologies could be re-isolated from the macerates of the leaves and roots grown on those media. The colonies exhibited different colors that varied from white to grey or green. Moreover, while some colonies showed fast growth, others underwent a moderately fast growth. A lifeless and powdery texture was observed. Further, the ITS rRNA genes were amplified and sequenced, and a BLAST search was performed, which led to a preliminary classification of our isolates as presented in Table 2. Further, phylogenetic analysis was performed to more precisely infer isolates' taxonomy using sequences published from collection strains, as references, during tree reconstruction. Our isolates could be clustered within two distinct groups. Group I included five strains isolated from the roots representing members of the Nectriaceae family. Two isolates presented identical ITS sequences (R3) and were related to *Fusarium equiseti* and three isolates were related to *Fusarium* sp. (R2) (Fig. 1). Group II contained eight strains isolated from the leaves representing members of the Pleosporaceae family. They consisted of five relatives of *Alternaria bokurai* (Fc3s: three isolates; Fc4s: two isolates) and three *Curvularia* sp. (Fc1s: three isolates) (Fig. 1). Palm roots were noted to harbor a number of different genera, with a prevalence of *Fusarium*. The leaves, on the other hand, were colonized by three major genera, with a predominance of *Alternaria* (Table 2). Out of 13 fungal isolates, 5 endophytic fungal taxa were represented. From a taxonomic perspective, the leaf and root samples were noted to differ in terms of endophytic fungal colonization. Interestingly, the strains assigned to *Fusarium* sp. were isolated from a healthy plant that did not show visible symptoms of disease, suggesting that they belonged to non-pathogenic *Fusarium* species. Accession numbers of our isolates (R2, R3, Fc1s, FC3s, Fc4s) are presented in Table 2.

3.2 Evaluation of the total diversity of the palm tree fungal endophytic flora by ITS rRNA cloning and sequencing of whole plant DNA

An ITS rDNA clone library analysis was employed to investigate the biodiversity and community structure within the leaves and roots of date palms. ITS ribosomal DNA clone libraries were constructed and complete sequence assembly of 100 clones per root and leaf sample was analyzed. The RFLP analysis of the ITS fungal clones using *Hae*III revealed the presence of 11 different profiles for the total 200 clones analyzed. Leaves presented eight different profiles (out of 100 clones) and roots showed five different profiles (out of 100 clones) (Fig. 2). Two profiles were common to leaves and roots.

Table 2 Closest match of ITS-rDNA sequences and phylogenetic affiliation from cultivable isolates from Tunisian date palm trees following BLAST searches in GenBank database

Taxonomic identification	Order	Strain	Closest BLAST match	Phylogenetic affiliation	Number of entophytic fungus isolates			Overlap (bp)
					Leaf	Root	Total	
Ascomycota	Pleosporales	Fc3s	"*Alternaria* sp."	*Alternaria bokurai*	2	0	2	565
		Fc4s	"*Alternaria gaisen*"	*Alternaria bokurai*	3	0	3	553
		Fc1s	"*Bipolaris tetramera*"	*Curvularia* sp.	3	0	3	562
Ascomycota	Hypocreales	R2	"*Fusarium* sp."	*Fusarium* sp.	0	3	3	552
		R3	"*Fusarium lacertarum*"	*Fusarium equiseti*	0	2	2	533

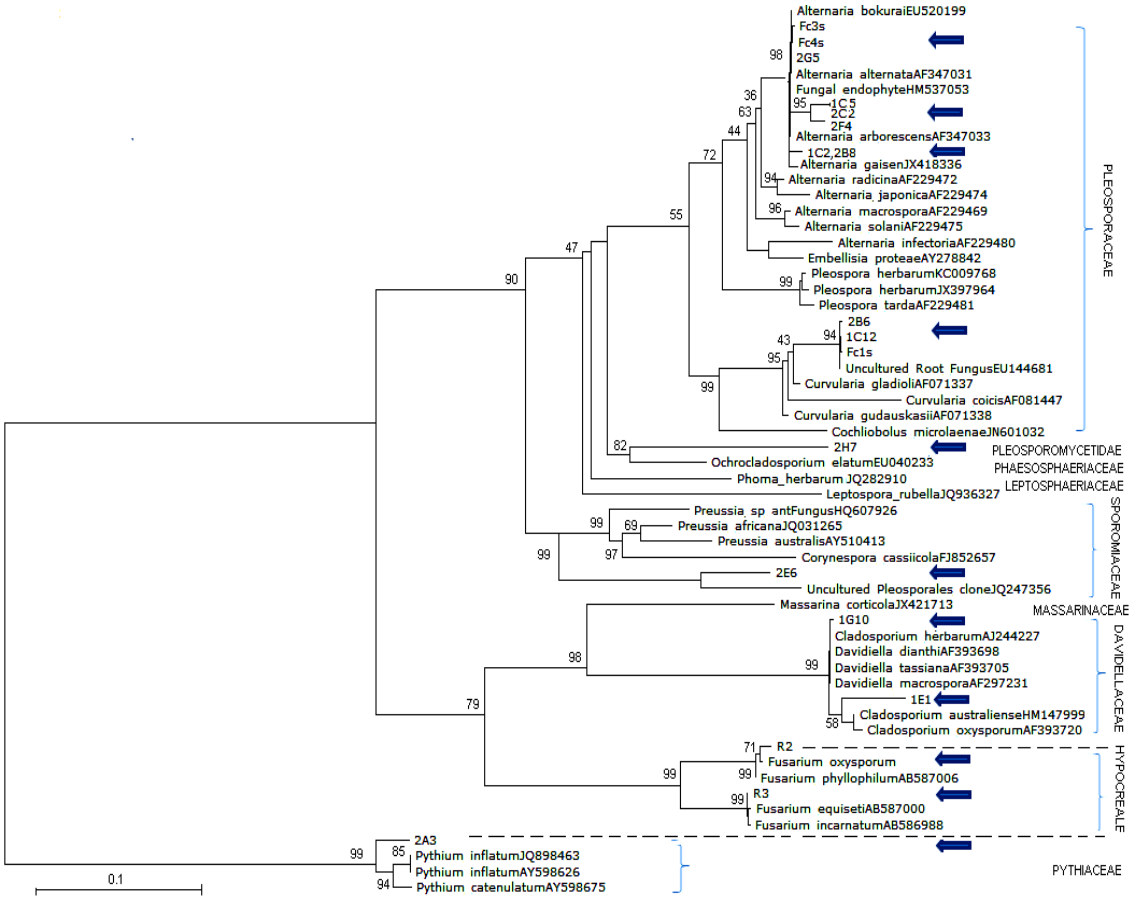

Fig. 1 Maximum parsimony (MP) tree showing the relationship between endophytic fungi and related fungi based on the sequences of ITS1-5.8S-ITS4 of rDNA

The tree was rooted with the *Pythiaceae* branch. Bootstraps values >50% (100 replicates) are shown at the nods

Indeed, not only 2C2 (root) and 1C5 (leaf) but also 2B8 (root) and 1C2 (leaf) were identical. Sequences of corresponding 13 clones 1C2, 2B8, 2G5, 2H7, 2C2, 2F4, 1G10, 1E1, 2B6, 1C12, 1C5, 2A3, and 2E6 were assigned and deposited in GenBank (non-redundant nucleotide database). Their accession numbers are listed in Table 3. The closest relative species were firstly determined for each clone sequence by direct BLAST searches in GenBank (Table 3). The results from the search for similarity matches revealed that 12 of the 13 clone sequences (1C2, 1C5, 2B8, 2G5, 2H7, 2C2, 2F4, 1G10, 1E1, 2B6, 1C12, and 2E6) showed the closest matches with Ascomycota and that one clone (2A3) showed a significant match with Oomycota. Table 3 shows the results of BLAST searches for clone sequences from palm leaves and roots. Their phylogenetic affiliation and relationship with other fungal strains were further determined by the construction of a phylogenetic tree, which is presented in Fig. 1. For confidence reasons, final phylogenetic affiliations were inferred by similarity with the closest ITS sequences from culture collection strains, when available, after curation of inappropriate taxonomical affiliation of sequences derived from BLAST searches. Obtained sequences were classified into four groups (Fig. 3). Group I was the most represented (78% of total root and leaf clones) and consisted of members from the Pleosporaceae family. Pleosporaceae appeared dominant in both roots and leaves samples with two genera *Alternaria* and *Curvularia* represented. Clones of Group I, assigned as 1C2, 1C5, 2B8, 2G5, 2C2, and 2F4, were related to *Alternaria* sp. and more precisely, for given clones, to *Alternaria alternata*. The *Alternaria* genus was globally representing an overall 61% of the 200 clones assessed with similar occurrence of the genus

Table 3 Closest match of ITS-rDNA sequences and phylogenetic affiliation from clones from Tunisian date palm trees following BLAST searches in GenBank database

Clone	Fragment size (bp)	Closest BLAST match	Phylogenetic affiliation*	Phylum	Family	%identity
Leaf						
1C2	580	"*Alternaria arborescens*"	*Alternaria* sp.	Ascomycota	Pleosporaceae	98
1C5	570	"*Alternaria gaisen*"	*Alternaria* sp.	Ascomycota	Pleosporaceae	98
1C12	570	"*Bipolaris tetramera*"	*Curvularia* sp.	Ascomycota	Pleosporaceae	99
1E1	548	"*Davidiella tassiana*"	*Cladosporium* sp.	Ascomycota	Davidiellaceae	99
1G10	546	"*Cladosporium* sp."	*Cladosporium herbarum*	Ascomycota	Davidiellaceae	99
Root						
2A3	573	"*Pythium* sp."	*Pythium* sp.	Oomycota	Pythiaceae	99
2B6	570	"*Bipolaris tetramera*"	*Curvularia* sp.	Ascomycota	Pleosporaceae	99
2E6	575	"Fungal endophyte"	Fungal endophyte	Ascomycota	Sporomiaceae	100
2G5	583	"*Alternaria abutilonis*"	*Alternaria alternata*	Ascomycota	Pleosporaceae	99
2F4	579	"*Alternaria gaisen*"	*Alternaria* sp.	Ascomycota	Pleosporaceae	98
2H7	563	"Fungal endophyte"	Fungal endophyte	Ascomycota	Pleosporomycetidae	98
2C2	570	"*Alternaria gaisen*"	*Alternaria* sp.	Ascomycota	Pleosporaceae	98
2B8	570	"*Alternaria arborescens*"	*Alternaria* sp.	Ascomycota	Pleosporaceae	98

* For confidence reasons, the final phylogenetic affiliation was derived by similarity with the closest culture collection isolates, when available, after curation of inappropriate taxonomical position of sequences obtained from the non-redundant nucleotide database

in leaves and roots. Indeed, *Alternaria* genus represented 55% of the root clones and 68% of the leaf clones. However, the distribution of the different *Alternaria* species varied between root and leaf as shown in Fig. 2c. Similar results were obtained for *Curvularia*, which represented an overall 17% of the total clone libraries, with 25% among leaf clones and 10% among root clones, respectively (Fig. 2c). However, as for *Alternaria*, root and leaf species of *Curvularia* differed, as suggested by the distribution of the 1C12 and 2B6 patterns among leaves and roots, respectively. Interestingly, our clones were related to obviously yet uncultivable *Curvularia* species in the databases (accession No. EU144681), but were also matching, with a highly significant score, a sequence obtained from one of our own cultivable isolates (Fc1s) (Fig. 1), suggesting that corresponding *Curvularia* species could, indeed, be cultured. Group II had a single representative of the Pythiaceae family, 2A3, distantly related to *Pythium catenulatum* which was further assigned as *Pythium* sp. (Fig. 1). It represented 10% of the root library but 0% from the leaves (Fig. 2c), representing an overall 5% of the total clones (Fig. 3). Group III consisted of members of the Davidiellaceae family and represented an average 10% of the total clones (Fig. 3). The clones of this group, represented by 1G10 and 1E1, were exclusively found among the leaf library (Fig. 2c) where they represented an overall 20% of the leaf clones. These clones were related to *Cladosporium herbarum* and *Cladosporium* sp., respectively (Fig. 1). Finally, remaining clones, related to uncultivable Pleosporales, were assembled in Group IV. These sequences represented an overall 7% of the total clones (Fig. 3). One clone, 2H7, was distantly related to *Ochrocladosporium* (Pleosporomycetidae). The second clone, 2E6, was also related to uncultivable Pleosporales but fell into the *Sporormiaceae*, as demonstrated by a significant bootstrap value of its node with *Preussia* and *Corynespora* species (Fig. 1).

Overall, both the ITS rDNA gene identification from clones amplified from total palm DNA and sequencing of ITS rDNA from cultivable strains yielded concordant results as the dominant species, *Alternaria* and *Curvularia*, were recovered by both methods. However, *Fusarium* species, that constituted the dominant flora among the cultivable fraction, were not detected by the cloning strategy. Conversely, some dominant species among the clones were not represented among the cultivable flora, such as *Cladosporium* (or its teleomorph *Davidiella*), but also *Pythium*. Of course, sequences related to uncultivable fungal endophytes were detected only by molecular techniques, except for *Curvularia* sp. (Table 4). Globally, the diversity recovered by the global DNA approach was broader than that obtained by the cultivable method.

Table 4 Diversities of the cultivable and uncultivable endophytic fungal floras associated to the date palm (*Phoenix dactylifera*) as estimated from sequencing of the ITS region

Strain/clone	Closest phylogenetic relatives	Cultivable fraction	ITS rDNA
R2	*Fusarium* sp. (Nectriaceae)	+	−
R3	*Fusarium equiseti* (Nectriaceae)	+	−
Fc1s	*Curvularia* sp. (Pleosporaceae)	+	+
Fc3s	*Alternaria bokurai* (Pleosporaceae)	+	−
Fc4s	*Alternaria bokurai* (Pleosporaceae)	+	−
1C2	*Alternaria* sp. (Pleosporaceae)	−	+
1G10	*Cladosporium rbarum* (Davidiellaceae)	−	+
1E1	*Cladosporium* sp. (Davidiellaceae)	−	+
1C12	*Curvularia* sp. (Pleosporaceae)	+	+
1C5	*Alternaria* sp. (Pleosporaceae)	−	+
2H7	Fungal endophyte (Pleosporomycetidae)	−	+
2C2	*Alternaria* sp. (Pleosporaceae)	−	+
2F4	*Alternaria* sp. (Pleosporaceae)	−	+
2B6	*Curvularia* sp. (Pleosporaceae)	+	+
2E6	Fungal endophyte (Sporomiaceae)	−	+
2A3	*Pythium* sp. (Pythiaceae)	−	+
2B8	*Alternaria* sp. (Pleosporaceae)	−	+
2G5	*Alternaria alternata* (Pleosporaceae)	−	+

+: species found; −: species not found

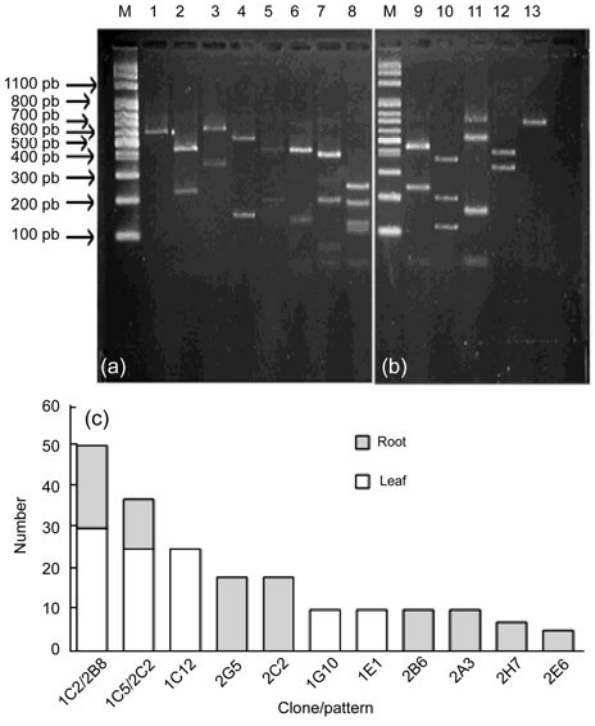

Fig. 2 RFLP profile following simple digestion with *Hae*III of representative clones from different groups
Clones from root tissues (2A3, 2B6, 2E6, 2G5, 2F4, 2H7, 2B8, and 2C2) (a) and leaf tissues (1C2, 1C12, 1E1, 1C5, and 1G10) (b) of *Phoenix dactylifera*. Bands 1–13: 2C2, 2B8, 2G5, 2F4, 2B6, 2A3, 2E6, 2H7, 1C2, 1C12, 1G10, 1E1, and 1C5, respectively. (c) Distribution of clones among patterns shows a peaked distribution

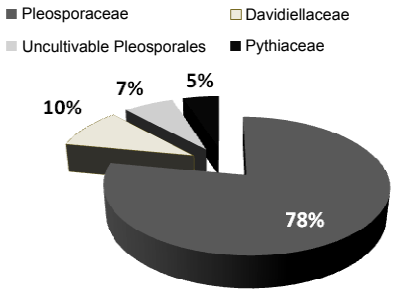

Fig. 3 Distribution of the fungi flora as determined by ITS rDNA cloning

4 Discussion

The present study constitutes the first attempt to extensively characterize the endophytic fungal flora associated with the date palm tree (*P. dactylifera*). Its aim was to develop an overview of the diversity of this mostly undeciphered flora, which may lay the ground for the construction of a more comprehensive description that covers other date palm varieties and field conditions. Our study shows that both DNA-based approaches and traditional cultivation techniques should be used to elucidate the diversity of the endophytic fungal flora associated with mature date palm trees, such as the Deglet Nour variety investigated

in this work, which accounts for 70 years of Tunisian date palm production. Both of our approaches (by culture and molecular techniques) demonstrated a high diversity of palm tree associated fungal flora in both root and leaf tissues of date palm (*P. dactylifera* L.). Overall, 11 sequence types (species equivalent) were recorded from the palm tree tissues. Six sequence types were obtained in the leaf samples among which five were obtained using the molecular approach (*Alternaria alternata*, *Alternaria* sp., *Curvularia* sp., *Cladosporium herbarum*, and *Cladosporium* sp.) and two using the culture approach (*Alternaria bokurai* and *Curvularia* sp.), *Curvularia* being common to clones and isolates. Ten sequence groups were detected in the palm roots with eight sequences identified from DNA cloning strategy (*Alternaria alternata*, three different *Alternaria* sp., *Curvularia* sp., and *Pythium* sp., and two types of uncultivable Pleosporales) and two from cultivation strategy (*Fusarium equiseti* and *Fusarium* sp.). Most of these fungi have been already reported as endophytes in other plants (Cao *et al.*, 2002; Gond *et al.*, 2007; Khan *et al.*, 2007), including wheat (Larran *et al.*, 2002a), soybeans (Larran *et al.*, 2002b), bananas (Pocasangre *et al.*, 2000; Cao *et al.*, 2002), and tomatoes (Larran *et al.*, 2001). Several endophytic fungi have recently been described from oil palms (Rungjindamai *et al.*, 2008), such as *Trametes elegans* and *Fomitopsis pinicola*. *Fusarium solani* and *Fusarium oxysporum* f. sp. *albedinis* were early reported in date palm trees (Lamberti, 1988; Hatimi, 1989; Fernandez *et al.*, 1995).

The endophytic fungi were not evenly distributed, with Pleosporaceae (not only *Alternaria* sp. and its teleomorph species *Bipolaris* sp., but also *Curvularia* sp.) constituting the dominant group in both the leaves and roots of the date palm tree. Indeed, *Alternaria* sp. constituted over 40% of the cultivable isolates and 61% of the clones with three species being identified (*Alternaria alternata* and two *Alternaria* sp. sequence types). To a lesser extent, *Curvularia* species represented 23% of the isolates and 17% of the total clone sequences. Several previous studies reported the occurrence of those species in other plants, including banana (Cao *et al.*, 2002), *Aegle marmelos* (Gond *et al.*, 2007), *Coffea Arabica* L. (Fernandes *et al.*, 2009), tangerine and lemons (Dini-Andreote *et al.*, 2009), *Deschampsia antarctica* Desv. (Rosa *et al.*, 2009), *Huperzia serrata* (Wang *et al.*, 2011), *Bletilla ochracea* (Tao *et al.*, 2008), wheat (Smit *et al.*, 1999), *Atriplex canescens* (Lucero *et al.*, 2011), and *Artemisia species* (Huang *et al.*, 2009). The group of Pleosporaceae represented by *Alternaria* species consists mainly of saprophytes that are commonly found in soil or on decaying plant tissues; however, some of them were reported to acquire pathogenic traits (Thomma, 2003). Species of *Alternaria* are prevalent in the mycoflora that infests and often parasitizes the seeds of a wide variety of food crops (Schroeder and Cole, 1977). Here, endophytic *Alternaria* and cultivable and uncultivable *Curvularia* species were not only isolated from tissue samples but also directly amplified by PCR amplification. The fact that these strains and clone sequences were isolated in 2010 from a healthy tree and still remained viable in 2012 (unpublished results) suggests, indeed, their GRAS status.

The second dominant group of endophytes, assigned as Nectriaceae (38.5%), was observed to be present exclusively in root tissues of date palm and in the cultivable fraction. Two species were identified, namely *Fusarium equiseti* and *Fusarium* sp. Those species are, in fact, reported to constitute the most important endophytes in a number of plant species, including *Aegle marmelos* (Gond *et al.*, 2007), banana (Cao *et al.*, 2002), *Apodytes dimidiate* (Shweta *et al.*, 2010), tomato (Hibar *et al.*, 2007), and maize (Gherbawy *et al.*, 2002). Pathogenic *Fusaria* such as *F. oxysporum* has long been known to be the causal agent of the Bayoud disease (El Hassni *et al.*, 2007). However, the *Fusarium* isolates from this study originated from a long-lasting healthy tree, suggesting their GRAS status. The lack of sequences belonging to *Fusaria* among the clones could however result from the difficulty to lyse the *Fusarium* cells (Vallaeys, unpublished), and reflect the inadequacy of plant DNA purification methods to assess the diversity of endophytic flora and further, underline the epidemiologic risk to underestimate or neglect given fungi populations, especially when referring to pathogens.

Furthermore, in this study, *Cladosporium* and its teleomorphic species *Davidiella* represented 10% of the clones. Although commonly cultivable, this genus was only detected in this study by clone sequencing and appropriate media should be optimized to isolate

and culture this genus in date palm. *Cladosporium* species are commonly found in many other plants (both herbaceous and woody) and in soil (Arx, 1949; Fisher *et al.*, 1995; Taylor *et al.*, 1999; Bensch *et al.*, 2010), including wheat (Smit *et al.*, 1999), pharmaceutical plants (*Heterosmilax japonica*) (Gao *et al.*, 2005), and *Atriplex canescens* (Lucero *et al.*, 2011) where they constitute an extremely large and important genus of plant pathogens. However, *Cladosporium* also includes cosmopolitan and ubiquitous saprotrophs. Here again, the fact that *Cladosporium*-related sequences were obtained from an old-aged healthy tree suggests their non-pathogenic status.

Finally, our molecular approach allowed us to enlighten the occurrence of uncultivable endophytes. In root samples, this firstly included *Pythium* (5% of the total clone sequence only), a common endophytic fungi, mainly reported as pathogenic, which can infect various hosts but remains undetectable using conventional culture techniques. Members of this genus act as pathogens towards a range of organisms, including plants, algae, fish, shrimp, mosquito larvae (Saunders *et al.*, 1988), and even humans (de Cock *et al.*, 1987). Some species of the genus can, however, be beneficial, functioning as biological control agents protecting against pathogenic fungi (Abdelghani *et al.*, 2004; Paul, 2004). Few species, such as *Pythium mycoparasiticum* (Jones and Deacon, 1995), *Pythium contiguanum* (Paul, 2004), and *Pythium paroecandrum* (Abdelghani *et al.*, 2004), have been reported to behave as mycoparasites. However, although modern taxonomy is widely developed for *Pythium* (Lévesque and de Cock, 2004), palm-derived sequences seem to belong to novel species, which do not allow for delineation of pathogenic versus GRAS or pathogenic status.

Furthermore, our molecular analysis data showed that palm tissues were also colonized by two sequence types hitting up to now uncultivable Pleosporales (6% of total clone libraries). Indeed, one clone showed distant similarity with *Preussia* sp. (Sporormiaceae) which comprises species from soil, wood, and plant debris (Cain, 1961; Arenal *et al.*, 2007). However no cultivable close relatives were available for this clone sequence. A clone sequence type, absent from the cultivable group, was affiliated distantly to *Ochrocladosporium*. Interestingly, another sequence types, presenting significant BLAST hits with given up to now uncultivable root fungal sequences, were detected both among our isolates and among our clones. This demonstrated that it could be cultured on traditional PDA agar. As previously reported, this sequence belonged to the dominant Pleosporales and thus appeared as a novel *Curvularia* species that could easily be further characterized. This result constitutes major progress in our knowledge of the palm-associated fungal flora.

Further, the preliminary characterization of endophytic fungi in this study revealed a variation in the type of indigenous fungi isolated from roots and leaves. Indeed, some genera were recorded in the leaf tissues but not registered in the root tissues collected from the same tree, which could be attributed to the different environments involved, namely the leaves, representing a source of nutrients, and the roots, representing a nutrient sink (Lim *et al.*, 2003). Among these, *Fusarium* sp. and *Fusarium equiseti* were recorded in the root but not in leaf tissues of the plant. Further comparisons between the endophytic communities obtained from the traditional and the molecular techniques indicated that the indigenous fungi isolated from the roots and leaves were different. In fact, some species, such as *Cladosporium* sp. (1G10 and 1E1), were only recorded in leaf tissues. Conversely, other species, such as *Pythium* sp. (2A3), were recorded in root but not in leaf tissues. These results suggest that the endophytic population of date palm varies depending on the plant organ analyzed. Nevertheless, some species, namely *Alternaria* sp. and *Curvularia* sp., were recorded in both tissues.

The comparison between the findings obtained for the endophytic fungal assemblages within *P. dactylifera* from the traditional and the culture independent molecular techniques suggests that some endophytic fungi could not be isolated from plant tissues using traditional cultural methods, presumably due to inherent limitations in those techniques. Conversely, the direct detection and identification of endophytic fungi from plant tissues using molecular techniques presents a number of limitations, such as those already suggested for *Fusaria*. In addition to lysis difficulties, as only sparse hyphae may exist within the plant tissues, underrepresented fungal DNA may be lost during the PCR process. This can be explained by competition with plant DNA present in excess in the sample, as universal primers also

amplify the plant ITS. This results in numerous plant-associated sequences in the clone library. Conversely, the primers may not completely match with some fungal template DNA. Another equally important point concerns the treatment of the host tissue (Guo *et al.*, 2001). It is important to thoroughly clean the surface of the host tissue (with ethanol, detergents, and reducing agents) to remove potential phylloplane fungi and other organisms present on the host surface. Though presenting a number of limitations, molecular techniques appear to offer a complementary method for the detection of endophytic fungi, particularly those that cannot be incubated on artificial media and can be directly amplified from plant tissues. With the improvement of DNA extraction and PCR molecular techniques, this method could yield rapid and accurate tools for the direct detection and identification of endophytic fungi within plant tissues.

In short, the present study demonstrated the occurrence and diversity of cultivable and uncultivable endophytes in date palm (*P. dactylifera* L.). The roles of these endophytes in this habitat remain to be elucidated. Further studies, some of which are currently underway, are needed to gain more comprehensive data with regards to the biodiversity of the fungal endophyte population in date palm suffering from troublesome diseases, such as the brittle leaf disease, and to compare them with data pertaining to healthy fungal endophyte populations. More generally, this study underlines the necessity to more extensively investigate the fungal diversity of the Mediterranean environment which, despite its importance, has not been subjected to exhaustive studies. Indeed, only a few disperse examples are addressed in the literature (Fisher *et al.*, 1992; Peláez *et al.*, 1998; Girlanda *et al.*, 2002), which clearly means, as underlined by this study, that in the Mediterranean environment, a large number of new species are likely to still be awaiting discovery.

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Compliance with ethics guidelines

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