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Soils of a Mediterranean hot spot of biodiversity and endemism (Sardinia, Tyrrhenian Islands) are inhabited by pan-European, invasive species of *Hypocrea/Trichoderma*

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Summary

We have used a Mediterranean hot spot of biodiversity (the Island of Sardinia) to investigate the impact of abiotic factors on the distribution of species of the common soil fungus *Trichoderma*. To this end, we isolated 482 strains of *Hypocrea/Trichoderma* from 15 soils comprising undisturbed and disturbed environments (forest, shrub lands and undisturbed or extensively grazed grass steppes respectively). Isolates were identified at the species level by the oligonucleotide BarCode for *Hypocrea/Trichoderma* (*Trich*OKEY), sequence similarity analysis (*Tricho*BLAST) and phylogenetic inferences. The majority of the isolates were positively identified as pan-European and/or pan-global *Hypocrea/Trichoderma* species from sections *Trichoderma* and *Pachybasium*, comprising *H. lixii/T. harzianum*, *T. gamsii*, *T. spirale*, *T. velutinum*, *T. hamatum*, *H. koningii/T. koningii*, *H. virens/T. virens*, *T. tomentosum*, *H. semiorbis*, *H. viridescens/T. viridescens*, *H. atroviridis/T. atroviride*, *T. asperellum*, *H. koningiopsis/T. koningiopsis and Trichoderma* sp. Vd2. Only one isolate represented a new, undescribed species belonging to the Harzianum–Catoptron Clade. Internal transcribed spacer sequence analysis revealed only one potentially endemic internal transcribed spacer 1 allele of *T. hamatum*. All other species exhibited genotypes that were already found in Eurasia or in other continents. Only few cases of correlation of species occurrence with abiotic factors were recorded. The data suggest a strong reduction of native *Hypocrea/Trichoderma* diversity, which was replaced by extensive invasion of species from Eurasia, Africa and the Pacific Basin.

Introduction

The mitosporic fungal genus *Trichoderma* (teleomorph *Hypocrea*, *Ascomycota*, *Hypocreales*) is a cosmopolitan and ubiquitous inhabitant of soils, decaying wood and plant debris where it plays a significant role in the turnover of plant polysaccharides (Klein and Eveleigh, 1998). Many of its species are also associated with the rhizosphere of plants or may occur as endophytes (Harman *et al*., 2004). Its species are therefore predominant components of the soil mycoflora in various ecosystems, such as agricultural fields, pastures, forests, salt marshes, prairies and deserts over a wide range of climatic zones (Widden and Abitbol, 1980; Nelson, 1982; Klein and Eveleigh, 1998; De Bellis *et al*., 2007). Consequently, a deeper understanding of the biodiversity, geography and speciation mechanisms of *Hypocrea/Trichoderma* is an interesting issue. Pioneering studies on the distribution of *Trichoderma* species in soil and rhizosphere ecosystems have been published by Danielson and Davey (1973), Widden and Abitbol (1980) and Nelson (1982). However, a critical evaluation of these studies is impossible because only a small portion of the *Hypocrea/Trichoderma* species known today (cf. Druzhinina *et al*., 2006) had been recognized at the time these studies were performed, and identification of species at that time had been based only on morphological species concept, which – because of the homoplasy of characters – is highly prone to errors. While molecular methods for correct species identification have now been established and used in several recent investigations on the mycogeography of the genus *Hypocrea/Trichoderma*

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(cf. Druzhinina *et al*., 2005; Zhang *et al*., 2005; and references therein), they have not yet been used in ecological studies on this genus.

In the present investigation, we have adopted the Tyrrhenian Island of Sardinia (Italy) – located in the Western Mediterranean Sea, between 38°51′52″ and 41°15′42″N and between 8°08′10″ and 9°50′08″E, with a surface area of 24 089 km^2 – as a model: Sardinia has since 35 Mio years ago become completely separated from the European mainland (today's Southern France/North-western Iberia), with an intermediary period of 5–7 Mio years when it was linked to today's Italy by a land bridge. Consequently, it is known for its high proportion of endemic vascular plant and animal species, and is one of Europe's last reserves of virgin evergreen oak (*Quercus ilex*) forests and dense Mediterranean maquis. On the basis of plant diversity – it is characterized by at least 50% of endemic species of vascular plants, which is a significantly higher proportion than e.g. for Australia (34%, IUCN, 2002) – it was identified as one of the biodiversity hot spots in the Mediterranean Basin and proposed to be included in EU and IUCN conservation policies (Grill *et al*., 2005). The great diversity and uniqueness of plant species also result in a broad chemical diversity and decomposability of the debris and metabolites from plants and provides a large number of trophic niches available for soil saprotrophs (Goberna *et al*., 2005). Thus, the investigation of such an ecosystem may provide basic insights into the mechanisms controlling the diversity of saprotrophic fungi like *Trichoderma* (Drake *et al*., 2002).

The aim of this study was therefore: (i) to obtain a representative number of samples from Sardinian ecosystems with different plant composition and influence of mankind to characterize the general diversity of *Hypocrea/Trichoderma* at a large (island) scale and identify dominant and subordinate species and (ii) to evaluate whether their distribution depends on abiotic factors, such as soil properties (carbon availability), altitude, climatic conditions and ecosystem disturbance.

Results

Diversity of Hypocrea/Trichoderma *in Sardinia*

A total of 482 isolates were obtained from the 15 sampled soils, purified and pre-screened by random amplification of polymorphic DNA (RAPD) analysis (Turner *et al*., 1997). Isolates with identical RAPD patterns (i.e. > 98% similarity) were considered to belong to the same species, which was verified by sequencing of internal transcribed spacer (ITS) of randomly selected pairs with such identical RAPD patterns. Therefore, only one or two strains representing each group of isolates from the same sampling location and yielding identical (> 98% similarity) RAPD fingerprints were subjected to species identification by gene sequence analysis. All cases with ambiguous RAPD results were also sequenced. Thus ITS sequences were obtained from 231 isolates and subjected to analysis by *TrichO*KEY (Druzhinina *et al*., 2005) and *Tricho*BLAST (Kopchinskiy *et al*., 2005). By this means, all but one isolate could be identified to represent already known species (Table 1), thus yielding 14 known taxa plus one unknown taxon. This yields a total biodiversity index (no. of species/no. of isolates) of 0.03.

H. lixii/T. harzianum was the most abundant species (57%) and present in 10 of the 15 sites investigated. It was the dominant species in seven of the locations where it was present (G1, S1, S3, F1, F3, EG3 and EG5) and the only species isolated from site EG2 (Fig. 1). These sites are equally distributed over the island, and differ in ecosystem types, soil properties including organic content, altitude and climatic conditions.

H. lixii/T. harzianum is known to possess several ITS alleles (I.S. Druzhinina and C.P. Kubicek, unpublished; http://www.isth.info). One ITS allele, which has already found in strains sampled in Austria, the Netherlands and Italy (C.P. Kubicek, unpublished), was predominant in the samples. Three additional alleles occurred at low frequency. One, representing the allele of the type strain CBS 226.95 and known to occur more abundantly in temperate climates, was found in samples F1 and G1, but also in S3 (Narbolia, shrub land) and EG7 (Pantaleo, pasture). In addition, a rare allele, so far only found in Malaysia (Kubicek *et al*., 2003), was detected in sampling sites S1 and G1. Finally, the third allele, which is very common in Central Africa, but also found in Central America, North America, China and the Pacific and Europe (C.P. Kubicek, unpublished), was noticed for two isolates in sample EG7.

After *T. harzianum*, *T. spirale* and *T. gamsii* were most abundant (7% and 11% of all isolates, and present in 7 and 9 of the 15 samples respectively). *T. spirale* was most abundant in the carbon-rich forest soil of Badde Salighes (F2), where it accounted for > 50% of the isolates, but it did not dominate any other location. *T. gamsii* was the exclusive species found in sample S2 (Torre de la Peña, shrub land), and accounted for the majority of isolates in G2 (Talana, grassland) and GE6 (San Nicolò). In S3 (Narbolia) it was the only species found together with *H. lixii/T. harzianum.*

Six more species occurred with a frequency of 5% or less but were detected in several soils (Fig. 1): *T. hamatum* (5%, 2 forest and 3 pasture locations on the eastern side of the island), *T. velutinum* (5%, 4 locations, mainly grazing grasslands), *H. koningii/T. koningii* (2%, 5 locations), *T. tomentosum* (2%, 3 distant locations), *H. viridescens/T. viridescens* (1.2%, 4 locations on the north-eastern side of the island) and *H. semiorbis* (1%, 2

Table 1. *Hypocrea*/*Trichoderma* species from Sardinian soils identified in this study and ITS rRNA gene cluster and *tef1* GenBank numbers of reference strains.

Soil	Species	Number of Isolates	Reference strains (C.P.K.)[a]	NCBI GenBank Accession Number	
				ITS1 and ITS2	*tef1*
F2	*Trichoderma* sp. C.P.K. 2657	1	2657	EF488139	EF488107
	T. hamatum	8	2820	EF596945	–
	T. spirale	19	2821	EF596946	–
	H. semiorbis	1	2819	EF596944	–
	H. viridescens/*T. viridescens*	1	2296	EF488140	EF488108
EG3	*T. gamsii*	1	2342	EF488141	EF488110
	T. hamatum	1	2343	EF488142	EF488111
	H. lixii/*T. harzianum*	17	2822	EF596947	–
	H. koningii/*T. koningii*	1	n/a	–	–
	Trichoderma sp. Vd2 *sensu* Jaklitsch *et al.* (2006)	12	2380	–	EF488109
F3	*H. lixii*/*T. harzianum*	11	2301	EF392738	EF392734
			2313	EF392739	EF392735
	H. koningii/*T. koningii*	2	2384		EF488112
	T. tomentosum	1	2373	EF488143	EF488116
S3	*T. gamsii*	6	2341	EF488146	EF488115
			2345	–	–
			2346	–	EF488117
	H. lixii/*T. harzianum*	10	2086	EF488145	EF488114
			2338	EF488144	EF488113
G2	*T. gamsii*	7	2331	–	EF488118
			2333	EF488147	EF488119
			2335	–	EF488120
	H. lixii/*T. harzianum*	3	2332	EF488148	EF488121
	H. viridescens/*T. viridescens*	1	2297	EF488149	EF488122
EG4	*T. gamsii*	2	2347	EF488151	EF488125
			2349	–	EF488126
	H. koningiopsis/*T. koningiopsis*	4	2298	EF488150	EF488123
			2348	–	EF488124
	H. semiorbis	4	2823	EF596948	–
	T. spirale	1	2824	EF596949	–
	T. velutinum	2	2825	EF596950	–
EG6	*T. gamsii*	7	2320	EF488153	EF488128
			2306	–	EF488129
			2323	–	–
	T. hamatum	5	2307	EF488152	–
			2315	–	EF488127
	T. spirale	1	2312	EF596943	EF596974
EG7	*T. gamsii*	4	2729	EF488154	–
	H. lixii/*T. harzianum*	7	2826	EF596951	–
			2827	EF596952	–
	T. spirale	6	2318	–	EF596975
	T. velutinum	9	2828	EF596953	–
	H. virens/*T. virens*	19	2829	EF596954	–
F1	*T. hamatum*	5	2830	EF596955	–
	H. lixii/*T. harzianum*	64	2831	EF596956	–
			2832	EF596957	–
			2834	EF596959	–
			2833	EF596958	–
	H. koningii/*T. koningii*	1	2068	–	EF488130
	T. spirale	1	2835	EF596970	–
	T. tomentosum	6	2066	EF596939	EF596971
	H. viridescens/*T. viridescens*	2	2069	EF488155	DQ790657
			2089	–	DQ790659
EG1	*T. hamatum*	3	2836	EF596960	–
	T. spirale	2	2308	EF596941	–
			2309	EF596942	EF596973
	H. viridescens/*T. viridescens*	2	2084	–	DQ790658
			2085	EF488156	–

Table 1. *cont.*

Soil	Species	Number of Isolates	Reference strains (C.P.K.)[a]	NCBI GenBank Accession Number	
				ITS1 and ITS2	*tef1*
S1	*T. asperellum*	3	2064	EF488157	–
			2063	–	EF48813
	T. gamsii	18	2070	–	DQ790645
			2090	EF488158	DQ790653
			1489	–	EF488132
			1495	–	EF488133
			2074	–	DQ790647
			2091	–	DQ790654
			2092	–	DQ790655
			2302	–	EF488134
			2076	–	DQ790649
			2077	–	DQ790650
			2078	EF488159	DQ790651
			2093	–	DQ790656
	H. lixii/T. harzianum	111	2845	EF596969	–
			2837	EF596961	–
			2838	EF596962	–
			2846	EF601683	–
	T. tomentosum	2	2067	EF596940	EF596972
	Trichoderma sp. Vd2 *sensu* Jaklitsch *et al.* (2006)	2	1488	DQ845431	DQ845416
EG2	*H. lixii/T. harzianum*	33	2839	EF596963	–
EG5	*H. lixii/T. harzianum*	14	2841	EF596965	–
			2842	EF596966	–
	T. velutinum	10	2840	EF596964	–
G1	*T. gamsii*	7	2303	–	EF488136
			2351	EF488161	EF488137
	H. lixii/T. harzianum	7	2844	EF596968	–
	H. koningii/T. koningii	2	2300	EF488160	EF488135
	T. spirale	4	2843	EF596967	–
	T. velutinum	2	n/a		–
S2	*T. gamsii*	7	2327	EF488162	EF488138
			2330		
Total		482			

a. C.P.K. number in the collection of fungal strains in Vienna University of Technology, Vienna, Austria.

locations). The latter fungus has so far only been found as its teleomorph in Australia and Tasmania (Chaverri and Samuels, 2003), and this is the first time that its anamorph is found in soil. Isolates of *T.* sp. Vd2 *sensu* Jaklitsch *et al*. (2006) were detected in soils S1 and EG3.

Three species were detected in single-soil samples only: *H. virens/T. virens* dominated site EG7, and *T. asperellum* and *T. koningiopsis* were detected in the most southern and most northern sites S1 and EG4 respectively.

A single isolate (C.P.K. 2657, soil F2), while clearly belonging to *Hypocrea/Trichoderma*, could not be identified. Based on its ITS1 and ITS2 sequences, a close similarity to species from the Harzianum–Catoptron Clade could be recognized. Figure 2 shows a phylogenetic analysis of this new species and members of the Harzianum–Catoptron clade, based on the sequence of the long intron of translation elongation factor 1-alpha (*tef1*) gene, which reveals it to be a sister species to *T. aggressivum.*

Most of the species identified in this inventory exhibited ITS1, ITS2 and *tef1* alleles already present in public databases. In fact, the Sardinian isolates of *T. tomentosum* exhibited the same *tef1* sequence as an isolate from Guatemala, indicating mixing over a large distance. However, in the case of *T. hamatum*, five isolates (C.P.K. 2314, C.P.K. 2315, C.P.K. 2316, C.P.K. 2317 and C.P.K. 2307) from EG6 (where it was a subdominant species) exhibited a new ITS1 sequence, which has not been found so far (Fig. 3). Interestingly, the purine transition occurred in the second position of a diagnostic $A_{(4)}$ stretch that is present in sequences of *T. hamatum* and closely related *T. pubescens* only.

The highest biodiversity of *Hypocrea/Trichoderma* was detected in site EG4 located on the slope of Punta La Marmora (about 1000 m above see level) close to the eastern coast of the island. The absence of *H. lixii/T. harzianum* in this soil was compensated by presence of a total of 5 species among the only 12 strains isolated. The next most diverse habitats were grassland G1 (5 species

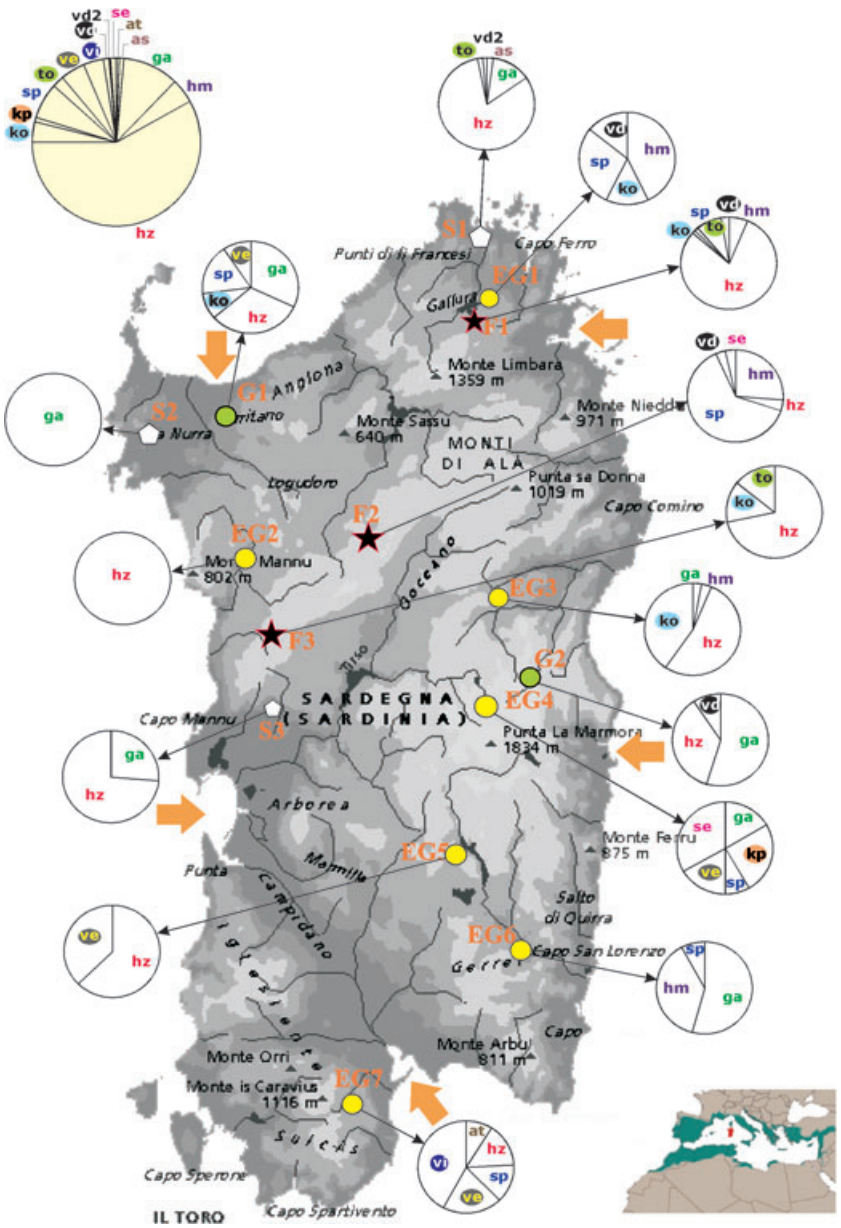

Fig. 1. Locations of sampling sites and diversity of *Hypocrea/Trichoderma* in Sardinia. F, S, G and EG correspond to forest, shrub land, grassland and extensively grazed grassland ecosystems. Individual species indicated as follows: hz – *H. lixii/T. harzianum*, ga – *T. gamsii*, sp. – *T. spirale*, ve – *T. velutinum*, hm – *T. hamatum*, ko – *H. koningii/T. koningii*, vi–*H. virens/T. virens*, to – *T. tomentosum*, se – *H. semiorbis*, vd – *H. viridescens/T. viridescens*, at – *H. atroviridis/T. atroviride*, as – *T. asperellum*, kp – *H. koningiopsis/T. koningiopsis* and vd2 – *Trichoderma* sp. Vd2 *sensu* Jaklitsch and colleagues (2006). Arrows indicate locations of main sea ports on the island. The diagram on the upper left shows the contribution of individual species to the total diversity of *Hypocrea/Trichoderma* in Sardinia. The scheme on the bottom right indicates the location of the island in the Mediterranean hot spot of biodiversity and endemism.

per 22 isolates) located close to Porto Torres and Sassari and pasture EG7 situated on the south of island near Golfo di Cagliari (5 species per 30 isolates). Two other locations where large numbers of individual species were detected (6 for S1 and 5 for F1) exhibit a relatively poor diversity of *Hypocrea/Trichoderma* because these are the soils from which the largest number of strains were isolated (136 and 80 respectively).

The lowest diversity of *Hypocrea/Trichoderma* was detected in three locations on the western side of the island where either exclusively *H. lixii/T. harzianum* or *T. gamsii* or a community of both species were detected. These were two lowland shrub lands (S2 and S3) and one pasture (EG2).

Ecology of Trichoderma *in soil*

In order to detect biotic and abiotic factors that may influence the occurrence of *Hypocrea/Trichoderma* diversity in soil and abundance of individual species, we characterized soil properties and microclimatic conditions and investigated their possible correlations with the species composition and the biodiversity index. Cluster analysis did not allow the separation of groups composed by samples collected from the same habitat (Fig. 4). Accordingly, the one-way ANOSIM test found no significant differences between habitat groups of samples ($R = -0.014$ and $P = 53.1\%$). The weighted Spearman coefficient (ρ_w) did not find any good agreement between

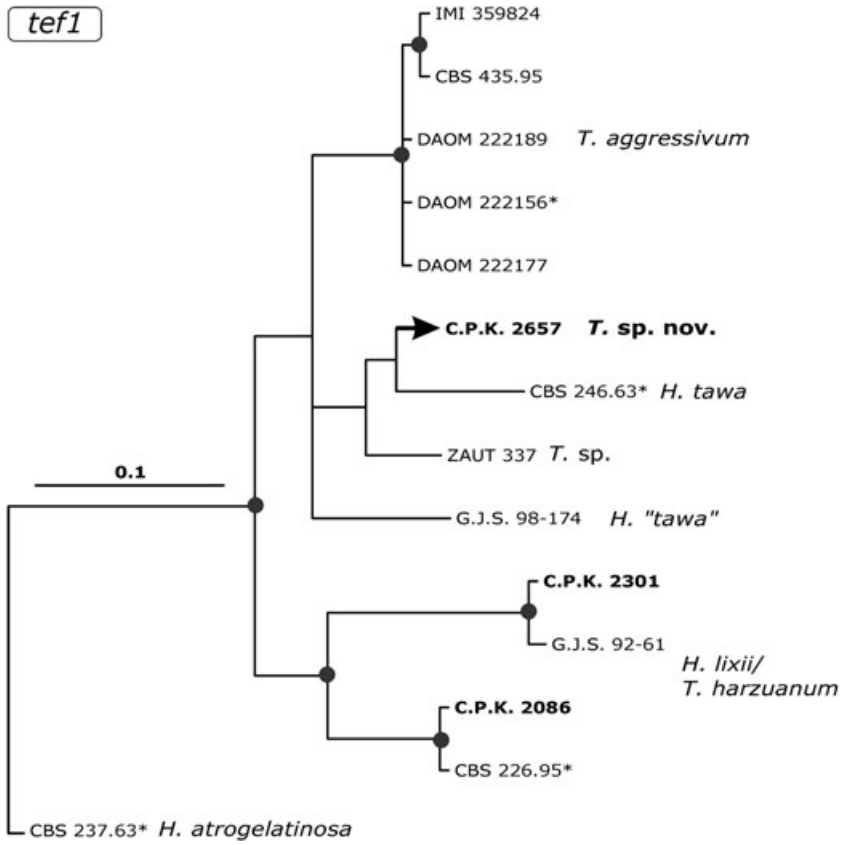

Fig. 2. Bayesian phylogram based on the large intron of *tef1* showing position of *Trichoderma* sp. C.P.K. 2657 in a vicinity of *T. aggressivum* and *H. tawa*. Black cycles indicate posterior probability values above 0.94. Asterisk shows position of *ex*-type strains for corresponding species. Bold font marks sequences obtained in this study, their accession numbers are given in Table 2. Other sequences may be retrieved from NCBI GenBank using '*Trichoderma* [strain number] + translation elongation' keywords.

distribution of species and environmental variables considered (no combination of environmental variables provided a value higher than $\rho = -0.27$ and $P = 95.2\%$). Finally, there was no correlation between the biodiversity index (which varied from 0.03 to 0.42; see Table 2) and any of the measured parameters.

A few exceptions to this rule were observed: *T. spirale* and *T. hamatum* showed a positive correlation with the content of organic compounds in the soil ($r = 0.60$ and $r = 0.57$ respectively; $n = 15$; $P < 5\%$). In addition, the occurrence of *H. viridescens/T. viridescens* was negatively correlated with the average annual temperature at the sampling site and correlated positively with the altitude of the location ($r = -0.70$ and $r = 0.65$ respectively; $n = 15$; $P < 5\%$). Also, this species exhibited a significant negative correlation with soil pH ($r = -0.57$; $P < 5\%$). In contrast, the occurrence of *T. velutinum* was positively correlated with pH ($r = 0.5$; $P < 5\%$), which coincides also with a pH optimum of this species at around pH 6.0 (plate growth experiments; data not shown).

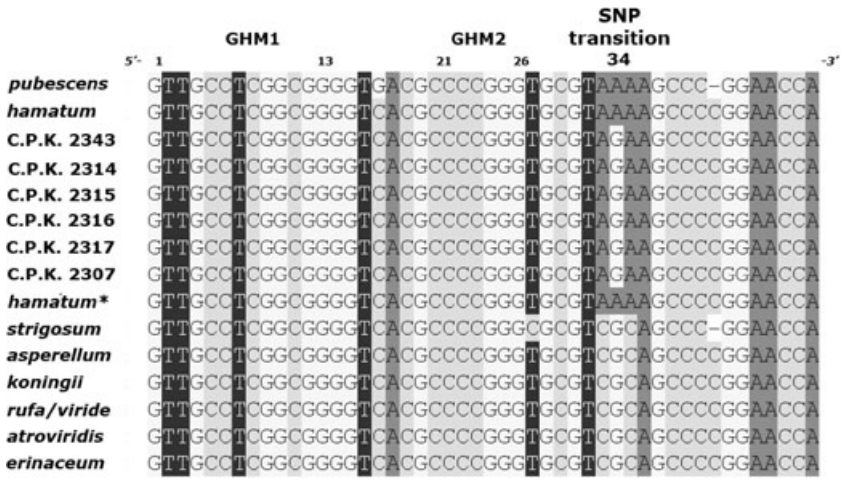

Fig. 3. Position of purine transition inside the species-specific ITS1 hallmark for *T. hamatum* and *T. pubescens*. GHM1 and GMH2 indicate positions of genus-specific hallmarks as indicated in Druzhinina and colleagues (2005). Numbers show regions of GHMs and the position of SNP, which may indicate the potentially endemic Sardinian allele of *T. hamatum*. Type sequences retrieved using accession numbers given in Druzhinina and colleagues (2005). **T. hamatum* G.J.S. 98-170, DQ109530.

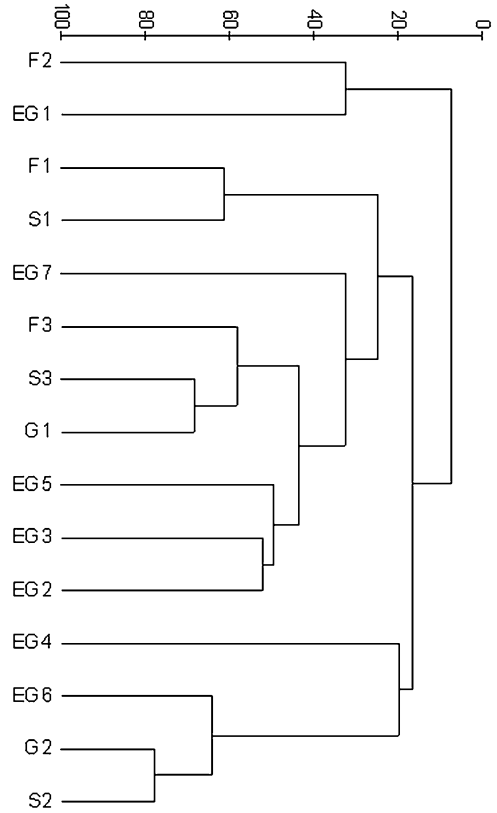

Fig. 4. Dendrogram obtained from Bray–Curtis similarities in species composition among samples.

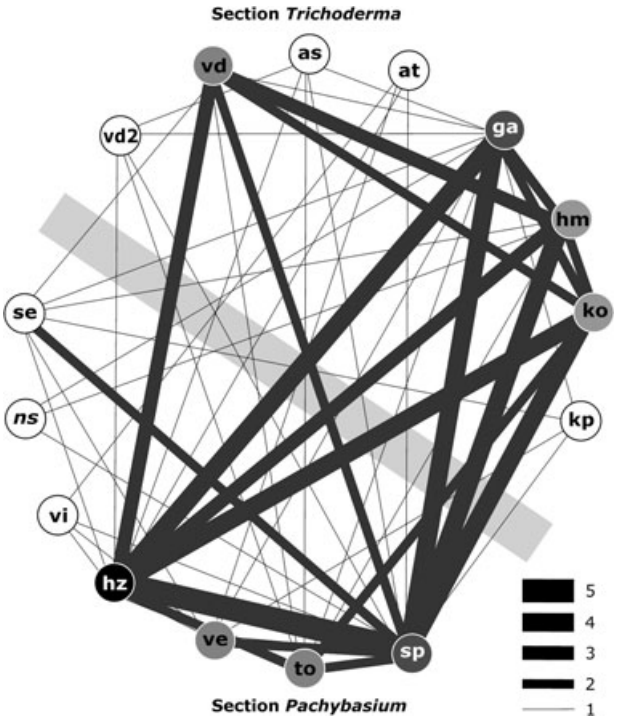

Fig. 5. Combined scheme of *Hypocrea/Trichoderma* spp. co-occurrence in Sardinian soils. Species are indicated as given on Fig. 1. Line thickness corresponds to the number of times when two species were isolated from the same soil sample.

When the simultaneous occurrence of the different *Hypocrea/Trichoderma* spp. was analysed (Fig. 5), we observed that in the majority of cases the dominant populations of *H. lixii/T. harzianum* and the subdominant populations of *T. gamsii*, *T. spirale* and *T. hamatum* occurred in the same soil and obviously did not compete each other. This may indicate either commensal relations between subdominant and dominant species of *Hypocrea/Trichoderma* or the ability to adapt to different trophic microniches within the samples that are specific for each of these species. In addition, the occurrence of *T. spirale* and *T. hamatum* also correlated ($r = 0.66$), most

Table 2. Global Positioning System (GPS) coordinates, climatic conditions and selected chemical and physical properties – texture, pH in water, percentage of soil organic carbon (C) and of soil organic matter (SOM) by dry weight – of sampled soils.

	GPS coordinates				*Hypocrea/Trichoderma*										
Soil code	N	E	Altitude (m)	Yearly rainfall (mm)	Biodiversity index	Species	Tmin °C	Tmax °C	Tave °C	Sand %	Silt %	Clay %	pH	C %	SOM %
EG1	41°00′29″	9°15′23″	246	877	0.42	3	6.2	22.6	13.8	75.2	13.3	11.5	5.2	6.2	10.7
EG2	40°19′24″	8°27′19″	45	657	0.03	1	6.5	23.9	14.6	71.6	12.0	16.3	6.0	5.9	10.2
EG3	40°18′29″	9°29′31″	378	663	0.16	5	6.7	24.4	14.8	86.3	6.3	7.3	5.4	3.4	5.9
EG4	40°07′16″	9°19′55″	958	970	0.38	5	8.0	25.1	15.7	72.8	15.0	12.2	5.0	9.3	16.1
EG5	39°36′07″	9°06′06″	314	741	0.08	2	7.1	23.8	14.7	41.7	22.1	36.2	7.0	1.6	2.7
EG6	39°29′12″	9°14′11″	605	638	0.23	3	8.0	26.2	16.2	49.7	30.6	19.7	4.8	6.7	11.6
EG7	39°05′29″	8°47′01″	204	874	0.11	5	10.0	25.5	17.2	74.7	14.2	11.0	6.0	2.5	4.3
F1	40°51′05″	9°08′59″	1080	800	0.07	6	5.3	22.3	13.1	83.2	8.6	8.2	4.5	6.0	10.5
F2	40°21′24″	8°55′13″	1019	738	0.17	5	4.8	22.4	12.8	67.8	20.0	12.2	5.0	15.3	26.3
F3	40°11′58″	8°35′09″	467	793	0.21	3	7.8	23.4	15.0	70.3	29.7	10.1	5.5	9.4	16.2
G1	40°37′14″	8°23′14″	128	622	0.23	5	6.5	22.7	14.0	55.6	25.2	19.2	5.4	4.1	7.0
G2	40°02′14″	9°28′30″	1093	978	0.27	3	3.5	21.0	11.4	69.5	17.3	13.2	4.2	8.3	14.4
S1	41°06′26″	9°05′11″	69	747	0.04	5	8.3	24.4	15.4	67.5	16.5	16.0	5.5	6.3	10.9
S2	40°35′04″	8°09′02″	193	650	0.14	1	10.0	24.9	16.9	30.5	43.4	25.6	7.0	4.2	7.3
S3	40°04′06″	8°32′15″	239	574	0.12	2	9.9	24.5	16.8	43.1	28.9	28.1	5.4	4.7	8.2

likely due to their bias towards high organic content in soils (see above).

Discussion

The initial idea of this investigation was to study *Hypocrea/Trichoderma* soil diversity on an island ecosystem known for its floristic richness in order to detect potentially endemic species and to reveal environmental factors controlling their distribution. However, in contrast to this expectation, we found common soil species that were already known to science by isolations from many locations in Eurasia, Africa and other continents almost exclusively.

Based on our data, the general soil diversity of *Hypocrea/Trichoderma* in Sardinia is relatively low: we identified 15 species among 482 isolates, corresponding to a diversity index (DI) of 0.03. As this is the first study of local diversity of the genus based on such a large sampling combined with molecular identification, we are limited in a comparison of our results with other biocenoses. Nevertheless, *Hypocrea/Trichoderma* biodiversity was assessed for such big continental fragments as South-East Asia (14 species/96 isolates, DI = 0.15; Kubicek *et al*., 2003), South America (10/53; DI = 0.20, Druzhinina *et al*., 2005) and China (13/135; DI = 0.096, Zhang *et al*., 2005). On a geographic scale most similar to the present study (Austrian virgin forest soil; Wuczkowski *et al*., 2003), 7 species were detected among 46 isolated strains (DI = 0.15). The highest diversity of *Hypocrea/Trichoderma* was so far found in the rhizosphere of *Coffea arabica* in Ethiopian highland forests (DI = 0.38; T.M. Belayneh and I.S. Druzhinina, unpublished).

Despite the generally low DI for *Hypocrea/Trichoderma* in Sardinia, some individual sampling sites showed relatively high DI values. The highest diversity was observed in soils sampled on the eastern rocky sites of the island (EG1 and EG4), which are located on the costal slope of the Limbara and of the Gennargentu mountains respectively. At the same time, pastures EG2 and EG5 and the shrub lands S2 and S3 located on the low western part of Sardinia had the smallest number of *Hypocrea/Trichoderma* species per sample unit.

Here and in previous studies (Kubicek *et al*., 2003; Druzhinina *et al*., 2005; Zhang *et al*., 2005; Hatvani *et al*., 2007), the ITS1 and ITS2 sequences were chosen as a diagnostic tool for detection of potentially endemic alleles because their alleles are known for all species of the genus (> 2200 sequences deposited in NCBI GenBank and > 2500 sequences stored in TU Vienna sequence database). The ITS1 and ITS2 sequences of ascomycetes have been estimated to have a mutation rate of 1 nt per 0.6 (± 0.5) per site and Mio years (Kasuga *et al*., 2002). As Sardinia has become isolated from the European mainland since at least 7 Mio years ago (Edel *et al*., 2001), we rationalized that if one or more of the *Hypocrea/Trichoderma* spp. found in this study would be endemic to Sardinia, this should be reflected by the presence of unique ITS1 and ITS2 alleles. Interestingly, only a single single nucleotide polymorphism (SNP) in the ITS1 sequence of *T. hamatum* fulfilled this expectation (Fig. 3). All the other species isolated in this study exhibited ITS1 and ITS2 alleles that are already known from pan-European and even pan-global species. In view of the above-cited mutation rate in ITS, these species must therefore have moved to Sardinia a long time after its separation from the mainland and must consequently be considered as 'invasive' species.

This hypothesis corresponds very well to the above-mentioned description of the unequal biodiversity on the island: e.g. the sampling site that exhibits the highest *Hypocrea/Trichoderma* biodiversity is the mountainous eastern side that is exposed to eastern and southern winds carrying spores from the closely located Italian peninsula (200 km) and South-eastern Europe. Fungal spores and bacteria have been detected in the stratosphere (Griffin *et al*., 2002; Wainwright *et al*., 2006), and carriage of fungal spores has been shown to be possible over distances such as from Africa to North America (Aylor, 2003). The more lowland western coast is exposed to Atlantic mistrals that potentially carry a smaller number of *Hypocrea/Trichoderma* propagules. The absence of species with large conidia (such as taxa from section *Longibrachiatum*), which may be less efficiently carried by clouds and dust, would be an argument in favour of airborne colonization.

Yet another possible explanation for the higher biodiversity of *Hypocrea/Trichoderma* on the eastern side – that they have been introduced by humans or other biota – cannot be excluded: the eastern, southern and northern locations contain the main sea ports of Sardinia (Fig. 1), and *Trichoderma* spp. have been reported as components of the human microflora (Kredics *et al*., 2003) and are frequent colonizers of the plant rhizosphere (Klein and Eveleigh, 1998). Such a scenario would be supported by the relatively large number of species found in northern G1 (close to Porto Torres, the ancient Roman colony of *Turris Lybissonis*) and southern pasture EG7 (located in the vicinity of Cagliari).

Our data do not completely exclude the probability that Sardinia may still contain endemic species of *Hypocrea/Trichoderma*. However, we hypothesize that they may be found in remaining native forests, probably being associated with the rhizosphere of relict plants, such as *Q. ilex* or *Taxus baccata*. If these endemic species indeed exist, however, they are probably not able to compete with the present dominant species *H. lixii/T. harzianum*, *T. gamsii*, *T. hamatum* and *T. spirale.* This conclusion is based on the fact that nearly all species detected in this study have

a high antagonistic potential and are known (and have been shown by us; V. Balmas, B. Scherm and Q. Migheli, unpubl. data) to be even successful agents of biological control against various plant pathogenic fungi (Vizcaino *et al*., 2005). In this respect it is interesting to note that other species such as *T. asperellum* and *H. atroviridis/T. atroviride*, which are widely used as biocontrol agents (see Seidl, 2006 for references) and usually dominate infrageneric communities of temperate soils (I. Druzhinina and C. P. Kubicek, unpublished), constituted only a minority in this study. The weak representation of these highly competitive species may be due to their inability to adapt to semi-arid climatic conditions on Sardinia, or to the fact that they are suppressed by other dominant species.

Another remarkable finding of this study was (with the exception of *T. hamatum* and *T. spirale* versus organic matter content; *T. velutinum* versus pH; *H. viridescens* versus altitude and inverse pH) the almost complete absence of correlation of species distribution with abiotic parameters of the soil samples. While it is possible that the distribution of these species is still correlated with a parameter that has not been taken into account, we nevertheless conclude that major environmental properties (such as soil type and climatic conditions) have no impact on the appearance of many *Hypocrea/Trichoderma* spp. This finding correlates well with the hypothesis of a highly invasive biodiversity of *Hypocrea/Trichoderma* in Sardinia that only highly adaptive species with a broad range of ecological niches would have been successful in colonizing this new territory.

The analysis of infrageneric communities showed that *H. lixii/T. harzianum*, *T. gamsii* and *T. spirale* frequently occurred together within the same soil. To the best of our knowledge, such a possibility has not been described for soil fungi so far. It is possible that some *Hypocrea/Trichoderma* species form commensalic or mutualistic relations in the degradation of polymeric materials and/or inhibitory components.

Finally, the detection of the anamorph of *H. semiorbis* in two Sardinian soils provides an interesting hint to the biogeography of *Hypocrea/Trichoderma*. The teleomorph *H. semiorbis* is believed to be restricted to Australia and Tasmania (Chaverri and Samuels, 2003), and its anamorph has been found in this study for the first time. A disparity in the distribution of the teleomorph and anamorph in *Hypocrea/Trichoderma* is not without precedent, and has been observed for *H. schweinitzii* and its anamorph *T. citrinoviride* (Turner *et al*., 1997) and *H. pilulifera* and its anamorph *T. piluliferum* (Hagn *et al*. 2003).

Terrestrial biodiversity is usually monitored by investigating species richness of macroorganisms such as vascular plants or vertebrates. Our study shows that the biodiversity of soil fungi such as *Hypocrea/Trichoderma* may very well serve as a bioindicator of a long-term ecosystem disturbance. From our data, we hypothesize that the modern *Hypocrea/Trichoderma* diversity in Sardinia reflects the general change of the Mediterranean environment in the course of the last millennia, when native forests were replaced by pastures and agricultural lands. We speculate that during these significant alterations, indigenuous species that were adapted to the specific conditions on the island finally were besieged by the constant invasion of environmental opportunists, which occupy broader ecological niches. This hypothesis is supported by finding the same DI in intensively grazed grasslands as in forest stands and shrub lands, thus suggesting that the anthropogenic pressures of the last millennia have not anymore affected the diversity of *Hypocrea/Trichoderma*.

Experimental procedures

Biological information on Sardinia

Topographically, Sardinia is described as 67.9% hilly and 13.6% mountainous areas that are divided by the Campidano Plain stretching from west to south (European Commission, 1994). It is characterized by a semi-arid climate and the majority of its ecosystems have developed under severe water limitation, less than 500 mm rainfall occurring only in some areas located in the south of the island, and an average annual rainfall of 700–900 mm occurring in the inner hilly areas (Vacca *et al*., 2003). The dominant ecosystems include evergreen forest and shrub (maquis) vegetation, mostly degraded into pastures. The soils of Sardinia also vary strongly by origin, their characteristics and properties depending on the type of parent material, topography, plant cover and land use. Almost 28% of the island's territory is an association of outcropping rock and leptosols, especially on the hard rocks (metamorphic, intrusive, effusive, dolomitic and calcareous) scattered throughout the island, mainly in sloping areas with irregular topography and devoid of trees and shrubs. In these areas extensive grazing, chiefly by sheep and goats, is practised. Only 18% of the island consists of different fluvisols, cambisols, vertisols, luvisols and nitisols (FAO-Unesco, http://www.fao.org), which have been formed in the inland and coastal plains, largely on Pleistocene and Holocene alluvial deposits and can sustain intensive irrigated and rainfed agriculture. Primarily cambisols, leptosols and regosols have formed in the remaining 54% of the territory depending on the parent rock, topography, extent and type of plant cover. These are marginal soils for intensive agriculture but are suitable for particular crops, grazing and forestry.

Sample sites and sampling procedure

Composite soil samples were collected between 2003 and 2006 at 15 sites in Sardinia, Italy. Fifteen non-cultivated sampling sites from different habitats were selected in a way to avoid costal, urban areas and rural settlements, major tourist pathways and all other ecosystems with specific devi-

ating properties (relic forests, river flood lands, salt marches and mountain peaks). The selected sampling sites comprised forest stands (3; F), shrub lands – maquis (3; S) and grassland – savannas (9; G). Seven of the nine grassland sites were extensively grazed (EG) and must therefore be considered as affected by anthropogenic disturbance. The prevalent vegetation in grassland was composed – although not exclusively – by *Asphodelus ramosus* L., *Cardus* spp., *Pteridium aquilinum* and various *Gramineae* spp. Shrub land comprised *Rubus fruticosus*, *Myrtus communis* L., *Pistacia lentiscus* L., *Chamaerops humilis* L., *Rosmarinus officialis* L., *Phyllirea* spp., *Cistus* spp., *Genista* spp. Forest mainly contained *Quercus suber* L., *Q. ilex* L., *Taxus baccata* L and *Olea europaea* L. var. *oleaster* Hoff. et Lk. Geographic coordinates, meterological conditions and soil properties of these habitats are given in Table 1. Sampling sites F1, F2, G2 and EG4 are all located at an altitude of more than 1000 m above sea level and therefore all are characterized by the lowest minimal, maximal and annual average temperatures. The soil of site F2 comprises the highest content of organic matter. Two lowland sites, S1 and EG2, are located in nearly identical climatic conditions and showed very similar soil properties. The main characteristics of each sampling site such as Global Positioning System coordinates, geographic location, altitude, average yearly rainfall, land-use type or disturbance, dominant vegetation are summarized in Table 2.

Soil samples (approximately 1000 g) were collected at the ends of two 20 m perpendicular transects and from the intersecting point at each site (Summerell *et al*., 1993), by using a weeding hoe to a depth of 5–15 cm. The location of transects was selected in a way avoiding the rhizosphere of big plants. The samples from each site were stored in paper bags at +4°C after they were sieved, air-dried and mixed thoroughly.

Characterization of soil properties

Soil samples were passed through a 0.2 cm sieve to separate coarse fragments and soil. Soil fractions were analysed for texture, i.e. percentage composition of sand, silt and clay, by using the Bouyoucos mechanical method (Day, 1965). The percentage of soil organic carbon and soil organic matter in each sample was determined according to Nelson and Sommers (1996).

Isolation, storage and substrata

The soil dilution plate method was used for isolation of *Trichoderma*. Approximately 200 g of soil from each sample was sieved through a stainless-steel wire mesh (710 μm diameter) and three 1 g subsamples of soil were suspended in 99 ml of 0.05% water agar, giving a 1:100 dilution; two successive dilutions (1:1000 and 1:2000) were prepared and aliquots of 1 ml of each suspension were evenly spread on 90-mm-diameter Petri plates containing *Trichoderma* selective medium (Elad *et al*., 1981), and on potato dextose agar (PDA; Merck and Co., Whitehouse Station, NY, USA) amended with streptomycin sulfate and oxytetracycline hydrochloride (100 + 100 μg ml^{-1}; Sigma, St Louis, MO, USA). Plates were incubated under near-ultra-violet light with a 12 h photoperiod at approximately 25°C. Three plates per suspension were prepared, giving a total of 36 plates per sample and per substrate (*Trichoderma* selective medium or PDA). After 7–10 days, all colonies resembling *Trichoderma* were transferred to plates containing PDA and incubated as above. *Trichoderma* colonies were subsequently subcultured using the single spore technique and stored at 4°C until DNA extraction. For long-term storage, plugs (0.5 cm diameter) were cut from PDA cultures and kept in 50% glycerol at –80°C. All the isolates described in the present work are conserved in the fungal collection of the Center for Biotechnology Development and Biodiversity Research, University of Sassari, Sardinia, Italy. Representative species have been deposited at CBS, Utrecht, the Netherlands.

DNA isolation and sequencing

Fungal genomic DNA was purified from fresh mycelium by following a standard method (Aljanabi and Martinez, 1997). Pre-screening of isolates by RAPD-PCR (Williams *et al*., 1990) was used with primer M13 (5′-GAGGGTGGCGGTTCT-3′) as described by Turner and colleagues (1997). Isolates from the same sample and sharing a similarity of > 98% (calculated as similarity index according to Nei and Li, 1979, as given in Turner *et al*., 1997) were considered as being the same species, and only one of them included in sequence analysis. For each species representative, a fragment of the nuclear rRNA comprising the ITS1 and ITS2 and the 5.8S rRNA gene was amplified and sequenced as previously described (Druzhinina *et al*., 2005). In addition, the fourth large intron of the *tef1* gene was also amplified if necessary as described by Druzhinina and colleagues (2004). Automated sequencing was performed with an ABI Prism 3100 DNA Sequencer (Applied Biosystems, Norwalk, CT, USA) at the sequencing core facility C.R.I.B.I. – Bio Molecular Research at the University of Padova, Italy.

Species identification

For species identification, ITS1 and ITS2 sequences were subjected to analysis by *TrichO*KEY (http://www.isth.info/tools/molkey/index.php; Druzhinina *et al*., 2005). In ambiguous cases, usually common for section *Trichoderma*, the result was re-checked by analysis of the large intron of *tef1* gene sequence using sequence similarity search against a database of type sequences implemented in *Tricho*BLAST (http://www.isth.info/tools/blast; Kopchinskiy *et al*., 2005). For analysis of unusual ITS1 and ITS2 or *tef1* alleles, sequences were automatically aligned with GeneDoc 2.6.002, manually edited and inspected by eye. Potentially unique alleles were then confirmed by sequence similarity search against NCBI GenBank and a database of fungal strains of Vienna University of Technology that currently contains more than 2700 *Hypocrea/Trichoderma* strains with more than 3300 sequences. A haplotype was considered to be unique if at least one allele (ITS1 and ITS2 or *tef1*) did not occur in any other strain isolated outside of Sardinia.

Phylogenetic analysis

DNA sequences were aligned using Genedoc 2.6.002, converted to an interleaved NEXUS file formatted for PAUP*4.0b10

and then manually edited in order for it to be recognized by MrBayes v3.0B4 program. The Bayesian approach to phylogenetic reconstructions (Rannala and Yang, 1996; Yang and Rannala, 1997) was implemented using MrBayes 3.0B4 (Huelsenbeck and Ronquist, 2001). The MODELTEST3-06 package (http://bioag.byu.edu/zoology/crandall_lab/modeltest.htm) was used to compare the likelihood of different nested models of DNA substitution and select the best-fit model for the investigated data set, using the AIC output strategy. Metropolis-coupled Markov chain Monte Carlo (MCMCMC) sampling was performed with four incrementally heated chains that were simultaneously run for one million of generations. To check for potentially poor mixing of MCMCMC, the analysis was repeated twice. The convergence of MCMCMC was monitored by examining the value of the marginal likelihood through generations. Convergence of substitution rate and rate heterogeneity model parameters was also checked. Bayesian posterior probabilities were obtained from the 50% majority rule consensus of 9600 trees sampled every 100 generations after removing the 400 first trees as the 'burn-in' stage. Values below 0.9 were not shown on the phylogram.

Statistical analysis

Similarities in species composition among samples have been calculated using the Bray–Curtis similarity measure and then shown by a cluster ordination. Formal significance tests for differences among habitats were performed using the one-way ANOSIM permutation/randomization test (Clarke and Warwick, 2001).

To assess the contribution of a set of 'best matching' environmental variables to dissimilarities among soil samples, the Spearman rank correlation coefficient was calculated by correlating Euclidean distance similarity matrices of environmental variables with Bray–Curtis similarity matrices from biological data.

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