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Trichoderma in the Maltese Islands

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Summary. This study assessed presence of *Trichoderma* spp. in the Maltese Islands. Isolates were identified using dichotomous keys and DNA barcoding. Ten distinct isolates were obtained from different soils and other substrates, and were identified as *T. virens, T. citrinoviride, T. gamsii,* and, in the former *T. harzianum* species complex, *T. breve, T. afroharzianum* and *T. atrobrunneum*. Five out of these six fungi are reported for the first time in the Maltese Islands, and *T. brevis* is reported for the first time in Europe.

Keywords. ITS, *tef1*.

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INTRODUCTION

The Maltese Islands are located in the central Mediterranean Sea, and together have a land area of 316 km^2 aligned in a NW-SE direction (Schembri, 1996). The climate of these islands is strongly bi-seasonal, with a hot, dry season from April to mid-September each year, and a mild wet season from mid-September to March. Relative humidity is high throughout the year, in the range of 65% to 80% (Galdies, 2011).

The known fungal diversity of the Maltese Islands includes approx. 400 macrofungal taxa, while the recorded list of microfungi species is incomplete and many remain unidentified or inadequately described. An extensive historical excursus for Maltese mycological studies was provided by Mifsud (2022), but only four studies have dealt with the microfungi on these islands (Saccardo, 1912, 1914, 1915; Porta-Puglia and Mifsud, 2006). Porta-Puglia and Mifsud (2006) reported for the first time the species *Trichoderma harzianum* Rifai (*Sordariomycetes*, *Hypocreales*, *Hypocreaceae*) as part of a checklist of microfungi of the Maltese Islands.

Trichoderma spp. are free-living, filamentous Ascomycetes with worldwide distributions. They grow rapidly, have bright green to white conidia and repeatedly branched conidiophores bearing phialides (Gams and Bissett, 1998). This genus was first described by Persoon (1794) and later by Rifai (1969). *Trichoderma* spp. can often occur on decaying wood and other sources of cellulose, including those occurring in soils (Kubicek *et al.*, 2008; Jaklitsch, 2009).

These fungi have also been isolated from unusual sources, including the guts of cellulose consuming insects such as cockroaches and termites, as well as marine mussels and sponges (Sallenave and Pouchus, 1999; Sallenave-Namont and Pouchus, 2000; Yoder *et al.*, 2008; Guswenrivo *et al.*, 2018; Yamada *et al.*, 2019). More than 360 species have been described within *Trichoderma*, and several new species are recognized using molecular taxonomy (Bissett *et al.*, 2015; Cai and Druzhinina, 2021). For *Trichoderma* taxonomy, the primary DNA barcoding loci for molecular identification are the complete sequences of the rRNA internal transcribed spacers 1 and 2 (ITS1 and ITS2), which also include the respective sequences of the genes encoding 5.8 S rRNA (Schoch *et al.*, 2012). Partial fragments of the translation elongation factor 1 alpha (tef1) gene (Druzhinina and Kubicek, 2005), and the RNA polymerase B subunit II (rpb2) gene (Liu *et al.*, 1999; Druzhinina *et al.*, 2006; Atanasova *et al.*, 2013) are generally used as secondary DNA barcodes. Phylogeny analyses within *Trichoderma* have led to separation of species into clades, that are groups of species which each include a common ancestor (Druzhinina *et al.*, 2006; Samuels *et al.*, 2012).

The cladistics system for *Trichoderma* has been revised, leading to the arrangement of all known *Trichoderma* species in different PhyloOrders based on the concept of genealogical concordance for phylogenetic species recognition (GCPSR) (Cai and Druzhinina, 2021). In the PhyloOrder system, species are ordered on a whole genus *rpb2* phylogram, and the PhyloOrder category determines neighbouring species. The taxonomy of *Trichoderma* currently accepted by the International Commission on *Trichoderma* Taxonomy (ICTT) assigns *Trichoderma* species to six PhyloOrders (https://trichokey.com/index.php/trichoderma-taxonomy-2020; last accessed 27 January, 2023).

The *Trichoderm*a species reported from the Maltese Islands are *T. harzianum* Rifai (*Sordariomycetes*, *Hypocreales*, *Hypocreaceae*) and *T. viride*, that were recorded by Porta-Puglia and Mifsud (2006). However, recent studies have discriminated several cryptic species based on molecular characterization, to the point where *Trichoderma* is referred to as a species complex, and its taxonomy is not considered as definitely set (Chaverri *et al.*, 2003; Samuels, 2006; Druzhinina *et al.*, 2010). Furthermore, it is probable that isolated areas, like islands, host *Trichoderma* strains or ecotypes with physiological and metabolic adaptations peculiar to the particular ecological and climatic features of each island.

The present study included a survey of occurrence of *Trichoderma* species in five soil samples and other organic substrates collected from distinct habitats in the Maltese Islands.

MATERIALS AND METHODS

Soil sampling for Trichoderma *spp.*

For isolation of *Trichoderma* spp., soil samples (each approx. 200 g) were collected from five locations in the Maltese Islands during the rainy season commencing from September 2017. The sampling locations selected were distinct habitats in the Maltese Islands, namely coastal garrigue (Ix-Xagħra l-Ħamra), the wet valley and ridge areas of a semi-natural woodland (Buskett garden), a man-made stand of *Pinus halepensis* (Floriana), and the Argotti Botanic Garden which hosts a large number of indigenous and exotic plant species. One gram sub-samples from each field soil sample were processed within 48 h from collection, and the remaining amount of each sample was preserved at 4°C in a pre-sterilized contain. Five other non-soil substrates were also sampled (Table 1 and Figure 1).

Table 1. Soil and other substrates assayed for *Trichoderma* isolates.

Sample	Origin	Sampling Location	Sample location coordinates
1	Soil	Ix-Xagħra l-Ħamra	35.95014°N; 14.34377°E
2	Soil	Floriana, Pinetum	35.89057°N; 14.50062°E
3	Soil	Buskett garden	35.85617°N; 14.39785°E
4	Soil	Buskett garden	35.85918°N; 14.39738°E
5	Coffee grounds	Argotti Botanic Garden	35.89239°N; 14.50300°E
6	*Aurificaria euphoriae* (Pat.) Ryvarden, basidiome	Argotti Botanic Garden	35.89239°N; 14.50300°E
7	Imported commercial compost (MXS Mikskaar, Tallinn, Estonia)	Argotti Botanic Garden	35.89239°N; 14.50300°E
8	*Euphorbia abyssinica* J.F. Gmel. trunk	Argotti Botanic Garden	35.89239°N; 14.50300°E
9	*Salsola melitensis* Botsch., trunk	Argotti Botanic Garden	35.89239°N; 14.50300°E
10	*Anacamptis pyramidalis* (L.) Rich., roots	Wied Babu	35.82191°N; 14.46021°E

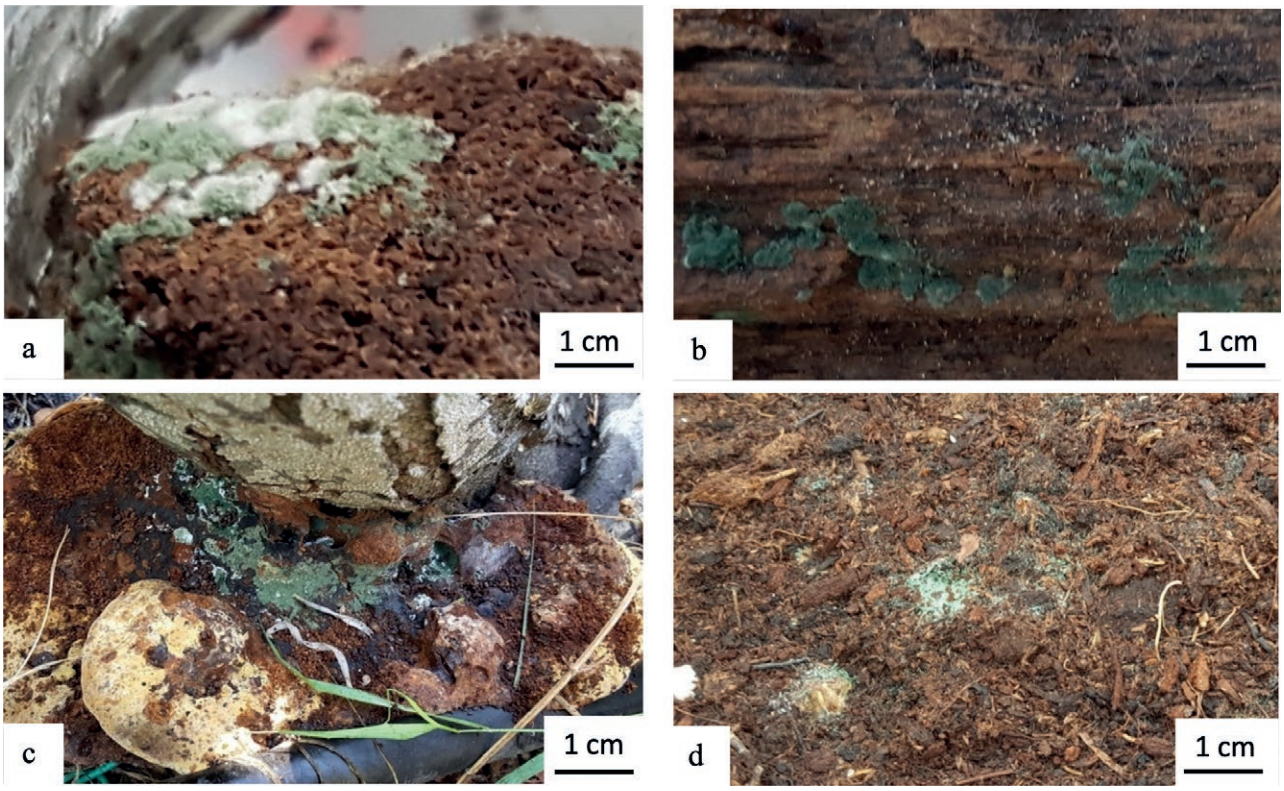

Figure 1. *Trichoderma* spp. growing on different substrates: (a) coffee grounds; (b) *E. abyssinica* trunk; (c) basidiome of *A. euphoriae*; (d) imported commercial compost.

Trichoderma *monoconidial isolations and isolate preservation*

For *Trichoderma* monoconidial isolations, 1 g of soil was dried at 60°C for 24 h, and then mixed with 500 mL of sterile water and allowed to rest for 24 h. Four 1:10 serial dilutions in sterile distilled water were then prepared, and 100 µL of each dilution was then spread onto a Petri dish containing modified Trichoderma selective medium prepared according to the recipe of Smith *et al.* (1990), except for the fungicides used, which were 2.5 mL L^{-1} Teldor (Fenhexamid 50% w/w; Bayer) and 2.5 mL L^{-1} Previcur (Propamocarb 60% w/w; Bayer). The Petri dishes were then incubated at 25°C for 24 to 48 h in the light, and were checked daily for colony growth. Single colonies were each transferred to a separate Petri dish containing potato dextrose agar, which had been prepared according to the manufacturer's instructions and supplemented with 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (Genesee Scientific). The isolation plates were then incubated at 25°C in light.

From each antibiotic medium culture, a small piece (2 mm^2) of mycelium was aseptically transferred to a labelled sterile tube containing 10 mL of sterile distilled water. The tube was vortexed for 20 sec and then serially diluted to 10^{-1} and 10^{-2}. Aliquots (100 µL) were evenly spread on 2% Water Agar in Petri dishes, which were placed in an incubator (MLR 352 PHCBI, Tokyo, Japan) at 25°C and 70% RH under 800 lux fluorescent lamps. After 24 h, the dishes were aseptically examined under a stereomicroscope and checked for individual germinated conidia that were separated from each other. A small piece of agar bearing a single germinated conidium was then excised with a sterile lancet and transferred onto Potato Dextrose Agar with antibiotics (as above), and incubated at 25°C. Colony growth was followed for 21 d, and the colony growth pattern, conidium colour, conidiation pattern, and reverse colour were recorded for each isolate. The micromorphological features of each isolate were also observed under a microscope, using fragments of colonies collected axenically from the conidiation area contour, and were suspended in distilled water. Monoconidial cultures of the isolated fungi were stored at 4°C in test tubes containing Synthetic Nutrient Agar prepared according to Elad *et al.* (1981). Long-term preservation of *Trichoderma* isolates was carried out in sterile 99% glycerol stored at -18°C, according to Stocco *et al.* (2010). Voucher specimens

and isolates are conserved in the collection of Maltese mycoflora, hosted at the Seed Bank of the Department of Biology of the University of Malta, Valletta, Malta, under the accession codes listed in Table 2.

Molecular identification of Trichoderma *isolates*

Molecular identification at species level of the *Trichoderma* isolates was carried out using gene sequencing. Single conidium cultures grown on PDA at 25°C for 6 d were preserved in ethanol, and the ethanol fixed tissues was aseptically dissected into small sections using a sterile scalpel. All samples were processed for DNA extraction using the NucleoSpin Plant Kit (Macherey-Nagel) according to manufacturer instructions.

The ribosomal region including internal transcribed spacers ITS1 and ITS2, and the small subunit ribosomal RNA 5.8S (ITS) were amplified by PCR using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3'), ITS2 (5'- GCTGCGTTCTTCTTCATCGATGC-3'), and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990). All amplifications were each carried out using the AccuStartTMII PCR ToughMix (Quantabio), in a final volume of 25 µL, containing 1 µL of each primer (10 pmol μL^{-1}) and 1 to 2 µL of DNA template. The PCR conditions were set to an initial denaturation temperature of 94°C for 5 min followed by 35 cycles each of 30 s at 94°C, 40 s at 48°C and 50 s at 72°C, with a final elongation phase of 7 min at 72°C. PCR products were visualized using electrophoresis on 1.5% agarose gels. For each successful PCR, 10 µL of PCR product were purified with a 2.5 µL mix containing exonuclease I (20 U μL^{-1}) and alkaline phosphatase (1 U μL^{-1}), using an incubation of 15 min at 37°C and 20 min at 75°C.

A fragment of the protein-coding translational elongation factor 1 alpha gene (tef1) was amplified by PCR using the primers EF1-1018F (5'-GAYTTCATCAAGAACATGAT-3') and EF1-1620R (5'-GACGTTGAADCCRACRTTGTC-3') (Stielow *et al*., 2015). All amplifications were each carried out using the AccuStartTMII PCR ToughMix (Quantabioin a final volume of 25 µL, containing 1 µL of each primer (10 pmol μL^{-1}) and 1 to 2 µL of DNA template. The PCRs were set to initial denaturation at 94 °C for 5 min followed by 35 cycles each of 30 s at 94°C, 40 s at 48°C and 50 s at 72°C, with a final elongation phase of 7 min at 72°C. PCR products were visualized using electrophoresis on 1.5% agarose gels. For each successful PCR, 10 µL of PCR product were purified with a 2.5 µL mix containing exonuclease I (20 U μL^{-1}) and alkaline phosphatase (1 U μL^{-1}) using incubation of 15 min at 37°C and then 20 min at 75°C. All purified PCR products were sequenced in both forward and reverse directions by Macrogen Inc. (Amsterdam, the Netherlands), using M13 universal primers.

Forward and reverse sequences were assembled using Geneious (v. R10, Biomatters), and were reciprocally verified to generate a complete contig of each sequenced fragment. All contigs were then exported in FASTA format and compared with the GenBank reference database for taxonomic assignment using the BLAST algorithmus (Altschul *et al*., 1990). The TrichOKey (http://isth.info/tools/molkey/index.php) and TrichoBLAST (http://www.isth.info/tools/blast/) tools were used to compare the ITS and tef1 sequences for species identification. In addition, a dataset of combined ITS and tef1 sequences was generated for eight *Trichoderma* isolates from the Maltese Islands. Furthermore, ten *Trichoderma* species reference strains, including *T. atrobrunneum* T57, *T. harzianum* CBS 226.95, *T. harzianum* HZA11, *T. afroharzianum* TB1-26, *T. breve* HMAS 248844, *T. zelobreve* CGMCC 3.19696, *T. virens Gv29-8, T. citrinoviride* HZA9, *T. neokoningii* CBS 120070, and *T. gamsii* GJS 05-111, were included in the analysis. *Cladobotryum heterosporum* CBS 719-88 was used as the outgroup. The multiple alignment of the combined sequence dataset (total 1226 nucleotide sites), performed with MUSCLE algorithm, and phylogenetic analysis using the Maximum Likelihood method were both carried out using MEGA11 software (Tamura *et al*., 2021). The accuracy of the analyses were assessed using the bootstrap method with 1000 replicates.

A small fungal collection was established at the Department of Biology, University of Malta, where cultures of *T. atrobrunneum*, *T. afroharzianum, T. gamsii, T. breve, T. citrinoviride, T. virens* and *T. gamsii* are maintained as a living collection on different substrates and as samples held at different storage temperatures.

RESULTS

Ten *Trichoderma* strains were isolated from different locations and substrates in the Maltese Islands. Four of the strains were isolated from soils from different sampling locations. Four strains were isolated from a basidiome of *Aurificaria euphoriae*, from wood of two different dead trees, and from a commercial potting compost, all originating from Argotti Botanic Garden. One strain was isolated from coffee grounds, and one was isolated from *A. pyramidalis* roots (Table 1 and Figure 1). Each sample yielded a single isolate. The growth patterns and colours of top and reverse sides of Petri dish cultures of the *Trichoderma* spp. isolates grown on PDA and recorded at 3 d intervals up to 11 d are shown in Figure 2.

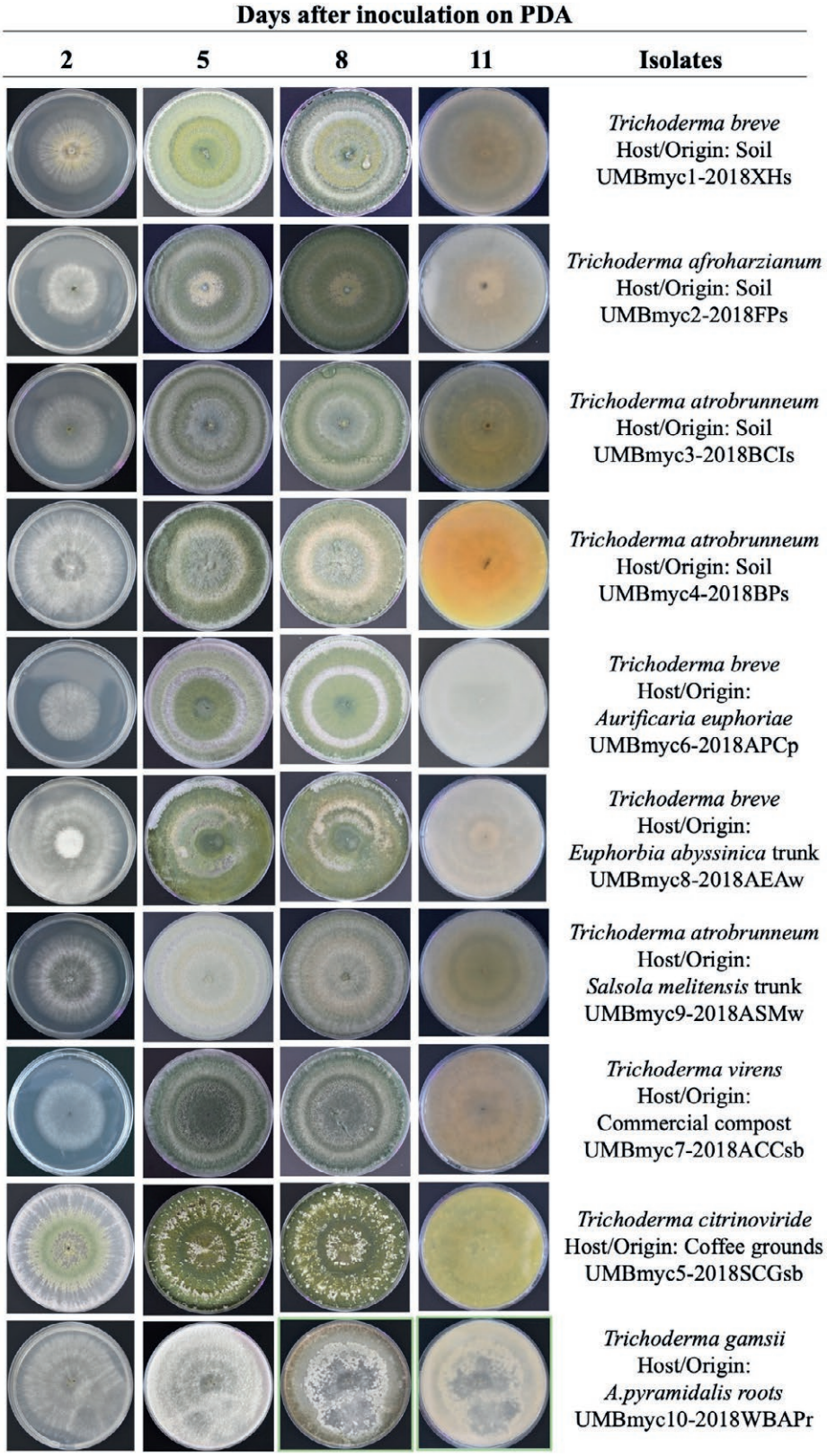

Figure 2. Top views of Petri plate cultures *Trichoderma* isolates grown on PDA for 2, 5, and 8 d. Colony reverse sides at day 11 are also shown, except for the *T. gamsii* colony, shown at day 21.

The isolates were identified using DNA sequencing. Sequences of ITS regions were used for preliminary identification at species level, based on BLAST analyses which only allowed definite identification of two isolates, UMBmyc5-2018SCGsb as *T. citrinoviride* Bissett, and UMBmyc7-2018ACCsb as *T. viride* Pers., with the remaining isolates identifying as *T. harzianum*.

Sequencing of tef1 gene was necessary to further differentiate within the *T. harzianum* species complex. Phylogenetic analysis of the combined ITS and tef1 sequences, compared with available sequences of *Trichoderma* species used as references, allowed identification of all the *T. harzianum* complex isolates as cryptic species, namely *T. breve* K. Chen & W.Y. Zhuang, *T. afroharzianum* P. Chaverri, F.B. Rocha & I. Druzhinina, and *T. atrobrunneum* F.B. Rocha, P. Chaverri & W. Jaklitsch. As shown in the phylogenetic tree in Figure 3, three isolates (UMBmyc3-2018BCIs, UMBmyc4-2018BPs and UMBmyc9-2018ASMw) were identified as *T. atrobrunneum*; isolate UMBmyc2-2018FPs clustered with the *T. afroharzianum* reference strain, and the isolates UMBmyc1-2018XHs, UMBmyc6-2018APCp and UMBmyc8-2018AEAw grouped with *T. breve* and *T. zelobreve*. The isolate UMBmyc10-2018WBAPr, from *Anacamptis pyramidalis* roots, was identified as *T. gamsii* Samuels & Druzhinina.

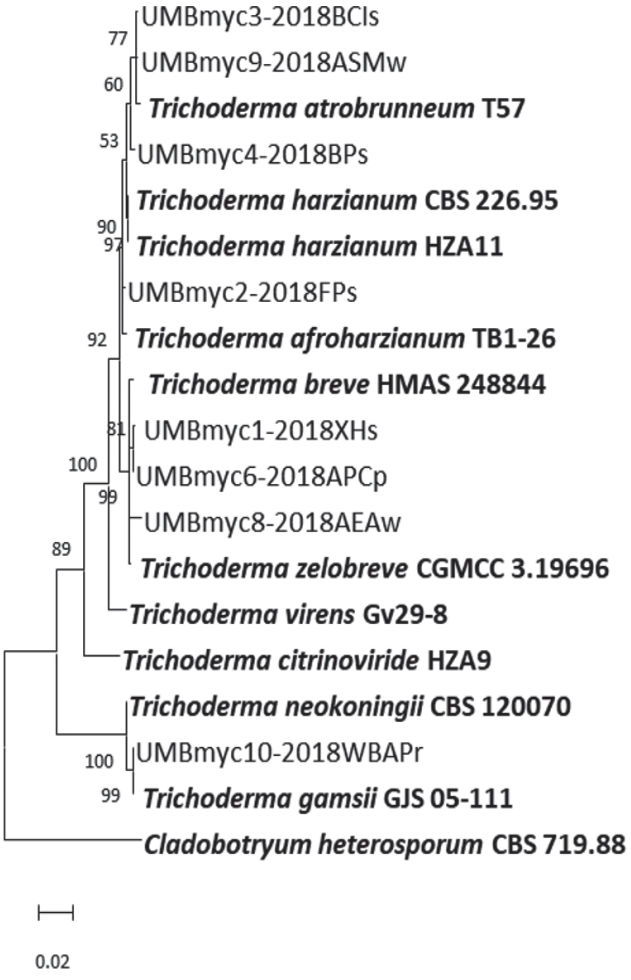

Figure 3. Phylogenetic tree for eight *Trichoderma* isolates, based on the combined sequences of ITS and tef1 gene fragments. The tree was obtained by using the Maximum Likelihood method and Tamura-Nei model. The proportions (%) in which the associated taxa clustered together are shown next to the branches, expressed as bootstrap values with 1000 replicates.

The assignment of species was carried out according to the current nomenclature defined by the International Commission on *Trichoderma* Taxonomy (ICTT; https://trichokey.com/index.php/trichoderma-taxonomy-2020, last accessed on 27 January, 2023). Among the six species identified in the Maltese Islands, listed in Table 2, *T. afroharzianum*, *T. atrobrunneum*, *T. breve* and *T. virens* belong to PhyloOrder clade 1, based on phylogeny of the currently rpb2-barcoded *Trichoderma* species. *Trichoderma citrinoviride* was assigned to PhyloOrder clade 3, and *T. gamsii* was assigned to PhyloOrder clade 5.

Nucleotide sequences were submitted to the GenBank Database with accession numbers from OQ378924 to OQ378933 for ITS (ten sequences) and from OQ384109 to OQ384116 for tef1 (eight sequences).

DISCUSSION

The Convention of Biological Diversity states that "Islands and their surrounding near-shore marine areas constitute unique ecosystems often comprising many plant and animal species that are endemic, and therefore found nowhere else on Earth" (Convention of Biological Diversity, https://www.cbd.int/island/). For these reasons, survey, cataloguing and preservation of biodiversity is important for small islands like the Maltase Islands. A multilocus identification system for *Trichoderma* (MIST), based on three phylogenetic marker databases (ITS, tef, and rpb2), is regarded as a valid tool for identification of *Trichoderma* species (Hatvani *et al*., 2014). The genealogical concordance for phylogenetic species recognition (GCPSR) (Cai and Druzhinina, 2021) is the most widely accepted approach for *Trichoderma* identification, mostly to detect cryptic species. Standardization of species recognition criteria and agreement between *Trichoderma* taxonomists allows unambiguous diagnoses of species (Cai and Druzhinina, 2021). According to ICTT nomenclature, the recognized species belonging to Harzianum and Virens Clades are joined in the same

Table 2. Species identification of the *Trichoderma* spp. isolates from the Maltase Islands, based on DNA barcoding, according to the International Commission on Trichoderma Taxonomy (ICTT).

Isolate No.[a]	Origin and sampling location	Species	PhyloOrder (ICTT)	GenBank sequence accession numbers	
				ITS	tef1
UMBmyc1-2018XHs	Soil, Ix- Xagħra l-Ħamra	*T. breve*	1	OQ378924	OQ384109
UMBmyc2-2018FPs	Soil Floriana pinetum	*T. afroharzianum*	1	OQ378925	OQ384110
UMBmyc3-2018BCIs	Soil, Buskett Garden	*T. atrobrunneum*	1	OQ378926	OQ384111
UMBmyc4-2018BPs	Soil, Buskett Garden	*T. atrobrunneum*	1	OQ378927	OQ384112
UMBmyc5-2018SCGsb	Spent coffee grounds	*T. citrinoviride*	3	OQ378928	-
UMBmyc6-2018APCp	*Aurificaria euphoriae* (Pat.) Ryvarden, ABG[b]	*T. breve*	1	OQ378929	OQ384113
UMBmyc7-2018ACCsb	Commercial compost, ABG	*T. virens*	1	OQ378930	-
UMBmyc8-2018AEAw	*Euphorbia abyssinica* J.F. Gmel., ABG	*T. breve*	1	OQ378931	OQ384114
UMBmyc9-2018ASMw	*Salsola melitensis* Botsch., ABG	*T. atrobrunneum*	1	OQ378932	OQ384115
UMBmyc10-2018WBAPr	*Anacampis pyramydalis* (L.), Wied Babu	*T. gamsii*	5	OQ378933	OQ384116

[a] Accession No. in the collection of Maltese mycoflora, Seed Bank of the Department of Biology, University of Malta, Valletta, Malta.
[b] ABG = Argotti Botanical Garden.

PhyloOrder clade, named 1 (Cai and Druzhinina, 2021). Two species (*T. citrinoviride* and *T. gamsii*) belonging, respectively, to PhyloOrder clades 3 and 5, were identified among the Maltese isolates.

The present study used the ITS and tef1 sequences, and subsequently the *ITS4* and *TEF1α* sequences, to define the biodiversity of *Trichoderma* in the Maltese Islands. These phylogenetic analyses allowed identification of Maltese isolates at species level. Although only ten isolates were studied, they were identified as four different phylogenetic Clades. Seven out of the belonged to the Harzianum Clade, which so far is the most common and widespread. Three other isolates were assigned to the Virens, Longibrachiatum and Viride Clades. Based on currently accepted nomenclature and taxonomy, the Maltese isolates belonged to six different PhyloOrders (Cai and Druzhinina, 2021). In particular, the isolate from coffee was identified as *T. citrinoviride* (Longibrachiatum Clade), a very common soil fungus and also detected as an opportunistic pathogen of immunocompromised humans (Hatvani *et al*., 2019). The isolate from compost was identified as *T. virens* (Virens Clade), a species commonly used as a biocontrol agent to protect various crops from a number of plant pathogens, and which has been utilized as a model for elucidating the mechanisms of biological control (Druzhinina *et al*., 2011). The endophytic isolate from orchid roots was identified as *T. gamsii* (Viride Clade). The Viride Clade is the largest and the most diverse group of *Trichoderma*, characterized by species producing a wide range of bioactive compounds (Marik *et al*., 2018).

Seven of the ten isolates, initially identified using ITS regions, belonged to the *T. harzianum* species complex, while the other three were *T. virens*, *T. gamsii* or *T. citrinoviride*. The seven isolates thus belonging to the *T. harzianum* species complex showed considerable phenotypic variation (Figure 2), which is consistent with findings of other authors (Chaverri and Samuels, 2003; Evans *et al*., 2003; Samuels, 2006; Hoyos-Carvajal *et al*., 2009; Jaklitsch, 2009; Gazis and Chaverri, 2010; Druzhinina *et al*., 2011). The subsequent molecular analyses including tef1 sequencing, allowed differentiation of the isolates into three cryptic species, namely *T. afroharzianum, T. atrobrunneum* or *T. breve*. These results confirm the importance of tef1 sequences for studies of phylogeny and taxonomic characterization in *Trichoderma*.

While all the species isolated in the Maltese Islands are ubiquitous and have been reported from many world regions, *T. breve* was previously reported only from China, where it was first described in 2017 (Chen and Zhuang, 2017), and from central Africa where it was recovered as an endophyte of *Coffea* (del Carmen H. Rodríguez *et al*., 2021). Thus, *T. breve* is reported here for the first time in Europe, and this report increases the list of *Trichoderma* species that occur in the European geographical areas (Jaklitsch, 2009, 2011; Jaklitsch and Voglmayr, 2015). Although similar to the *T. harzianum* species complex for morphology and culture traits, *T. breve* is phylogenetically more closely related to *T. bannaense*, another newly described species from China, than to *T. harzianum* (Chen and Zhuang, 2017). The Maltese isolates of *T. breve* were from soil, from a dead branch of *E. abyssinica*, and from the polypore fungus *A. euphoriae* growing on *Prunus cerasifera,* suggesting that *T. breve* may exhibit more than one ecological habit.

All of the other *Trichoderma* species isolated in the present study, namely *T. afroharzianum*, *T. atrobrunneum*, *T. citrinoviride*, *T. virens*, and *T. gamsii*, have been extensively described and isolated from a number of geographical areas and substrates (Chaverri and Samuels, 2003; Jaklitsch *et al*., 2006; Chaverri *et al*., 2015). Due to the peculiar environmental and climatic features of the Maltese Islands, these isolates may have beneficial properties and also resilience to abiotic stresses that occur in the Mediterranean basin, such as drought, heat stress and salinity, making them suitable for applications where climate change and global warming prescribe potential biotechnology applications.

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LITERATURE CITED

Altschul S.F., Gish W., Miller W., Myers E.W., 1990. Basic Local Alignment Search Tool. *Journal of Molecular Biology* 215.3: 403-410.

Atanasova L., Druzhinina I.S., Jaklitsch W.M., 2013. Two hundred *Trichoderma* species recognized on the basis of molecular phylogeny. In: *Trichoderma: Biology and Applications*, CABI, 10–42, Wallingford, UK.

Bissett J., Gams W., Jaklitsch W., Samuels G.J., 2015. Accepted *Trichoderma* names in the year 2015. *IMA Fungus* 6: 263–295. https://doi.org/10.5598/imafungus.2015.06.02.02

Cai F., Druzhinina I.S., 2021. In honor of John Bissett: authoritative guidelines on molecular identification of *Trichoderma*. *Fungal Diversity* 107: 1–69. https://doi.org/10.1007/s13225-020-00464-4

Chaverri P., Castlebury L.A., Samuels G.J., Geiser D.M., 2003a. Multilocus phylogenetic structure within the *Trichoderma harzianum*/*Hypocrea lixii* complex. *Molecular Phylogenetics and Evolution* 27: 302–313. https://doi.org/10.1016/S1055-7903(02)00400-1

Chaverri P., Samuels G.J., 2003b. *Hypocrea*/*Trichoderma* (Ascomycota, Hypocreales, Hypocreaceae): species with green ascospores. *Studies in Mycology* 48: 1–116.

Chaverri P., Branco-Rocha F., Jaklitsch W., Gazis R., Degenkolb T., Samuels G.J., 2015. Systematics of the *Trichoderma harzianum* species complex and the re-identification of commercial biocontrol strains. *Mycologia* 107: 558–590. https://doi.org/10.3852/14-147

Chen K., Zhuang W.-Y., 2017. Discovery from a large-scaled survey of *Trichoderma* in soil of China. *Scientific Reports* 7: 9090. https://doi.org/10.1038/s41598-017-07807-3

del Carmen H. Rodríguez M., Evans H.C., de Abreu L.M., de Macedo D.M., Ndacnou M.K., Barreto R.W., 2021. New species and records of *Trichoderma* isolated as mycoparasites and endophytes from cultivated and wild coffee in Africa. *Scientific Reports* 11: 5671. https://doi.org/10.1038/s41598-021-84111-1

Druzhinina I., Kubicek C.P., 2005. Species concepts and biodiversity in *Trichoderma* and *Hypocrea*: from aggregate species to species clusters? *Journal of Zhejiang University-SCIENCE B* 6: 100–112. https://doi.org/10.1631/jzus.2005.B0100

Druzhinina I.S., Kopchinskiy A.G., Kubicek C.P., 2006. The first 100 *Trichoderma* species characterized by molecular data. *Mycoscience* 47: 55–64. https://doi.org/10.1007/S10267-006-0279-7

Druzhinina I.S., Kubicek C.P., Komon-Zelazowska M., Belayneh Mulaw T., Bissett J., 2010. The *Trichoderma harzianum* demon: complex speciation history resulting in coexistence of hypothetical biological species, recent agamospecies and numerous relict lineages. *BMC Evolutionary Biology* 10: 94. https://doi.org/10.1186/1471-2148-10-94

Druzhinina I.S., Seidl-Seiboth V., Herrera-Estrella A., Horwitz B.A., Kenerley C.M., … Kubicek C.P., 2011. *Trichoderma*: the genomics of opportunistic success. *Nature Reviews Microbiology* 9: 749–759. https://doi.org/10.1038/nrmicro2637

Elad Y., Chet I., Henis Y., 1981. A selective medium for improving quantitative isolation of *Trichoderma* spp. from soil. *Phytoparasitica* 9: 59–67. https://doi.org/10.1007/BF03158330

Evans H.C., Holmes K.A., Thomas S.E., 2003. Endophytes and mycoparasites associated with an indigenous forest tree, *Theobroma gileri*, in Ecuador and a preliminary assessment of their potential as biocontrol agents of cocoa diseases. *Mycological Progress* 2: 149–160. https://doi.org/10.1007/s11557-006-0053-4

Galdies C., 2011. The Climate of Malta: statistics, trends and analysis 1951-2010 – Valletta: National Statistics

Office, 2011 viii, 45p. Available at: https://nso.gov.mt/wp-content/uploads/The_Climate_of_Malta.pdf

Gams W., Bissett J., 1998. Morphology and identification of *Trichoderma*. In: *Trichoderma and Gliocladium* (G.E. Harmann, C.P. Kubicek, ed.). Taylor and Francis ed., London, England, 3–34.

Gazis R., Chaverri P., 2010. Diversity of fungal endophytes in leaves and stems of wild rubber trees (*Hevea brasiliensis*) in Peru. *Fungal Ecology* 3: 240–254. https://doi.org/10.1016/j.funeco.2009.12.001

Guswenrivo I., Nagao H., Lee C.Y., 2018. The Diversity of Soil Fungus in and Around Termite Mounds of *Globitermes sulphureus* (Haviland) (Blattodea: Termitidae) and Response of Subterranean Termite to Fungi. In: *Sustainable Future for Human Security* (B. McLellan, ed.), Singapore, Springer Singapore, Japan 37–52.

Hatvani L., Homa M., Chenthamara K., Cai F., Kocsubé S., … Kredics L., 2019. Agricultural systems as potential sources of emerging human mycoses caused by *Trichoderma*: a successful, common phylotype of *Trichoderma longibrachiatum* in the frontline. *FEMS Microbiology Letters* 366: fnz246. https://doi.org/10.1093/femsle/fnz246

Hatvani L., Vágvölgyi C., Druzhinina I., 2014. Chapter 3 - DNA Barcode for Species Identification in *Trichoderma*. In: *Biotechnology and Biology of Trichoderma*, Elsevier, 41–55. https://doi.org/10.1016/B978-0-444-59576-8.01001-8

Hoyos-Carvajal L., Orduz S., Bissett J., 2009. Genetic and metabolic biodiversity of *Trichoderma* from Colombia and adjacent neotropic regions. *Fungal Genetics and Biology* 46: 615–631. https://doi.org/10.1016/j.fgb.2009.04.006

Jaklitsch W.M., 2009. European species of *Hypocrea* Part I. The green-spored species. *Studies in Mycology* 63: 1–91. https://doi.org/10.3114/sim.2009.63.01

Jaklitsch W.M., Samuels G.J., Dodd S.L., Lu B.-S., Druzhinina I.S., 2006. *Hypocrea rufa*/*Trichoderma viride*: a reassessment, and description of five closely related species with and without warted conidia. *Studies in Mycology* 56: 135–177. https://doi.org/10.3114/sim.2006.56.04

Jaklitsch W.M., 2011. European species of *Hypocrea* part II: species with hyaline ascospores. *Fungal Diversity* 48: 1–250. https://doi.org/10.1007/s13225-011-0088-y

Jaklitsch W.M., Voglmayr H., 2015. Biodiversity of *Trichoderma* (*Hypocreaceae*) in Southern Europe and Macaronesia. *Studies in Mycology* 80: 1–87. https://doi.org/10.1016/j.simyco.2014.11.001

Kubicek C.P., Komon-Zelazowska M., Druzhinina I.S., 2008. Fungal genus *Hypocrea*/*Trichoderma*: from barcodes to biodiversity. *Journal of Zhejiang University SCIENCE B* 9: 753–763. https://doi.org/10.1631/jzus.B0860015

Liu Y.J., Whelen S., Hall B.D., 1999. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerse II subunit. *Molecular Biology and Evolution* 16: 1799–1808. https://doi.org/10.1093/oxfordjournals.molbev.a026092

Marik T., Tyagi C., Racić G., Rakk D., Szekeres A., Kredics L., 2018. New 19-Residue Peptaibols from *Trichoderma* Clade Viride. *Microorganisms* 6: 85. https://doi.org/10.3390/microorganisms6030085

Mifsud S., 2022. *An Annotated Checklist of Macrofungi Occurring in Gozo*. MSc Thesis, University of Malta, Msida, Malta.

Persoon C.H., 1794. *Disposita methodica fungorum*. *Römers Neues Mag Bot*, 1 (1794), pp. 81-128.

Porta-Puglia A., Mifsud D., 2006. Fungal and fungal-like plant pathogens of the Maltese Islands. *Petria* 16: 163–256.

Rifai A., 1969. A revision of the genus *Trichoderma. Mycological Papers* 116: 1–56.

Saccardo P.A., 1912. Fungi ex Insula Melita (Malta) lecti a Doct. A. Caruana-Gatto et Doct. G. Borg. *Bullettino Società Botanica Italiana 1912* 19: 314–326.

Saccardo P.A., 1914. Fungi ex Insula Melita (Malta) lecti a Doct. A. Caruana-Gatto et Doct. G. Borg. *Nuovo Giornale Botanico Italiano* 21: 110–126.

Saccardo P.A., 1915. Fungi ex Insula Melita (Malta) lecti a Doct. A. Caruana-Gatto et Doct. G. Borg. *Nuovo Giornale Botanico Italiano* 22: 24–76.

Sallenave C., Pouchus Y.F., 1999. Bioaccumulation of mycotoxins by shellfish: Contamination of mussels by metabolites of a *Trichoderma koningii* strain isolated in the marine environment. *Toxicon* 37(1): 77-83. https://doi.org/10.1016/s0041-0101(98)00135-4

Sallenave-Namont C., Pouchus Y.F., 2000. Toxigenic saprophytic fungi in marine shellfish farming areas. *Mycopathologia* 149(1): 21-5. https://doi.org/10.1023/A:1007259810190

Samuels G.J., 2006. *Trichoderma*: Systematics, the Sexual State, and Ecology. *Phytopathology* 96: 195–206. https://doi.org/10.1094/PHYTO-96-0195

Samuels G.J., Ismaiel A., Mulaw T.B., Szakacs G., Druzhinina I.S., … Jaklitsch W.M., 2012. The Longibrachiatum Clade of *Trichoderma*: a revision with new species. *Fungal Diversity* 55: 77–108. https://doi.org/10.1007/s13225-012-0152-2

Schembri P.J., 1996. The Maltese Islands: climate, vegetation and landscape. *GeoJournal* 41.2: 115–125.

Schoch C.L., Seifert K.A., Huhndorf S., Robert V., Spouge J.L., … Schindel D., 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA

barcode marker for *Fungi*. *Proceedings of the National Academy of Sciences* 109: 6241–6246. https://doi.org/10.1073/pnas.1117018109

Smith V.L., Wilcox W.F., Harman G.E., 1990. Potential for biological control of *Phytophthora* root and crown rots of apple by *Trichoderma* and *Gliocladium* spp. *Phytopathology* 80: 880–885.

Stielow J.B., Lévesque C.A., Seifert K.A., Meyer W., Irinyi L., Robert V., 2015. One fungus, which genes? Development and assessment of universal primers for potential secondary fungal DNA barcodes. *Persoonia - Molecular Phylogeny and Evolution of Fungi* 35: 242–263. https://doi.org/10.3767/003158515X689135

Stocco M., Mónaco C., Cordo C., 2010. A comparison of preservation methods for *Trichoderma harzianum* cultures. *Revista Iberoamericana de Micología* 27: 213. https://doi.org/10.1016/j.riam.2010.06.001

Tamura K., Stecher G., Kumar S., 2021. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution,* 38 I7 3022–3027, https://doi.org/10.1093/molbev/msab120

White T.J., Bruns T., Lee S., Taylor J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols, a Guide to Methods and Applications*., Academic Press, New York, 315–322.

Yamada T., Fujii A., Kikuchi T., 2019. New Diterpenes with a Fused 6-5-6-6 Ring System Isolated from the Marine Sponge-Derived Fungus *Trichoderma harzianum*. *Marine Drugs* 17: 480. https://doi.org/10.3390/md17080480

Yoder J.A., Glenn B.D., Benoit J.B., Zettler L.W., 2008. The giant Madagascar hissing-cockroach (*Gromphadorhina portentosa*) as a source of antagonistic moulds: concerns arising from its use in a public setting. *Mycoses* 51: 95–98. https://doi.org/10.1111/j.1439-0507.2007.01470.x