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Axenic Culture and DNA Barcode Identification of Wood Decay Fungi from the Maltese Islands

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Abstract: Wood-decaying fungi are important study subjects for their ecological role as well as for their biotechnological applications. They break down lignin, cellulose, and hemicelluloses using enzymes that modify the chemical structure of these complex macromolecules. Due to their ability to degrade wood, these fungi can create structural damage to wooden structures and to trees, especially those with very low level of fitness. Previous studies on wood decay fungi in the Maltese Islands are limited to records and checklists described by a handful of authors. The aim of this study was to provide a comprehensive description of wood decay fungal diversity in the Maltese Islands including an updated checklist based on DNA barcoding, as well as to establish the first wood-decay fungal culture collection at the Biology Department Seed Bank of the University of Malta. Several surveys were carried out during the rainy season along wooded areas of the Maltese Islands as well as in historical gardens. Isolates were identified using macro- and micro-morphological features, dichotomous keys, as well as molecular data. Basidiomes were recorded growing on 14 different host plant species, 11 axenic cultures have been made and 9 species of wood decay fungi have been conclusively identified by DNA barcoding. The collection of the axenic isolates includes one of *Aurificaria* cf. *euphoria*, three of *Ganoderma resinaceum sl.*, two of *Laetiporus sulphureus*, one of *Inonotus sp.*, one of *Inonotus rickii* anamorph, one of *Inocutis tamaricis*, one of *Stereum hirsutum*, and one of *Pleurotus eryngii.* However, the mycelium of *Coriolopsis gallica*, though collected and identified, could not be isolated.

Keywords: conservation; wood decay fungi; DNA barcoding; Maltese Islands

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1. Introduction

According to the International Union for the Conservation of Nature (IUCN), more than 150,300 species of the world's biota are on the IUCN Red List, of which 44,000 species, are threatened with extinction [1]. The UN's 2030 Agenda for Sustainable Development calls for the preservation of biodiversity, which is threatened due to many factors [2]. The estimated fungal biodiversity is between 2.2 million and 3.8 million species, many of which have yet to be identified and described [3]. The Global Fungal Red List Initiative is a further step towards conservation of fungal diversity [4]. Fungal biodiversity preservation can be achieved through ex situ culture collections, which can help to preserve and manage these biological resources [5,6].

An important group of fungi in forest ecosystems as well as in urban environments, are the wood decay fungi (WDF), which, through their enzymes, degrade lignocellulosic substrates and boost the nutrient cycles [7,8]. The WDF derive their nutrients from wood, being purely necrotrophic to biotrophic, and act as primary or as opportunistic pathogens. Depending on the fungal and host species, as well as the environmental conditions, WDF can affect both hardwood and sapwood, causing severe damage [9,10]. WDF can be divided

into three categories: white-rot fungi able to degrade all wood components, in contrast to brown-rot fungi which can predominantly degrade cellulose and hemicellulose through cellulase-degrading enzymes but not lignin. Soft-rot fungi differ from brown-rot and white-rot by growing mainly inside the lignified S2 layer of the cell wall in wood-forming tissue and colonize via the wood rays [9,11–13].

With regards to wood production in forest, stands affected by WDF experience a reduction in their yield. WDF usually compromise the overall stability of trees by reducing the structural integrity of wood. When present on trees in public and private green areas, WDF can indirectly harm people and objects in the close vicinity of the trees, especially if at an advanced stage of infection. The formation of decaying cavities is an additional contributor to lack of stability in compromised trees, though this is sometimes taken advantage of in healthy monumental trees, where hollowing out of the main trunk helps to increase the tree's ability to survive once-in-a-lifetime storms [14]. On a positive note, WDF have huge potential for biotechnology applications, some are edible, and several WDF species are sources of bioactive secondary metabolites [15]. WDF are additionally used in primary biomass degradation for production of bioethanol [16,17], production of enzymes for industry [18], in bioaccumulation and bioremediation [19–21], as well as biomaterials [22–25].

Fungal diversity represents a valuable ecological and biotechnological asset facing threats such as anthropogenic pressure and habitat loss. Therefore, preserving fungal strains through ex situ conservation is crucial to ensure the accessibility of materials for both fundamental and practical research purposes [26]. The WFCC (World Federation for Culture Collections), along with collections presents at universities, research institutes, partnerships, like MIRRI (Microbial Resource Research Infrastructure) or BCCM (Belgian Coordinated Collections of Microorganisms), and companies, play a fundamental role in preserving fungal diversity and species richness at different scales [27–30].

The Maltese Islands are part of the Mediterranean region, where the climate 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% [31,32]. The intensive human use of the Islands over thousands of years, has considerably reduced the forest cover of the Maltese Islands and at present this is very low; remnant woodland remains are small and scattered, but some ancient trees are also present in the urban environment [32,33]. Climate change modelling for the Mediterranean predicts strong impacts [34], with negative consequences for biodiversity, especially as these regions may host unique WDF ecotypes. An extensive historical excursus for Maltese mycological studies was carried out by [35], with approximately 50 species of WDF present in Mifsud's checklist. However, the total number of fungal species currently cited for the Maltese Islands that have been analyzed through DNA barcoding with sequence deposits amount to only 47, of which only three can be considered as WDF, namely *Crepidotus neotrichocystis, Xilaria melitensis* and *Schizophyllum amplum* [36–38]. This means that, for WDF, positive genetic identification is still limited.

The present work aimed to study the occurrence of WDF on trees and shrubs in semi-natural areas of the Maltese Islands and in public gardens in order to provide an updated checklist based on confirmed records by DNA barcoding, together with a comprehensive description including host species and their status. An additional aim included the establishment of the first WDF culture collection at the Biology Department Seed Bank of the University of Malta (BDUM), which adds to the already existing axenic culture collection of *Trichoderma* spp. and soil microbial species [39].

2. Materials and Methods

2.1. Sampling of Wood Decay Fungi

We conducted several walk-through surveys to observe the presence of WDF in different locations of the Maltese Islands during the rainy season from September 2020 to March 2023. The sampling locations were specifically selected for the presence of medium

to large-size indigenous and exotic phanerophytes in semi-natural woodland and public gardens. All the sampling locations were geotagged with an IPhone 13 Pro smartphone and host plant species noted and identified against the available literature [33]. Any basidiomes spotted on the host species were carefully collected with a sharp knife and placed in a paper bag and processed for axenic isolation within 1 h from collection. A portion of the context, which is the mycelium layer between the hymenium and the pileus, was removed for axenic culture isolation, with the remaining part of each basidiome placed in a paper bag, given an accession number and dried at 50 °C in a drying cabinet to produce an *exsiccate* collection.

2.2. Wood Decay Fungi Isolation and Isolate Preservation

An aseptic technique was used throughout for WDF isolation. A slice measuring 3 × 1 × 1 cm of fresh context was removed with a sharp blade, rinsed under tap water for 3 min then gently dried with clean tissue paper. The context slice was next cut into 6 pieces using a sterile blade, and the pieces were immersed in 400 mL sterile distilled water with two squirts of Tween 20 and gently stirred for 5 min. The same process was repeated twice, followed by a third passage using 400 mL of 3% hydrogen peroxide for 3 min. After this step, the pieces were transferred to the laminar flow hood (Biobase), rinsed again in sterile distilled water for 1 min followed by 1 min in 3% hydrogen peroxide, and then left for another minute in a sterile solution with 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (Genesee Scientific, Morrisville, NC, USA). Pieces of hymenium were rinsed in sterile distilled water before being plated on Potato Dextrose Agar (PDA, Oxoid, Ireland) prepared according to the manufacturer's instructions. The sealed Petri dishes were incubated at 25 °C and 70% RH under 800 lux fluorescent lamps in a growth chamber (MLR 352 PHCBI, Tokyo, Japan) with a 12L/12D light cycle and checked on alternate days for colony growth and possible contaminants. Once the mycelium had developed from the pieces of hymenium, a small piece (2 mm^2) of mycelium was aseptically transferred onto a new PDA plate with 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (Genesee Scientific). After ensuring that a clear pure mycelium culture growing on the substrate with antibiotic was again obtained, sections of the isolates were transferred onto PDA and allowed to grow before final preservation. Cultures of the isolated fungi were also stored at 4 °C in test tubes containing Synthetic Nutrient Agar prepared according to Elad et al. (1981) [40]. The basidiome and the isolates have been assigned a voucher specimen, and they are conserved in the collection of Maltese mycoflora, hosted at the Biology Department Seed Bank of the University of Malta, under the accession codes UMBmyc, which stands for University of Malta, Biology Mycotheca Bank.

2.3. Morphological Observations

Morphological observations of the mycelium, as well as colours, were made according to Kornerup and Wanscher (1981) [41]. Sections of the basidiomes were incubated for 1 h at 40 °C in 3% NaOH solution, then carefully dissected under a stereomicroscope (OPTIKA SZO) and examined in 3% NaOH solution at room temperature [42,43]. To study the basidiospores and hyphae, sections of the basidiomes were examined in Melzer's reagent, lactic acid cotton blue and KOH 4% [44], using an optical microscope (Nikon Ci-L) and identified through macro- and micro-morphological characters using manuals and dichotomous keys [45,46]. The morphological identification was then confirmed by DNA barcoding.

2.4. DNA Extraction and Molecular Identification of WDF Isolates

Genomic DNA was extracted from fresh tissues or dry specimens using a CTAB isolation procedure adapted from Doyle and Doyle [47]. PCR amplification of the ITS region (nuclear ribosomal internal transcribed spacer) and LSU (large subunit ribosomal DNA) was performed using the primer pairs ITS1/ITS4 and LR0R/LR5, respectively (http://biology.duke.edu/fungi/mycolab/primers.htm accessed on 30 January 2024) [48].

For tef1-α, a fragment between exons 4 and 8 was amplified with the primer pair 983F and 2218R [49,50]. A touchdown PCR was used with an initial annealing temperature of 60 °C [51]. Table 1 shows PCR primers used in this study.

Table 1. PCR primers used.

Genes	Primers	Primer Sequences (5′–3′)	Notes	Reference
ITS	ITS1	TCCTCCGCTTATTGATATGC	For the ITS primers, an initial denaturation at 94 °C for 3 min was followed by 30 cycles at the following conditions: 90 s at 94 °C, 90 s at 55 °C and 2 min at 72 °C. A final extension at 72 °C for 10 min completed the PCR.	[52]
	ITS4	TCCGTAGGTGAACCTGCGG		
nrLSU	LR0R	ACCCGCTGAACTTAAGC	For the nrLSU primers, an initial denaturation at 94 °C for 5 min was followed by 35 cycles at the following conditions: 60 s at 94 °C, 90 s at 50 °C and 4 min at 72 °C. A final extension at 72 °C for 10 min completed the PCR.	[48]
	LR6	CGCCAGTTCTGCTTACC		
EF1-α	EF1-983F	GCYCCYGGHCAYCGTGAYTTYAT	For the EF1-α primers, a touchdown PCR was performed where an initial denaturation at 94 °C for 3 min was followed by 10 cycles of 30 s at 94 °C, 60 s starting at 60 °C and dropping by 1 °C per cycle until a temperature of 50 °C was reached and a 90 s extension at 72 °C. The initial 10 cycles were then followed by 35 cycles of 30 s at 94 °C, 60 s at 56 °C and 90 s at 72 °C. A final extension at 72 °C for 10 min completed the PCR.	[50]
	EF1-2212R	CCRACRGCRACRGTYYGTCTCAT		

IUPAC codes: W = A or T, R = A or G, Y = C or T, N = A or T or C or G, D = G or A or T, M = A or C. ITS, internal transcribed spacer region; nrLSU, large nuclear ribosomal RNA subunit; EF1-α, translation elongation factor 1 alpha.

Successful PCR reactions resulted in a single band on a 0.8% agarose gel. PCR products were purified by adding 1 U of Exonuclease I and 0.5 U FastAP Alkaline Phosphatase (Thermo Scientific, St. Leon-Rot, Germany) and incubating at 37 °C for 1 h, followed by inactivation at 80 °C for 15 min. All the sequencing reactions were performed by Macrogen Inc. (Seul, Korea and Maastricht, The Netherlands), with primers LROR, LR3, LR3R, LR5 for the 28S; ITS1, ITS4 for the ITS-5.8S region; and 2212R, 1577F, 983F, 1567R for *tef1-α* gene [50,53]. Sequences were assembled and edited with SequencherTM 4.8 software (Gene Codes Corporation, Ann Arbor, MI, USA).

2.5. Cloning

The amplification of ITS sequences were problematic for 2 strains, viz. UMBmyc11-2021FL* and UMBmyc17-2021FL*, whose amplicons produced chromatograms with strong signal but many overlapping peaks, making base calling impossible for these chromatograms. To solve this issue, these strains were subjected to further cloning.

The purified DNA amplicons of the aforementioned were cloned into pJET1.2/blunt vector using a CloneJET PCR cloning kit (Fermentas, Waltham, MA, USA), following the manufacturer's instructions. Ligated plasmids were transformed into DH5α™ Chemically Competent *Escherichia coli* cells (Invitrogen, Waltham, MA, USA) by heat shock following the manufacturer's instructions. Eight randomly chosen clones were re-amplified by direct colony PCR and sequenced with primers pair ITS1-ITS4. The assembled sequences were compared with homologous sequences retrieved from GenBank and identified through BLAST searches in the NCBI database to confirm identification (we considered a threshold value higher than 97% suitable for positive species identification).

3. Results

From the 28 samples collected on 14 different dead and live host plant species, a total of 11 isolates were successfully obtained, 3 from semi-natural environments and 7 from public gardens, as shown in Table 2. Whereas, Table 3 shows the WDF species identified in this study based on DNA barcoding. Sampling locations are given in Figure 1. Note that for some samples, it was not possible to carry out any isolation due to basidiome deterioration.

Table 2. Species identification of the wood decay fungi isolates from the Maltase Islands, based on DNA barcoding.

Samples	Wood Decay Fungi	Isolate Accession Number	Host Plant	Host Status	Sampling Location	Coordinates
1	*Aurificaria* cf. *euphoriae*	UMBmyc11-2021FL*	*Olea europaea* L.	Dead	Floriana—PG	35.89448° N; 14.49919° E
2			*Casuarina equisetifolia* L.	Living	Msida—PG	35.90283° N; 14.48573° E
3			*Carya illinoinensis* (Wangenh.) K. Koch.	Living	Rabat—SNW	35.85512° N; 14.39665° E
4	*Ganoderma resinaceum sl*	UMBmyc12-2021FL*	*Ceratonia siliqua* L.	Living	Floriana—PG	35.88915° N; 14.49964° E
5		UMBmyc13-2021SL*	*Tamarix africana* Poir.	Dead	Sliema—PG	35.91664° N; 14.50259° E
6		UMBmyc14-2021VL*	*Olea europaea* L.	Living	La Valletta—PG	35.89504° N; 14.51216° E
7			*Casuarina equisetifolia* L.	Living	St Julian's—PG	35.92257° N; 14.48683° E
8	*Laetiporus sulphureus*	UMBmyc15-2021MS*	*Ceratonia siliqua* L.	Living—Monumental	Msida—PG	35.90187° N; 14.48258° E
9	*Laetiporus sulphureus*	UMBmyc16-2021RB*	*Quercus robur* L.	Living—highly damaged— Monumental	Rabat—SNW	35.85672° N; 14.39864° E
10	*Inonotus* sp.	UMBmyc17-2021FL*	*Maclura pomifera* (Raf.) Schneid.	Dead	Floriana—PG	35.89242° N; 14.50293° E
11	*Inonotus rickii* (*Ptychogaster cubensis* anmr)	UMBmyc18-2021RB*	*Quercus ilex* L.	Living—damaged—Monumental	Rabat—SNW	35.85512° N; 14.39665° E
12			*Celtis australis* L.	Living—damaged	Hal Lija—PG	35.90482° N;14.44770° E
13			*Celtis australis* L.	Living—damaged	Attard—PG	35.89673° N; 14.44781° E
14			*Casuarina equisetifolia* L.	Living	Ta' Xbiex—PG	35.89992° N; 14.49462° E
15			*Pistacia lentiscus* L.	Living	Rabat—PG	35.85756° N; 14.39730° E
16			*Carya illinoinensis* (Wangenh.) K. Koch.	Living—damaged	Attard—PG	35.89673° N; 14.44781° E
17			*Sapindus saponaria* L.	Living	Floriana—PG	35.89242° N; 14.50312° E
18			*Harpullia pendula* Planch. ex F.Muell.	Living	Attard—PG	35.89673° N; 14.44781° E
19	*Inocutis tamaricis*	UMBmyc19-2021BP*	*Tamarix africana* Poir.	Living—highly damaged	Sliema—PG	35.91513° N; 14.50521° E
20			*Tamarix africana* Poir.	Living—damaged	Ta' Xbiex—PG	35.90032° N; 14.49998° E
21			*Tamarix africana* Poir.	Living—damaged	Marsaskala—PG	35.85738° N; 14.56850° E
22			*Tamarix africana* Poir.	Living—damaged	St. Paul's Bay—PG	35.94535° N; 14.38621° E
24			*Tamarix africana* Poir.	Living—damaged	Mellieha—SNW	35.98269° N; 14.33281° E
25	*Stereum hirsutum*	UMBmyc20-2021BP*	*Acacia saligna* (Labill.) H.L.Wendl.	Dead	St. Paul's Bay—SNW	35.93750° N; 14.38508° E
26	*Pleurotus eryngii*	UMBmyc21-2021BP*	*Ferula melitensis* (Brul., C.Brul., Cambr., Giusso, Salme and Bacch.)	Living	Siggiewi—G	35.84734° N; 14.39249° E
27	*Coriolopsis gallica*	UMBmyc22-2021BP	*Acacia saligna* (Labill.) H.L.Wendl.	Dead	Manoel Island—SNW	35.90441° N; 14.50421° E
28			Log	Dead	St. Paul's Bay—PG	35.93856° N; 14.38514° E
29			*Olea europaea* L.	Dead	Floriana—PG	35.89448° N; 14.49919° E

*: Isolated; PG: public garden; SNW: semi-natural woodland; G: garigue.

Table 3. Species identification of the WDF isolates, based on DNA barcoding.

Isolate No. [(1)]	Species	- Gene Bank Sequence Accession Numbers - Amplicons Length - Percentage of Identity with the First BLAST Result		
		ITS	**LSU**	**Tef 1**
UMBmyc11-2021FL*	*Aurificaria* cf. *euphoriae*	PP512944 346 bps (*Phellinus* sp. 92.36%)	1109 bps (*Fulvifomes subindicus* 97.16%)	1169 bps (*Fulvifomes halophilus* 94.76%)
UMBmyc12-2021FL*	*Ganoderma resinaceum sl*	PP512939 616 bps (*Ganoderma resinaceum* 99.83%)	PP512951 1083 bps (*Ganoderma resinaceum* 99.91%)	PP531582 1157 bps (*Ganoderma resinaceum* 100%)
UMBmyc13-2021SL*	*Ganoderma resinaceum sl*	PP512940 620 bps (*Ganoderma resinaceum* 100%)	PP512952 1119 bps (*Ganoderma resinaceum* 100%)	PP531583 1157 bps (*Ganoderma resinaceum* 100%)
UMBmyc14-2021VL*	*Ganoderma resinaceum sl*	PP512942 616 bps (*Ganoderma resinaceum* 100%)	PP512954 1119 bps (*Ganoderma resinaceum* 100%)	PP531585 1157 bps (*Ganoderma resinaceum* 100%)
UMBmyc15-2021MS*	*Laetiporus sulphureus*	PP512936 576 bps (*Laetiporus sulphureus* 100%)	PP512948 1099 bps (*Laetiporus sulphureus* 100%)	PP531579 1154 bps (*Laetiporus sulphureus* 98.70%)
UMBmyc16-2021RB*	*Laetiporus sulphureus*	PP512946 577 bps (*Laetiporus sulphureus* 100%)	PP512956 1099 bps (*Laetiporus sulphureus* 100%)	PP531587 1154 bps (*Laetiporus sulphureus* 98.70%)
UMBmyc17-2021FL*	*Inonotus* sp.	PP512943 741 bps (*Phellinus* sp. 94.40%)	1114 bps	-
UMBmyc18-2021RB*	*Inonotus rickii* anamorph: *Ptychogaster cubensis*	PP512935 735 bps (*Inonotus rickii* 100%)	PP512947 1143 bps (*Inonotus rickii* 99.64%)	PP531578 1166 bps (*Inonotus rickii* 99.50%)
UMBmyc19-2021BP*	*Inocutis tamaricis*	PP512941 481 bps (*Inocutis tamaricis* 99.17%)	PP512953 1116 bps (*Inocutis tamaricis* 99.58%)	PP531584 1169 bps (*Arambarria destruens* 82.30%)
UMBmyc20-2021BP*	*Stereum hirsutum*	PP512938 609 bps (*Stereum hirsutum* 100%)	PP512950 1079 bps (*Stereum hirsutum* 99.81%)	PP531581 1191 bps (*Stereum hirsutum* 96.24%)
UMBmyc21-2021BP*	*Pleurotus eryngii*	PP512937 650 bps (*Pleurotus eryngii* 99.54%)	PP512949 1079 bps (*Pleurotus eryngii* 99.91%)	PP531580 1148 bps (*Pleurotus eryngii* 99.49%)
UMBmyc22-2021BP	*Coriolopsis gallica*	PP512945 612 bps (*Coriolpsis gallica* 99.84%)	PP512955 943 bps (*Coriolpsis gallica* 100%)	PP531586 535 bps (*Coriolpsis gallica* 99.84%)

[(1)] Accession Nos. in the collection of Maltese mycoflora, Seed Bank of the Department of Biology, University of Malta, Valletta, Malta. * Isolated.

From the two public gardens sampled in Floriana, sample 1 UMBmyc11-2021FL* collected on a dead stump of *Olea europaea sl.* gave an isolate identified as *Inonotus indicus*, whereas sample 10 UMBmyc17-2021FL* collected on a dead stump of *Maclura pomifera* gave an isolate identified as *Inonotus* sp. Moreover, sample 4 UMBmyc12-2021FL* collected on *Ceratonia siliqua* in Floriana, sample 6 UMBmyc14-2021VL* collected on *Olea europaea* growing public gardens in Valletta, and sample 5 UMBmyc13-2021SL* collected on a dead stump of *Tamarix africana* in Sliema were all identified as *Ganoderma resinaceum sl*. Sample 8 UMBmyc15-2021MS* collected *Ceratonia siliqua* in Msida and sample 9 UMBmyc16-2021RB* obtained from a highly damaged *Quercus robur* gave isolates identified as *Laetiporus sulphureus*. From the semi-natural environments present at Buskett limits of Rabat, sample 11 (UMBmyc18-2021RB*) was obtained from a mature *Quercus ilex*, whose isolate was identified as *Inonotus rickii* (anamorph: *Ptychogaster cubensis* Pat.). Sample 19 (UMBmyc19-2021BP*) isolated from growth on *Tamarix africana* in Sliema was identified as *Inocutis tamaricis*. The stump of *Acacia saligna*, an invasive alien plant species, hosted isolate

27 (UMBmyc20-2021BP*) identified as *Stereum hirsutum*. Lastly, sample 28 (UMBmyc21-2021BP*) was found growing on the endemic *Ferula melitensis* in a garrigue habitat limits of Siggiewi and identified as *Pleurotus eryngii*.

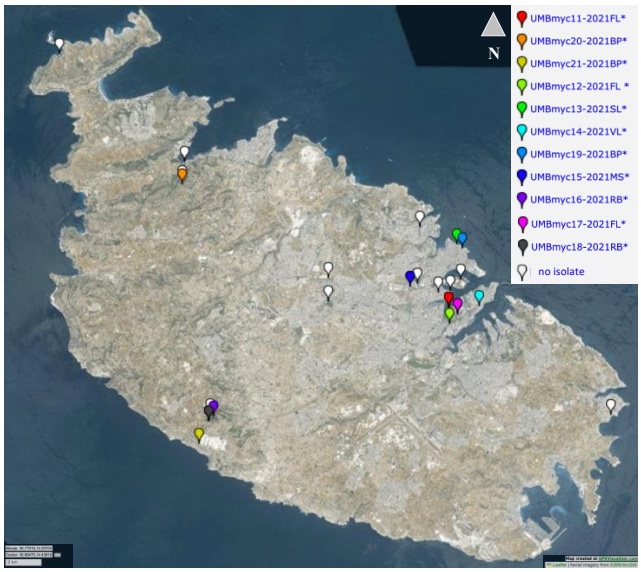
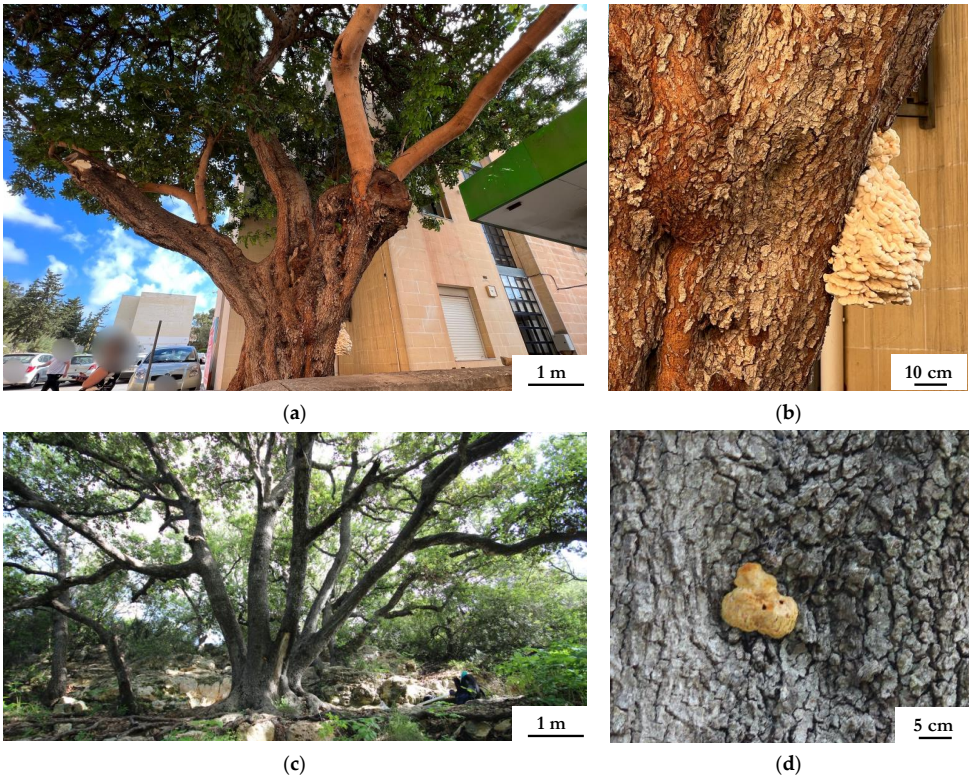


Figure 1. Sampling locations for WDF isolates are shown in colored waypoints, whereas white waypoints show location where WDF were also encountered. Some sampling locations gave more than one sample. Source: GPS Visualiser, Aerial imagery from ESRIArcGIS.

It is worth noting that *Laetiporus sulphureus* and *Inonotus rickii* were isolated on two monumental trees, namely *Ceratonia siliqua* and *Quercus ilex* respectively, shown in Figure 2.



(**a**) (**b**)

(**c**) (**d**)

Figure 2. WDF on monumental trees. *Laetiporus sulphureus* on *Ceratonia siliqua* growing at University Campus, Msida (**a**,**b**); *Inonotus rickii* (anamorph: *Ptychogaster cubensis* Pat.) on *Quercus ilex* growing at Buskett, Rabat (**c**,**d**).

The habit, growth patterns and colors of the WDF isolated cultures on PDA were noted and photographed after 4 weeks from the top and reverse sides of the Petri dishes. These details, as well as the species identification, are shown in Figure 3. On the other hand, Figure 4 shows damage occurring on trees infected with WDF. Figure 5 also shows the orders' percentages isolated in the present study.

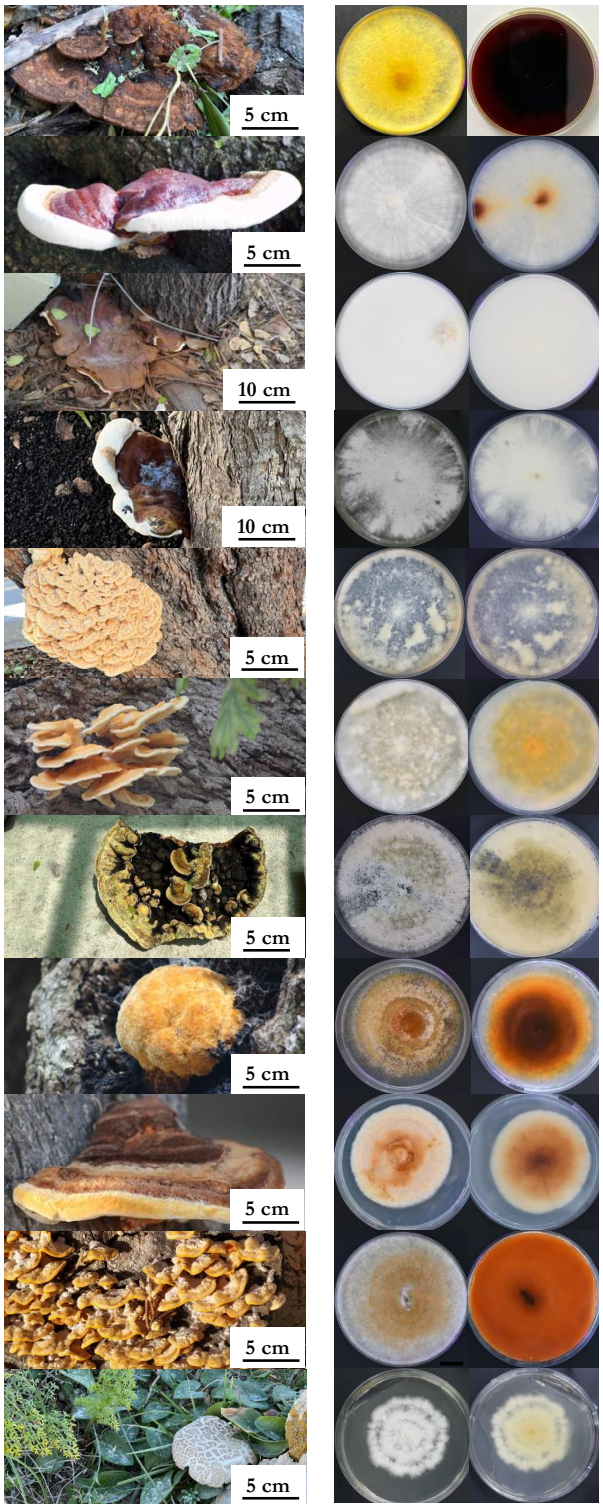


Figure 3. Wood decay fungi isolated in Malta. (**a**) Basidiomes; (**b**) isolates' growth on PDA top and bottom sides; (**c**) species name, accession number and isolate number.

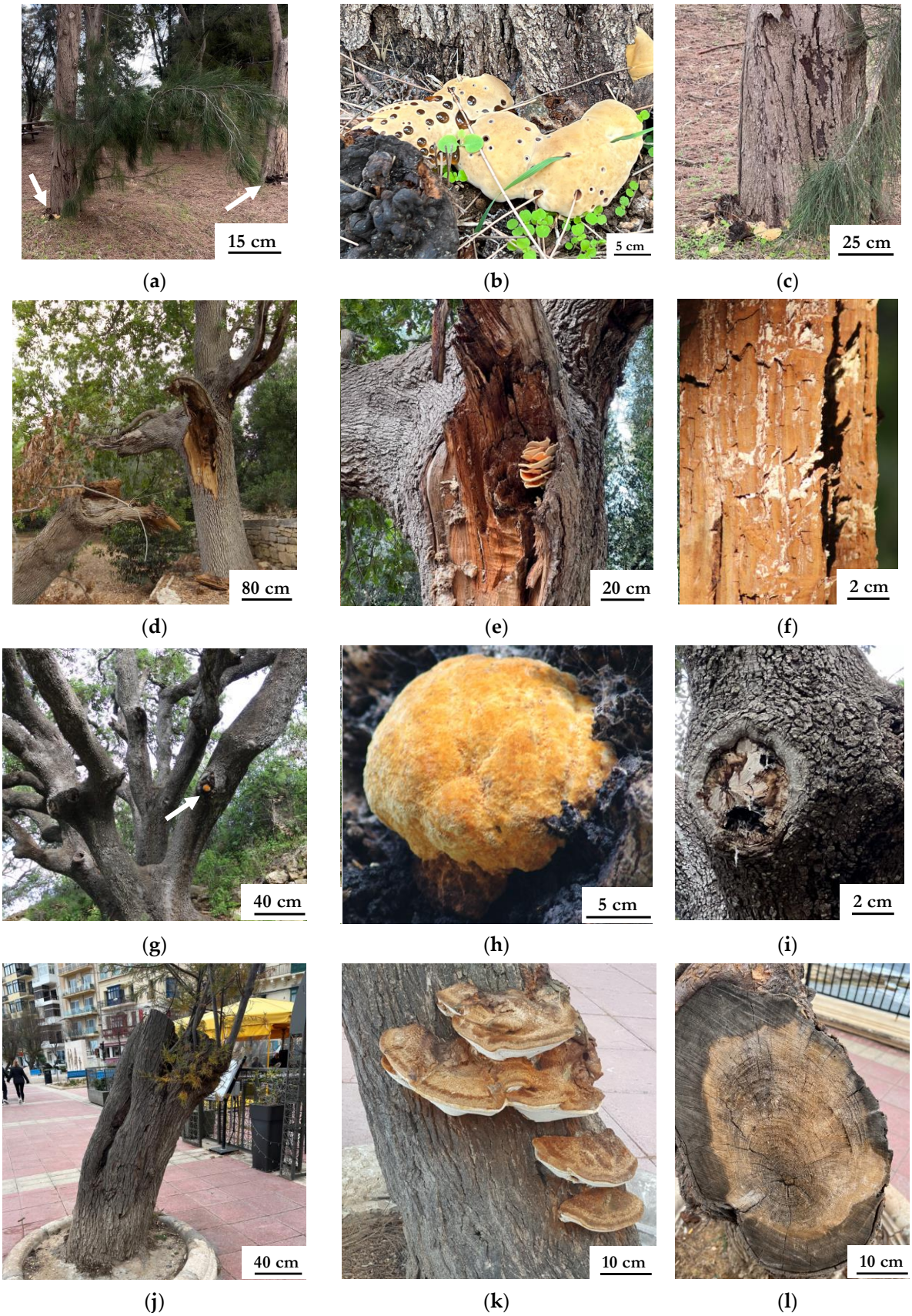


Figure 4. Wood decay fungi affecting host individuals with very low fitness. (**a**–**c**) *Aurificaria* cf. *euphoria*; (**d**–**f**) *Laetiporus sulphureus*; (**g**–**i**) *Inonotus rickii* (anamorph: *Ptychogaster cubensis* Pat.); (**j**–**l**) *Inocutis tamaricis*.

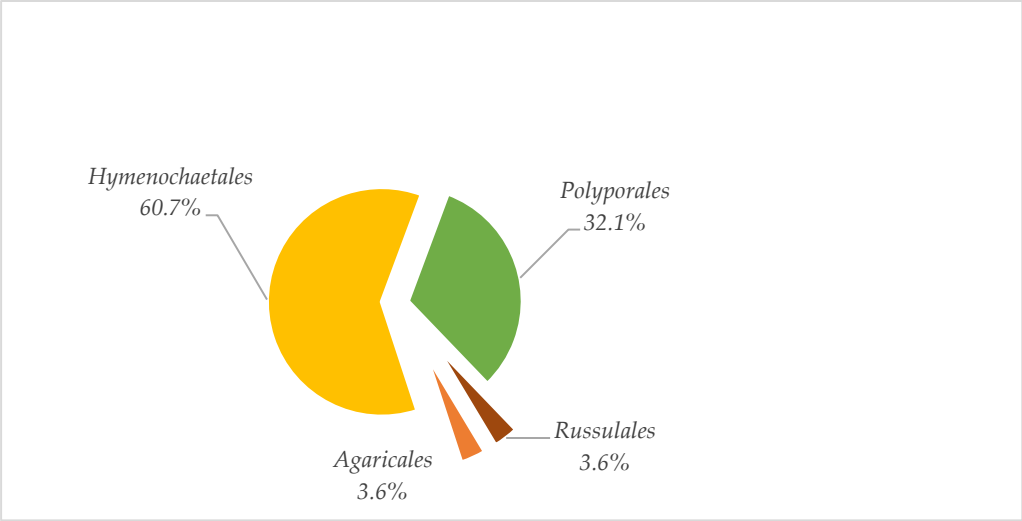


Figure 5. WDF orders isolated in Malta from this study shown as a percentage.

4. Discussion

The Convention on Biological Diversity underscores the importance of islands and their adjacent near-shore marine areas. It emphasizes their role as unique ecosystems hosting numerous plant and animal species that are often endemic, with species existing exclusively in these locations and nowhere else on the planet [54] Associated with these endemic macro-species, there is a huge variety of other organisms, often at microbial level, that have an essential and crucial role in supporting the ecosystem. The holistic surveying, cataloguing, and preservation of biodiversity becomes of paramount importance for small islands like the Maltese Islands.

A multi-locus identification system for WDF based on phylogenetic marker databases (ITS, 28S, and *tef1-α*), coupled with morphological observation, is regarded as a valid tool for identification for fungal species; however, if sequences are missing from public databases, and morphology is not clear enough, some collections would remain unidentified at species level and would require further studies. The present study used the DNA sequences to define the diversity of WDF in the Maltese Islands. The high homoplasy and the phenotypic plasticity in key morphological characters make species delimitations often fuzzy in different fungal clades, e.g., *Ganodermataceae*. This translates into an extremely high level of misidentification of species, and possible error in sequences deposited in the public repositories, such as GenBank. The scattered woodland cover, some of which occurs on private land with restricted access, as well as the very short wet seasons, limited the study to 28 samples, from which only 11 mycelial isolates were possible. The authors acknowledge the possibility that other methodologies of isolation could have been employed to increase the number of pure mycelial isolates [31,32,55]. These were identified as nine different species, namely, *Aurificaria* cf. *euphoriae, Ganoderma resinaceum sl.*, *Laetiporus sulphureus*, *Inonotus* sp., *Inonotus rickii* anamorph: *Ptychogaster cubensis, Inocutis tamaricis*, *Pleurotus eryngii, Stereum hirsutum,* and *Coriolopsis gallica*. Seven species out of nine had already been recorded in the Maltese Islands and identified through macro- and micro-morphological characters by various authors [35,37,56–58], but the identification was never confirmed with the DNA barcoding.

The multi-locus system identified the Isolate UMBmyc11-2021FL* as *Phellinus* sp. 92.36% for ITS, *Fulvifomes subindicus* 97.16% for LSU, and *Fulvifomes halophilus* 94.76% for Tef1, suggesting that further studies are needed. However, using the micro- and macro- morphological observations, the authors found higher similarities with *Aurificaria* cf. *euphoriae* [45]. The same species was also recorded by Briffa as *Inonotus indicus* on *Ceratonia siliqua* [58]. In the present study, it was recorded also on *Olea europaea*, *Casuarina equisetifolia* and *Carya illinoinensis*.

Regarding the genus *Ganoderma* in Malta, the species *G. applanatum, G. australe and G. lucidum* have been documented by different authors [56–58], growing on different plant hosts, such as *Eriobotrya japonica, Ceratonia siliqua, Laurus nobilis, Populus alba, Fraxinus angustifolia, Ulmus minor* and *Carya olivaeformis*. In our study, the isolates UMBmyc12-2021FL*, UMBmyc13-2021SL* and UMBmyc14-2021VL* were identified as *G. resinaceum sl*. growing respectively on *Ceratonia siliqua, Tamarix africana, Olea europaea* and *Casuarina equisetifolia*. For the Maltese Islands, *G. resinaceum sl*. is a new addition. Moreover, isolates UMBmyc15-2021MS* and UMBmyc16-2021RB* were identified as *Laetiporus sulphureus*, which were previously recorded on *Ceratonia siliqua* [56,58]. In our study this species was also recorded on *Quercus robur.* Furthermore, while [58] previously recorded *Inocutis tamaricis* on *Tamarix* spp., in our studies this was only sampled on *Tamarix africana.*

Interestingly, *Stereum hirsutum,* which was recorded growing on *Quercus* wood by Saccardo in 1914, had not been reported for 150 years until our present study, where it was found on a dead stump of *Acacia saligna*. Lastly, *Pleurotus eryngii* var. *ferulae*, first recorded by [59], in our study was simply identified as *Pleurotus eryngii.* All the identifications carried out in our study were confirmed through DNA barcoding. *Coriolopsis gallica,* previously recorded by [60] as *Trametes hispida* on rotting wood of *Quercus* sp, in the present study was encountered on *Acacia saligna*, *Olea europaea*, and on a log of unidentified wood. However, from the basidiomes, it was not possible to obtain any living mycelium, but it was still possible to obtain DNA, and through the sequencing the identity was confirmed as *Coriolopsis gallica*.

Noteworthy, for the isolate UMBmyc17-2021FL* collected from a basidiomes in Argotti Botanic Garden growing on a dead stump of *Maclura pomifera,* even with the use of the DNA barcoding, it was not possible to identify it at the species level (Blast results showed a percentage identity lower than 94.40% with sequences) and, therefore, we stopped at genus identification as *Inonotus sp.,* suggesting that further studies are needed.

A new record for the Maltese Islands was discovered, namely *Inonotus rickii* anamorph: *Ptychogaster cubensis,* which was recorded growing on several tree species, namely, *Quercus ilex, Celtis australis, Casuarina equisetifolia, Pistacia lentiscus, Carya illinoinensis, Sapindus saponaria* and *Harpullia pendula.* All the specimens recorded were ascribed to the anamorph described in Europe with a basidiome semi-spherical or cushion shaped, soft and fleshy at first, yellowish brown to golden brown, then dark brownish [61,62], and not like the species described in Florida as plumose and bearded-like [63]. *Inonotus rickii* anamorph: *Ptychogaster cubensis* is an infecting basidiomycete of urban trees, posing a significant threat globally by parasitizing a variety of hosts [64]. The fungus's impact is particularly pronounced, where infected ornamental trees not only lose their visual appeal, but also become a substantial danger to other members of the urban tree population due to the exhibited disease symptoms [65].

The WDF identified in our study can be further categorized into two types: white-rot and brown-rot [13]. The white-rot fungi causing heartwood rot are *Aurificaria* cf. *euphoriae, Inonotus rickii*, *Inonotus* sp., *Inocutis tamaricis* and *Pleurotus eryngii,* whereas *Stereum hirsutum* causes sapwood rot and, *Ganoderma resinaceum* causes butt and root rot [66]. The sole brown-rot species which causes heartwood rot is *Laetiporus sulphureus* [66]. As shown in Figure 1, the sampled trees are growing in highly urbanized areas, were usually their fitness is not at the very best, and can be further decreased by the WDF, therefore making the infected trees more sensitive to windthrow, and harming the surrounding environment.

All the WDF species identified in the current study have been extensively documented in various geographical areas, including the Mediterranean region, along with associations with different plant species [45,66–72]. With regards to the Maltese Islands, although we consider the previous records of high historical value, our study confirms some former identifications through the DNA barcoding technique.

Furthermore, the culture collection, including 11 indigenous WDF, holds significant potential for various biotechnology applications. Some of these WDF, like *Laetiporus sulphureus* and *Pleurotus eryngii,* are edible, while all species are sources of bioactive compounds re-

lated to either primary or secondary metabolism [15,68,73]. These fungi are also valuable for primary biomass degradation for production of bioethanol [16], production of enzymes for industry [18], and bioaccumulation, bioremediation [19–21]. Notably, *G.resinaceum* may serve as biomaterial with various applications [22–25].

Considering the environmental and climatic conditions of the Maltese Islands, these isolated fungi may possess properties and enhanced adaptation to abiotic stresses common in the Mediterranean basin, such as drought, heat stress, and salinity. This makes them particularly suitable for potential biotechnological applications in the context of climate change and global warming.

5. Conclusions

Culture collections serve as ex situ preservation of fungal specimens and assume also a pivotal scientific role. The repository of 11 indigenous fungal strains at the Seed Bank at the Biology Department, University of Malta specifically contributes to the ex situ conservation endeavors concerning Mediterranean WDF. These strains serve as a robust foundation, along with the *Trichoderma*, for prospective scientific inquiry, spanning both theoretical and practical applications. Future research aims at their cultivation on selected lignocellulosic substrates under axenic conditions, as well as to further investigate the taxonomy of Isolates UMBmyc17-2021FL* and UMBmyc11-2021FL*. It is crucial to pay attention to the presence of WDF, not just for the preservation of monumental trees, but also for the overall well-being of forests and ecosystems. Additionally, this attention is essential from a health and safety standpoint, particularly concerning infected trees in public areas. By prioritizing the monitoring and management of WDF, we are not only safeguarding the longevity of monumental trees, but also contributing to the broader health, safety, and sustainability of our natural environments.

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