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Titolo della tesi

DNA barcoding and integrative taxonomy on animal taxa characterized by a high cryptic diversity

SSD **BIO/05 ZOOLOGIA**

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Riassunto

Lo studio della biodiversità è ancora oggi una materia estremamente attuale. Il DNA barcoding, proposto da Hebert et al. (2003), si è rivelato uno strumento potente e rapido per l'identificazione di campioni e la scoperta di nuove specie. Questo approccio molecolare trova applicazioni in diversi campi, tra i quali l'ecologia delle comunità, biologia della conservazione, monitoraggio della salute pubblica, farmacognosia, industria alimentare e medicina legale. Il successo del DNA barcoding si basa su due premesse principali: (i) il barcoding gap che divide le divergenze genetiche intra-specifiche da quelle inter-specifiche, permettendo di distinguere campioni conspecifici da quelli appartenenti a specie diverse; (ii) la disponibilità di librerie di sequenze di riferimento associate a campioni identificati con precisione da tassonomi esperti. Nei casi in cui le analisi di DNA barcoding non forniscono identificazioni certe, spesso a causa di lacune nella libreria di riferimento, diventa necessaria un'indagine tassonomica integrata, che incorpora molteplici set di caratteri indipendenti come la morfologia, l'ecologia e, la biogeografia, per la validazione e l'identificazione delle specie. Partendo da ipotesi tassonomiche preliminari basate su metodi di delimitazione delle specie in unità tassonomiche operative molecolari (MOTU) è possibile guidare studi di tassonomia integrata per ottenere una conoscenza più approfondita sulla biodiversità.

La tesi di dottorato ha avuto come scopo l'integrazione di tecniche molecolari di DNA barcoding e tassonomia integrata per lo studio della biodiversità di due gruppi animali, entrambi caratterizzati da un difficile riconoscimento morfologico delle specie, ma per i quali è disponibile un diverso grado di conoscenza tassonomica: la tribù Alticini Newman, 1834 e la famiglia Ostreidae Rafinesque, 1815. Gli Alticini sono caratterizzati da un'elevata diversità con circa 10.000 specie distribuite in 601 generi. Questo gruppo presenta una tassonomia ben definita, tuttavia, l'identificazione delle specie richiede un'elevata competenza tassonomica. Al contrario, la famiglia Ostreidae, composta da 78 specie distribuite in tutto il mondo, presenta una tassonomia poco definita. L'identificazione tassonomica delle specie è complessa a causa della plasticità morfologica delle conchiglie e dall'assenza di caratteristiche morfologiche che possano distinguere con precisione le specie.

Nella prima parte della tesi, è stato sviluppato lo studio sulla biodiversità degli Alticini. L'approccio di DNA barcoding è stato applicato generando una libreria di riferimento per 99 specie appartenenti a 21 generi basata sull'identificazione morfologica e sequenziamento di 278 campioni da 119 siti lungo l'intera dorsale appenninica. Il tasso di corretta identificazione di questi campioni basato su librerie di sequenze disponibili BOLD e GB è risultato elevato (87%). Tuttavia, sono emersi 684 casi di errata identificazione nella libreria BOLD-GB per le specie studiate, che sono state *a*

posteriori corrette sulla base della libreria di riferimento generata nella tesi. Questo studio ha corroborato l'importanza dell'accuratezza delle librerie di riferimento e del ruolo cruciale del tassonomo sia nella generazione delle librerie sia nella loro validazione a posteriori. La solida tassonomia disponibile per gli Alticini rende il DNA barcoding uno strumento efficiente per l'identificazione molecolare delle specie, con un numero limitato di gruppi che richiedono una rivalutazione tassonomica. Successivamente, nella tesi è stato applicato un approccio di tassonomia integrata sul gruppo di specie del *Longitarsus candidulus*. In questo caso, la validità di tre specie morfologicamente molto simili è stata confermata utilizzando un approccio filogenetico multi-locus. In particolare, le specie *L. laureolae* e *L. leonardii* mostrano una peculiare distribuzione disgiunta appennino-pirenaica che riflette probabilmente un evento di speciazione durante il Pleistocene associato ad un periodo di significativi cambiamenti climatici in Europa.

Nella seconda parte della tesi è stato sviluppato lo studio sulla biodiversità delle ostriche a scala mondiale attraverso l'analisi di 813 campioni depositati in due delle principali collezioni disponibili al mondo: Muséum National d'Histoire Naturelle di Parigi (MNHN) e il Florida Museum of Natural History (FLMNH) della Florida. Lo studio di DNA barcoding basato su 424 sequenze ottenute dai campioni museali ha dimostrato che le librerie di sequenze BOLD-GB sono poco efficaci per l'identificazione dei campioni, specialmente per le Ostreinae e delle regioni tropicali. La diversità delle ostriche dell'Oceano Indo-pacifico è, infatti, in larga parte ancora sconosciuta. Molti campioni museali, assegnati con un approccio filogenetico a 38 MOTU, non corrispondono a specie note per le quali sono disponibili sequenze nei database BOLD-GB. Molte di queste MOTU rappresentano verosimilmente specie nuove per la scienza e richiedono un ulteriore studio tassonomico con approccio integrativo. Questo approccio è stato applicato in questa tesi in tre casi di studio. Il primo si basa su dati molecolari degli olotipi e dei topotipi di due specie recentemente descritte su base morfologica: *Magallana markuschumberi* e *M. valentichscotti*. I risultati dimostrano che queste due nuove specie non sono valide, bensì corrispondono a due specie molto comuni: *M. angulata* e *M. belcheri*, rispettivamente, delle quali rappresentano quindi dei "junior synonyms". Nel secondo caso studio, è stato applicato un approccio molecolare multi-locus per la validazione molecolare e sistematica di una specie abissale, *Neopycnodonte zibrowii*, descritta attraverso comparazioni morfologiche. Infine, nel terzo caso studio è stata descritta una nuova specie raccolta in Kuwait combinando dati morfologici, molecolari e di distribuzione. Questo studio ha anche portato alla definizione di nuove caratteristiche morfologiche della sottofamiglia Ostreinae.

In conclusione, questa tesi corrobora l'estrema utilità dell'integrazione dei dati molecolari e morfologici negli studi sulla biodiversità e fornisce una pipeline per sviluppare questi studi in gruppi animali morfologicamente omogenei o criptici e per i quali è disponibile un diverso grado di

conoscenza tassonomica. Nel caso degli Alticini viene sviluppata un’accurata libreria di riferimento per le applicazioni di barcoding in Italia e in Europa, mentre per le ostriche vengono identificate le principali aree di incertezza tassonomica del gruppo a scala globale che richiedono un approccio tassonomico integrato. I risultati di questa tesi forniranno pertanto una guida e faciliteranno i futuri studi sulla biodiversità di entrambi i gruppi animali.

Abstract

The study of biodiversity is still a hot research topic today. DNA barcoding, proposed by Hebert et al. (2003), has proven to be a powerful and rapid tool for sample identification and species discovery. This molecular approach finds applications in various fields including community ecology, conservation biology, public health monitoring, pharmacognosy, food industry, and forensic medicine. The success of DNA barcoding relies on two main premises: (i) the barcoding gap, which separates intra-specific genetic divergences from inter-specific genetic divergences, allowing to distinguish conspecific samples from those belonging to different species; (ii) the availability of reference sequence libraries associated with accurately identified samples by expert taxonomists. In cases where DNA barcoding analyses fails to provide certain identifications (often due to gaps in the reference library), it become necessary an integrative taxonomic investigation, incorporating independent characters sets such as morphology, ecology, and biogeography, for species validation and identification. Starting from preliminary taxonomic hypotheses, based on species delimitation methods to define molecular operational taxonomic units (MOTUs), integrative taxonomic studies can allow reaching robust taxonomic conclusions and in-depth understanding of biodiversity.

The aim of the PhD thesis is to combine DNA barcoding and integrative taxonomy to assess the taxonomic diversity of two selected animal groups for which morphological diagnosis can be extremely challenging. The two animal groups selected as study model are the flea beetles (tribe Alticini Newman, 1834) and the true oysters, that belong to the families Chrysomelidae Latreille, 1802 and Ostreidae Rafinesque, 1815, respectively. Alticini are characterized by high diversity with approximately 10,000 species distributed across 601 genera. This group exhibits well-defined taxonomy; however, taxonomic experts on regional faunas are often required for correct identification at species-level. In contrast, the Ostreidae family, composed of 78 species distributed worldwide, has a poorly defined taxonomy. Taxonomic identification of most species is hampered by their phenotypic plasticity and the absence of reliable diagnostic characters.

In the first part, the thesis develops studies on the biodiversity of Alticini. The DNA barcoding approach was applied by generating a reference library for 99 species belonging to 21 genera based on morphological identification and sequencing of 278 samples from 119 sites along the entire Apennine chain. The rate of correct identification of these samples based on available BOLD and GB sequence libraries was high (88%). However, 684 cases of misidentification were found in the BOLD-GB library for the studied species, which were subsequently corrected based on the reference library generated in the thesis. This study substantiates the importance of accurate reference libraries and the key role of taxonomists both in generating libraries and in their posterior validation. The solid taxonomy available for Alticini makes DNA barcoding an efficient tool for molecular species identification, with a limited number of groups requiring taxonomic reassessment. Subsequently, an integrated taxonomy approach was applied in the thesis to the *Longitarsus candidulus* species group. In this case, the validity of three morphologically very similar species was confirmed using a multi-locus phylogenetic approach. In particular, the species *L. laureolae* and *L. leonardii* show a peculiar disjunct Apennine-Pyrenean distribution, likely reflecting a speciation event during the Pleistocene associated with a period of severe climate change in Europe.

In the second part of the thesis, the study of global oysters' diversity was developed through the analysis of 813 samples deposited in two of the world's major collections: the Muséum National d'Histoire Naturelle in Paris (MNHN) and the Florida Museum of Natural History (FLMNH). DNA barcoding analysis based on 424 sequences obtained from museum specimens demonstrated that BOLD-GB sequence libraries are ineffective for specimen identification, especially for Ostreinae and for specimens collected in tropical regions. The diversity of Indo-Pacific oysters is largely unknown. Many museum specimens, identified through a phylogenetic approach into 38 MOTUs, do not correspond to known species for which sequences are available in BOLD-GB databases. Many of these MOTUs likely represent new species to science and require further taxonomic study with an integrative approach. This approach was applied in this thesis in three case studies. The first is based on molecular data from holotypes and topotypes of two species recently described morphologically: *Magallana markuschumberi* and *M. valentichscotti*. The results demonstrate that these two new species are not valid but correspond to two very common species: *M. angulata* and *M. belcheri*, respectively, of which they represent junior synonyms. In the second study, a multilocus molecular approach was applied for molecular validation and systematic assessment of an abyssal species *Neopycnodonte zibrowii* described through morphological comparisons. Finally, in the third case study, a new species collected in Kuwait was described combining morphological, molecular, and distributional data. This study also led to documentation of new morphological characteristics of the Ostreinae subfamily.

In conclusion, this thesis corroborates the extreme utility of integrating molecular and morphological data in biodiversity studies and provides a pipeline for developing these studies in morphologically homogeneous or cryptic animal groups for which varying degrees of taxonomic knowledge are available. In the case of Alticini, an accurate reference library for barcoding applications in Italy and Europe is developed, while for oysters, the main areas of taxonomic uncertainty are identified at a global scale and will require the application of integrated taxonomic approaches. The results of this thesis will therefore provide guidance and facilitate future studies on the biodiversity of both animal groups.

GENERAL INTRODUCTION

The exploration and discovery of biodiversity is still a hot topic of research. Catalogue of Life (CoL) has inventoried over 1.9 million terrestrial and marine eukaryotes (Bánki et al., 2023). However, local and global biodiversity knowledge is far to be achieved and estimates of the number of existing species range from 2 to 10 million according to the more conservative models (Costello et al., 2012; Mora et al., 2011). One of the most useful and rapid tool for biodiversity assessments and species discovery is the DNA barcoding (DeSalle et al., 2005; Hebert et al., 2003). DNA barcoding is a molecular method that uses DNA sequence data from a short gene fragment for specimen identification and species discovery (Hebert et al., 2003). This revolutionary molecular approach was proposed for the first time by Hebert et al. (2003) that established the standard cytochrome c oxidase I (COI) gene fragment as a reliable barcode for animals. Barcode of Life Data System (BOLD) (Ratnasingham & Hebert, 2007) and the Barcode Index Number (BIN) System (Ratnasingham & Hebert, 2013) have been development to boost the practise of DNA barcoding. To date, more than 250 thousand of animal species are present in BOLD with millions of barcodes sequences. DNA barcoding can be applied in a wide range of fields: biodiversity assessment, community ecology, conservation biology, biosecurity, public health monitoring, pharmacognosy, food industry and forensics (Fišer Pečnikar & Buzan, 2014; Shadrin, 2021).

The success of DNA barcoding is based on two main premises: (i) the barcoding gap which divides the intra-specific genetic divergence from the inter-specific divergence and help to distinguish specimens belonging to the same species from those belonging to different species (Hebert et al., 2004); (ii) the existence of comprehensive sequence libraries composed of individuals correctly identified by expert taxonomist that provide reference for specimen identification (Floyd et al., 2010). The second premise is still far from being achieved because many taxonomic groups lack of specimens correctly identified at species-level (Kvist, 2013; Kwong et al., 2012). The lack of reliable taxonomic identifications in sequence libraries derived by seven main issues: (i) taxonomic mislabelling, (ii) sequencing errors, (iii) sequence conflict, (iv) taxonomic conflict, (v) low taxonomic resolution, (vi) missing taxa and (vii) missing intraspecific variants (Keck et al., 2023). An inadequate a priori identification of specimens can negatively affect present and future DNA barcoding studies (Collins & Cruickshank, 2013).

Therefore, the DNA barcoding is not meant as an alternative tool to the morphological identification in taxonomy, as it is specifically based on the morphological identification of reference specimens made by expert taxonomists to build a reference sequence library, which is used for the downstream molecular identification of specimens to the species level. However, many animal groups are characterized by a challenging morphological diagnosis and they require highly specialised taxonomists for species identifications (Will et al., 2005). In addition, morphology alone cannot provide a reliable identification for cryptic species diversity for which speciation is not accompanied by discrete morphological changes (Bickford et al., 2007). In these cases, morphological identification of reference specimens might be incorrect and the association between morphologically identified specimens and DNA barcodes might generate taxonomic inconsistencies, thus undermining one of the main premises of the DNA barcoding approach (Salvi et al., 2021). It is also possible that DNA barcoding analysis does not provide straightforward identifications of specimens because the query sequences do not match anything deposited in the reference library (Meier et al., 2006). Phylogenetic clusters of these unidentified specimens represent ‘unassigned’ molecular operational taxonomic units (MOTUs) and they might represent either known species that have not yet been sequenced (and therefore not represented in reference library) or species new to science that needs to be characterised and described. Therefore, for all those organisms with unclear morphological diagnosis, or extensive cryptic diversity, the DNA barcoding approach is particularly useful for the detection of taxonomic inconsistencies and for the identification of unassigned MOTUs that require in-depth taxonomic assessments.

Integrative taxonomy is an effective multidisciplinary approach for species identification that include several independent variables as morphology, multilocus phylogeny, biogeography, ecology and ethology (Dayrat, 2005; Padial et al., 2010). In this framework, molecular species delimitation methods applied to unassigned MOTUs are useful to propose primary species hypotheses (PSHs) (Puillandre et al., 2012). Subsequently, the integration of additional information from independent sets of characters (morphological, ecological, behavioural, biogeographical and so on) under an integrative taxonomy approach can be applied to determine conclusive secondary species hypotheses (SSHs) and validate the true species diversity (Pante et al., 2015; Puillandre et al., 2012; Will et al., 2005; Zamani et al., 2022).

MODEL ORGANISMS, AIMS AND OBJECTIVES OF THE THESIS

The aim of the PhD thesis is to combine DNA barcoding and integrative taxonomy to assess the taxonomic diversity of two selected animal groups for which morphological diagnosis can be extremely challenging. The two animal groups selected as study model are the flea beetles (tribe Alticini Newman, 1834) and the true oysters, that belong to the families Chrysomelidae Latreille, 1802 and Ostreidae Rafinesque, 1815, respectively.

Alticini is a tribe characterized by a high diversity with over 10,000 species and 601 genera (Douglas et al., 2023). The high species diversity is accompanied with a shared morphology with subtle differences differentiating several sympatric species, therefore taxonomic experts on regional faunas are often required for correct identification at species-level. Previous studies on Alticini combining taxonomic expertise and molecular approaches have proven the utility of DNA barcoding for fast and reliable specimen identifications (Coral Şahin et al., 2019; Salvi et al., 2020).

Ostreidae is a small bivalve family with 78 recognised species distributed worldwide (MolluscaBase, 2024). Despite the systematics of ostreid subfamilies is well established (Salvi & Mariottini, 2021), the diversity and distribution of living oysters are far from being fully understood (Guo et al., 2018; Lam & Morton, 2003; Li et al., 2017; Sekino & Yamashita, 2016). Most of the oyster species were described using morphological characters alone. However, taxonomic identification of most species is hampered by their phenotypic plasticity and the absence of reliable diagnostic characters. Several molecular studies have shown how shell morphology is both unreliable and misleading for oyster classification, with misidentifications that extend up to the subfamily level (Salvi et al., 2021; Wang et al., 2004). Misidentifications also propagate in public repositories of DNA sequences (e.g. GenBank), thus generating taxonomic inconsistencies when using DNA barcoding approach. For these reasons, museum collections represent a valuable source both for implementing a reliable DNA reference library based on vouchers and type specimens and for the assessment of the global oyster diversity.

The main aim of the PhD thesis is to combine DNA barcoding methods and integrative taxonomy approaches to assess species diversity in these two taxonomically challenging animal groups. To fulfil this goal, the thesis implemented the following objectives:

1. Perform a comprehensive sampling of target taxa across Italy and Spain (Apennine and Pyrenees ranges) for flea beetles, and at a global scale for oysters, based on both fieldwork and museum collections.
2. Generate a comprehensive DNA sequence dataset to implement reference libraries for the assessments of flea beetle and oyster diversity.
3. Assess the diversity and distribution of these two groups with a DNA barcoding approach.
4. Implement an integrative taxonomic pipeline to validate morphological similar species, resolve taxonomic uncertainties, and turn species discovery (based on DNA barcoding) into species description.

The experimental design follows the workflow depicted in Figure 1. The first step (A) consists of a typical DNA barcoding approach of the sequenced specimens, based on reference libraries improved by a large amount of new DNA sequences, and validated by expert taxonomists. In many cases, this step is enough to reach an unambiguous identification of the specimen at the species level (A1), but in some others is not (A2). In such cases, the pipeline proceeds through a second step, that consists of an integrative taxonomic assessment of these specimens to disentangle whether the 'unassigned' specimens belong to known species that are not represented in the reference library (B1) or to new (i.e. undescribed) species (B3).

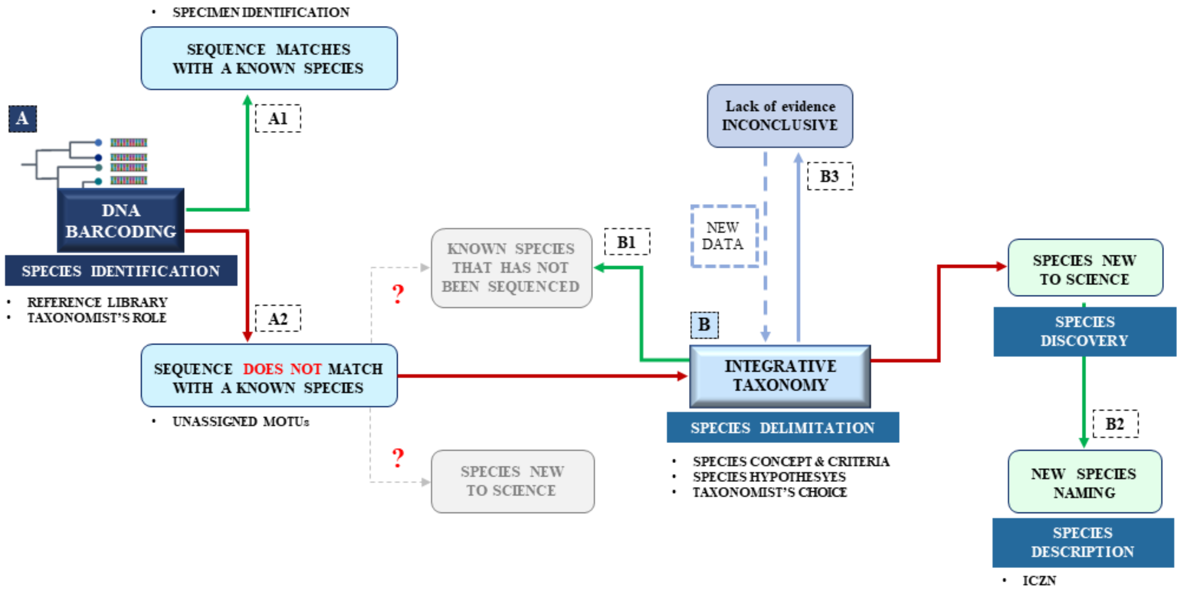

Figure 1. Pipeline of the PhD thesis. Step A refers to the DNA barcoding assessment. Green arrows bring to conclusive results. Red arrows indicate ongoing investigation. Result A1 indicates a correct specimen identification. Result A2 indicates a lack of information in reference library that is followed by two possible hypotheses (question marks). Step B refers to the integrative taxonomic assessment. Result B1 assigns molecular data to an extant species that has not been sequenced before. Result B2 indicates the description of a new species. Result B3 indicates a lack of evidence and the need of new data to reach a taxonomic conclusion.

Species identification based on a simple DNA barcoding approach (A) was possible mainly in flea beetles, an animal groups for which a robust taxonomic framework is available, both in terms of taxonomic knowledge and availability of DNA sequence data. On the other hand, animal groups characterised by poor taxonomy such as oysters, often required an integrative taxonomic step (B) to identify the source of the taxonomic uncertainty (for ambiguous matches) or to resolve unassigned MOTUs, either with an available species name (B1) or with a new species description (B2). The following four chapters of the thesis illustrate the DNA barcoding and integrative taxonomic approaches implemented for flea beetles (Part I) and for oysters (Part II) along with selected cases studies.

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Part I

ALTICINI AS STUDY MODEL

CHAPTER 1

DNA barcoding assessment of Alticini diversity across the Apennines (Coleoptera, Chrysomelidae)

Introduction

The Italian biodiversity is one of the richest in Europe with around 60,000 species, among which 8.6% are endemic (Biondi, 2006). The high species richness in a relatively small territory, about 300,000 km^2, is explained by various biogeographic factors such as: (i) its location at the center of the Mediterranean Sea, allowing exchanges with neighbouring peninsulas and the African continent; (ii) the presence of mountain ranges and latitudinal extension, determining high environmental and climatic heterogeneity; (iii) the complex paleogeographic and paleoclimatic history, strongly impacted by Quaternary climate changes. The two main mountain systems are the Alps and the Apennines which host a mosaic of different ecosystems of conservation interest. Such high number of species combined with the geographical and ecological heterogeneity of Italy poses significant challenges for biodiversity assessments.

In line with a general pattern, in Italy most of the studies and conservation efforts focus on vertebrates. However, insect diversity represents the 65% of the Italian species, and 12,000 species belongs to the order Coleoptera Linnaeus, 1758 (Ruffo & Stoch, 2006). The family Chrysomelidae Latreille, 1802 is a well-represented group in the Italian beetles which is characterized by species with low dispersal ability and selective plant feeding behaviours. These factors had a role on the rate of endemicity and speciation, and for these reasons, leaf beetles represent an interesting study system for evolutionary and biodiversity studies (Baselga et al., 2015; Gómez-Zurita et al., 2016). The tribe Alticini Newman, 1834 is one of the most represented groups within the family Chrysomelidae. There are 340 Alticini species in Italy belonging to 25 genera with a rate of endemicity of 11.8% (Biondi et al., 2013; Biondi, 2006). High diversity and limited number of diagnostic characters make the morphological identification of Alticini challenging and often requires taxonomists with expertise on fauna from specific regions. Previous studies on Alticini combining taxonomic expertise and molecular approaches have proven the utility of DNA barcoding for fast and reliable specimen identifications (Coral Şahin et al., 2019; Salvi et al., 2020).

In this study, we performed a comprehensive collection and DNA sequencing of Alticini along the Apennine chain, and we applied a DNA barcoding approach to assess their taxonomic diversity.

The aims of this study are (i) to assess whether public repositories of DNA sequences such as BOLD (Ratnasingham & Hebert, 2007) and GenBank (Sayers et al., 2021) provide a reliable reference library for the identification of Alticini from Italy , (ii) to produce the first well-annotated reference barcoding library of Apenine Alticini combining the taxonomic expertise of the specialist to solve ambiguous identifications retrieved from barcoding analysis and (iii) to identify taxonomic uncertainties that could be solved with integrative taxonomic and evolutionary studies.

Materials and methods

Sample collection and morphological identification

Specimens of Alticini were collected on their respective host plants by sweep net and the aid of aspirator from 2017 to 2022 during the PhD project of Dr. Emanuele Berrilli and further implemented during this PhD thesis. Sampling sites were numbered using serial numbers from northern to southern localities. The specimens were collected from 119 sampling sites among all the principal massifs, from Emilia-Romagna, northern part of Apennine, to the southernmost regions of the Apennine in Calabria and Sicily. The altitudinal range of the sites varies from 31m in the northern sector of the Apennine chain (Emilia-Romagna) up to 2624 m on Maiella Mountains (Abruzzo).

Collected specimens were immediately stored in 95% ethanol. Morphological identification at the species level was performed by a taxonomist specialist of this group Maurizio Biondi through the dissection and study of the genitalia, median lobe of the aedeagus for males and spermatheca for females. These highly reliable taxonomic identifications can be referred to “Level 1” *sensu* Steinke & Hanner (2011). Dissection of the specimens were carried out using a Leica M205C binocular microscope.

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted using a standard high-salt protocol (Sanbrook et al., 1989) using the non-invasive method described in Salvi et al. (2020). We amplified the *cox1* gene fragment with the primers Lon-LCO-F (5’-CTC AGC CAT TTT ACC GAA TAA ATG-3’) and LonHCO-R (5’-GGA TTT GGI ATA ATT TCY CATA TTG-3’) (Salvi, D’Alessandro, et al., 2019). PCR amplification protocol followed Salvi, Maura, et al. (2019). PCR products were purified and sequenced by an external service (Genewitz, Germany). The obtained chromatograms of each sequence were manually edited using Geneious Prime 11 (Biomatters Ltd., Auckland, New Zealand). The complete standard barcode region of the mitochondrial cytochrome c oxidase I (*cox1*) gene (658 bp) was obtained trimming the sequences using the primers LCO1490 and HCO2198 (Folmer et al., 1994). Sequences were submitted in BOLD and GenBank (GB) databases (IDs of BOLD and accession numbers of GenBank are requested).

Sequence dataset building

We built two non-redundant datasets of *cox1* sequences of Alticini. The first dataset (DT1) includes a total of 278 sequences of the specimens collected along Apennine chain and identified by the expert taxonomist. The second dataset (DT2) includes the DT1 and all the sequences of Alticini available in BOLD and in GenBank (data updated to 10/12/2023). We retrieved a total of 9505 sequences from BOLD database from the workbench section of the website with the following filters: (i) Taxonomy = alticini, (ii) Marker = COI-5P; (iii) Minimum sequence length = 300 bp. From these 9505 sequences we selected a total of 7612 sequences identified at species-level. We retrieved a total of 8573 sequences from GB using NCBI-nucleotide interface of Geneious Prime 11.0.18+10 (Biomatters Ltd., Auckland, New Zealand) with the keywords “("Alticini"[Organism] AND cytochrome[All Fields])”, including 162 complete, or partial, mitochondrial genomes. We didn’t use the specific keywords “alticini cytochrome c oxidase subunit I”, because GB improved the automatic feature annotation of metazoan *cox1* gene only in the last years (Sayers et al., 2021). We use the command *Map to Reference(s)* in Geneious to map all the GB sequences on the complete *cox1* gene of the well-annotated mitochondrial genome of *Psylliodes chlorophana* (MT644112). A total of 7697 sequences were obtained after trimming the mapped dataset using the primers LCO1490 and HCO2198 (Folmer et al., 1994). From these 7697 sequences we selected a total of 5922 sequences which have at least 300 bp and an identification at species-level. Because both BOLD and GB periodically mine sequences from each other, we dereplicate the merged dataset deleting sequences with identical GB accession number. We obtained a total of 6810 sequences from BOLD and GB in which are included 276 sequences that were already published in previous works by our lab (Berrilli, Biondi, D’Alessandro, et al., 2023; Berrilli, Biondi, Garzia, et al., 2023; Salvi, D’Alessandro, et al., 2019; Salvi et al., 2020). The final DT2 was composed by a total of 7088 sequences, including sequences of DT1. The DT2 was divided into 8 sub-datasets according to the seven most represented genera in BOLD (*Altica*, DT2_Alt; *Aphthona*, DT2_Aph; *Chaetocnema*, DT2_Chae; *Crepidodera*, DT2_Cre; *Longitarsus*, DT2_Lon; *Phyllotreta*, DT2_Phy; *Psylliodes*, DT2_Psy) and one with all the other genera (DT2_others). All the cases in which the gender of the specific epithets of GB or BOLD sequences were not correctly annotated, we edited the names following updated nomenclature.

Table 1. Number of sequences included in each of the eight sub-datasets of DT2.

Genus	**Sub-dataset**	**N. of sequences from DT1**	**N. of sequences from BOLD and GenBank**	**Total amount of sequences in DT2**
Altica	DT2_Alt	20	812	832
Aphthona	DT2_Aph	31	192	222
Chaetocnema	DT2_Chae	29	321	350
Crepidodera	DT2_Cre	8	447	455
Longitarsus	DT2_Lon	94	1605	1669
Phyllotreta	DT2_Phy	26	1099	1125
Psylliodes	DT2_Psy	20	938	958
Other genera	DT2_others	50	1396	1446

Molecular taxonomic assessment

For the taxonomic assessment of sequences we followed the pipeline described in (Salvi et al., 2020). All the following analyses were performed in Rstudio version 2023.9.0.463 (Posit team, 2023). The thresholds within barcoding gaps of DT1 and each DT2 sub-datasets were calculated using *localMinima* function in *spider* R package (Brown et al., 2012). We assessed the barcoding identification efficiency of each dataset using the *bestCloseMatch* analyses (BCM) (Brown et al., 2012) *sensu* Meier et al. (2006) and we identified the optimal threshold for each dataset according to the number of correct/incorrect/ambiguous identifications of each putative threshold. The BCM analysis flagged as “correct” identifications the sequences within the same cluster that bear the same taxonomic name. The BCM analysis flagged as “incorrect” identifications the sequences within the same cluster that bear different taxonomic names. The BCM analysis flagged as “ambiguous” the conspecific sequences that form distinct sister clusters. Finally, the BCM analysis flagged as “no ID” the unidentified sequences without conspecific sequence in dataset. Furthermore, we used the automated tool TAxCI (Rulik et al., 2017) to better explore the occurrence of taxonomic uncertainties within DT2. We combined the outputs of *BCCluster* (BCM clustering) and *tci* present in *TaxCI* R package (Rulik et al., 2017) (results not shown). In particular, the *tci* analyses can calculate the taxonomic consistency index (tci) that is an expression of “how polyphyletic” a species is in each tree ($0 \leq \text{tci} \leq 1$). The *tci* analyses can also highlight if there are more than one species in the same cluster, if the species is present in more than one cluster, and if the species has a low abundance in the cluster. The *tci* analysis is based on tree topology; we generated a Neighbour-Joining (NJ) tree for each dataset using the K2P model in MEGA11 (Tamura et al., 2021). Each case of taxonomic uncertainty in BOLD-GB sequences (misidentifications) detected by BCM and TAxCI analyses was resolved in a correct species identification based on the DT1 reference library, after a further check of all data associated to the reference voucher specimens (molecular data, metadata, geographical information and morphological data) (Salvi et al., 2020). We listed all the BOLD-GB misidentifications with the correct nomenclature in supplementary Table S1.

Diversity patterns

To illustrate the main patterns of species diversity along the Apennine chain and along the altitudinal gradient, we used QGIS v3.28.13 software (QGIS Development Team, 2022). We mapped the 119 sampling localities along Apennine chain and we defined four main Apennine sectors according to the CKmap project for the peninsular and insular Italy (Stoch & Vigna Taglianti, 2006): Northen, Central, Southern and Sicilian. We retrieved the number of specimens and species collected in each sector using the command *intersect* in QGIS. We used Rstudio to produce the bar plots using the R package *ggplot2* (Wickham, 2016) to show the distribution of species in each sector and along altitudinal ranges.

Results

Taxonomic assessment

The DT1 reference library includes 99 species, unevenly distributed among 21 genera, and represented by 1 to 12 sequences (average number of sequences for morphospecies: 5). The most represented genera within the library are, *Longitarsus*, *Psylliodes*, *Phyllotreta*, *Aphthona*, and *Chaetocnema*. The number of species for each genus in the final library is reported in Figure 1. The DT2 includes 547 species in 101 genera, represented by 1 to 593 sequences (average number of sequences for species: 18). The number of sequences distributed among genera in DT1 and in each DT2 sub-dataset are reported in Table 2.

Table 2. Number of genera and species in DT1 and each DT2 sub-datasets. Minimum, maximum, median and mean of specimens per species.

Datasets	**Genera**	**Species**	**Min**	**Max**	**Median**	**Mean**
DT1	21	99	1	12	2	2
DT2_Alt	1	37	1	196	6	22
DT2_Aph	1	28	1	29	2	8
DT2_Chae	1	33	1	77	5	11
DT2_Cre	1	17	2	111	9	27
DT2_Lon	1	85	1	202	6	20
DT2_Phy	1	35	1	593	9	32
DT2_Psy	1	65	1	138	2	15
DT2_others	94	246	1	68	2	6

The barcoding identification efficiency (*i.e.* percentage of correct identifications) of the DT2 library, based on BCM analysis and applied to each DT2 sub-datasets is reported in Table 3. The average proportion of correct sequence identifications across the eight DT2 sub-datasets is 86% +/- 11.8. Correct identifications concern 86 species (87%) among 19 genera (90%).

Within the BOLD-GB dataset, we identified 684 sequences with a wrong taxonomic identification. These sequences belong to 25 species within 7 genera: *Altica*, *Aphthona*, *Chaetocnema*, *Longitarsus*, *Psylliodes*, *Derocrepis*, and *Neocrepidodera*. The highest number of misidentifications concern *Altica brevicollis* which is been misidentified with 11 different species. The second highest number of misidentifications regards the species *Longitarsus pratensis* and *Longitarsus luridus.* The complete list of the sequences with a wrong taxonomic identification is reported in the supplementary Table S1.

Table 3. BCM output for each sub-dataset of DT2 (BOLD + GenBank + sequences generated in our study). In bold, the percentage of correct identification of our sequences within each DT2 sub-dateset.

Dataset	correct	incorrect	ambiguous	unissigned (no ID)	% correct	Optimal Threashold
DT2_Alt	554	39	233	2	66.9	2.0%
DT2_Alt_ours	13	2	1	0	**81.3**	
DT2_Aph	207	4	4	9	92.4	6.9%
DT2_Aph_ours	30	1	0	0	**96.8**	
DT2_Chae	218	1	0	5	97.3	6.0%
DT2_Chae_ours	28	1	0	0	**96.6**	
DT2_Cre	400	8	45	2	87.9	4.4%
DT2_Cre_ours	7	1	0	0	**87.5**	
DT2_Lon	1447	24	224	10	84.9	5.2%
DT2_Lon_ours	85	6	4	1	**88.5**	
DT2_Phy	803	14	302	6	71.4	7.1%
DT2_Phy_ours	22	0	3	1	**84.6**	
DT2_Psy	892	25	17	24	93.1	4.3%
DT2_Psy_ours	12	5	0	3	**60.0**	
DT2_others	1300	21	9	120	89.7	5.5%
DT2_others_ours	48	1	0	3	**92.3**	

Table 4. List of 278 specimens included in the final barcoding library of Apennine Alticini. For each specimen is reported voucher code, taxonomic identification, coordinates, locality code, altitude, and Apennine sector.

Voucher	Species	Latitude	Longitude	Locality code	Altitudine (m)	Sector
106A	*Altica brevicollis*	42°32'10.9"N	13°27'14.9"E	27	862	C-Ap
106B	*Altica brevicollis*	42°32'10.9"N	13°27'14.9"E	27	862	C-Ap
90A	*Altica brevicollis*	42°29'51.3"N	13°23'12.5"E	33	1133	C-Ap
307A	*Altica brevicollis*	42°09'34.4"N	14°02'58.6"E	64	637	C-Ap
189A	*Altica oleracea*	42°8.538'N	13°44.606'E	65	476	C-Ap
562A	*Altica oleracea*	39°55'51.6"N	16°09'45.2"E	92	1665	S-Ap
130B	*Altica oleracea*	44°27'12"N	11°17'08"E	9	65	N-Ap
130A	*Altica oleracea*	44°27'12"N	11°17'08"E	9	65	N-Ap
117A	*Altica oleracea*	44°30'01"N	11°23'10"E	5	49	N-Ap
117B	*Altica oleracea*	44°30'01"N	11°23'10"E	5	49	N-Ap
135A	*Altica oleracea*	44°10'25"N	11°28'54"E	11	285	N-Ap
135B	*Altica oleracea*	44°10'25"N	11°28'54"E	11	285	N-Ap
88A	*Altica tamaricis*	42°46.97'N	13°45.89'E	18	103	C-Ap
88B	*Altica tamaricis*	42°46.97'N	13°45.89'E	18	103	C-Ap
554A	*Altica tamaricis*	40°35'58.2"N	16°12'06.6"E	85	210	S-Ap

554B	*Altica tamaricis*	40°35'58.2"N	16°12'06.6"E	85	210	S-Ap
308A	*Aphthona cyparissiae*	42°10'45.1"N	13°24'18.1"E	53	1826	C-Ap
243A	*Aphthona euphorbiae*	42°10'31.7"N	13°48'15.7"E	56	431	C-Ap
243B	*Aphthona euphorbiae*	42°10'31.7"N	13°48'15.7"E	56	431	C-Ap
520A	*Aphthona euphorbiae*	40°31'00.8"N	16°07'28.4"E	87	914	S-Ap
520B	*Aphthona euphorbiae*	40°31'00.8"N	16°07'28.4"E	87	914	S-Ap
528A	*Aphthona euphorbiae*	39°09'13.0"N	16°45'27.1"E	97	888	S-Ap
528B	*Aphthona euphorbiae*	39°09'13.0"N	16°45'27.1"E	97	888	S-Ap
518A	*Aphthona euphorbiae*	40°59'55.5"N	14°40'24.2"E	84	715	S-Ap
549A	*Aphthona euphorbiae*	42°09'51.0"N	13°18'45.2"E	63	800	C-Ap
686001A	*Aphthona euphorbiae*	37°55'36.2"N	14°40'59.2"E	113	1697	Sicilian
534A	*Aphthona euphorbiae*	37°58'22.8"N	15°03'04.5"E	110	1238	Sicilian
534B	*Aphthona euphorbiae*	37°58'22.8"N	15°03'04.5"E	110	1238	Sicilian
129A	*Aphthona flava*	44°27'12"N	11°17'08"E	9	65	N-Ap
305B	*Aphthona herbigrada*	42°20'14.7"N	13°36'55.9"E	45	1293	C-Ap
305A	*Aphthona herbigrada*	42°20'14.7"N	13°36'55.9"E	45	1293	C-Ap
126A	*Aphthona herbigrada*	44°10'22"N	11°28'48"E	12	340	N-Ap
126B	*Aphthona herbigrada*	44°10'22"N	11°28'48"E	12	340	N-Ap
306A	*Aphthona illigeri*	42°20'14.7"N	13°36'55.9"E	45	1293	C-Ap
306B	*Aphthona illigeri*	42°20'14.7"N	13°36'55.9"E	45	1293	C-Ap
283A	*Aphthona lutescens*	42°40'21.8"N	13°29'22.1"E	21	858	C-Ap
283B	*Aphthona lutescens*	42°40'21.8"N	13°29'22.1"E	21	858	C-Ap
40A	*Aphthona lutescens*	42°46.97'N	13°45'89'E	19	103	C-Ap
40B	*Aphthona lutescens*	42°46.97'N	13°45'89'E	19	103	C-Ap
40C	*Aphthona lutescens*	42°46.97'N	13°45'89'E	19	103	C-Ap
527001A	*Aphthona nigriceps*	39°44'38.0"N	16°06'29.8"E	95	959	S-Ap
527001B	*Aphthona nigriceps*	39°44'38.0"N	16°06'29.8"E	95	959	S-Ap
278001A	*Aphthona venustula*	42°40'20.3"N	13°27'57.3"E	22	1356	C-Ap
517A	*Aphthona venustula*	41°22'31.6"N	14°30'59.7"E	80	1340	C-Ap
103A	*Aphthona venustula*	42°10'30"N	13°19'19"E	57	1300-1500	C-Ap
535A	*Aphthona venustula*	37°58'22.8"N	15°03'04.5"E	110	1238	Sicilian
535B	*Aphthona venustula*	37°58'22.8"N	15°03'04.5"E	110	1238	Sicilian
258001A	*Chaetocnema concinna*	42°31'12.2"N	13°22'08.4"E	30	1322	C-Ap
258001B	*Chaetocnema concinna*	42°31'12.2"N	13°22'08.4"E	30	1322	C-Ap
249A	*Chaetocnema concinna*	42°10'39.7"N	13°46'06.9"E	54	722	C-Ap
249B	*Chaetocnema concinna*	42°10'39.7"N	13°46'06.9"E	54	722	C-Ap
287001A	*Chaetocnema concinna*	42°38'33.5"N	13°28'20.9"E	25	974	C-Ap
209B	*Chaetocnema concinna*	42°12'50.8"N	12°58'03.6"E	49	545	C-Ap
209A	*Chaetocnema concinna*	42°12'50.8"N	12°58'03.6"E	49	545	C-Ap
132A	*Chaetocnema conducta*	44°27'12"N	11°17'08"E	9	65	N-Ap
132B	*Chaetocnema conducta*	44°27'12"N	11°17'08"E	9	65	N-Ap
124A	*Chaetocnema conducta*	44°28'53"N	11°18'29"E	7	155	N-Ap
120B	*Chaetocnema conducta*	44°32'18"N	11°19'55"E	2	31	N-Ap
61A	*Chaetocnema hortensis*	42°26'5.88"N	13°36'47.82"E	37	1669	C-Ap
454A	*Chaetocnema hortensis*	42°06'08.7"N	14°06'42.2"E	69	2556	C-Ap
454B	*Chaetocnema hortensis*	42°06'08.7"N	14°06'42.2"E	69	2556	C-Ap
604A	*Chaetocnema hortensis*	39°22'30.3"N	16°41'49.0"E	96	1206	S-Ap
612B	*Chaetocnema hortensis*	38°32'07.7"N	16°18'19.2"E	99	1035	S-Ap

612A	*Chaetocnema hortensis*	38°32'07.7"N	16°18'19.2"E	99	1035	S-Ap
119A	*Chaetocnema hortensis*	44°32'18"N	11°19'55"E	2	31	N-Ap
581B	*Chaetocnema hortensis*	41°25'22.6"N	14°22'52.4"E	77	1012	C-Ap
637A	*Chaetocnema hortensis*	37°53'01.6"N	13°23'51.1"E	121	810	Sicilian
626A	*Chaetocnema montenegrina*	37°56'31.6"N	14°40'16.3"E	112	1450	Sicilian
626B	*Chaetocnema montenegrina*	37°56'31.6"N	14°40'16.3"E	112	1450	Sicilian
246A	*Chaetocnema obesa*	42°10'39.7"N	13°46'06.9"E	54	722	C-Ap
246B	*Chaetocnema obesa*	42°10'39.7"N	13°46'06.9"E	54	722	C-Ap
92A	*Chaetocnema semicoerulea*	42°46.97'N	13°45.89'E	18	103	C-Ap
92B	*Chaetocnema semicoerulea*	42°46.97'N	13°45.89'E	18	103	C-Ap
621A	*Chaetocnema subcoerulea*	37°52'12.4"N	14°40'41.1"E	123	1262	Sicilian
621B	*Chaetocnema subcoerulea*	37°52'12.4"N	14°40'41.1"E	123	1262	Sicilian
237A	*Chaetocnema tibialis*	42°25'51"N	13°17'16"E	38	694	C-Ap
278A	*Crepidodera aurata*	42°40'20.3"N	13°27'57.3"E	22	1356	C-Ap
89A	*Crepidodera aurea*	42°29'51.3"N	13°19'43.2"E	34	1133	C-Ap
225D	*Crepidodera aurata*	44°27’19’’N	11°17’09’’E	8	66	N-Ap
225C	*Crepidodera aurea*	44°27’19’’N	11°17’09’’E	8	66	N-Ap
225A	*Crepidodera aurea*	44°27’19’’N	11°17’09’’E	8	66	N-Ap
225B	*Crepidodera aurea*	44°27’19’’N	11°17’09’’E	8	66	N-Ap
247A	*Crepidodera pluta*	42°10'39.7"N	13°46'06.9"E	54	722	C-Ap
247B	*Crepidodera pluta*	42°10'39.7"N	13°46'06.9"E	54	722	C-Ap
46A	*Longitarsus aeneicollis*	42°30’26.40’'N	13°33'14.46’’E	31	1344	C-Ap
491A	*Longitarsus aeneicollis*	40°30'31.4"N	16°07'01.7"E	89	1055	S-Ap
654A	*Longitarsus aeneicollis*	39°22'30.3"N	16°41'49.0"E	96	1206	S-Ap
661A	*Longitarsus aeneicollis*	38°25'10.3"N	16°10'36.0"E	101	955	S-Ap
503A	*Longitarsus aeneicollis*	37°58'22.8"N	15°03'04.5"E	110	1238	Sicilian
507A	*Longitarsus aeneicollis*	37°54'36.9"N	13°59'25.2"E	115	861	Sicilian
294A	*Longitarsus aenicollis*	42°10'36.9"N	13°23'42.2"E	55	2117	C-Ap
694A	*Longitarsus albineus*	37°54'57.1"N	13°58'06.0"E	114	786	Sicilian
185E	*Longitarsus ballotae*	42°8.538'N	13°44.606'E	65	445	C-Ap
185B	*Longitarsus ballotae*	42°8.538'N	13°44.606'E	65	445	C-Ap
649A	*Longitarsus cerinthes*	39°55'51.6"N	16°09'45.2"E	92	1665	S-Ap
208A	*Longitarsus codinai*	42°12'50.8"N	12°58'03.6"E	49	545	C-Ap
683A	*Longitarsus codinai*	37°52'12.4"N	14°40'41.1"E	123	1262	Sicilian
629A	*Longitarsus codinai*	37°54'10.1"N	14°04'11.6"E	116	1245	Sicilian
671002A	*Longitarsus codinai*	38°11'55.9"N	15°29'31.4"E	104	682	Sicilian
696A	*Longitarsus codinai*	37°52'47.5"N	14°01'46.3"E	122	1635	Sicilian
253A	*Longitarsus corynthius*	42°23'58.25"N	13°18'19.96"E	42	669	C-Ap
229A	*Longitarsus echii*	42°23'58.25"N	13°18'19.96"E	42	669	C-Ap
664A	*Longitarsus exsoletus*	38°20'54.9"N	16°09'00.6"E	102	946	S-Ap
504A	*Longitarsus exsoletus*	37°58'22.8"N	15°03'04.5"E	110	1238	Sicilian
500A	*Longitarsus exsoletus*	38°11'55.9"N	15°29'31.4"E	104	682	Sicilian
500B	*Longitarsus exsoletus*	38°11'55.9"N	15°29'31.4"E	104	682	Sicilian
701A	*Longitarsus exsoletus*	37°53'44.8"N	14°04'01.0"E	118	1395	Sicilian

14A	*Longitarsus fallax*	42°44.51’N	13°43.97’E	19	191	C-Ap
43A	*Longitarsus foudrasi*	42°25'01.4"N	13°11'58.2"E	40	1126	C-Ap
660A	*Longitarsus foudrasi*	39°09'06.1"N	16°44'01.3"E	98	746	S-Ap
502A	*Longitarsus foudrasi*	38°02'32.0"N	15°21'31.7"E	107	495	Sicilian
502B	*Longitarsus foudrasi*	38°02'32.0"N	15°21'31.7"E	107	495	Sicilian
821A	*Longitarsus ganglbaueri*	44°13'03.1"N	10°41'22.0"E	10	1289	N-Ap
286A	*Longitarsus ganglbaueri*	42°39'59.17"N	13°29'28.56"E	23	889	C-Ap
820B	*Longitarsus gracilis*	44°07'18.7"N	10°48'55.0"E	15	1641	N-Ap
820A	*Longitarsus gracilis*	44°07'18.7"N	10°48'55.0"E	15	1641	N-Ap
273A	*Longitarsus gracilis*	42°25'02.5"N	13°45'43.9"E	39	1525	C-Ap
273B	*Longitarsus gracilis*	42°25'02.5"N	13°45'43.9"E	39	1525	C-Ap
404A	*Longitarsus gracilis*	42°10'01.5"N	13°24'07.1"E	62	2198	C-Ap
440A	*Longitarsus gracilis*	42°28'26.2"N	12°59'52.2"E	35	2168	C-Ap
439A	*Longitarsus gracilis*	42°28'22.1"N	12°59'51.2"E	36	2188	C-Ap
262A	*Longitarsus juncicola*	42°33'34.3"N	13°20'41.4"E	26	1315	C-Ap
484A	*Longitarsus juncicola*	41°25'22.6"N	14°22'52.4"E	77	1012	C-Ap
505A	*Longitarsus lateripunctatus*	37°52'12.4"N	14°40'41.1"E	123	1262	Sicilian
12A	*Longitarsus linnaei*	42°19'22"N	13°28'24"E	47	584	C-Ap
12B	*Longitarsus linnaei*	42°19'22"N	13°28'24"E	47	584	C-Ap
60A	*Longitarsus luridus*	42°26'5.88"N	13°36'47.82"E	37	1669	C-Ap
287B	*Longitarsus luridus*	42°38'33.5"N	13°28'20.9"E	25	974	C-Ap
646A	*Longitarsus luridus*	40°29'40.3"N	16°06'49.0"E	91	1109	S-Ap
655A	*Longitarsus luridus*	39°22'30.3"N	16°41'49.0"E	96	1206	S-Ap
486A	*Longitarsus luridus*	41°08'04.8"N	14°34'08.2"E	83	1090	S-Ap
123A	*Longitarsus luridus*	44°28'53"N	11°18'29"E	7	155	N-Ap
123B	*Longitarsus luridus*	44°28'53"N	11°18'29"E	7	155	N-Ap
683001A	*Longitarsus luridus*	37°52'12.4"N	14°40'41.1"E	123	1262	Sicilian
683002A	*Longitarsus luridus*	37°52'12.4"N	14°40'41.1"E	123	1262	Sicilian
501A	*Longitarsus luridus*	38°02'06.1"N	15°21'54.9"E	108	377	Sicilian
513A	*Longitarsus luridus*	37°53'04.1"N	13°23'50.8"E	120	801	Sicilian
128B	*Longitarsus luridus*	44°10'22"N	11°28'48"E	12	340	N-Ap
44A	*Longitarsus lycopi*	42°29’52.56’’N	13°23'12.72’’E	32	1118	C-Ap
483A	*Longitarsus lycopi*	41°24'25.5"N	14°24'22.4"E	79	1011	C-Ap
128A	*Longitarsus membranaceus*	44°10'22"N	11°28'48"E	12	340	N-Ap
298A	*Longitarsus minimus*	42°10'36.9"N	13°23'42.2"E	55	2117	C-Ap
298B	*Longitarsus minimus*	42°10'36.9"N	13°23'42.2"E	55	2117	C-Ap
408A	*Longitarsus monticola*	41°41'20.0"N	13°56'15.5"E	72	2217	C-Ap
186A	*Longitarsus niger*	42°8.538'N	13°44.606'E	65	446	C-Ap
498A	*Longitarsus niger*	39°22'30.3"N	16°41'49.0"E	96	1206	S-Ap
506A	*Longitarsus niger*	37°54'36.9"N	13°59'25.2"E	115	861	Sicilian
476A	*Longitarsus nigrofasciatus*	41°26'01.0"N	14°25'57.5"E	76	1632	C-Ap
499A	*Longitarsus obliteratoides*	38°09'56.3"N	15°46'49.0"E	106	549	S-Ap
676A	*Longitarsus obliteratoides*	38°02'06.1"N	15°21'54.9"E	108	377	Sicilian

820001A	*Longitarsus obliteratus*	44°07'18.7"N	10°48'55.0"E	15	1641	N-Ap
57A	*Longitarsus obliteratus*	42°19'29.91"N	13°20'24.87"E	46	1011	C-Ap
210A	*Longitarsus obliteratus*	42°14'27.8"N	12°58'59.6"E	48	1063	C-Ap
185A	*Longitarsus ordinatus*	42°8.538'N	13°44.606'E	65	445	C-Ap
230A	*Longitarsus parvulus*	42°25'51"N	13°17'16"E	38	694	C-Ap
230B	*Longitarsus parvulus*	42°25'51"N	13°17'16"E	38	694	C-Ap
510A	*Longitarsus parvulus*	37°53'04.1"N	13°23'50.8"E	120	801	Sicilian
494A	*Longitarsus pellucidus*	40°29'56.3"N	16°06'54.9"E	90	1133	S-Ap
653001A	*Longitarsus pellucidus*	39°44'38.0"N	16°06'29.8"E	95	959	S-Ap
630A	*Longitarsus pellucidus*	37°54'02.2"N	14°04'08.3"E	117	1245	Sicilian
163A	*Longitarsus pinguis*	42°10'08"N	13°32'02"E	60	1689	C-Ap
36A	*Longitarsus pratensis*	42°25'11.6"N	13°17'50.0"E	78	690	C-Ap
125A	*Longitarsus pratensis*	44°28'53"N	11°18'29"E	7	155	N-Ap
125E	*Longitarsus pratensis*	44°28'53"N	11°18'29"E	7	155	N-Ap
121A	*Longitarsus pratensis*	44°30'27"N	11°23'10"E	4	45	N-Ap
121B	*Longitarsus pratensis*	44°30'27"N	11°23'10"E	4	45	N-Ap
211B	*Longitarsus pratensis*	42°14'27.8"N	12°58'59.6"E	48	1063	C-Ap
31A	*Longitarsus strigicollis*	42°29'51.3"N	13°23'12.5"E	33	1133	C-Ap
51B	*Longitarsus succineus*	42°31’24.18’"N	13°29'34.62’’E	29	1370	C-Ap
51A	*Longitarsus succineus*	42°31’24.18’"N	13°29'34.62’’E	29	1370	C-Ap
487A	*Longitarsus succineus*	40°30'39.6"N	16°07'12.9"E	88	1003	S-Ap
653001B	*Longitarsus succineus*	39°44'38.0"N	16°06'29.8"E	95	959	S-Ap
662A	*Longitarsus succineus*	38°25'25.5"N	16°09'41.2"E	100	940	S-Ap
683B	*Longitarsus succineus*	37°52'12.4"N	14°40'41.1"E	123	1262	Sicilian
681001A	*Longitarsus succineus*	39°44'38.0"N	16°06'29.8"E	109	1151	Sicilian
672A	*Longitarsus succineus*	38°10'56.8"N	15°28'34.1"E	105	838	Sicilian
508A	*Longitarsus succineus*	37°54'36.9"N	13°59'25.2"E	115	861	Sicilian
160A	*Longitarsus tabidus*	42°10'02"N	13°31'33"E	61	1499	C-Ap
652A	*Longitarsus tabidus*	39°44'38.0"N	16°06'29.8"E	95	959	S-Ap
287A	*Longitarsus zangherii*	42°38'33.5"N	13°28'20.9"E	25	974	C-Ap
234A	*Phyllotreta atra*	42°25'51"N	13°17'16"E	38	694	C-Ap
616A	*Phyllotreta cruciferae*	38°02'06.1"N	15°21'54.9"E	108	377	Sicilian
445A	*Phyllotreta ganglbaueri*	42°10'22.3"N	13°18'53.4"E	58	1201	C-Ap
445B	*Phyllotreta ganglbaueri*	42°10'22.3"N	13°18'53.4"E	58	1201	C-Ap
611A	*Phyllotreta nemorum*	38°32'07.7"N	16°18'19.2"E	99	1035	S-Ap
820003A	*Phyllotreta nigripes*	44°07'18.7"N	10°48'55.0"E	15	1641	N-Ap
184A	*Phyllotreta nigripes*	42°11.60'N	13°40.50'E	50	636	C-Ap
274A	*Phyllotreta nigripes*	42°25'02.5"N	13°45'43.9"E	39	1525	C-Ap
595A	*Phyllotreta nigripes*	39°54'15.8"N	16°10'55.7"E	93	2128	S-Ap
595B	*Phyllotreta nigripes*	39°54'15.8"N	16°10'55.7"E	93	2128	S-Ap
449A	*Phyllotreta nigripes*	41°26'59.8"N	14°22'19.9"E	73	2047	C-Ap
618A	*Phyllotreta nigripes*	37°58'19.3"N	15°03'14.4"E	111	1248	Sicilian
112A	*Phyllotreta ochripes*	42°48.17'N	13°40.51'E	17	317	C-Ap
112B	*Phyllotreta ochripes*	42°48.17'N	13°40.51'E	17	317	C-Ap
191A	*Phyllotreta procera*	42°8.538'N	13°44.606'E	65	476	C-Ap
191B	*Phyllotreta procera*	42°8.538'N	13°44.606'E	65	476	C-Ap
194A	*Phyllotreta punctulata*	42°10'45.76"N	13°49'13.35"E	52	265	C-Ap
450A	*Phyllotreta punctulata*	42°06'37.0"N	14°06'52.7"E	68	2624	C-Ap

278002A	*Phyllotreta punctulata*	42°40'20.3"N	13°27'57.3"E	22	1356	C-Ap
710B	*Phyllotreta punctulata*	41°26'03.4"N	14°25'25.9"E	74	1857	C-Ap
579A	*Phyllotreta punctulata*	41°26'03.1"N	14°25'17.8"E	75	1923	C-Ap
579B	*Phyllotreta punctulata*	41°26'03.1"N	14°25'17.8"E	75	1923	C-Ap
263A	*Phyllotreta undulata*	42°33'34.3"N	13°20'41.4"E	26	1315	C-Ap
263B	*Phyllotreta undulata*	42°33'34.3"N	13°20'41.4"E	26	1315	C-Ap
580A	*Phyllotreta undulata*	41°25'22.6"N	14°22'52.4"E	77	1012	C-Ap
580B	*Phyllotreta undulata*	41°25'22.6"N	14°22'52.4"E	77	1012	C-Ap
753A	*Psylliodes algirica*	37°54'36.9"N	13°59'25.2"E	115	861	Sicilian
670A	*Psylliodes algirica*	38°13'01.2"N	15°30'40.2"E	103	468	Sicilian
670B	*Psylliodes algirica*	38°13'01.2"N	15°30'40.2"E	103	468	Sicilian
751A	*Psylliodes ausoniae*	37°52'12.4"N	14°40'41.1"E	123	1262	Sicilian
710E	*Psylliodes brisouti*	41°26'03.4"N	14°25'25.9"E	74	1857	C-Ap
436A	*Psylliodes chalcomera*	41°10'54.6"N	14°37'46.2"E	82	803	S-Ap
724A	*Psylliodes chalcomera*	42°09'51.0"N	13°18'45.2"E	63	800	C-Ap
724B	*Psylliodes chalcomera*	42°09'51.0"N	13°18'45.2"E	63	800	C-Ap
192A	*Psylliodes chrysocephala*	42°10'45.76"N	13°49'13.35"E	52	265	C-Ap
740A	*Psylliodes heikertingeri*	37°53'06.9"N	13°23'50.0"E	119	786	Sicilian
244A	*Psylliodes instabilis*	42°10'31.7"N	13°48'15.7"E	56	431	C-Ap
796A	*Psylliodes isatidis*	43°35'46.0"N	12°13'25.3"E	16	1173	C-Ap
555A	*Psylliodes laevifrons*	40°31'00.8"N	16°07'28.4"E	87	914	S-Ap
751E	*Psylliodes laevifrons*	37°52'12.4"N	14°40'41.1"E	123	1262	Sicilian
568A	*Psylliodes laevifrons*	37°58'22.8"N	15°03'04.5"E	110	1238	Sicilian
561E	*Psylliodes libertii*	38°09'56.3"N	15°46'49.0"E	106	549	S-Ap
561F	*Psylliodes libertii*	38°09'56.3"N	15°46'49.0"E	106	549	S-Ap
751F	*Psylliodes luteola*	37°52'12.4"N	14°40'41.1"E	123	1262	Sicilian
64A	*Psylliodes napi*	42°26'5.88"N	13°36'47.82"E	37	1669	C-Ap
240A	*Psylliodes obscuroaenea*	42°10'31.7"N	13°48'15.7"E	56	431	C-Ap
708A	*Apteropoda orbiculata*	40°32'46.5"N	16°05'45.2"E	86	484	S-Ap
516A	*Arrhenocoela lineata*	44°07'22"N	10°15'08"E	14	1073	N-Ap
250A	*Batophila aerata*	42°10'46.9"N	13°45'24.8"E	51	1013	C-Ap
250B	*Batophila aerata*	42°10'46.9"N	13°45'24.8"E	51	1013	C-Ap
284A	*Batophila aerata*	42°40'21.8"N	13°29'22.1"E	21	858	C-Ap
284B	*Batophila aerata*	42°40'21.8"N	13°29'22.1"E	21	858	C-Ap
111A	*Derocrepis sodalis*	42°32'10.9"N	13°27'14.9"E	27	862	C-Ap
111B	*Derocrepis sodalis*	42°32'10.9"N	13°27'14.9"E	27	862	C-Ap
300A	*Derocrepis sodalis*	42°07'48"N	13°33'38"E	66	1400	C-Ap
432A	*Derocrepis sodalis*	44°07'26.2"N	10°14'57.0"E	13	1143	N-Ap
432B	*Derocrepis sodalis*	44°07'26.2"N	10°14'57.0"E	13	1143	N-Ap
941A	*Dibolia alpestris*	42°07'12.9"N	14°09'25.4"E	67	2043	C-Ap
941B	*Dibolia alpestris*	42°07'12.9"N	14°09'25.4"E	67	2043	C-Ap
313A	*Dibolia cryptocephala*	42°24'53"N	13°45'53"E	41	1497	C-Ap
183A	*Dibolia femoralis_erythrogaster*	42°11.60'N	13°40.50'E	50	635	C-Ap
171A	*Dibolia femoralis_erythrogaster*	42°20'40"N	13°26'28"E	44	758	C-Ap
171B	*Dibolia femoralis_erythrogaster*	42°20'40"N	13°26'28"E	44	758	C-Ap

195A	*Dibolia occultans*	42°10'45.76"N	13°49'13.35"E	52	265	C-Ap
266A	*Dibolia occultans*	42°33'34.3"N	13°20'41.4"E	26	1315	C-Ap
942A	*Dibolia timida*	42°22'0.8"N	13°43'22"E	43	1350	C-Ap
600A	*Epitrix atropae*	39°45'27.0"N	16°04'29.3"E	94	1373	S-Ap
600B	*Epitrix atropae*	39°45'27.0"N	16°04'29.3"E	94	1373	S-Ap
86A	*Hermaeophaga cicatrix*	42°44.06'N	13°38.83'E	20	563	C-Ap
86B	*Hermaeophaga cicatrix*	42°44.06'N	13°38.83'E	20	563	C-Ap
584A	*Hermaeophaga cicatrix*	41°10'54.6"N	14°37'46.2"E	82	803	S-Ap
796001A	*Hermaeophaga mercurialis*	43°35'46.0"N	12°13'25.3"E	16	1173	C-Ap
105A	*Hippuriphila modeeri*	42°29'51.3"N	13°23'12.5"E	33	1133	C-Ap
261A	*Hippuriphila modeeri*	42°33'34.3"N	13°20'41.4"E	26	1315	C-Ap
299A	*Hippuriphila modeeri*	42°31'39"N	13°20'40"E	28	1260	C-Ap
279A	*Mniophila muscorum*	42°39'45.6"N	13°27'46.8"E	24	1412	C-Ap
279B	*Mniophila muscorum*	42°39'45.6"N	13°27'46.8"E	24	1412	C-Ap
296A	*Neocrepidodera corpulenta*	42°10'36.9"N	13°23'42.2"E	55	2117	C-Ap
296B	*Neocrepidodera corpulenta*	42°10'36.9"N	13°23'42.2"E	55	2117	C-Ap
100A	*Neocrepidodera ferruginea*	42°25'01.4"N	13°11'58.2"E	41	1126	C-Ap
602B	*Neocrepidodera ferruginea*	39°22'30.3"N	16°41'49.0"E	96	1206	S-Ap
602A	*Neocrepidodera ferruginea*	39°22'30.3"N	16°41'49.0"E	96	1206	S-Ap
609A	*Neocrepidodera ferruginea*	39°09'06.1"N	16°44'01.3"E	98	746	S-Ap
631A	*Neocrepidodera impressa*	37°53'04.1"N	13°23'50.8"E	120	801	Sicilian
583A	*Neocrepidodera transversa*	41°21'25.8"N	14°34'52.4"E	81	1104	S-Ap
628A	*Ochrosis ventralis*	37°54'36.9"N	13°59'25.2"E	115	861	Sicilian
442A	*Orestia kraatzi*	42°10'14.1"N	13°18'40.6"E	59	1034-1116	C-Ap
99A	*Podagrica malvae*	42°48.17'N	13°40.51'E	17	317	C-Ap
99B	*Podagrica malvae*	42°48.17'N	13°40.51'E	17	317	C-Ap
136B	*Podagrica malvae*	44°29'47"N	11°23'08"E	6	51	N-Ap
136A	*Podagrica malvae*	44°29'47"N	11°23'08"E	6	51	N-Ap
599A	*Sphaeroderma rubidum*	39°45'27.0"N	16°04'29.3"E	94	1373	S-Ap
608B	*Sphaeroderma rubidum*	39°09'06.1"N	16°44'01.3"E	98	746	S-Ap
608A	*Sphaeroderma rubidum*	39°09'06.1"N	16°44'01.3"E	98	746	S-Ap
131A	*Sphaeroderma rubidum*	44°27'12"N	11°17'08"E	9	65	N-Ap
565A	*Sphaeroderma testaceum*	38°09'56.3"N	15°46'49.0"E	106	549	S-Ap
585B	*Sphaeroderma testaceum*	41°08'04.8"N	14°34'08.2"E	83	1090	S-Ap
585A	*Sphaeroderma testaceum*	41°08'04.8"N	14°34'08.2"E	83	1090	S-Ap

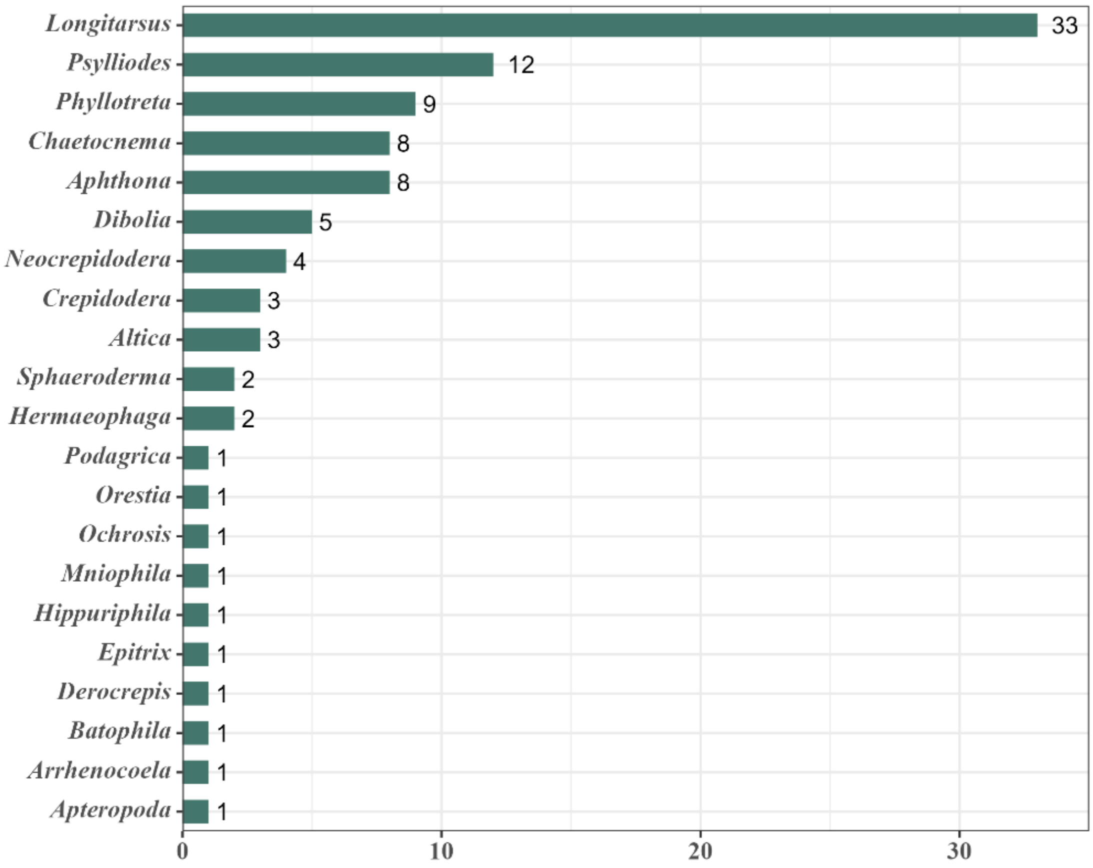

Figure 1. Number of species for each genus in the final library.

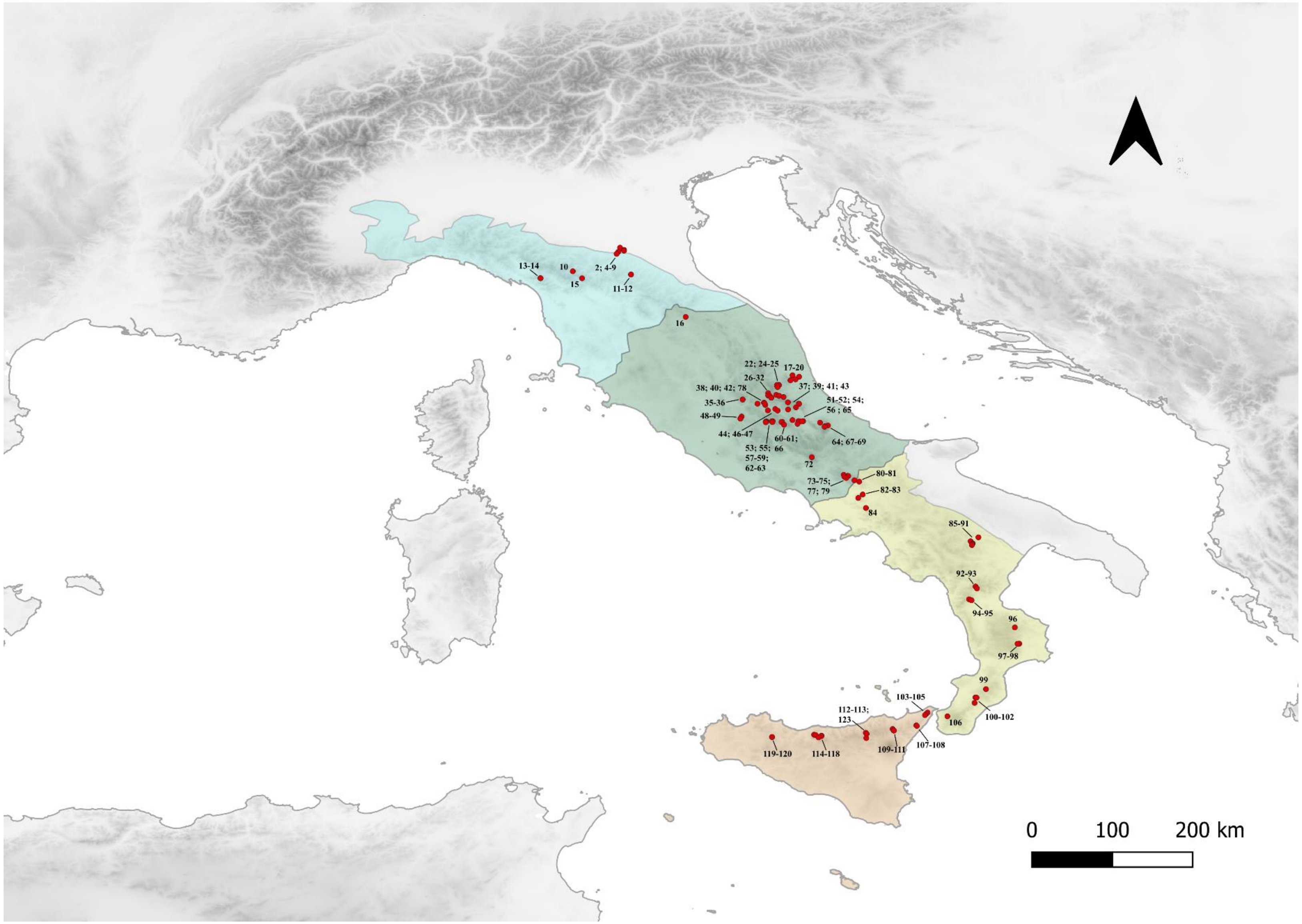

Figure 2. Geographical distribution of the 119 sampling localities. The four Apennine sectors are shown un different colours: Northern (blue), Central (green), Southern (yellow), and Sicilian (orange).

Geographical distribution and altitudinal range

The geographical distribution of the 119 sampling localities is reported in Figure 2. The number of specimens and species collected in each of the four Apennine sectors is reported in Figure 3. The highest number is relative to the Central Apennine with 141 specimens within 71 species. Collected specimens cover an altitudinal range from 31 to 2624 meter. The highest number of species was collected in the altitudinal range from 1200 to 1399 meter. The number of species dropped above 1400 meter. The distribution of the number of species along altitudinal ranges is showed in Figure 4.

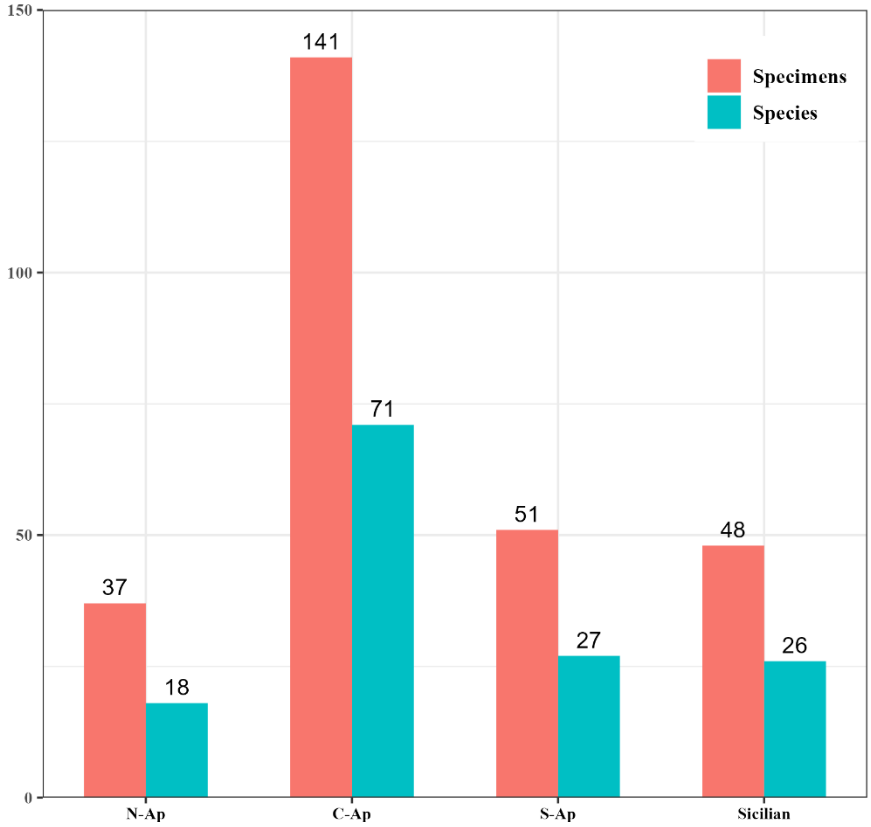

Figure 3. Number of specimens and species collected in the four Apennine sectors: Northern (N-Ap), Central (C-Ap), Southern (S-Ap), and Sicilian.

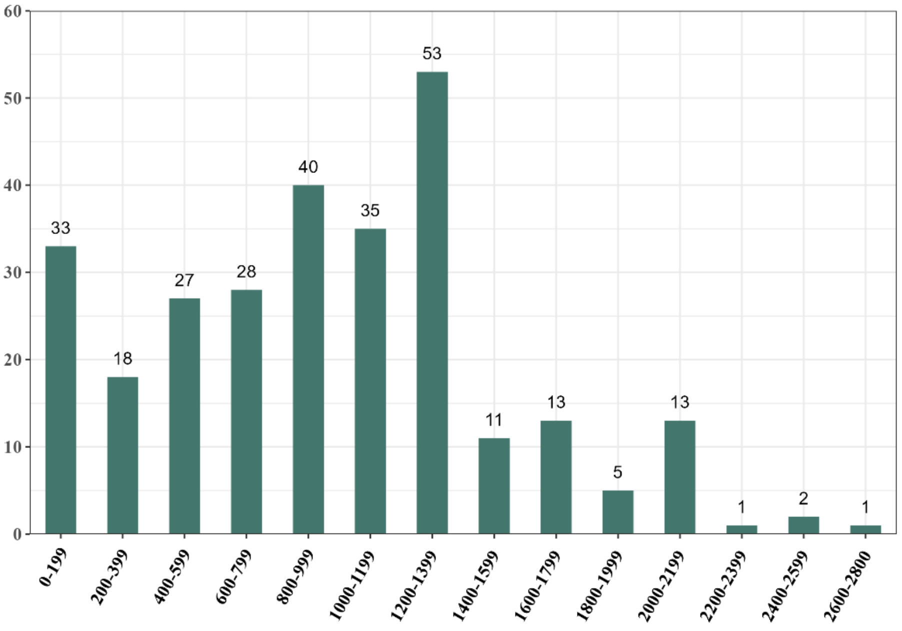

Figure 4. Number of species collected along altitudinal ranges (meters).

Discussion

The DNA barcoding can be a useful tool for specimen identification (Hebert et al., 2003). However, the successful of DNA barcoding depends on the presence of a reliable reference libraries to assign correct identification to new query sequences (Floyd et al., 2010; Will et al., 2005).

We produced a reliable barcoding library (DT1) of 278 sequences belong to 99 species within 21 genera (see Table 4). Based on the DT1 reference library we assessed the reliability of BOLD and GenBank libraries for accurate identifications of Alticini sequences, and we obtained a rate of correct identification of 87% of the sequences concerning 86 species in 19 genera. This finding is in agreement with previous DNA barcoding studies on European invertebrates (Hendrich et al., 2015; Morinière et al., 2017). Taxonomic uncertainties (wrong identifications) that were resolved based on DT1 reference library, regards 684 BOLD-GB sequences. These misidentified sequences belong to 25 species within 7 genera: *Altica*, *Aphthona*, *Chaetocnema*, *Longitarsus*, *Psylliodes*, *Derocrepis*, and *Neocrepidodera*. Most of these taxonomic uncertainties in BOLD-GB concerns *Altica brevicollis* and *longitarsus pratensis* and *L. luridus*. These results corroborate the utility of *a posteriori* taxonomic revision of uncertain identifications and allow identifying species and genera (*e. g. Altica* and *Longitarsus*) that require further taxonomic investigations (Salvi et al., 2020)

In Italy, there are 340 species in 25 genera within of Alticini, so, our barcoding library (DT1) covers one third of the Italian biodiversity within 84% of the genera (Biondi et al., 2013; Biondi, 2006). The Apennine Alticini biodiversity is one of the richest in Europe if we consider that the total amount of European species within this tribe is about 400 (85%). The most represented genus in the DT1 library is *Longitarsus* with 33 species. This result reflects the high biodiversity of the most represented genus in Alticini tribe that counts over 700 species distributed in all zoogeographical regions (Salvi et al., 2020). Furthermore, the DT1 library contains reference sequences of one endemic species *Longitarsus zangherii* present in the Central Apennine sector (Biondi et al., 2013), and three glacial relicts in Apennine chain *Aphthona cyparissiae* (Koch, 1803), *Dibolia alpestris* Mohr, 1981, *Longitarsus minimus* Kutschera, 1863.

The highest diversity of species is recorded in the Central Apennine sector with 71 species. This region hosts around half of the entire Alticini species diversity of Italy (Biondi, 2006). The Apennine Alticini biodiversity consist of 85% of the European species (Jong et al., 2014) which share genera and species with the rest of Palearctic regions. Within the Apenine range, the highest species diversity is found below 1400 m altitude a.s.l, and in particular in between 1200 and 1400 m altitude a.s.l. where 53 species are recorded. Above 1400 meters of altitude the number of species drastically drop. Larvae and adult of flea beetles feed respectively on roots and leaves of plants of different angiosperm families, with levels of trophic specialization ranging from strictly monophagous to widely polyphagous (Salvi, D'Alessandro, et al., 2019). The decrease of species diversity with increasing altitude could follow the decrease of habitat suitability for plan hosts. Indeed, the same trend was already documented in plant diversity at the Apennine (Di Musciano et al., 2018) and the European scale (Körner & Kèorner, 1999).

In conclusion, this study provides the first taxonomically curated barcoding library of Alticini species from the Apennines validated by a taxonomist specialised in this group and region. Our study underlined the crucial role of taxonomists in the study of biodiversity and in multiple steps of the DNA barcoding pipeline, form the library construction to the library validation. Indeed, beside the utility of this library for Apennine biodiversity assessment, this library provided a reference for a number of species for which it was possible to resolve taxonomic uncertainties and the correct nomenclatures of sequences available in BOLD and GB repositories. For this reason, our barcoding library will play a key role as reference for future biodiversity assessments at regional, national, and European scale.

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Table S1. List of 684 sequences retrieved from BOLD-GB repositories and for which our taxonomic re-assessment allowed to correct the identification using our specimens as references. For each sequence, we reported the genus, the cluster-name of the BCM analyses, the original taxonomic identification (Species-1), the accession number, and the new classification based on our reference library (Species-2).

Genus	**Cluster**	**Species-1**	**Accession Number**	**Species-2**
Altica	result.3	*Altica ericeti*	KX943460	*Altica brevicollis*
Altica	result.3	*Altica aenescens*	KM449496	*Altica brevicollis*
Altica	result.3	*Altica engstroemi*	KJ961936	*Altica brevicollis*
Altica	result.3	*Altica chamaenerii*	KJ962255	*Altica brevicollis*
Altica	result.3	*Altica lythri*	MZ629781	*Altica brevicollis*
Altica	result.3	*Altica quercetorum*	KU913613	*Altica brevicollis*
Altica	result.3	*Altica aenescens*	KU915281	*Altica brevicollis*
Altica	result.3	*Altica aenescens*	KU917046	*Altica brevicollis*
Altica	result.3	*Altica quercetorum*	KU917146	*Altica brevicollis*
Altica	result.3	*Altica lythri*	MH322700	*Altica brevicollis*
Altica	result.3	*Altica koreana*	MK138593	*Altica brevicollis*
Altica	result.3	*Altica lythri*	MH322698	*Altica brevicollis*
Altica	result.3	*Altica lythri*	MZ630668	*Altica brevicollis*
Altica	result.3	*Altica engstroemi*	KJ962940	*Altica brevicollis*
Altica	result.3	*Altica engstroemi*	KJ962965	*Altica brevicollis*
Altica	result.3	*Altica chamaenerii*	KJ963283	*Altica brevicollis*
Altica	result.3	*Altica longicollis*	KJ963312	*Altica brevicollis*
Altica	result.3	*Altica chamaenerii*	KJ963827	*Altica brevicollis*
Altica	result.3	*Altica chamaenerii*	KJ964709	*Altica brevicollis*
Altica	result.3	*Altica engstroemi*	KJ964883	*Altica brevicollis*
Altica	result.3	*Altica chamaenerii*	KJ965187	*Altica brevicollis*
Altica	result.3	*Altica engstroemi*	KJ965591	*Altica brevicollis*
Altica	result.3	*Altica chamaenerii*	KJ965872	*Altica brevicollis*
Altica	result.3	*Altica engstroemi*	KJ965900	*Altica brevicollis*
Altica	result.3	*Altica engstroemi*	KJ966317	*Altica brevicollis*

Altica	result.3	*Altica chamaenerii*	KJ967485	*Altica brevicollis*
Altica	result.3	*Altica aenescens*	KM440707	*Altica brevicollis*
Altica	result.3	*Altica aenescens*	KM440760	*Altica brevicollis*
Altica	result.3	*Altica aenescens*	KM445529	*Altica brevicollis*
Altica	result.3	*Altica aenescens*	KM450094	*Altica brevicollis*
Altica	result.3	*Altica aenescens*	KM451178	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF656458	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF652810	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF652811	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF652813	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF652812	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF652824	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF652814	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF652825	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF652827	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF652826	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF652835	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF652863	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF652873	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF652865	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF652883	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF652887	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF652893	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF652917	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF652944	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF652945	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF652958	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF652987	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF652988	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF653131	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF653133	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF653132	*Altica brevicollis*

Altica	result.3	*Altica ampelophaga*	KF653160	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF653161	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF653162	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF653175	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF653177	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF653179	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF653176	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF653178	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF653180	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF653181	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF653182	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF653183	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF653205	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF653190	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF653191	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF653192	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF653206	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF653184	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF653207	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF653208	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF653219	*Altica brevicollis*
Altica	result.3	*Altica ericeti*	KF653227	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF653809	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF653811	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF653812	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF653813	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF653810	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF653816	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF653814	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF653819	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF653818	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF653820	*Altica brevicollis*

Altica	result.3	*Altica ampelophaga*	KF653817	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF653821	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF653878	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF654333	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF654699	*Altica brevicollis*
Altica	result.3	*Altica ampelophaga*	KF654701	*Altica brevicollis*
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Altica	result.3	*Altica aenescens*	KF163263	*Altica brevicollis*
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Altica	result.3	*Altica ampelophaga*	MH510730	*Altica brevicollis*
Altica	result.12	*Altica lythri*	MH322701	*Altica oleracea*
Altica	result.12	*Altica helianthemi*	JF890764	*Altica oleracea*
Altica	result.12	*Altica lythri*	MH322699	*Altica oleracea*
Altica	result.12	*Altica lythri*	MH322697	*Altica oleracea*
Altica	result.12	*Altica opacifrons*	KJ962078	*Altica oleracea*
Altica	result.12	*Altica opacifrons*	KJ964966	*Altica oleracea*
Altica	result.12	*Altica opacifrons*	KJ966716	*Altica oleracea*
Aphthona	result.2	*Aphthona cyparissiae*	MH322711	*Aphthona flava*
Aphthona	result.2	*Aphthona cyparissiae*	MH322709	*Aphthona flava*
Aphthona	result.2	*Aphthona cyparissiae*	MH322708	*Aphthona flava*
Aphthona	result.2	*Aphthona cyparissiae*	MH322707	*Aphthona flava*
Aphthona	result.3	*Aphthona atrovirens*	KU915359	*Aphthona euphorbiae*
Aphthona	result.3	*Aphthona atrovirens*	KU912356	*Aphthona euphorbiae*
Aphthona	result.5	*Aphthona melancholica*	OR184757	*Aphthona venustula*
Aphthona	result.5	*Aphthona melancholica*	OR184708	*Aphthona venustula*
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Aphthona	result.5	*Aphthona melancholica*	KF656295	*Aphthona venustula*
Aphthona	result.5	*Aphthona melancholica*	KF656294	*Aphthona venustula*
Aphthona	result.6	*Aphthona pallida*	KU913237	*Aphthona nigriceps*
Aphthona	result.6	*Aphthona pallida*	KU919347	*Aphthona nigriceps*
Aphthona	result.6	*Aphthona pallida*	KU919367	*Aphthona nigriceps*
Aphthona	result.6	*Aphthona pallida*	KU910476	*Aphthona nigriceps*
Chaetocnema	result.5	*Chaetocnema scheffleri*	KX943431	*Chaetocnema tibialis*
Chaetocnema	result.5	*Chaetocnema scheffleri*	KF655250	*Chaetocnema tibialis*
Chaetocnema	result.8	*Chaetocnema picipes*	MG061195	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	KR489583	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	KR483868	*Chaetocnema concinna*

Chaetocnema	result.8	*Chaetocnema picipes*	KR486739	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	MG059550	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	MH407426	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	KU912793	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	KU913343	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	KU913947	*Chaetocnema concinna*
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Chaetocnema	result.8	*Chaetocnema picipes*	KU917335	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	JF889786	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	MZ631977	*Chaetocnema concinna*
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Chaetocnema	result.8	*Chaetocnema picipes*	KU908128	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	KU909739	*Chaetocnema concinna*
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Chaetocnema	result.8	*Chaetocnema picipes*	KM443706	*Chaetocnema concinna*
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Chaetocnema	result.8	*Chaetocnema picipes*	KR488237	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	KR484332	*Chaetocnema concinna*

Chaetocnema	result.8	*Chaetocnema picipes*	KR480158	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	MG061102	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	KR490071	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	KR483195	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	MG058761	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	MF636001	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	KR486919	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	MG054156	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	MG057844	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	MG057138	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	KR487156	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	MG058388	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	KR479957	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	MG059410	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	KR480768	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	KR483109	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	KR491430	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	KR489800	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	MG057427	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	MG054370	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	BIOUG10305-E11	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	BIOUG12434-C06	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	BIOUG16091-H06	*Chaetocnema concinna*
Chaetocnema	result.8	*Chaetocnema picipes*	BIOUG16127-H02	*Chaetocnema concinna*
Chaetocnema	result.21	*Chaetocnema subcoerulea*	KU911969	*Chaetocnema montenegrina*
Chaetocnema	result.21	*Chaetocnema subcoerulea*	KM446162	*Chaetocnema montenegrina*
Chaetocnema	result.21	*Chaetocnema subcoerulea*	KM441683	*Chaetocnema montenegrina*
Longitarsus	result.5	*Longitarsus scutellaris*	KR130105	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR480203	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR485790	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR482604	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	MG060660	*Longitarsus pratensis*

Longitarsus	result.5	*Longitarsus scutellaris*	KR480761	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR487716	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR488814	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR483422	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR482097	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR480834	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus reichei*	KU914666	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus reichei*	KU917139	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	HQ954158	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	MG062601	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR482841	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR484199	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR484810	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR485404	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR486228	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR486278	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR487457	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR488474	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR488608	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR490324	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR490475	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus reichei*	KU918316	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus reichei*	KU907319	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KM451412	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	MG060904	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR491188	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR126393	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR125821	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR485678	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR486529	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	MG055637	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	MG056813	*Longitarsus pratensis*

Longitarsus	result.5	*Longitarsus scutellaris*	KR489500	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR480557	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR485032	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	MG054361	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	MG058392	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR122926	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	MG055432	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR480124	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	MG054759	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	MG055102	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR488280	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR119941	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR486740	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR490677	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR489964	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	MG055711	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	MG054632	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR489548	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR489279	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	MG054537	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR481516	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR480130	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	MG058363	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	MG062432	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	MG059994	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR491284	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	MG053928	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	MG058030	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR480364	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR488627	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR122849	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	MG061708	*Longitarsus pratensis*

Longitarsus	result.5	*Longitarsus scutellaris*	KR487634	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR481305	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	MG060285	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	HQ954159	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	MG060737	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	KR484826	*Longitarsus pratensis*
Longitarsus	result.5	*Longitarsus scutellaris*	MG058553	*Longitarsus pratensis*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653730	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF652436	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653324	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653358	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653361	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653357	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653360	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653359	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653363	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653365	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653364	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653367	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653366	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653368	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653369	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653379	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653380	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653537	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653726	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653728	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653729	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653727	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653731	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653733	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653732	*Longitarsus juncicola*

Longitarsus	result.8	*Longitarsus ordinatus*	KF653735	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653734	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653737	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653736	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653738	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653741	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653739	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653742	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653743	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653744	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653745	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653325	*Longitarsus juncicola*
Longitarsus	result.8	*Longitarsus ordinatus*	KF653740	*Longitarsus juncicola*
Longitarsus	result.23	*Longitarsus pellucidus*	MT372408	*Longitarsus codinai*
Longitarsus	result.23	*Longitarsus pellucidus*	MT372407	*Longitarsus codinai*
Longitarsus	result.23	*Longitarsus pellucidus*	MT372406	*Longitarsus codinai*
Longitarsus	result.23	*Longitarsus pellucidus*	MT372405	*Longitarsus codinai*
Longitarsus	result.23	*Longitarsus pellucidus*	MG054895	*Longitarsus codinai*
Longitarsus	result.23	*Longitarsus pellucidus*	MG056145	*Longitarsus codinai*
Longitarsus	result.23	*Longitarsus pellucidus*	MG053748	*Longitarsus codinai*
Longitarsus	result.23	*Longitarsus pellucidus*	MG057935	*Longitarsus codinai*
Longitarsus	result.23	*Longitarsus pellucidus*	MG059377	*Longitarsus codinai*
Longitarsus	result.23	*Longitarsus pellucidus*	MG060779	*Longitarsus codinai*
Longitarsus	result.23	*Longitarsus pellucidus*	MG054415	*Longitarsus codinai*
Longitarsus	result.23	*Longitarsus pellucidus*	MG057469	*Longitarsus codinai*
Longitarsus	result.23	*Longitarsus pellucidus*	MG055065	*Longitarsus codinai*
Longitarsus	result.23	*Longitarsus pellucidus*	KU914889	*Longitarsus codinai*
Longitarsus	result.23	*Longitarsus pellucidus*	KR480207	*Longitarsus codinai*
Longitarsus	result.23	*Longitarsus pellucidus*	KR487382	*Longitarsus codinai*
Longitarsus	result.23	*Longitarsus pellucidus*	KR488383	*Longitarsus codinai*
Longitarsus	result.23	*Longitarsus pellucidus*	KU906283	*Longitarsus codinai*
Longitarsus	result.23	*Longitarsus pellucidus*	KU908543	*Longitarsus codinai*

Longitarsus	result.23	*Longitarsus pellucidus*	MG055292	*Longitarsus codinai*
Longitarsus	result.23	*Longitarsus pellucidus*	MG056417	*Longitarsus codinai*
Longitarsus	result.23	*Longitarsus pellucidus*	KR484411	*Longitarsus codinai*
Longitarsus	result.28	*Longitarsus atricillus*	KM450237	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KU913100	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KU915650	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus apicalis*	JF890820	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	OR184799	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	OR184800	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	OR184798	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	OR184792	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	OR184790	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	OR184774	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	OR184768	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	OR184771	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	OR184767	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	OR184766	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	OR184763	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	MZ629596	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KU906420	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KU911269	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	HQ953558	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF653025	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF652312	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF652303	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF652624	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF134546	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF134548	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF134545	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF134547	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF134549	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF134554	*Longitarsus aeneicollis*

Longitarsus	result.28	*Longitarsus bedeli*	KF134558	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus bedeli*	KF134559	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF134560	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF134561	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF134566	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF134569	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF134570	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF134574	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF134575	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF134581	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF652300	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF652305	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF652308	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF652306	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF652307	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF652311	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF652315	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF652385	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF652410	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF652466	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF652479	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF652489	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF652488	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF652787	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF652805	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF653024	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF653023	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF653026	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF653101	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF653618	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF653622	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF653628	*Longitarsus aeneicollis*

Longitarsus	result.28	*Longitarsus atricillus*	KF653626	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF653631	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF653630	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF653670	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF653876	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF653877	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF653900	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF654232	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF654235	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF654236	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF654447	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF654486	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF654487	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF654664	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF655009	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF655011	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF655024	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF655350	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF655357	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KF656131	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	MIS_LEM_MS_229	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	MIS_LEM_MS_231	*Longitarsus aeneicollis*
Longitarsus	result.28	*Longitarsus atricillus*	KX943363	*Longitarsus aeneicollis*
Longitarsus	result.33	*Longitarsus testaceus*	KR128939	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR122263	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR481184	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR490736	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus atricillus*	MZ066511	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR481430	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR128307	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR120663	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR128206	*Longitarsus luridus*

Longitarsus	result.33	*Longitarsus testaceus*	KR121993	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR124936	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR123264	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR131101	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR130867	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR484403	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR126756	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR119567	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR484048	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR128362	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR126242	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR125515	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR120371	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR119120	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR489660	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR122822	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR128910	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR130528	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR123152	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR128969	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR119207	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR124289	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR128356	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR127953	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR126712	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR123652	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR127636	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR130297	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR123803	*Longitarsus luridus*
Longitarsus	result.33	*Longitarsus testaceus*	KR122373	*Longitarsus luridus*
Longitarsus	result.39	*Longitarsus helvolus*	KU913464	*Longitarsus membranaceus*
Longitarsus	result.39	*Longitarsus helvolus*	KU916133	*Longitarsus membranaceus*

Longitarsus	result.39	*Longitarsus helvolus*	KU916246	*Longitarsus membranaceus*
Longitarsus	result.39	*Longitarsus helvolus*	KU916658	*Longitarsus membranaceus*
Longitarsus	result.44	*Longitarsus curtus*	KX943501	*Longitarsus monticola*
Longitarsus	result.44	*Longitarsus curtus*	OR184780	*Longitarsus monticola*
Longitarsus	result.44	*Longitarsus curtus*	OR184709	*Longitarsus monticola*
Longitarsus	result.44	*Longitarsus curtus*	OR184713	*Longitarsus monticola*
Longitarsus	result.44	*Longitarsus curtus*	KF656258	*Longitarsus monticola*
Longitarsus	result.47	*Longitarsus suturellus*	KJ965495	*Longitarsus ganglbaueri*
Longitarsus	result.47	*Longitarsus suturellus*	KU913427	*Longitarsus ganglbaueri*
Longitarsus	result.47	*Longitarsus suturellus*	KU915636	*Longitarsus ganglbaueri*
Longitarsus	result.47	*Longitarsus suturellus*	KU915687	*Longitarsus ganglbaueri*
Longitarsus	result.47	*Longitarsus suturellus*	KU919071	*Longitarsus ganglbaueri*
Longitarsus	result.47	*Longitarsus suturellus*	KU909995	*Longitarsus ganglbaueri*
Longitarsus	result.47	*Longitarsus suturellus*	KU912271	*Longitarsus ganglbaueri*
Phyllotreta	result.5	*Phyllotreta aerea*	MG056036	*Phyllotreta punctulata*
Phyllotreta	result.7	*Phyllotreta cruciferae*	KX943506	*Phyllotreta atra*
Phyllotreta	result.7	*Phyllotreta astrachanica*	MH407435	*Phyllotreta atra*
Phyllotreta	result.7	*Phyllotreta cruciferae*	KF655856	*Phyllotreta atra*
Psylliodes	result.2	*Psylliodes hyoscyami*	KM450066	*Psylliodes chalcomera*
Psylliodes	result.2	*Psylliodes hyoscyami*	MW254861	*Psylliodes chalcomera*
Psylliodes	result.6	*Psylliodes fiorellae*	MW254856	*Psylliodes obscuroaenea*
Psylliodes	result.7	*Psylliodes milleri*	OR184760	*Psylliodes napi*
Psylliodes	result.7	*Psylliodes milleri*	OR184758	*Psylliodes napi*
Psylliodes	result.7	*Psylliodes milleri*	OR184755	*Psylliodes napi*
Psylliodes	result.7	*Psylliodes milleri*	KF652933	*Psylliodes napi*
Psylliodes	result.10	*Psylliodes hispanus*	KX943503	*Psylliodes instabilis*
Psylliodes	result.10	*Psylliodes brisouti*	MH323327	*Psylliodes instabilis*
Psylliodes	result.10	*Psylliodes brisouti*	MH323330	*Psylliodes instabilis*
Psylliodes	result.10	*Psylliodes brisouti*	MH323329	*Psylliodes instabilis*
Psylliodes	result.10	*Psylliodes brisouti*	MH323328	*Psylliodes instabilis*
Psylliodes	result.10	*Psylliodes hispanus*	KF654806	*Psylliodes instabilis*
Psylliodes	result.10	*Psylliodes pyritosa*	MW254881	*Psylliodes instabilis*

Derocrepis	result.33	*Derocrepis rufipes*	KU913529	*Derocrepis sodalis*
Derocrepis	result.33	*Derocrepis rufipes*	KU914645	*Derocrepis sodalis*
Derocrepis	result.33	*Derocrepis rufipes*	MH323076	*Derocrepis sodalis*
Derocrepis	result.33	*Derocrepis rufipes*	MH323075	*Derocrepis sodalis*
Derocrepis	result.33	*Derocrepis rufipes*	KU906863	*Derocrepis sodalis*
Derocrepis	result.33	*Derocrepis rufipes*	KU908494	*Derocrepis sodalis*
Derocrepis	result.33	*Derocrepis rufipes*	KU912447	*Derocrepis sodalis*
Derocrepis	result.33	*Derocrepis rufipes*	KM441088	*Derocrepis sodalis*
Derocrepis	result.33	*Derocrepis rufipes*	KM441676	*Derocrepis sodalis*
Derocrepis	result.33	*Derocrepis rufipes*	KM448729	*Derocrepis sodalis*
Derocrepis	result.33	*Derocrepis rufipes*	KM449435	*Derocrepis sodalis*
Derocrepis	result.33	*Derocrepis rufipes*	KM451481	*Derocrepis sodalis*
Derocrepis	result.33	*Derocrepis rufipes*	HQ948262	*Derocrepis sodalis*
Derocrepis	result.33	*Derocrepis rufipes*	KM444306	*Derocrepis sodalis*
Derocrepis	result.33	*Derocrepis rufipes*	KM441135	*Derocrepis sodalis*
Derocrepis	result.33	*Derocrepis rufipes*	KM442002	*Derocrepis sodalis*
Derocrepis	result.33	*Derocrepis rufipes*	MH020467	*Derocrepis sodalis*
Neocrepidodera	result.137	*Neocrepidodera transversa*	MH323248	*Neocrepidodera impressa*
Neocrepidodera	result.137	*Neocrepidodera transversa*	MH323247	*Neocrepidodera impressa*

CHAPTER 2

Apennine-Pyrenees disjunct distribution: an unusual biogeographic pattern revealed in flea beetles of the *Longitarsus candidulus* species-group (Coleoptera, Chrysomelidae)

Introduction

The Quaternary's climatic changes determined significant large-scale range shift in many species (Avise, 2000; G. Hewitt, 2000, 2003). These species distribution shifts have been strongly influenced by mountain areas which, depending on their geographical location, spatial orientation, local biotic context and species' ecological requirements, can serve either as both dispersal barriers or environmental suitable areas (Rahbek et al., 2019; Schmitt, 2009; Schmitt et al., 2016). The role of the major European mountain ranges, such as Alps, Pyrenees, and Balkans, in structuring the distribution and genetic diversity of fauna and flora is well known (G. M. Hewitt, 1999, 2004; Schmitt, 2009, 2017).

Many phylogeographic studies have highlighted how mountain systems have acted as important dispersion barriers for temperate species for postglacial expansions both northward, from the three Mediterranean peninsulas (Iberian, Italian and Balkan peninsulas) and southward, from extra-Mediterranean refuges (Taberlet et al., 1998; Hewitt, 2001, 2004, 2011; Stewart et al., 2010; Schmitt and Varga, 2012). However, several studies have demonstrated a wide plethora of species-specific responses to Pleistocene climate oscillations by temperate species explained by differences in ecological requirements, dispersal capacity and their interplay with a complexity of additional factors operating at regional scale and shaping population demography and species ranges (Stewart and Lister, 2001; Deffontaine et al., 2005; Pauls et al., 2006; Schmitt and Varga, 2012; Maura et al., 2014; Salvi et al., 2014, 2016; Senczuk et al., 2019). During glacial periods, temperate species may have lived at lower altitudes in periglacial areas, and at higher altitudes during dry interglacial periods (Pauls et al., 2006; Zeisset and Beebee, 2008; Maura et al., 2014; Morales-Barbero et al., 2018). For these species, mountain systems might have provided a sort of island archipelago refugium rather than a barrier to dispersal during interglacial periods (Mardulyn et al., 2009; Martinet et al., 2018; Schmitt, 2009). Such range fragmentation has determined gene flow disruption among populations isolated on distinct mountains, causing allopatric divergence and possibly speciation (Schmitt, 2017; Schmitt et al., 2016). Traces of such processes are today visible as shared biogeographic patterns for mountain species with strongly allopatric distributions of genetic diversity across major mountain ranges of Pyrenees, Alps, Apennines, Balkans and Caucasus (Pauls et al., 2006; Varga and Schmitt, 2008; Valtueña et al., 2012; Štundlová et al., 2019; Lucati et al., 2020; Ehl et al., 2021; Sistri et al., 2022).

Among these biogeographic patterns, the Apennine-Pyrenees disjunct is very uncommon in literature. These two mountain systems have very different geological histories. The Pyrenees evolved since the early Mesozoic by the collision of the Iberian and Eurasian plates, and they extend nearly 500 kilometers from the Bay of Biscay to the Gulf of Lion on the Mediterranean coast (Vergés et al., 2002). Instead, the Apennine, extending north to south along the Italian Peninsula for 1,200 kilometers, evolved over several tectonic phases, most of which occurred during the Cenozoic Era, and culminated in the Miocene and Pliocene epochs (about 23 to 2.6 million years ago) by the collision of the African and Eurasian plates and the rotation of the Corso-Sardinian block (Bosellini, 2005; Corrado et al., 1997). Nowadays, Pyrenees and Apennines are about 500 kilometers apart, with the Massif Central and the Alps providing elements of environmental connectivity between them. Indeed, many plant and animal species associated with medium-high mountain environments have a range that extends across these four major mountain ranges, demonstrating the historical biogeographic connection between western European mountains (Dixon et al., 2009; Todisco et al., 2010; Dillenberger and Kadereit, 2013; Sanz et al., 2014; Feuda et al., 2015; Schmitt et al., 2016; Zlatkov and Huemer, 2017; Hinojosa et al., 2019). However, cases of species or closely related taxa with a disjunct Apennines-Pyrenean distribution are uncommon, and are found only in some plants, such as *Adenostyles alpina* (Dillenberger and Kadereit, 2013), *Androsace vitaliana* (Dixon et al., 2009), or *Saponaria bellidifolia* (Csergö et al., 2009), and in a few animals such as the ground beetles *Nebria orsinii* (Ruffo, 1971) or the iconic case of the Pyrenean chamois *Rupicapra pyrenaica* (Fioravanti et al., 2019; Masseti & Salari, 2017; Salari et al., 2014).

To investigate the Apennine-Pyrenees biogeographic pattern, the flea beetles *Longitarsus laureolae* Biondi and *L. leonardii* Doguet (Coleoptera, Chrysomelidae) offer a suitable study system. These two species, together with *Longitarsus candidulus* (Foudras), constitute a homogeneous group (species-group *sensu* Bergeal and Doguet, 1991) sharing similar external, aedeagic and spermathecal morphology (Figure 1), as well as being mainly associated with host plants of the family Thymelaeaceae (Biondi, 1988). This species-group probably also includes *Longitarsus arnoldi* described from Algeria, for which (Bergeal and Doguet, 1991), however, the host plant is not yet known. *Longitarsus laureolae* and *L. leonardii* occur in medium-mountain environments, the former in the central-southern Apennines, including the Sicilian range, and the latter along the Cantabrian-Pyrenean chain (Figure 2). Beside living in similar environments, these two species also share the same host plant, *Daphne laureola* (Biondi, 1988, 1991). Differently, *L. candidulus* is a thermophilus element with a Holo-Mediterranean distribution. It occurs mainly on coastal and sub-coastal environments (only extends into inland continental areas in the Iberian Peninsula and in the Maghreb) and is associated with the host plants *Thymelaea hirsuta* and *Daphne gnidium* (Biondi, 1988).

Here we investigated the evolutionary history of the *Longitarsus candidulus* species-group, with a focus on the origin of the Apennine-Pyrenees biogeographic pattern of *L. laureolae* and *L. leonardii*. First, we assessed the monophyly of the *candidulus* species-group within a phylogenetic framework including other Western Palaearctic *Longitarsus* species. Second, we estimated the divergence time of cladogenetic events within the *candidulus* specie-group using a multispecies coalescent model to determine whether speciation events were caused by isolation on distinct mountain ranges throughout the Pleistocene glacial cycles. The main aim of this study is to identify the biogeographic and evolutionary processes underlying the diversification within the *candidulus* species-group and the origin of the Apennine-Pyrenees pattern.

Materials and methods

Sampling and morphological identification

For this study, 27 specimens of *L. candidulus*, *L. laureolae* and *L. leonardii* were collected from eight localities in central and southern Italy, Sicily, Sardinia, Spain and Portugal (Table 1). Specimens were collected on their respective host plants by sweep net and the aid of aspirator and then stored in 95% ethanol. Morphological identification at the species level was performed by Maurizio Biondi through the dissection and study of the genitalia, median lobe of the aedeagus for males and spermatheca for females. Dissection of the specimens were carried out using a Leica M205C binocular microscope. Photographs of the habitus and spermatheca were taken employing a Leica DMC5400 camera and composed using the Zerene Stacker software, version 1.04. Scanning electron micrographs of the median lobe of the aedeagus were taken using a Hitachi TM-1000.

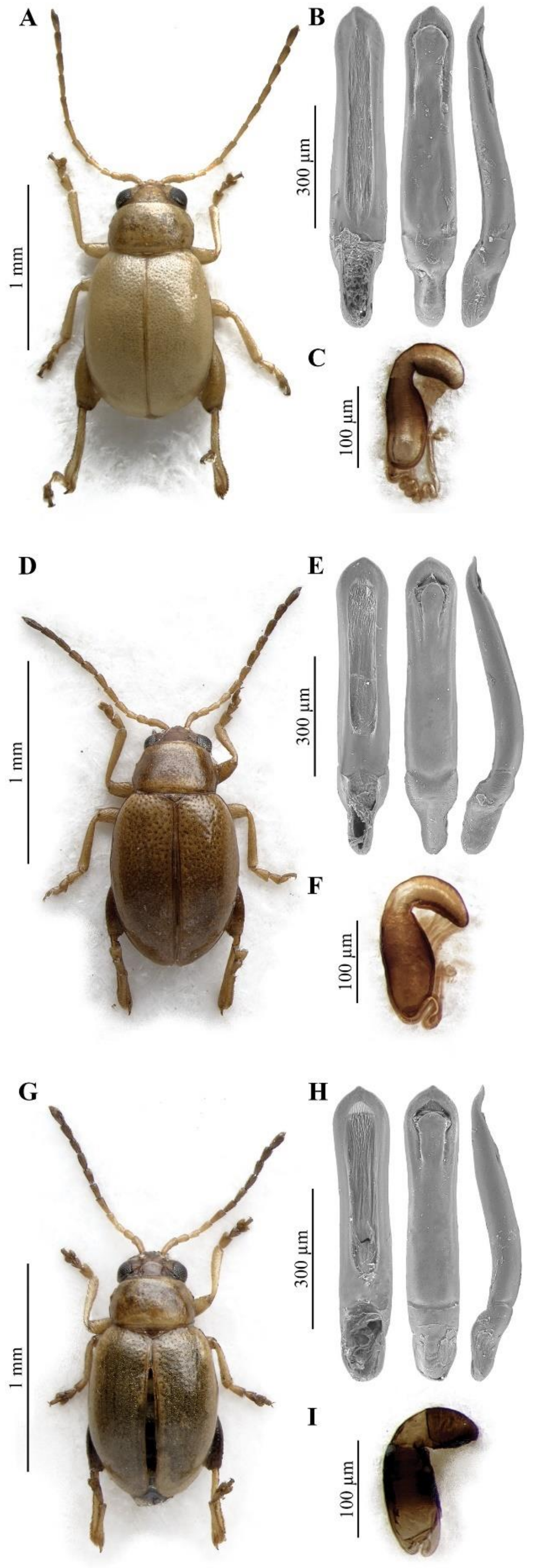

Figure 1. Habitus (A, D, G), median lobe of the aedeagus (B, E, H) in ventral, dorsal, and lateral view (from left to right), and spermatheca (C, F, I), of members of the *Longitarsus candidulus* species-group: *L. candidulus* (A–C), *L. laureolae* (D–F), and *L. leonardii* (G–I).

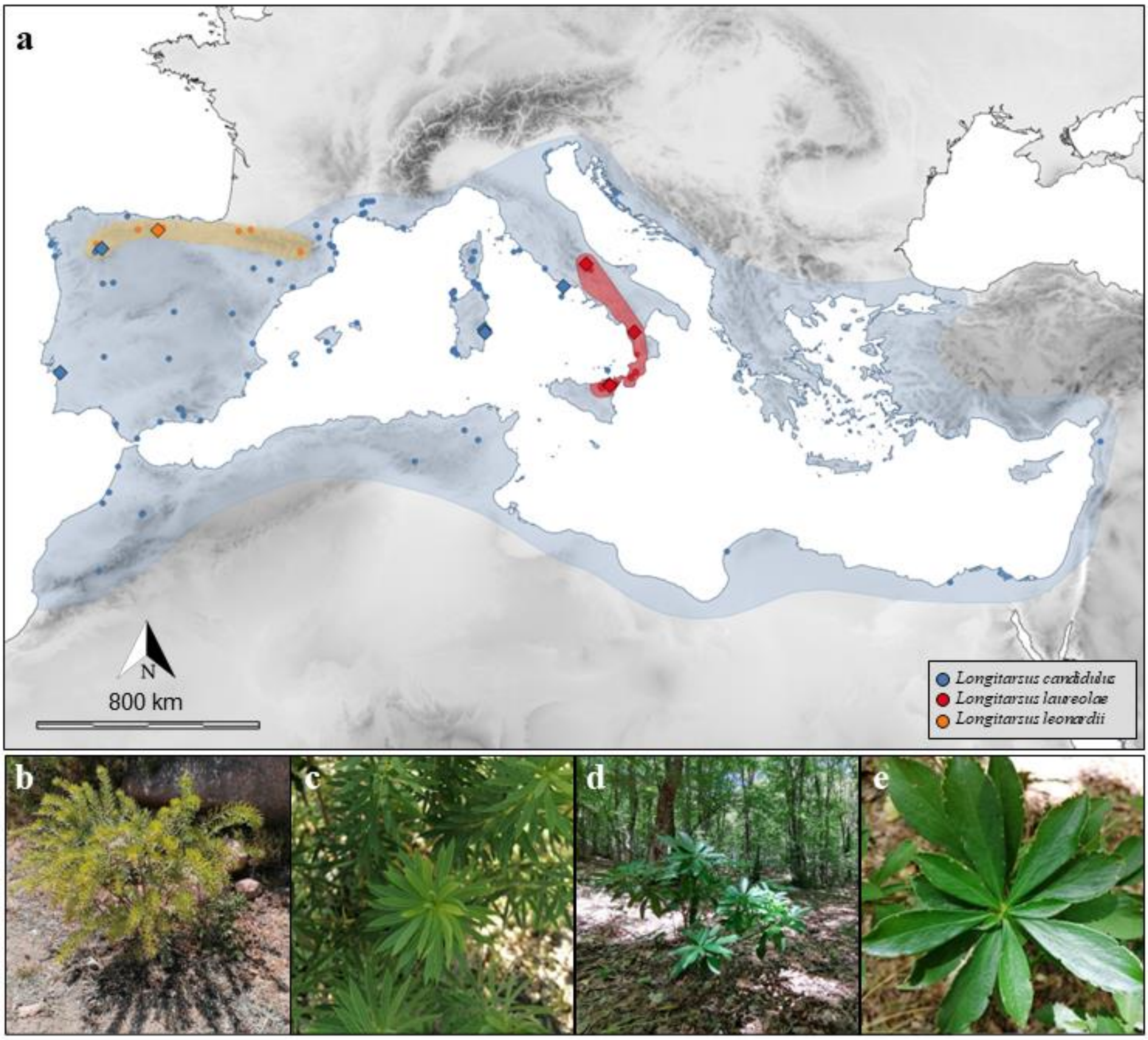

Figure 2. Map with geographical range of *L. candidulus* (light-blue), *L. laureolae* (red) and *L. leonardii* (orange) along with historical records for the 3 species (small circles) and sampling localities (squares) (A). Host plants of *L. candidulus*: *Thymelaea hirsuta* from Sardinia (B) and *Daphne gnidium* from Portugal (C). Host plant of *L. laureolae* and *L. leonardii*: *Daphne laureola* from Sicily (D) with the detail of foliar damage on the leaf margin caused by feeding of *L. laureolae* (E).

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted using a standard high-salt protocol (Sanbrook et al., 1989) with two different methods to allow subsequent morphological analysis: (i) using the three left legs of each specimen, and (ii) using the non-invasive method proposed in Salvi et al. (2020). This method involves the separation of the head-prothorax portion of the animal from the rest of the body with the use of an entomological pin and the immersion of the two parts directly in lysis buffer and proteinase K, allowing the subsequent reassembled of the animal on an entomological card point. We amplified the standard barcode region of the mitochondrial *cytochrome c oxidase I* gene (*cox1*; primer pairs: LCO1490Lon-F/HCO2198Lon-R; Salvi et al., 2019). For selected individuals of the three species, we amplified one additional mitochondrial gene fragment, the 16S ribosomal DNA (16S rDNA; primer pairs: 16Sdir-Lon CACCTGTTTAWTAAAAACAT/16Srev; this study and Palumbi et al., 1991), and three protein-coding single copy nuclear genes: Carbamoylphosphate synthase (*CAD*; primer pairs: CAD1bFw_mod/CD-668Rlon; Berrilli et al., 2023), Crossveinless 2 (*Cv2*; primer pairs: Cv2-F/Cv2-R; Gikonyo, 2021), and Wingless (*Wg*; primer pairs: Wg550F/WgAbRZ-R; Wild and Maddison, 2008). Successful amplification was determined by gel electrophoresis and PCR products were purified and sequenced by an external service (Genewitz, UK). The obtained chromatograms of each sequence were manually edited and assembled into a consensus sequence using Geneious Prime 2021 (Biomatters Ltd., Auckland, New Zealand). Heterozygous positions for the nuclear coding gene fragments were identified based on the presence of two peaks at a single site in the chromatograms and were coded in the alignment using IUPAC ambiguity codes. Consensus sequences were deposited in GenBank database (GenBank accession: OR373124-OR373138; OR378514-OR378524; OR397789- OR397817).

Phylogenetic analyses

Prior to phylogenetic analyses we performed species delimitation analysis to infer putative species boundaries within the *candidulus* species-group. We implemented the multirate Poisson Tree Processes model (mPTP; Kapli et al., 2017) on a Maximum likelihood (ML) tree based on *cox1* sequence data of *L. candidulus*, *L. laureolae* and *L. leonardii* (*cox1* dataset; see below). Analyses were performed using mPTP v. 0.2.4 with 10 runs of 100 million MCMC generations each, sampling every 10,000 (burn-in = 10%). The convergence of the independent runs was assessed through the average standard deviation of delimitation support values (ASDDSV) and the overall support for the ML estimate calculated computing the mean of the average support values (ASV) over the ten runs.

Phylogenetic analyses were performed in two steps. To determine whether the *candidulus* species-group formed a monophyletic clade, consistently with morphological characters, we built a dataset (mitochondrial dataset) including sequences of the two mitochondrial markers *cox1* and 16S of 52 *Longitarsus* species and the outgroup *Batophila aurata* (Marsham) obtained from Salvi et al. (2019), that currently provides the most comprehensive datasets for Western Palaearctic *Longitarsus* species. Once the group's monophyly within the genus *Longitarsus* was established, we used the sequences of both mitochondrial and nuclear markers (mito-nuclear dataset) to assess the phylogenetic relationship within the *candidulus* species-group, using *Longitarsus pellucidus* (Foudras) as an outgroup based on the results of the first analysis.

Multiple sequences alignment was performed separately for each gene with MAFFT v7.450 using the G-INS-I progressive method algorithm. The *cox1* alignment (*cox1* dataset) and concatenated sequence alignments (mitochondrial dataset and mito-nuclear dataset) were used to infer ML trees in IQ-TREE 1.6.12 (Nguyen et al., 2015) using the W-IQ-TREE webserver (Trifinopoulos et al., 2016). The best substitution models of each gene partition were determined by the ModelFinder module, including flexible rate heterogeneity across sites models (Kalyaanamoorthy et al., 2017), based on the Bayesian Information Criterion (BIC). We used the Edge Linked partition model to allow each partition to have its own evolutionary rate. Branch support was assessed by 1000 replicates of ultrafast bootstrapping (uBS) (Hoang et al., 2018; Minh et al., 2013) and SH-like approximate likelihood ratio test (SH- aLRT) (Guindon et al., 2010). FigTree v1.3.1 (Rambaut and Drummond, 2009) was used to depict the trees.

The close phylogenetic relationship between *L. laureolae* and *L. leonardii* recovered in ML analyses was further investigated using a phylogenetic network approach based on the five markers sequence data (Supplementary Tab 1). Haplotype phase of nuclear genes was determined using the algorithm PHASE (Stephens et al., 2001; Stephens and Donnelly, 2003) as implemented in DnaSP v5 (Librado and Rozas, 2009) with the initial 1000 iterations discarded as burn-in, 1 as thinning interval and 1000 post-burnin iterations. Phylogenetic relationships among haplotypes were inferred through the median-joining distance method (Bandelt et al., 1999) using PopArt 1.7 (Leigh and Bryant, 2015).

To estimate the species tree and divergence times of the *candidulus* species-group we used the multispecies coalescent method implemented in the StarBeast2 packages of BEAST2 v.2.7.1 (R. Bouckaert et al., 2019; Ogilvie et al., 2017). For this analysis we used alignments of the two mitochondrial genes and phased alignments of the three nuclear genes. We unlinked substitution models and clock models of gene partitions, with the exception of the trees of the mitochondrial genes *cox1* and 16S. We used a relaxed clock model (uncorrelated lognormal clock) calibrated using the available 16S substitution rate (clock.rate = 0.0054, stdev = 0.0009) estimated for beetles by Papadopoulou et al. (2010). The remaining settings were as follows: (unlinked) models of nucleotide substitution for each gene partition, we set HKY as substitution model and frequencies to empirical for all the gene partition; we unchecked the estimate box of substitution rate and checked the estimate box of clock rate of each partition, excepted for the 16S; we set a constant population model and a Yule process as species tree prior. StarBeast2 was run two times, with 300 million generations, sampling every 30,000 generations. We used Tracer v.1.7.1 (Rambaut et al., 2018) to check the runs for convergence (burn-in = 25%), LogCombiner and TreeAnnotator to combine runs and summarize the trees in a Maximum Clade Credibility Tree representing the posterior distribution. We used DensiTree and FigTree to visualized the results (Rambaut and Drummond, 2009; Bouckaert, 2010).

Results

Phylogenetic relationships and molecular dating

Species delimitation analyses based on mPTP retrieved convergence between runs (ASDDSV < 0.01) and supports the three species of the *candidulus* species-group as distinct species clusters (ASV median: 80%).

ML analyses based on *cox1* and 16S resolved the phylogenetic position of *L. candidulus*, *L. laureolae* and *L. leonardii* in a monophyletic group within the genus *Longitarsus* (SH-aLRT = 89, uBS = 85; Figure 3). The phylogenetic tree of the *candidulus* species-group based on mitochondrial and nuclear marker shows a sister relationship of *L. candidulus* to the clade composed by *L. laureolae* and *L. leonardii* (SH-aLRT = 100, uBS = 100; Figure 3). The three species were recovered as reciprocally monophyletic (SH-aLRT = 100, uBS = 100; Figure 3).

The median-joining network based on 30 sequences of *cox1* data shows three distinct haplogroups corresponding to the species *L. candidulus, L. laureolae,* and *L. leonardii*, with a closer relationship between the latter (Figure 4). A similar pattern is observed in the 16S network based on 9 sequences, although with a lower number of mutational steps. The three species are reciprocally monophyletic also in the network based on the 12 sequences of nuclear marker *CAD*, whereas a lack of sorting between *L. laureolae* and *L. leonardii* is apparent in the *Cv2* and *Wg* networks, based respectively on 16 and 12 sequences (Figure 4).

Also in the coalescent species tree, relationships within the *candidulus* species-group are well resolved (Figure 5), with a high level of nodal support and a topology consistent with the ML analysis. *L. candidulus* is supported as sister to the clade composed by *L. laureolae* and *L. leonardii* (Bayesian posterior probabilities, BPP = 0.97). The split between *L. candidulus* and the *L. laureolae*/*L. leonardii* clade is estimated to have occurred during the Middle Pliocene (3.08 Mya, million years ago; 95% High Posterior Density interval, HPD95: 3.98–2.20 Mya). The phylogenetic relationship between *L. laureolae* and *L. leonardii* received the maximum support (BPP = 1), with the separation between these two species estimated to have occurred in the late Middle Pleistocene (0.86 Mya; HPD95: 1.29–0.46 Mya).

Table 1. Details on sampling localities for each studied species (see also Figure 2).

Species	Locality	N. specimens	Coordinates	Elevation (m)
L. candidulus	Italy, Lazio, Sabaudia (LT)	3	41.25, 13.04	8
	Italy, Sardegna, Gairo (NU)	2	39.74, 9.67	67
	Italy, Sardegna, Bari sardo (NU)	2	39.84, 9.63	255
	Portugal, Monte Novo do Sul, Alcácer do Sal	4	38.40, -8.67	6
	Spain, Parque Natural Serra da Enciña da Lastra, Ourense*	8	42.47, -6.87	1,095
L. laureolae	Italy, Sicily, Malabotta (ME)	5	37.97, 15.05	1,230
	Italy, Calabria, Lungro (CS)	2	39.75, 16.09	1,241
	Italy, Abruzzo, Bosco di Sant’Antonio (AQ)	2	41.97, 14.02	1,164
L. leonardii	Spain, Piedrasluengas, Palencia	2	43.04, -4.45	1,340

*Sequence data from Baselga et al. (2015)

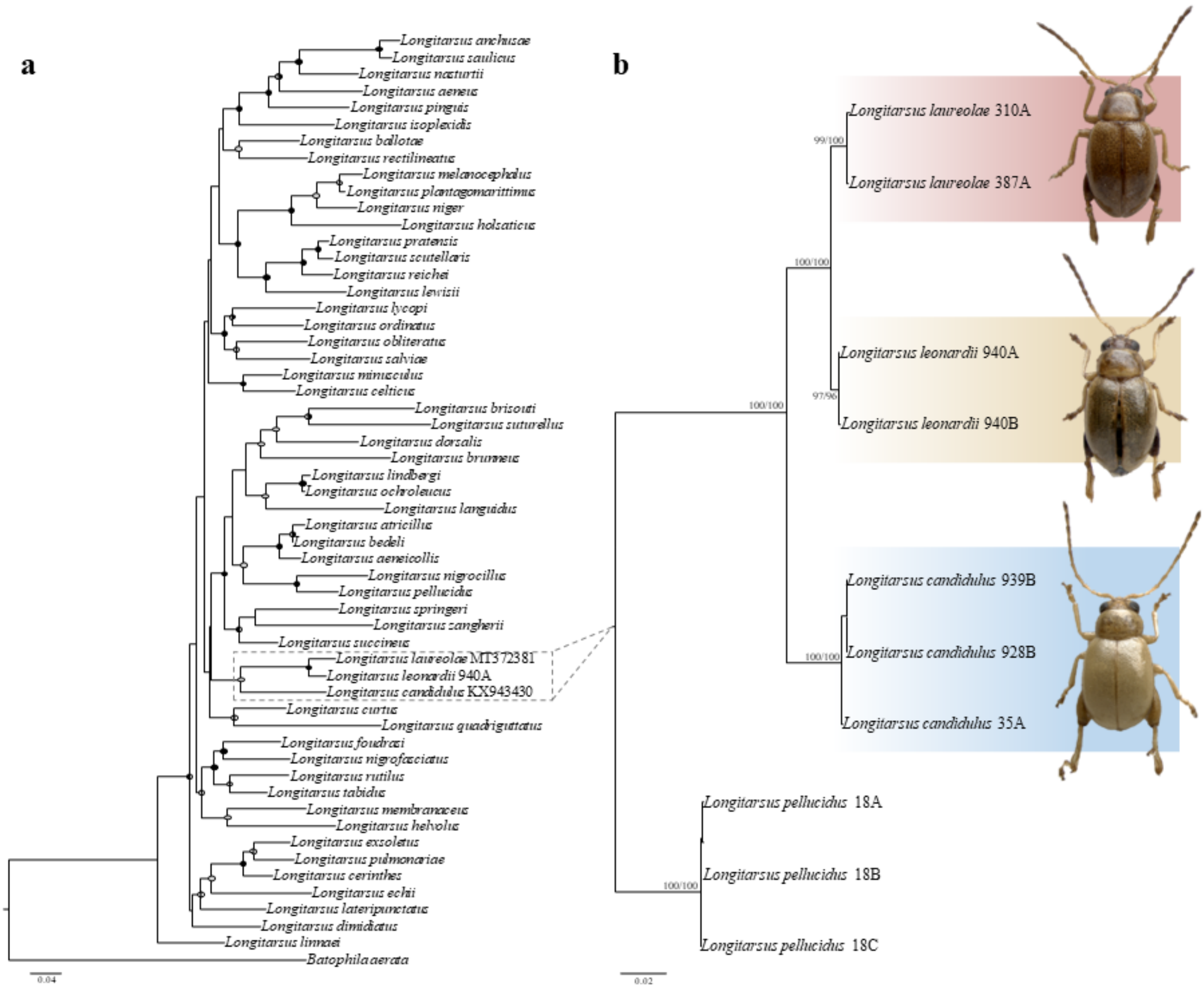

Figure 3. Maximum likelihood phylogenetic tree of Western Palaearctic *Longitarsus* species based on the *cox1* and 16S gene fragments (A). Circles in correspondence of nodes represent SH-aLRT support (SH-aLRT, left half) and ultrafast bootstrapping support (uBS, right half): black for uBS > 90 and SH-aLRT > 90; gray for uBS of 70–90 and SH-aLRT of 70–90. Half white circles or no circles represent nodes with <70 uBS and <70 SH-aLRT values. The *candidulus* species-group is highlighted by a dashed rectangle. Maximum likelihood phylogenetic tree of the *candidulus* species-group based on 2 mitochondrial (*cox1* and 16S) and 3 nuclear (CAD, Cv2, and Wg) gene fragments (B). The SH-aLRT support and uBS support is reported on the nodes. For each sequence the voucher code is reported.

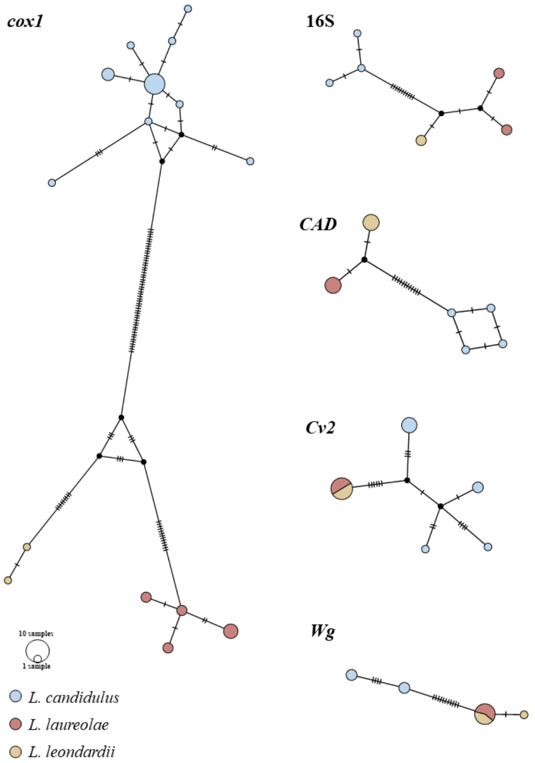

Figure 4. Haplotype networks showing the phylogenetic relationships within the candidulus species-group based on two mitochondrial (*cox1* and 16S) and 3 nuclear (CAD, Cv2 and Wg) markers. Haplotypes are represented by circles colored according to the 3 species and with a size proportional to their frequency (see the size reference inset in the bottom left corner). Vertical bars represent mutational steps (nucleotide substitutions).

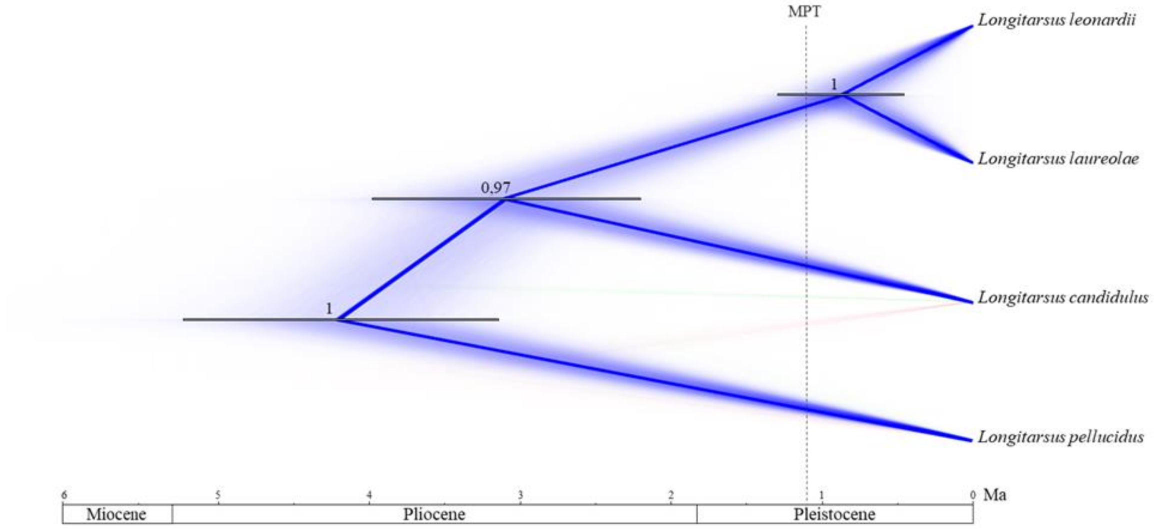

Figure 5. Time-calibrated species tree of the *candidulus* species-group estimated in BEAST showing the consensus tree topology (dark blue line) and trees from the posterior distribution visualized using the software DensiTree. Bayesian posterior probability values are reported on each node along with light blue bars representing the 95% high posterior density interval (HPD95) of node age. Time scale is in millions of years (Ma). The dashed line highlights the onset of the Mid-Pleistocene transition (MPT, ~ 1.25–0.7 Mya).

Discussion

The phylogenetic analysis of *candidulus* species-group allowed clarifying the systematics and the evolutionary history of these species and provided insights into the main paleoclimatic events that have influenced the diversification of this group in the Mediterranean region and the origin of the uncommon Apennine-Pyrenees biogeographic pattern.

Phylogenetic analyses recovered the *candidulus* species-group as a distinct lineage within the genus *Longitarsus* with a closer relationship between *L. laureolae* and *L. leonardii* endemic to Apennine and Pyrenees respectively (Figure 3). This result is consistent with the shared morphological features among species in this group, especially between the Apennine-Pyrenees species (Biondi, 1988, 1991), and with their association to host plants of the family Thymelaeaceae (Biondi, 1988). Such association is unique among *Longitarsus* flea beetles and further corroborate the finding that host-use patterns are phylogenetically constrained in *Longitarsus*, with a clear association between closely related species and specific plant families (Salvi et al., 2019).

The timing of speciation events in the *candidulus* species-group seems associated to major Plio-Pleistocene climatic events that resulted in significant environmental changes in Europe. Indeed, the split between *L. candidulus* and the ancestor of *L. laureolae* and *L. leonardii* is estimated around three million years ago (HPD95: 3.98-2.20 Mya), during the transition from the Pliocene to the Pleistocene (Piacenzian age). During this period, a change from warmer and humid to colder and dry climates took place in Europe, culminating in the establishment of the Quaternary glacial-interglacial climatic cycles (Bertini and Combourieu-Nebout, 2023). This climatic transition resulted in a significant change in the distribution of ecosystems across Europe, with the partial disappearance of the subtropical vegetation present during the Zanclean age (5.3-3.6 Myr), the expansion of the temperate "mixed mesophytic" forest (with *Picea* and *Fagus*) and the concomitant southward shift of more thermophilic species (Bertoldi et al., 1989; Bertini, 2010; Birks and Tinner, 2016). These major climatic and environmental changes have resulted in the emergence of new niches, ecological barriers, and increased habitat complexity, providing occasion for dispersal and isolation processes that ultimately lead to speciation (G. Hewitt, 2000; Schluter, 2009). According to this scenario, the divergence between the thermophilic species *L. candidulus* and the ancestor of the temperate species *L. laureolae* and *L. leonardii* would have been triggered by habitat segregation, with the former associated to xerophilous vegetation in peri-Mediterranean coastal and southern areas, and the latter tracking temperate environments. A similar case of ecological speciation associated with the environmental changes taking place during the Pliocene-Pleistocene transition was suggested for the Mediterranean endemic land snail *Murella muralis* (Fiorentino et al., 2013).

The diversification between *L. laureolae* and *L. leonardii* is estimated at 0.86 Mya (HPD95: 1.29–0.46 Mya), during the Mid-Pleistocene Transition (1.25–0.7 Mya). The recent speciation between these species is further corroborated by their reciprocally monophyly observed in fast-evolving genes (i.e., genes with short coalescent time) opposed to the lack of sorting observed at slow-evolving loci (Figure 4). During this period, the intensification of glacial cycles from a 41,000-year periodicity to a strongly asymmetric 100,000-year cycle, with long-duration cooling of the climate and build-up of thick ice sheets, caused significant environmental changes (Head et al., 2008). In Alpine regions, the enhanced glacier development, with average depression of the snowline to 1200 m, disrupted the continuity of temperate forest cover and of associated biotas (Blondel et al., 2010; Muttoni et al., 2010; Nagl, 1972; Thompson, 2020). These factors would explain how the establishment of unfavorable conditions for the persistence of temperate flea beetles in western Alps and the Massif Central, have led to the isolation of two lineages in the Pyrenees and Apennines respectively. This scenario is consistent with that proposed for the *Rupicapra pyrenaica*, whose Pyrenean and Apennine populations were probably separated during the latest middle Pleistocene due to environmental changes and competition with the northern chamois *Rupicapra rupicapra* (Fioravanti et al., 2019; Masseti & Salari, 2017). Indeed, for several other species, current disjunct distribution on different mountain ranges have been explained as the result of allopatric divergence associated with the environmental changes occurring during the Middle-Pleistocene Transition, such as in the case of butterflies of the genera *Zerynthia* (Dapporto, 2010) and *Erebia* (Hinojosa et al., 2019) or in the case of the leaf beetles of the *Cryptocephalus flavipes* species complex (Montagna et al., 2016) and the genera *Oreina* (Borer et al., 2010). This pattern of allopatric divergence can also be found in species related to mountain deciduous forests, such as in the case of Fire-coloured beetles of the genera *Pyrochroa* (Molfini et al., 2023). On the other hand, the origin of the Apennine-Pyrenees disjunct pattern observed in the perennial herbaceous plant *Adenostyles alpina* has been explained by a long-distance dispersion event from southern Italy (subsp. *macrocephala*) to the Pyrenees (subsp. *pyrenaica*) (Dillenberger and Kadereit, 2013; see also the case of *Androsace vitaliana* in Dixon et al., 2009). Long-distance dispersal is unlikely in the case of *L. laureolae* and *L. leonardii* because flea beetles have subapter wings and extremely low dispersal capacity. These findings corroborate the idiosyncratic nature of species response to Pleistocene climatic oscillation and reinforce the notion that shared biogeographic pattern do not always equate shared evolutionary histories (Provan and Bennett, 2008; Stewart et al., 2010; Canestrelli et al., 2012; Salvi et al., 2016).

Finally, the strict association between flea beetles in the *candidulus* species-group and distinct host plants raises the question on whether diversification processes in this group were associated to the historical availability of host plants. While today the distribution of *Daphne laureola*, *D. gnidium*, and *Thymelaea hirsuta* is continuous across the West Palearctic, past fragmentation of their ranges might have trigger the allopatric divergence between flea beetles. According to this hypothesis speciation in flea beetles might have been associated to the historical range changes of their host plants throughout the Pleistocene climatic cycles. Further study using climatic suitability reconstructions for both *Longitarsus* flea beetles and Thymelaeaceae host plants applied to current and past bioclimatic envelops would allow testing this hypothesis.

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Table S1. GenBank accession numbers of the DNA sequences used in this study.

Voucher	**Species**	***Cox1***	**16S**	***Wg***	***CAD***	***Cv2***
928A	*L. candidulus*	OR373124	-	-	-	-
928B	*L. candidulus*	OR373125	-	-	OR397793	OR397802
930A	*L. candidulus*	OR373126	-	-	-	-
931A	*L. candidulus*	OR373127	-	-	-	-
939A	*L. candidulus*	OR373128	-	-	-	OR397803
939B	*L. candidulus*	OR373129	OR378515	OR397813	-	OR397804
939C	*L. candidulus*	OR373130	-	-	-	-
939D	*L. candidulus*	OR373131	-	-	-	-
35A	*L. candidulus*	MT372347*	OR378514	OR397812	OR397792	OR397801
35B	*L. candidulus*	MT372346*	-	-	-	-
35D	*L. candidulus*	MT372345*	-	-	-	-
BMNH:851156	*L. candidulus*	KF654907*	-	-	-	-
BMNH:851157	*L. candidulus*	KF654908*	-	-	-	-
BMNH:851199	*L. candidulus*	KF654944*	-	-	-	-
BMNH:851200	*L. candidulus*	KF654945*	-	-	-	-
BMNH:851201	*L. candidulus*	KF654946*	-	-	-	-
BMNH:851209	*L. candidulus*	KF654954*	-	-	-	-
BMNH:851210	*L. candidulus*	KF654955*	-	-	-	-
	L. candidulus	KX943430*	KX943430*	-	-	-
31A	*L. laureolae*	MT372384*	-	-	-	-
31B	*L. laureolae*	MT372381*	OR378517	-	-	-
31C	*L. laureolae*	MT372383*	-	-	-	-
31D	*L. laureolae*	MT372382*	-	-	-	-
387B	*L. laureolae*	OR373134	-	-	-	-
387A	*L. laureolae*	OR373135	OR378518	OR397817	OR397797	OR397808
310A	*L. laureolae*	OR373138	OR378516	OR397816	OR397796	OR397807
362B	*L. laureolae*	OR373136	OR378519	-	-	-
362A	*L. laureolae*	OR373137	-	-	-	-
940A	*L. leonardii*	OR373132	OR378520	OR397814	OR397794	OR397805
940B	*L. leonardii*	OR373133	OR378521	OR397815	OR397795	OR397806
18A	*L. pellucidus*	MT372405*	OR378522	OR397809	OR397789	OR397798
18B	*L. pellucidus*	MT372406*	OR378523	OR397810	OR397790	OR397799
18C	*L. pellucidus*	MT372407*	OR378524	OR397811	OR397791	OR397800

*GenBank sequences of other studies.

Part II

OYSTERS AS STUDY MODEL

CHAPTER 3

Museum of Natural History Collections as a gold mine for taxonomic studies: the hidden diversity of oysters (Ostreidae Rafinesque, 1815)

Introduction

The marine biodiversity is still heavily underestimated due to the challenging environmental conditions that limited the exploration in the past (Appeltans et al., 2012; Bouchet, 2006). The main depositories of the marine biodiversity knowledge are Natural History Museums (NHMs) such as the Muséum National d'Histoire Naturelle of Paris (MNHN) and the Florida Museum of Natural History (FLMNH). These NHMs preserve specimens collected during a long period of time across the world's oceans and seas, and have an active role in studies on species richness and diversity, and to map species distributions and areas of conservation importance (Bakker et al., 2020; O'connell Jr. et al., 2004). Several studies demonstrated the powerful combination of NHMs collections and new molecular approaches to study the marine life diversity, and discover new species (Miller et al., 2017; Puillandre et al., 2012; Zaharias et al., 2024).

A successful approach for studying marine diversity at large scale can start with a single-gene approach derived from DNA barcoding pipeline to assess primary species hypotheses (PSH). This first step can allow to explore the level of intra- and inter-specific divergences of the molecular operational taxonomic units (MOTUs). A second step can integrate additional information from independent sets of characters (morphological, ecological, behavioural, biogeographical and so on) under an integrative taxonomy approach to determine secondary species hypotheses (SSHs) and validate the true species diversity (Pante et al., 2015; Puillandre et al., 2012; Will et al., 2005; Zamani et al., 2022). Integrative approaches have fuelled a growing trend on species discovery among marine fauna, especially in animals characterized by higher levels of cryptic diversity including oysters (Adams et al., 2014; Brasier et al., 2016; Lam & Morton, 2003; Zou & Li, 2016).

The Ostreidae Rafinesque, 1815 is a bivalve family, commonly named true oysters, well known for their important role in aquaculture (Botta et al., 2020). This family currently comprises almost 80 extant species distributed worldwide (MolluscaBase, 2024). However, most of the knowledge of this family is relative to cultured species, whereas wild species are understudied. The taxonomy and species identification of this family is challenging due to low availability of informative morphological characters, and the extensive phenotypic plasticity of shells (e.g. Salvi et al., 2022). In the last two decades, several molecular studies documented hidden cryptic diversity indicating that the diversity of this group is largely underestimated (e.g., Al-Kandari et al., 2021; Hu et al., 2019; Lam & Morton, 2003; Salvi et al., 2022; Sigwart et al., 2021; Xia et al., 2014). However, most of the studies are focused on assessment of relatively small regions (Al-Kandari et al., 2021; Crocetta et al., 2015; Kirkendale et al., 2004; Lam & Morton, 2004; Salvi et al., 2022; Sekino & Yamashita, 2016). Therefore, are knowledge on the global diversity of oysters and on their distribution is still limited.

The aim of our study is to assess the biodiversity of Ostreidae at a global scale using hundreds of specimens from two large NHM collections hosted at the MNHN (Paris) and FLMNH (Florida). Our goals are (i) to test the effectiveness of BOLD (Ratnasingham & Hebert, 2007) and GenBank (Bethesda, 1988) libraries as references for identifications of museum specimens, (ii) to explore the diversity and distribution of oyster species, and (iii) to identify putative new species to guide future integrative taxonomic studies.

Materials and methods

Museum collections

A total of 813 specimens were analysed in this study, gathered from the Muséum National d'Histoire Naturelle (MNHN) collection (N=493) and the Florida Museum of Natural History (FLMNH) collection (N=320). The proportion of morphologically identified specimens at the species-level and genus level were of 7% and 2% for the MNHN collection, and 20% and 54% for the FLMNH collection. For each specimen, we collected a small tissue sample stored in 95% ethanol for molecular analyses.

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted using the Epmotion 5075 robot (Eppendorf), following the manufacturers' recommendations at the SSM laboratory of MNHN, or using the standard high-salt protocols at the Molecular Systematics of Department of MeSVA at L'Aquila University (Sambrook et al., 1989). We amplified the mitochondrial cytochrome oxidase subunit I (*cox1*) gene fragment by polymerase chain reaction (PCR). For the COI fragment we designed new primers specific to

Ostreidae using Geneious Prime 11.0.18+10 (Biomatters Ltd., Auckland, New Zealand). We optimised the base match of the new primers' sequences according to consensus sequence generated from 3844 of *cox1* sequences retrieved from GenBank (01/04/2021) and mapped to the complete *cox1* sequence of *Magallana ariakensis* (HQ700934). New primers were: osLCO1490 (5' – TIT CIA CIA AYC AYY TRG AYA TTG G - 3') and osHCO998-R (5' – ACR GTI GCI GCI CTR AAR TAA GCI CG - 3'). The forward primer osLCO1490 is a modification of the degenerated primer jgLCO1490 proposed by Geller et al. (2013) and target the same site as the universal barcode primer LCO1490 (Folmer et al., 1994). The reverse primer osHCO998-R binds a region located downstream of the target region of the universal barcode primer HCO2198 (Folmer et al., 1994). Sequencing of PCR products was carried out by the company Genewiz® (https://www.genewiz.com), using the same primers employed for amplification.

Sequence dataset building

We built two non-redundant datasets including *cox1* sequences of museum specimens from MNHN and FLMNH collections. The first dataset (MNHN_DS1) includes a total of 252 sequences generated in the SSM lab of MNHN. The second dataset (FLMNH_DS1) includes a total of 172 sequences generated in our Molecular Systematics Lab. The sequences of the two datasets were named using the museum voucher code relative to each specimen due to the limited number of morphological identifications available for both museums' collections. Finally, the two datasets were merged into a single dataset of 424 *cox1* sequences (Museums_DS1).

We mined *cox1* sequences from the two data repositories BOLD and GenBank (GB) the 1st of January 2024. We retrieved a total of 3003 sequences from the BOLD database from the workbench section with the following filters: (i) Taxonomy = Ostreidae, (ii) Marker = COI-5P; (iii) Minimum sequence length = 300 bp. We retrieved a total of 4855 sequences from GB using the NCBI-nucleotide interface in Geneious using the query "("Ostreidae"[Organism] AND cytochrome [All Fields]) AND (animals[filter] AND mitochondrion[filter]) AND ("300"[SLEN] : "100000"[SLEN])". This search retrieved also 104 *cox1* from mitochondrial genome sequences. We use the command *Map to Reference(s)* in Geneious to map all GB sequences on the complete *cox1* gene of the annotated mitochondrial genome of *Magallana gigas* (EU672831). After excluding contaminant sequences, we obtained a total of 4018 sequences. Both BOLD and GB periodically mine sequences from each other, so we dereplicate the merged dataset deleting sequences with identical GB accession number, and we obtained a total of 4029 unique sequences. The final dataset (GB-BOLD) was trimmed using the standard barcode primers LCO1490 and HCO2198 (Folmer et al., 1994). We split the GB-BOLD dataset into four sub-datasets according to subfamilies. The four sub-datasets of Crassostreinae, Ostreinae, Saccostreinae and Striostreinae subfamilies consisted of 2684, 422, 918 and 9 sequences respectively. We manually edited the taxonomic names of sequences' names according to the most recent taxonomic nomenclature in MolluscaBase, (2024) to standardize the nomenclature with the reference library built based on newly generated sequences.

Molecular taxonomic assessment

We applied a *blastn* analysis (Johnson et al., 2008) to assign Museums_DS1 sequences at the subfamily-level or genus-level based on GB-BOLD sequences as references. After this preliminary analysis, we split the Museums_DS1 dataset into the four subfamily sub-datasets of Crassostreinae, Ostreinae, Saccostreinae and Striostreinae subfamilies consisting of 31, 235, 141, and 17 sequences respectively. Finally, for each subfamily we merged the Museums_DS1 and GB-BOLD sub-datasets and we obtained the final datasets of Crassostreinae, Ostreinae, Saccostreinae and Striostreinae with 2715, 657, 1059, and 26 sequences respectively.

We analysed the final subfamily datasets with ASAP (Puillandre et al., 2021) in default mode with a K80 model of substitution to estimate molecular operational taxonomic units (MOTUs) and we used the GB-BOLD sequences as references for naming the MOTUs. We retrieved four categories of MOTUs: i. MOTUs that reflect valid taxonomic identification at species-level, ii. MOTUs that belong to *Saccostrea* lineages (Lam & Morton, 2003; Sekino & Yamashita, 2016), iii. MOTUs that matched more than one species name in the GB-BOLD library (*i.e.* ambiguous MOTUs), and iv. MOTUs that are composed only by museum sequences, and, for this reason, cannot be assigned to any species name (*i.e.* unassigned MOTUs). The unassigned MOTUs found within Ostreinae subfamily were named according to the subfamily name following to a serial number. For the Ostreinae subfamily, we didn't assign names of MOTUs according to genus-level because previous multilocus phylogenetic studies revealed the polyphyly of the genera *Ostrea* and *Dendostrea* (Li et al., 2021; Salvi et al., 2014).

Distribution of MOTUs across marine ecoregions

We dereplicated the sampling sites associated to the specimens hosted in the museum collections and we obtained a non-redundant dataset of 217 localities. We used QGIS v3.28.13 software (QGIS Development Team, 2022) to map these localities, to assign them to the marine ecoregions of MEOW database (Spalding et al., 2007) and to illustrate the geographic coverage of the two museum collections. For each marine realm and province, we retrieved the number of species collected using the command *intersect* in QGIS. One voucher specimen IM-2013-62625 was excluded from the analysis because lacking geographic information on the MNHN database. We used RStudio v. 2023.09.0 to plot the number of specimens and MOTUs found in each marine realms using the R package *ggplot2* (Wickham, 2016).

Results

We obtained 424 unique *cox1* sequences from the 813 specimens analysed, corresponding to a sequencing success rate of 52%. The 424 sequenced specimens cluster in 67 unique MOTUs, among which 17 corresponds to known species, 7 to known *Saccostrea* lineages, 5 are ambiguous MOTUs, and 38 are unassigned MOTUs. In terms of museum specimens, we successfully identified 119 museum specimens as 17 known species (28.1%), and 70 specimens as 7 *Saccostrea* lineages (16.5%) out of ten lineages described in Lam & Morton (2003) and Sekino & Yamashita (2016). Whereas 86 specimens belong to the 5 ambiguous MOTUs (20.3%), and 149 specimens belong to the 38 unassigned MOTUs (35.1%). The highest number of ambiguous and unassigned MOTUs are within the Ostreinae subfamily with 3 and 34 MOTUs respectively. We retrieved only 1 ambiguous MOTU within Crassostreinae and 4 unassigned MOTUs within Saccostreinae.

The map of all the 217 sampling localities, for each museum collection, is reported in Figure 1. A total of 43 museum specimens were sampled in temperate regions (10%) and 380 in tropical regions (90%). Temperate realms registered higher rate of identifications at species-level (88.4%) than the tropical realms (21.1%). The number of specimens and distinct MOTUs among temperate and tropical ecosystems are listed in Table 1. For the complete list of all the museum specimens, the MOTUs and the geographical information see Table 2. The list of coordinates of each sampling localities are reported in the supplementary Table S1.

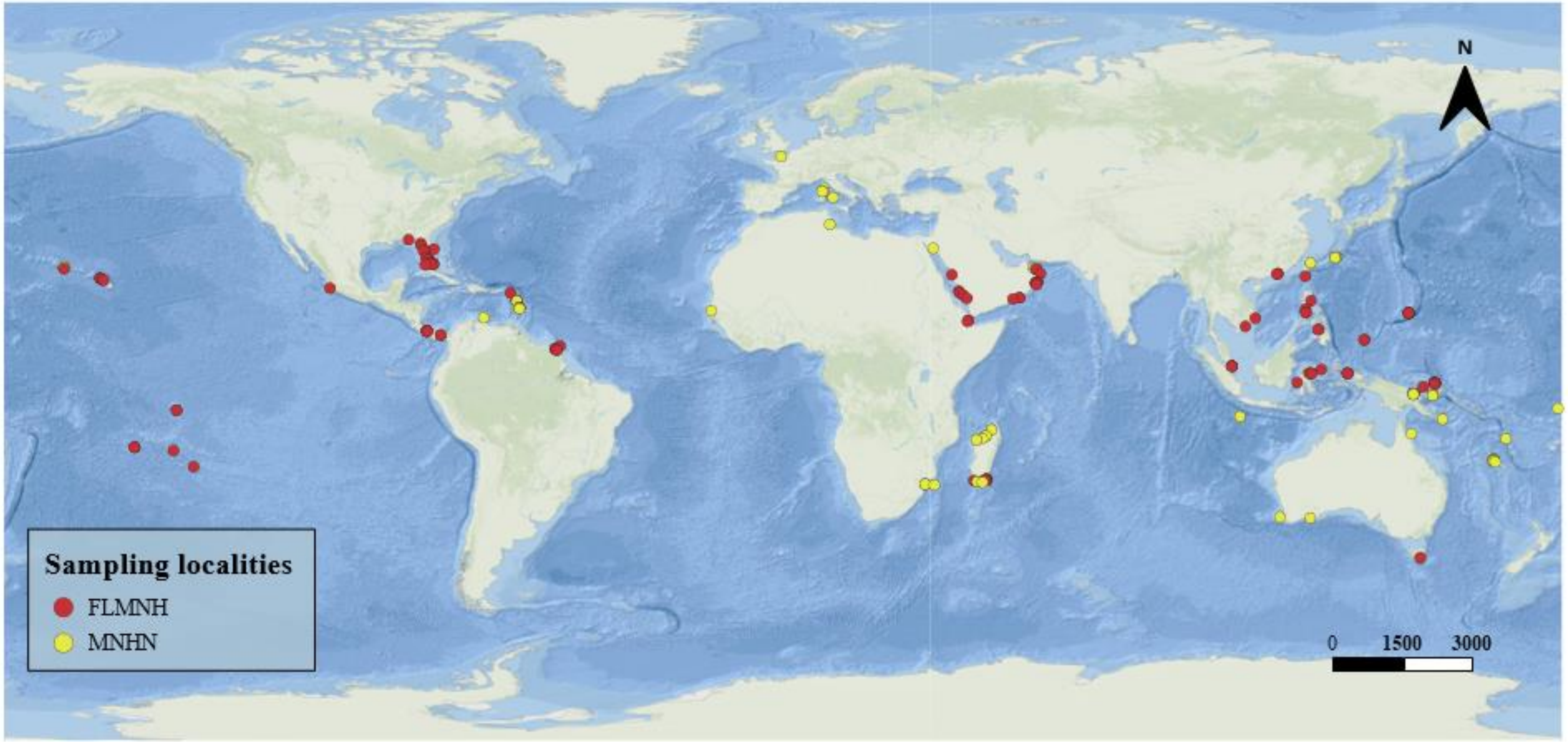

Figure 1. Distribution of sampling sites of MNHN and FLMNH collections.

Table 1. Numbers of museum specimens and number of distinct MOTUs in the temperate and tropical regions according to MEOW database (Spalding et al., 2007). "Correct" refers to identifications at species-level; "Lineage" refers to the *Saccostrea* MOTUs described in Lam & Morton (2003) and Sekino & Yamashita, (2016); "Ambiguous" refers to specimens that matched BOLD-GB sequences with more than one taxonomic name; and "Unassigned" refers to specimens that didn't match any GB-BOLD sequence. The sum of the specimens is 423 because the voucher IM-2013-62625 of MNHN does not have geographical information in the museum database (see Table 2).

Identifications	**N. of specimens**	**% of specimens**	**N. of MOTUs**	**% of MOTUs**
Temperate region	**43**	**100**	**9**	**100**
Correct	38	88.4%	7	77.8%
Lineage	0	0%	0	0%
Ambiguous	3	7.0%	1	11.1%
Unassigned	2	4.7%	1	11.1%
Tropical region	**380**	**100**	**63**	**100**
Correct	70	18.4%	13	20.6%
Lineage	84	22.1%	7	11.1%
Ambiguous	146	38.4%	5	8%
Unassigned	80	21.1%	38	60.3%

Table 2. List of all the museum specimens used in our analysis. Vouchers that start with "UF" are from the FLMNH collection, and vouchers that start with "IM" are from the MNHN collection. Other codes in the vouchers' column refer to specimens that still need to be registered in the MNHN collection. We also reported subfamilies, MOTUs, locality code, marine realm, marine province, and latitudinal zones.

Voucher	**Subfamily**	**MOTU**	**Loc**	**Realm**	**Province**	**Lat_Zone**
UF446711	Crassostreainae	*Crassostrea virginica*	64	Temperate Northern Atlantic	Warm Temperate Northwest Atlantic	Temperate
UF462088	Crassostreainae	*Crassostrea virginica*	67	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-62629	Crassostreinae	*Crassostrea brasiliana/gasar/tulipa*	104	Tropical Atlantic	West African Transition	Tropical
IM-2013-62630	Crassostreinae	*Crassostrea brasiliana/gasar/tulipa*	104	Tropical Atlantic	West African Transition	Tropical
IM-2013-62631	Crassostreinae	*Crassostrea brasiliana/gasar/tulipa*	104	Tropical Atlantic	West African Transition	Tropical
IM-2013-57218	Crassostreinae	*Crassostrea brasiliana/gasar/tulipa*	116	Tropical Atlantic	North Brazil Shelf	Tropical
IM-2013-57219	Crassostreinae	*Crassostrea brasiliana/gasar/tulipa*	116	Tropical Atlantic	North Brazil Shelf	Tropical
IM-2013-57220	Crassostreinae	*Crassostrea brasiliana/gasar/tulipa*	116	Tropical Atlantic	North Brazil Shelf	Tropical
IM-2013-57221	Crassostreinae	*Crassostrea brasiliana/gasar/tulipa*	116	Tropical Atlantic	North Brazil Shelf	Tropical
IM-2013-57222	Crassostreinae	*Crassostrea brasiliana/gasar/tulipa*	116	Tropical Atlantic	North Brazil Shelf	Tropical
IM-2013-20890	Crassostreinae	*Crassostrea rhizophorae*	93	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-20129	Crassostreinae	*Crassostrea rhizophorae*	93	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-20965	Crassostreinae	*Crassostrea rhizophorae*	94	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-20955	Crassostreinae	*Crassostrea rhizophorae*	94	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2019-16315	Crassostreinae	*Magallana belchieri*	54	Central Indo-Pacific	Sunda Shelf	Tropical
IM-2019-16316	Crassostreinae	*Magallana belchieri*	54	Central Indo-Pacific	Sunda Shelf	Tropical
IM-2019-16317	Crassostreinae	*Magallana belchieri*	54	Central Indo-Pacific	Sunda Shelf	Tropical
IM-2009-7743	Crassostreinae	*Magallana gigas*	1	Temperate Northern Atlantic	Northern European Seas	Temperate

IM-2009-7742	Crassostreinae	*Magallana gigas*	1	Temperate Northern Atlantic	Northern European Seas	Temperate
IM-2009-7741	Crassostreinae	*Magallana gigas*	1	Temperate Northern Atlantic	Northern European Seas	Temperate
IM-2009-7845	Crassostreinae	*Magallana gigas*	5	Temperate Northern Atlantic	Mediterranean Sea	Temperate
IM-2009-7844	Crassostreinae	*Magallana gigas*	5	Temperate Northern Atlantic	Mediterranean Sea	Temperate
IM-2009-7843	Crassostreinae	*Magallana gigas*	5	Temperate Northern Atlantic	Mediterranean Sea	Temperate
A	Crassostreinae	*Magallana gigas*	51	Central Indo-Pacific	Sunda Shelf	Tropical
B	Crassostreinae	*Magallana gigas*	51	Central Indo-Pacific	Sunda Shelf	Tropical
C	Crassostreinae	*Magallana gigas*	51	Central Indo-Pacific	Sunda Shelf	Tropical
IM-2000-32035	Crassostreinae	*Magallana gigas*	51	Central Indo-Pacific	Sunda Shelf	Tropical
IM-2013-62640	Crassostreinae	*Magallana gigas*	209	Temperate Australasia	Southeast Australian Shelf	Temperate
IM-2013-57320	Crassostreinae	*Talonostrea talonata*	117	Tropical Atlantic	North Brazil Shelf	Tropical
IM-2013-57202	Crassostreinae	*Talonostrea talonata*	118	Tropical Atlantic	North Brazil Shelf	Tropical
IM-2013-57201	Crassostreinae	*Talonostrea talonata*	118	Tropical Atlantic	North Brazil Shelf	Tropical
UF463996	Ostreinae	*Alectrionella plicatula*	60	Central Indo-Pacific	Sunda Shelf	Tropical
UF327545	Ostreinae	*Alectrionella plicatula*	119	Central Indo-Pacific	Western Coral Triangle	Tropical
UF327543	Ostreinae	*Alectrionella plicatula*	119	Central Indo-Pacific	Western Coral Triangle	Tropical
UF327544	Ostreinae	*Alectrionella plicatula*	119	Central Indo-Pacific	Western Coral Triangle	Tropical
UF280282	Ostreinae	*Alectrionella plicatula*	128	Central Indo-Pacific	Western Coral Triangle	Tropical
IM-2013-50437	Ostreinae	*Alectrionella plicatula*	130	Central Indo-Pacific	Eastern Coral Triangle	Tropical
UF322096	Ostreinae	*Alectrionella plicatula*	138	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-10441	Ostreinae	*Alectrionella plicatula*	148	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-16366	Ostreinae	*Alectrionella plicatula*	149	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-10249	Ostreinae	*Alectrionella plicatula*	150	Central Indo-Pacific	Eastern Coral Triangle	Tropical
UF323383	Ostreinae	*Alectrionella plicatula*	161	Central Indo-Pacific	Eastern Coral Triangle	Tropical
UF323686	Ostreinae	*Alectrionella plicatula*	162	Central Indo-Pacific	Eastern Coral Triangle	Tropical
UF467652	Ostreinae	*Dendostrea* cf *frons*	35	Western Indo-Pacific	Red Sea and Gulf of Aden	Tropical
UF521082	Ostreinae	*Dendostrea* cf *frons*	36	Western Indo-Pacific	Red Sea and Gulf of Aden	Tropical
UF500135	Ostreinae	*Dendostrea* cf *frons*	44	Central Indo-Pacific	Western Coral Triangle	Tropical
UF455813	Ostreinae	*Dendostrea* cf *frons*	52	Western Indo-Pacific	Red Sea and Gulf of Aden	Tropical

IM-2007-39358	Ostreinae	*Dendostrea* cf *frons*	173	Western Indo-Pacific	Western Indian Ocean	Tropical
UF445387	Ostreinae	*Dendostrea folium/sandvichensis*	49	Central Indo-Pacific	Tropical Northwestern Pacific	Tropical
IM-2013-17303	Ostreinae	*Dendostrea folium/sandvichensis*	144	Central Indo-Pacific	Eastern Coral Triangle	Tropical
UF445594	Ostreinae	*Dendostrea frons/sandvichensis*	9	Central Indo-Pacific	South Kuroshio	Tropical
UF445556	Ostreinae	*Dendostrea frons/sandvichensis*	12	Central Indo-Pacific	South Kuroshio	Tropical
UF463039	Ostreinae	*Dendostrea frons/sandvichensis*	13	Central Indo-Pacific	South Kuroshio	Tropical
UF510950	Ostreinae	*Dendostrea frons/sandvichensis*	22	Central Indo-Pacific	South China Sea	Tropical
UF511059	Ostreinae	*Dendostrea frons/sandvichensis*	23	Central Indo-Pacific	South China Sea	Tropical
UF544829	Ostreinae	*Dendostrea frons/sandvichensis*	24	Central Indo-Pacific	South China Sea	Tropical
UF544831	Ostreinae	*Dendostrea frons/sandvichensis*	24	Central Indo-Pacific	South China Sea	Tropical
UF544830	Ostreinae	*Dendostrea frons/sandvichensis*	24	Central Indo-Pacific	South China Sea	Tropical
UF299446	Ostreinae	*Dendostrea frons/sandvichensis*	46	Central Indo-Pacific	Tropical Northwestern Pacific	Tropical
UF459364	Ostreinae	*Dendostrea frons/sandvichensis*	47	Central Indo-Pacific	Tropical Northwestern Pacific	Tropical
UF463046	Ostreinae	*Dendostrea frons/sandvichensis*	50	Central Indo-Pacific	Tropical Northwestern Pacific	Tropical
UF463053	Ostreinae	*Dendostrea frons/sandvichensis*	50	Central Indo-Pacific	Tropical Northwestern Pacific	Tropical
UF463975	Ostreinae	*Dendostrea frons/sandvichensis*	59	Central Indo-Pacific	Sunda Shelf	Tropical
UF463986	Ostreinae	*Dendostrea frons/sandvichensis*	61	Central Indo-Pacific	Sunda Shelf	Tropical
UF508000	Ostreinae	*Dendostrea frons/sandvichensis*	85	Eastern Indo-Pacific	Hawaii	Tropical
UF508474	Ostreinae	*Dendostrea frons/sandvichensis*	86	Eastern Indo-Pacific	Hawaii	Tropical

UF508475	Ostreinae	*Dendostrea frons/sandvichensis*	86	Eastern Indo-Pacific	Hawaii	Tropical
UF508476	Ostreinae	*Dendostrea frons/sandvichensis*	86	Eastern Indo-Pacific	Hawaii	Tropical
UF508471	Ostreinae	*Dendostrea frons/sandvichensis*	86	Eastern Indo-Pacific	Hawaii	Tropical
UF508473	Ostreinae	*Dendostrea frons/sandvichensis*	86	Eastern Indo-Pacific	Hawaii	Tropical
UF508018	Ostreinae	*Dendostrea frons/sandvichensis*	87	Eastern Indo-Pacific	Hawaii	Tropical
UF303671	Ostreinae	*Dendostrea frons/sandvichensis*	88	Eastern Indo-Pacific	Hawaii	Tropical
UF303677	Ostreinae	*Dendostrea frons/sandvichensis*	89	Eastern Indo-Pacific	Hawaii	Tropical
IM-2013-20047	Ostreinae	*Dendostrea frons/sandvichensis*	92	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-20052	Ostreinae	*Dendostrea frons/sandvichensis*	92	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-9579	Ostreinae	*Dendostrea frons/sandvichensis*	92	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-9578	Ostreinae	*Dendostrea frons/sandvichensis*	92	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-20061	Ostreinae	*Dendostrea frons/sandvichensis*	92	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-20168	Ostreinae	*Dendostrea frons/sandvichensis*	93	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-71095	Ostreinae	*Dendostrea frons/sandvichensis*	96	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-71199	Ostreinae	*Dendostrea frons/sandvichensis*	99	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-71548	Ostreinae	*Dendostrea frons/sandvichensis*	100	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-71625	Ostreinae	*Dendostrea frons/sandvichensis*	101	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-71626	Ostreinae	*Dendostrea frons/sandvichensis*	101	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical

IM-2013-70701	Ostreinae	*Dendostrea frons/sandvichensis*	102	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-70347	Ostreinae	*Dendostrea frons/sandvichensis*	103	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF526145	Ostreinae	*Dendostrea frons/sandvichensis*	107	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF526144	Ostreinae	*Dendostrea frons/sandvichensis*	107	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF508728	Ostreinae	*Dendostrea frons/sandvichensis*	109	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF526150	Ostreinae	*Dendostrea frons/sandvichensis*	110	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF526151	Ostreinae	*Dendostrea frons/sandvichensis*	111	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF444901	Ostreinae	*Dendostrea frons/sandvichensis*	123	Central Indo-Pacific	Western Coral Triangle	Tropical
UF444886	Ostreinae	*Dendostrea frons/sandvichensis*	123	Central Indo-Pacific	Western Coral Triangle	Tropical
UF310047	Ostreinae	*Dendostrea frons/sandvichensis*	126	Central Indo-Pacific	Western Coral Triangle	Tropical
UF423753	Ostreinae	*Dendostrea frons/sandvichensis*	166	Western Indo-Pacific	Western Indian Ocean	Tropical
UF400974	Ostreinae	*Dendostrea frons/sandvichensis*	215	Eastern Indo-Pacific	Southeast Polynesia	Tropical
UF400963	Ostreinae	*Dendostrea frons/sandvichensis*	215	Eastern Indo-Pacific	Southeast Polynesia	Tropical
UF400952	Ostreinae	*Dendostrea frons/sandvichensis*	215	Eastern Indo-Pacific	Southeast Polynesia	Tropical
UF322520	Ostreinae	*Lopha cristagalli*	137	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-62634	Ostreinae	*Ostrea angasi*	209	Temperate Australasia	Southeast Australian Shelf	Temperate
IM-2013-62635	Ostreinae	*Ostrea angasi*	209	Temperate Australasia	Southeast Australian Shelf	Temperate
IM-2013-62636	Ostreinae	*Ostrea angasi*	209	Temperate Australasia	Southeast Australian Shelf	Temperate
IM-2013-62637	Ostreinae	*Ostrea angasi*	209	Temperate Australasia	Southeast Australian Shelf	Temperate
IM-2013-62638	Ostreinae	*Ostrea angasi*	209	Temperate Australasia	Southeast Australian Shelf	Temperate
IM-2013-62639	Ostreinae	*Ostrea angasi*	209	Temperate Australasia	Southeast Australian Shelf	Temperate
IM-2013-62641	Ostreinae	*Ostrea angasi*	209	Temperate Australasia	Southeast Australian Shelf	Temperate

IM-2009-7744	Ostreinae	*Ostrea edulis*	1	Temperate Northern Atlantic	Northern European Seas	Temperate
IM-2019-4910	Ostreinae	*Ostrea edulis*	2	Temperate Northern Atlantic	Mediterranean Sea	Temperate
IM-2019-4908	Ostreinae	*Ostrea edulis*	2	Temperate Northern Atlantic	Mediterranean Sea	Temperate
IM-2019-14340	Ostreinae	*Ostrea edulis*	3	Temperate Northern Atlantic	Mediterranean Sea	Temperate
IM-2019-14341	Ostreinae	*Ostrea edulis*	3	Temperate Northern Atlantic	Mediterranean Sea	Temperate
IM-2013-62633	Ostreinae	*Ostrea edulis*	210	Temperate Australasia	Southeast Australian Shelf	Temperate
UF511031	Ostreinae	*Ostrea equestris*	24	Central Indo-Pacific	South China Sea	Tropical
UF511096	Ostreinae	*Ostrea equestris*	24	Central Indo-Pacific	South China Sea	Tropical
UF286693	Ostreinae	*Ostrea equestris*	70	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF367498	Ostreinae	*Ostrea equestris*	75	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF367509	Ostreinae	*Ostrea equestris*	75	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF298640	Ostreinae	*Ostrea equestris*	75	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF298639	Ostreinae	*Ostrea equestris*	76	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF367484	Ostreinae	*Ostrea equestris*	76	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF298645	Ostreinae	*Ostrea equestris*	76	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF298643	Ostreinae	*Ostrea equestris*	79	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF508472	Ostreinae	*Ostrea equestris*	86	Eastern Indo-Pacific	Hawaii	Tropical
IM-2013-62592	Ostreinae	*Ostrea equestris*	186	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62529	Ostreinae	*Ostrea equestris*	186	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62599	Ostreinae	*Ostrea equestris*	207	Temperate Australasia	Southwest Australian Shelf	Temperate
IM-2013-62595	Ostreinae	*Ostrea equestris*	208	Temperate Australasia	Southwest Australian Shelf	Temperate
IM-2013-62605	Ostreinae	*Ostrea equestris*	208	Temperate Australasia	Southwest Australian Shelf	Temperate
UF341294	Ostreinae	*Ostrea futamiensis*	62	Central Indo-Pacific	Sunda Shelf	Tropical
IM-2019-13386	Ostreinae	*Ostrea neostentina*	4	Temperate Northern Atlantic	Mediterranean Sea	Temperate
IM-2019-13387	Ostreinae	*Ostrea neostentina*	4	Temperate Northern Atlantic	Mediterranean Sea	Temperate
IM-2019-13388	Ostreinae	*Ostrea neostentina*	4	Temperate Northern Atlantic	Mediterranean Sea	Temperate

IM-2013-32056	Ostreinae	*Ostrea neostentina*	6	Temperate Northern Atlantic	Mediterranean Sea	Temperate
IM-2013-32057	Ostreinae	*Ostrea neostentina*	6	Temperate Northern Atlantic	Mediterranean Sea	Temperate
IM-2013-32058	Ostreinae	*Ostrea neostentina*	6	Temperate Northern Atlantic	Mediterranean Sea	Temperate
IM-2013-32059	Ostreinae	*Ostrea neostentina*	6	Temperate Northern Atlantic	Mediterranean Sea	Temperate
IM-2013-32060	Ostreinae	*Ostrea neostentina*	6	Temperate Northern Atlantic	Mediterranean Sea	Temperate
IM-2013-62598	Ostreinae	*Ostrea neostentina*	207	Temperate Australasia	Southwest Australian Shelf	Temperate
IM-2013-62603	Ostreinae	*Ostrea neostentina*	208	Temperate Australasia	Southwest Australian Shelf	Temperate
IM-2013-62601	Ostreinae	*Ostrea neostentina*	208	Temperate Australasia	Southwest Australian Shelf	Temperate
IM-2013-56656	Ostreinae	*Ostrea permollis*	114	Tropical Atlantic	North Brazil Shelf	Tropical
UF463229	Ostreinae	Ostreinae 1	35	Western Indo-Pacific	Red Sea and Gulf of Aden	Tropical
UF467654	Ostreinae	Ostreinae 1	35	Western Indo-Pacific	Red Sea and Gulf of Aden	Tropical
UF467655	Ostreinae	Ostreinae 1	35	Western Indo-Pacific	Red Sea and Gulf of Aden	Tropical
UF574575	Ostreinae	Ostreinae 1	37	Western Indo-Pacific	Somali/Arabian	Tropical
UF423883	Ostreinae	Ostreinae 1	167	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62492	Ostreinae	Ostreinae 1	191	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62495	Ostreinae	Ostreinae 1	191	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62494	Ostreinae	Ostreinae 1	191	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62514	Ostreinae	Ostreinae 1	197	Western Indo-Pacific	Western Indian Ocean	Tropical
UF569962	Ostreinae	Ostreinae 10	19	Western Indo-Pacific	Somali/Arabian	Tropical
IM-2007-39238	Ostreinae	Ostreinae 10	171	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2007-39237	Ostreinae	Ostreinae 10	171	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2007-39236	Ostreinae	Ostreinae 10	171	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2007-39235	Ostreinae	Ostreinae 10	171	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2007-39234	Ostreinae	Ostreinae 10	171	Western Indo-Pacific	Western Indian Ocean	Tropical
UF476052	Ostreinae	Ostreinae 11	7	Western Indo-Pacific	Red Sea and Gulf of Aden	Tropical
UF570190	Ostreinae	Ostreinae 11	15	Western Indo-Pacific	Somali/Arabian	Tropical
UF571177	Ostreinae	Ostreinae 11	18	Western Indo-Pacific	Somali/Arabian	Tropical
IM-2007-33654	Ostreinae	Ostreinae 11	56	Central Indo-Pacific	Western Coral Triangle	Tropical
UF505493	Ostreinae	Ostreinae 11	108	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical

IM-2013-62498	Ostreinae	Ostreinae 11	190	Western Indo-Pacific	Western Indian Ocean	Tropical
UF322404	Ostreinae	Ostreinae 12	133	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-11257	Ostreinae	Ostreinae 12	146	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-11258	Ostreinae	Ostreinae 12	146	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-11308	Ostreinae	Ostreinae 12	156	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-16523	Ostreinae	Ostreinae 12	159	Central Indo-Pacific	Eastern Coral Triangle	Tropical
UF461404	Ostreinae	Ostreinae 13	216	Eastern Indo-Pacific	Southeast Polynesia	Tropical
UF461476	Ostreinae	Ostreinae 13	217	Eastern Indo-Pacific	Southeast Polynesia	Tropical
UF461479	Ostreinae	Ostreinae 13	217	Eastern Indo-Pacific	Southeast Polynesia	Tropical
UF445881	Ostreinae	Ostreinae 14	8	Central Indo-Pacific	South Kuroshio	Tropical
UF428377	Ostreinae	Ostreinae 14	168	Central Indo-Pacific	Northeast Australian Shelf	Tropical
UF445819	Ostreinae	Ostreinae 15	11	Central Indo-Pacific	South Kuroshio	Tropical
UF445864	Ostreinae	Ostreinae 15	11	Central Indo-Pacific	South Kuroshio	Tropical
UF445720	Ostreinae	Ostreinae 15	13	Central Indo-Pacific	South Kuroshio	Tropical
UF327541	Ostreinae	Ostreinae 16	126	Central Indo-Pacific	Western Coral Triangle	Tropical
UF327542	Ostreinae	Ostreinae 16	126	Central Indo-Pacific	Western Coral Triangle	Tropical
UF310077	Ostreinae	Ostreinae 16	126	Central Indo-Pacific	Western Coral Triangle	Tropical
UF414075	Ostreinae	Ostreinae 17	81	Eastern Indo-Pacific	Hawaii	Tropical
UF414049	Ostreinae	Ostreinae 17	82	Eastern Indo-Pacific	Hawaii	Tropical
UF413913	Ostreinae	Ostreinae 17	84	Eastern Indo-Pacific	Hawaii	Tropical
UF323789	Ostreinae	Ostreinae 18	160	Central Indo-Pacific	Eastern Coral Triangle	Tropical
UF347795	Ostreinae	Ostreinae 19	163	Eastern Indo-Pacific	Marshall, Gilbert and Ellis Islands	Tropical
UF436261	Ostreinae	Ostreinae 2	65	Temperate Northern Atlantic	Warm Temperate Northwest Atlantic	Temperate
UF449256	Ostreinae	Ostreinae 2	66	Temperate Northern Atlantic	Warm Temperate Northwest Atlantic	Temperate
UF510818	Ostreinae	Ostreinae 2	68	Temperate Northern Atlantic	Warm Temperate Northwest Atlantic	Temperate
UF450595	Ostreinae	Ostreinae 2	69	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF446815	Ostreinae	Ostreinae 2	71	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF350115	Ostreinae	Ostreinae 2	72	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF286654	Ostreinae	Ostreinae 2	73	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF286719	Ostreinae	Ostreinae 2	74	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical

UF437453	Ostreinae	Ostreinae 2	78	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF437454	Ostreinae	Ostreinae 2	78	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF437456	Ostreinae	Ostreinae 2	78	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF437451	Ostreinae	Ostreinae 2	78	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF437452	Ostreinae	Ostreinae 2	78	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF437455	Ostreinae	Ostreinae 2	78	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF323746	Ostreinae	Ostreinae 2	80	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF414061	Ostreinae	Ostreinae 2	83	Eastern Indo-Pacific	Hawaii	Tropical
UF451251	Ostreinae	Ostreinae 2	91	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-8631	Ostreinae	Ostreinae 2	93	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-8632	Ostreinae	Ostreinae 2	93	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-8633	Ostreinae	Ostreinae 2	93	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-8634	Ostreinae	Ostreinae 2	93	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-72639	Ostreinae	Ostreinae 2	97	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-71145	Ostreinae	Ostreinae 2	98	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-71146	Ostreinae	Ostreinae 2	98	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-71152	Ostreinae	Ostreinae 2	100	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-70702	Ostreinae	Ostreinae 2	102	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-70346	Ostreinae	Ostreinae 2	103	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF551105	Ostreinae	Ostreinae 2	105	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF372447	Ostreinae	Ostreinae 2	106	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF445582	Ostreinae	Ostreinae 20	9	Central Indo-Pacific	South Kuroshio	Tropical
UF445619	Ostreinae	Ostreinae 20	9	Central Indo-Pacific	South Kuroshio	Tropical
UF446728	Ostreinae	Ostreinae 20	9	Central Indo-Pacific	South Kuroshio	Tropical
UF445733	Ostreinae	Ostreinae 20	10	Central Indo-Pacific	South Kuroshio	Tropical
UF521024	Ostreinae	Ostreinae 21	34	Western Indo-Pacific	Red Sea and Gulf of Aden	Tropical
IM-2013-62620	Ostreinae	Ostreinae 21	205	Western Indo-Pacific	Western Indian Ocean	Tropical
UF304339	Ostreinae	Ostreinae 22	26	Central Indo-Pacific	South Kuroshio	Tropical
UF493157	Ostreinae	Ostreinae 22	43	Central Indo-Pacific	Western Coral Triangle	Tropical
IM-2013-55780	Ostreinae	Ostreinae 22	134	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-12625	Ostreinae	Ostreinae 22	151	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-11333	Ostreinae	Ostreinae 22	154	Central Indo-Pacific	Eastern Coral Triangle	Tropical

IM-2013-11315	Ostreinae	Ostreinae 22	156	Central Indo-Pacific	Eastern Coral Triangle	Tropical
UF366014	Ostreinae	Ostreinae 22	165	Central Indo-Pacific	Eastern Coral Triangle	Tropical
UF310072	Ostreinae	Ostreinae 23	121	Central Indo-Pacific	Western Coral Triangle	Tropical
UF310708	Ostreinae	Ostreinae 24	63	Central Indo-Pacific	Western Coral Triangle	Tropical
IM-2013-16417	Ostreinae	Ostreinae 26	139	Central Indo-Pacific	Eastern Coral Triangle	Tropical
UF584958	Ostreinae	Ostreinae 3	27	Western Indo-Pacific	Somali/Arabian	Tropical
UF584907	Ostreinae	Ostreinae 3	28	Western Indo-Pacific	Somali/Arabian	Tropical
UF367977	Ostreinae	Ostreinae 3	29	Western Indo-Pacific	Somali/Arabian	Tropical
UF367875	Ostreinae	Ostreinae 3	31	Western Indo-Pacific	Somali/Arabian	Tropical
UF585063	Ostreinae	Ostreinae 3	32	Western Indo-Pacific	Somali/Arabian	Tropical
UF297605	Ostreinae	Ostreinae 3	33	Western Indo-Pacific	Somali/Arabian	Tropical
UF280251	Ostreinae	Ostreinae 3	120	Central Indo-Pacific	Western Coral Triangle	Tropical
IM-2013-62594	Ostreinae	Ostreinae 3	189	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-20007	Ostreinae	Ostreinae 30	95	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-20176	Ostreinae	Ostreinae 30	95	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-20178	Ostreinae	Ostreinae 30	95	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-57043	Ostreinae	Ostreinae 30	115	Tropical Atlantic	North Brazil Shelf	Tropical
IM-2013-51330	Ostreinae	Ostreinae 31	136	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-17314	Ostreinae	Ostreinae 31	141	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-53571	Ostreinae	Ostreinae 32	127	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-5665	Ostreinae	Ostreinae 32	142	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2019-10054	Ostreinae	Ostreinae 32	181	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
IM-2019-8308	Ostreinae	Ostreinae 32	183	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
IM-2007-39012	Ostreinae	Ostreinae 33	174	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2019-8726	Ostreinae	Ostreinae 34	178	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
IM-2013-62527	Ostreinae	Ostreinae 35	201	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2007-38587	Ostreinae	Ostreinae 36	169	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2007-39288	Ostreinae	Ostreinae 36	170	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62490	Ostreinae	Ostreinae 36	202	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2007-38312	Ostreinae	Ostreinae 37	206	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2007-38311	Ostreinae	Ostreinae 37	206	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2007-38309	Ostreinae	Ostreinae 37	206	Western Indo-Pacific	Western Indian Ocean	Tropical

IM-2007-38308	Ostreinae	Ostreinae 37	206	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-81374	Ostreinae	Ostreinae 38	177	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
UF298644B	Ostreinae	Ostreinae 4	77	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF298644C	Ostreinae	Ostreinae 4	77	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
UF569985	Ostreinae	Ostreinae 5	20	Western Indo-Pacific	Somali/Arabian	Tropical
UF569992	Ostreinae	Ostreinae 5	20	Western Indo-Pacific	Somali/Arabian	Tropical
UF576596	Ostreinae	Ostreinae 6	38	Western Indo-Pacific	Somali/Arabian	Tropical
UF576693	Ostreinae	Ostreinae 7	16	Western Indo-Pacific	Somali/Arabian	Tropical
UF585152	Ostreinae	Ostreinae 8	28	Western Indo-Pacific	Somali/Arabian	Tropical
UF585836	Ostreinae	Ostreinae 9	40	Western Indo-Pacific	Somali/Arabian	Tropical
UF585837	Ostreinae	Ostreinae 9	40	Western Indo-Pacific	Somali/Arabian	Tropical
IM-2013-62604	Saccostreinae	*Saccostrea glomerata*	207	Temperate Australasia	Southwest Australian Shelf	Temperate
IM-2013-62597	Saccostreinae	*Saccostrea glomerata*	207	Temperate Australasia	Southwest Australian Shelf	Temperate
IM-2013-62596	Saccostreinae	*Saccostrea glomerata*	207	Temperate Australasia	Southwest Australian Shelf	Temperate
UF445625	Saccostreinae	*Saccostrea mordax/scyphophilla*	12	Central Indo-Pacific	South Kuroshio	Tropical
UF445617	Saccostreinae	*Saccostrea mordax/scyphophilla*	12	Central Indo-Pacific	South Kuroshio	Tropical
UF449037	Saccostreinae	*Saccostrea mordax/scyphophilla*	14	Temperate Northern Pacific	Warm Temperate Northwest Pacific	Temperate
UF449035	Saccostreinae	*Saccostrea mordax/scyphophilla*	14	Temperate Northern Pacific	Warm Temperate Northwest Pacific	Temperate
UF367888	Saccostreinae	*Saccostrea mordax/scyphophilla*	17	Western Indo-Pacific	Somali/Arabian	Tropical
UF577625	Saccostreinae	*Saccostrea mordax/scyphophilla*	21	Western Indo-Pacific	Somali/Arabian	Tropical
UF455840	Saccostreinae	*Saccostrea mordax/scyphophilla*	53	Western Indo-Pacific	Red Sea and Gulf of Aden	Tropical
IM-2013-11264	Saccostreinae	*Saccostrea mordax/scyphophilla*	157	Central Indo-Pacific	Eastern Coral Triangle	Tropical
UF296246	Saccostreinae	*Saccostrea mordax/scyphophilla*	164	Central Indo-Pacific	Java Transitional	Tropical
IM-2013-62586	Saccostreinae	*Saccostrea mordax/scyphophilla*	185	Western Indo-Pacific	Western Indian Ocean	Tropical

IM-2013-62500	Saccostreinae	*Saccostrea mordax/scyphophilla*	188	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62501	Saccostreinae	*Saccostrea mordax/scyphophilla*	188	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62563	Saccostreinae	*Saccostrea mordax/scyphophilla*	188	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62578	Saccostreinae	*Saccostrea mordax/scyphophilla*	188	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62580	Saccostreinae	*Saccostrea mordax/scyphophilla*	188	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62519	Saccostreinae	*Saccostrea mordax/scyphophilla*	198	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62562	Saccostreinae	*Saccostrea mordax/scyphophilla*	198	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62497	Saccostreinae	*Saccostrea mordax/scyphophilla*	198	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62555	Saccostreinae	*Saccostrea mordax/scyphophilla*	198	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62558	Saccostreinae	*Saccostrea mordax/scyphophilla*	198	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62561	Saccostreinae	*Saccostrea mordax/scyphophilla*	198	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62545	Saccostreinae	*Saccostrea mordax/scyphophilla*	200	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62584	Saccostreinae	*Saccostrea mordax/scyphophilla*	200	Western Indo-Pacific	Western Indian Ocean	Tropical
UF445952	Saccostreinae	*Saccostrea mordoides*	10	Central Indo-Pacific	South Kuroshio	Tropical
IM-2013-15987	Saccostreinae	*Saccostrea mordoides*	145	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-15988	Saccostreinae	*Saccostrea mordoides*	145	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-15989	Saccostreinae	*Saccostrea mordoides*	145	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-15990	Saccostreinae	*Saccostrea mordoides*	145	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-15991	Saccostreinae	*Saccostrea mordoides*	145	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-17964	Saccostreinae	*Saccostrea mordoides*	154	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-17963	Saccostreinae	*Saccostrea mordoides*	157	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-17968	Saccostreinae	*Saccostrea mordoides*	158	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-16524	Saccostreinae	*Saccostrea mordoides*	159	Central Indo-Pacific	Eastern Coral Triangle	Tropical

IM-2013-80138	Saccostreinae	*Saccostrea mordoides*	180	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
IM-2013-80136	Saccostreinae	*Saccostrea mordoides*	180	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
IM-2013-80137	Saccostreinae	*Saccostrea mordoides*	180	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
UF445604	Saccostreinae	*Saccostrea* non-*mordax* LinC	12	Central Indo-Pacific	South Kuroshio	Tropical
IM-2013-85269	Saccostreinae	*Saccostrea* non-*mordax* LinC	175	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
IM-2013-81373	Saccostreinae	*Saccostrea* non-*mordax* LinC	177	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
IM-2013-74912	Saccostreinae	*Saccostrea* non-*mordax* LinC	211	Eastern Indo-Pacific	Marquesas	Tropical
IM-2013-74911	Saccostreinae	*Saccostrea* non-*mordax* LinC	211	Eastern Indo-Pacific	Marquesas	Tropical
IM-2013-74913	Saccostreinae	*Saccostrea* non-*mordax* LinC	211	Eastern Indo-Pacific	Marquesas	Tropical
IM-2013-74914	Saccostreinae	*Saccostrea* non-*mordax* LinC	211	Eastern Indo-Pacific	Marquesas	Tropical
IM-2013-74915	Saccostreinae	*Saccostrea* non-*mordax* LinC	211	Eastern Indo-Pacific	Marquesas	Tropical
IM-2013-74910	Saccostreinae	*Saccostrea* non-*mordax* LinC	212	Eastern Indo-Pacific	Marquesas	Tropical
UF401100	Saccostreinae	*Saccostrea* non-*mordax* LinC	214	Eastern Indo-Pacific	Southeast Polynesia	Tropical
UF401077	Saccostreinae	*Saccostrea* non-*mordax* LinC	214	Eastern Indo-Pacific	Southeast Polynesia	Tropical
IM-2007-41450	Saccostreinae	*Saccostrea* non-*mordax* LinD	41	Central Indo-Pacific	Western Coral Triangle	Tropical
IM-2007-37607	Saccostreinae	*Saccostrea* non-*mordax* LinD	42	Central Indo-Pacific	Western Coral Triangle	Tropical
IM-2007-37606	Saccostreinae	*Saccostrea* non-*mordax* LinD	42	Central Indo-Pacific	Western Coral Triangle	Tropical
IM-2007-33688	Saccostreinae	*Saccostrea* non-*mordax* LinD	55	Central Indo-Pacific	Western Coral Triangle	Tropical
IM-2013-47860	Saccostreinae	*Saccostrea* non-*mordax* LinD	131	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-47586	Saccostreinae	*Saccostrea* non-*mordax* LinD	132	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-47588	Saccostreinae	*Saccostrea* non-*mordax* LinD	132	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-47587	Saccostreinae	*Saccostrea* non-*mordax* LinD	132	Central Indo-Pacific	Eastern Coral Triangle	Tropical
UF445607	Saccostreinae	*Saccostrea* non-*mordax* LinF	12	Central Indo-Pacific	South Kuroshio	Tropical
UF459198	Saccostreinae	*Saccostrea* non-*mordax* LinF	45	Central Indo-Pacific	Tropical Northwestern Pacific	Tropical
UF445389	Saccostreinae	*Saccostrea* non-*mordax* LinF	49	Central Indo-Pacific	Tropical Northwestern Pacific	Tropical
UF526143	Saccostreinae	*Saccostrea* non-*mordax* LinF	107	Tropical Atlantic	Tropical Northwestern Atlantic	Tropical
IM-2013-16846	Saccostreinae	*Saccostrea* non-*mordax* LinF	143	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-16847	Saccostreinae	*Saccostrea* non-*mordax* LinF	143	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-12056	Saccostreinae	*Saccostrea* non-*mordax* LinF	152	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-10917	Saccostreinae	*Saccostrea* non-*mordax* LinF	153	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-10918	Saccostreinae	*Saccostrea* non-*mordax* LinF	153	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-10919	Saccostreinae	*Saccostrea* non-*mordax* LinF	153	Central Indo-Pacific	Eastern Coral Triangle	Tropical

IM-2013-10915	Saccostreinae	*Saccostrea* non-*mordax* LinF	153	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-10916	Saccostreinae	*Saccostrea* non-*mordax* LinF	153	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-10866	Saccostreinae	*Saccostrea* non-*mordax* LinF	155	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-80784	Saccostreinae	*Saccostrea* non-*mordax* LinF	179	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
IM-2013-80371	Saccostreinae	*Saccostrea* non-*mordax* LinF	182	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
IM-2013-84668	Saccostreinae	*Saccostrea* non-*mordax* LinF	184	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
IM-2013-84669	Saccostreinae	*Saccostrea* non-*mordax* LinF	184	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
UF445618	Saccostreinae	*Saccostrea* non-*mordax* LinG	12	Central Indo-Pacific	South Kuroshio	Tropical
IM-2013-85271	Saccostreinae	*Saccostrea* non-*mordax* LinG	175	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
IM-2013-85275	Saccostreinae	*Saccostrea* non-*mordax* LinG	175	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
IM-2013-85272	Saccostreinae	*Saccostrea* non-*mordax* LinG	175	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
IM-2013-85276	Saccostreinae	*Saccostrea* non-*mordax* LinG	175	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
IM-2013-81375	Saccostreinae	*Saccostrea* non-*mordax* LinG	177	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
UF434832	Saccostreinae	*Saccostrea* non-*mordax* LinH	57	Central Indo-Pacific	Tropical Northwestern Pacific	Tropical
UF434904	Saccostreinae	*Saccostrea* non-*mordax* LinH	58	Central Indo-Pacific	Tropical Northwestern Pacific	Tropical
UF458945	Saccostreinae	*Saccostrea* non-*mordax* LinH	90	Tropical Eastern Pacific	Tropical East Pacific	Tropical
UF444904	Saccostreinae	*Saccostrea* non-*mordax* LinH	122	Central Indo-Pacific	Western Coral Triangle	Tropical
UF444893	Saccostreinae	*Saccostrea* non-*mordax* LinH	124	Central Indo-Pacific	Western Coral Triangle	Tropical
UF444889	Saccostreinae	*Saccostrea* non-*mordax* LinH	125	Central Indo-Pacific	Western Coral Triangle	Tropical
IM-2013-17307	Saccostreinae	*Saccostrea* non-*mordax* LinH	144	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-17308	Saccostreinae	*Saccostrea* non-*mordax* LinH	144	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-17309	Saccostreinae	*Saccostrea* non-*mordax* LinH	144	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-12053	Saccostreinae	*Saccostrea* non-*mordax* LinH	152	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-12052	Saccostreinae	*Saccostrea* non-*mordax* LinH	152	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-12055	Saccostreinae	*Saccostrea* non-*mordax* LinH	152	Central Indo-Pacific	Eastern Coral Triangle	Tropical
UF446727	Saccostreinae	*Saccostrea* non-*mordax* LinI	12	Central Indo-Pacific	South Kuroshio	Tropical
IM-2013-54675	Saccostreinae	*Saccostrea* non-*mordax* LinI	129	Central Indo-Pacific	Eastern Coral Triangle	Tropical
UF445341	Saccostreinae	*Saccostrea* non-*mordax* LinJ	48	Central Indo-Pacific	Tropical Northwestern Pacific	Tropical
UF445342	Saccostreinae	*Saccostrea* non-*mordax* LinJ	48	Central Indo-Pacific	Tropical Northwestern Pacific	Tropical
UF444903	Saccostreinae	*Saccostrea* non-*mordax* LinJ	123	Central Indo-Pacific	Western Coral Triangle	Tropical
IM-2013-47861	Saccostreinae	*Saccostrea* non-*mordax* LinJ	131	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-54203	Saccostreinae	*Saccostrea* non-*mordax* LinJ	135	Central Indo-Pacific	Eastern Coral Triangle	Tropical

IM-2013-16855	Saccostreinae	*Saccostrea* non-*mordax* LinJ	143	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-13665	Saccostreinae	*Saccostrea* non-*mordax* LinJ	147	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2007-33656	Saccostreinae	*Saccostrea* non-*mordax* LinJ	172	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
IM-2013-85277	Saccostreinae	*Saccostrea* non-*mordax* LinJ	175	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
IM-2013-85288	Saccostreinae	*Saccostrea* non-*mordax* LinJ	175	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
IM-2013-85292	Saccostreinae	*Saccostrea* non-*mordax* LinJ	175	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
IM-2013-81968	Saccostreinae	*Saccostrea* non-*mordax* LinJ	176	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
IM-2013-80135	Saccostreinae	*Saccostrea* non-*mordax* LinJ	180	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
IM-2013-80370	Saccostreinae	*Saccostrea* non-*mordax* LinJ	182	Central Indo-Pacific	Tropical Southwestern Pacific	Tropical
UF380186	Saccostreinae	*Saccostrea palmula*	112	Tropical Eastern Pacific	Tropical East Pacific	Tropical
UF372723	Saccostreinae	*Saccostrea palmula*	113	Tropical Eastern Pacific	Tropical East Pacific	Tropical
IM-2013-62615	Saccostreinae	*Saccostrea palmula*	204	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62616	Saccostreinae	*Saccostrea palmula*	204	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62617	Saccostreinae	*Saccostrea palmula*	204	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62624	Saccostreinae	*Saccostrea palmula*	204	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62613	Saccostreinae	*Saccostrea palmula*	204	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62625	Saccostreinae	*Saccostrea palmula*	NO DATA	NO DATA	NO DATA	NO DATA
UF521195	Saccostreinae	Saccostreinae 1	25	Western Indo-Pacific	Red Sea and Gulf of Aden	Tropical
UF368004	Saccostreinae	Saccostreinae 1	30	Western Indo-Pacific	Somali/Arabian	Tropical
UF521062	Saccostreinae	Saccostreinae 1	39	Western Indo-Pacific	Red Sea and Gulf of Aden	Tropical
UF521063	Saccostreinae	Saccostreinae 1	39	Western Indo-Pacific	Red Sea and Gulf of Aden	Tropical
UF521064	Saccostreinae	Saccostreinae 1	39	Western Indo-Pacific	Red Sea and Gulf of Aden	Tropical
UF521061	Saccostreinae	Saccostreinae 1	39	Western Indo-Pacific	Red Sea and Gulf of Aden	Tropical
UF521065	Saccostreinae	Saccostreinae 1	39	Western Indo-Pacific	Red Sea and Gulf of Aden	Tropical
UF400729	Saccostreinae	Saccostreinae 1	213	Eastern Indo-Pacific	Southeast Polynesia	Tropical
IM-2013-62567	Saccostreinae	Saccostreinae 2	187	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62552	Saccostreinae	Saccostreinae 2	187	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62573	Saccostreinae	Saccostreinae 2	187	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62517	Saccostreinae	Saccostreinae 2	188	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62525	Saccostreinae	Saccostreinae 2	193	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62589	Saccostreinae	Saccostreinae 2	193	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62581	Saccostreinae	Saccostreinae 2	193	Western Indo-Pacific	Western Indian Ocean	Tropical

IM-2013-62526	Saccostreinae	Saccostreinae 2	193	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62590	Saccostreinae	Saccostreinae 2	193	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62521	Saccostreinae	Saccostreinae 2	194	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62614	Saccostreinae	Saccostreinae 2	203	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62618	Saccostreinae	Saccostreinae 3	203	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62619	Saccostreinae	Saccostreinae 3	203	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62621	Saccostreinae	Saccostreinae 3	203	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62623	Saccostreinae	Saccostreinae 3	203	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-12653	Saccostreinae	Saccostreinae 4	140	Central Indo-Pacific	Eastern Coral Triangle	Tropical
IM-2013-62509	Striostreinae	*Striostrea margaritacea*	191	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62574	Striostreinae	*Striostrea margaritacea*	191	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62499	Striostreinae	*Striostrea margaritacea*	192	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62506	Striostreinae	*Striostrea margaritacea*	192	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62539	Striostreinae	*Striostrea margaritacea*	192	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62540	Striostreinae	*Striostrea margaritacea*	192	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62542	Striostreinae	*Striostrea margaritacea*	192	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62551	Striostreinae	*Striostrea margaritacea*	192	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62557	Striostreinae	*Striostrea margaritacea*	192	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62560	Striostreinae	*Striostrea margaritacea*	192	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62579	Striostreinae	*Striostrea margaritacea*	192	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62583	Striostreinae	*Striostrea margaritacea*	192	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62502	Striostreinae	*Striostrea margaritacea*	195	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62513	Striostreinae	*Striostrea margaritacea*	196	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62575	Striostreinae	*Striostrea margaritacea*	198	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62507	Striostreinae	*Striostrea margaritacea*	198	Western Indo-Pacific	Western Indian Ocean	Tropical
IM-2013-62546	Striostreinae	*Striostrea margaritacea*	199	Western Indo-Pacific	Western Indian Ocean	Tropical

Museum specimens are distributed in 8 marine realms: 3 temperate realms (*i.e.* Temperate Northern Atlantic, Temperate Northern Pacific and Temperate Australasia), and 5 tropical realms (*i.e.* Tropical Atlantic, Central Indo-Pacific, Eastern Indo-Pacific, Western Indo-Pacific, Tropical Eastern Pacific). The number of specimens and MOTUs within each realm is showed in Figure 2. The highest number of specimens and MOTUs is found in the Central Indo-Pacific realm with 159 and 35 (37.5% and 52%), followed by the Western Indo-Pacific realm with 112 and 23 (26% and 34%), and the Tropical Atlantic realm with 76 and 12 (18% and 18%). The marine realms in which are present the specimens identified at species-level are: Temperate Northern Atlantic, Temperate Australasia, Central Indo-Pacific, Tropical Atlantic, Eastern Indo-Pacific, Tropical Eastern Pacific, and Western Indo-Pacific. The marine realms in which are present specimens identified as *Saccostrea* lineages are: Central Indo-Pacific, Tropical Atlantic, Eastern Indo-Pacific, and Tropical Eastern Pacific. The marine realms in which are present specimens with ambiguous identifications are: Temperate Northern Pacific, Tropical Atlantic, Central Indo-Pacific, Eastern Indo-Pacific, and Western Indo-Pacific. The marine realms in which are present unassigned MOTUs are: Temperate Northern Atlantic, Tropical Atlantic, Central Indo-Pacific, Eastern Indo-Pacific, and Western Indo-Pacific.

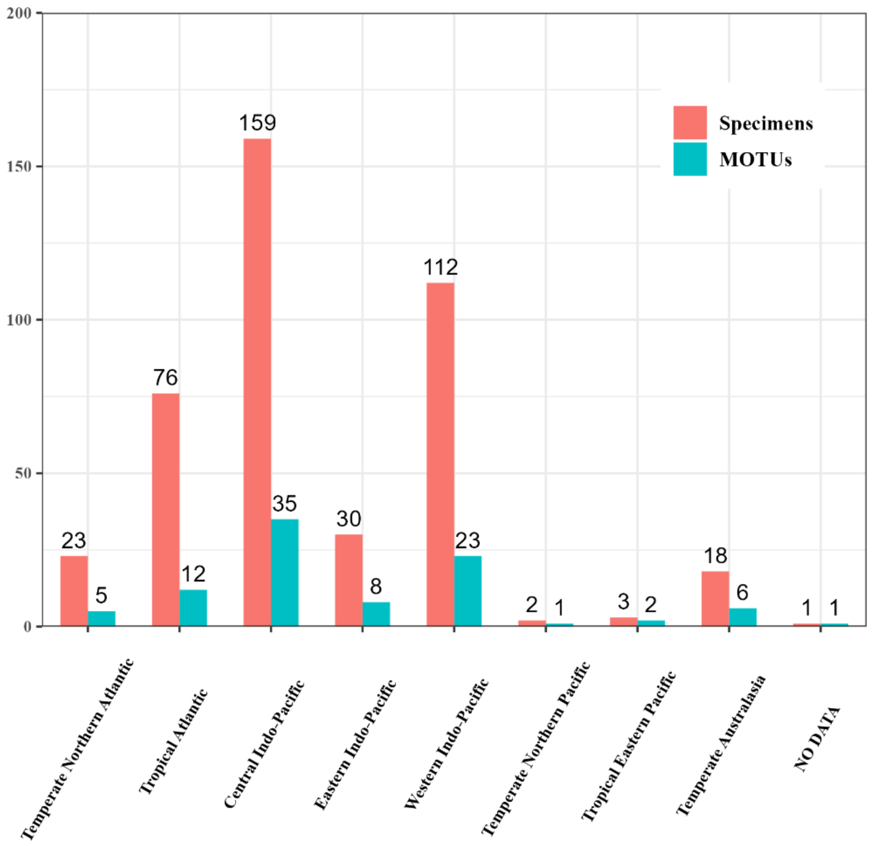

Figure 2. Number of specimens and MOTUs in each marine realm based on MEOW database (Spalding et al., 2007).

Discussion

Collections hosted at Natural History Museums provide a window with a wide view on the biodiversity of Earth. We had the privilege to access two of the main collections of Ostreidae species in the world, hosted at the MNHN and FLMNH, and the observed level of diversity has clearly met the expectations.

Among the over eight hundred museum specimens tested about half of them (424), from 217 sites spanning all world's oceans, were successfully sequenced for the standard barcode marker *cox1* (Hebert et al., 2003). This sequencing rate is relatively high for museum specimens, considering that these samples have been stored at room temperature, many of them for more than a decade, and in some cases conservation conditions are not known. Moreover, genomic DNA extraction from mollusc tissues can be challenging due to the abundance of mucopolysaccharides in their tissues (Panova et al., 2016; Pereira et al., 2011; Sokolov, 2000). To overcome these drawbacks, we took advantage of the experience and infrastructure of the MNHN, and we implemented specific primers and improved PCR protocols that can serve as standard for *cox1* barcoding assessments on oysters.

Based on BOLD-GB sequences library we could identify at the species-level less than a third of museum specimens (28%) representing 17 species. Such a low identification rate indicates that public repositories do not provide an efficient barcode library for molecular identification of oyster species as already documented in previous studies (*e.g.* Crocetta et al., 2015). A great majority of specimens represent undescribed lineages of *Saccostrea* (16.5%), ambiguous MOTUs (20.3%), or unassigned MOTUs (35.1%) demonstrating that the taxonomy of oyster is still far from being fully resolved.

We performed a DNA barcoding analysis using our sequences as queries, and a dataset of to assign an identification at species-level to the specimens. We identified a total of 67 MOTUs and we classified them according to the level of taxonomic certainty/uncertainty. The rate of identification at species-level was of 28%, with a total of. This result underlines that BOLD-GB repositories are not valuable reference libraries for DNA barcoding studies on oyster biodiversity.

The higher rate of identification at species-level was on temperate regions (88.4%) highlighting a severe gap of knowledge in tropical regions: 38 unassigned MOTUs occur in tropical regions (60.3%), and only 2 in a temperate region (*i.e.* Temperate Northern Atlantic realm). Most of the unassigned MOTUs (34 corresponding to 50.7%) belong to the Ostreinae subfamily. Since at least 15 recognised species are not represented in BOLD-GB libraries, it is likely that some of these unassigned MOTUs represent these species that have not yet been sequenced before. However, there is still a large number of MOTUs that might represent species new to science indicating that the diversity of this subfamily is highly underestimated. To clarify the taxonomic status of all these unassigned MOTUs it will be necessary an integrative taxonomic approach (Dayrat, 2005; Puillandre et al., 2012; Will et al., 2005). This approach on taxa has already proved fruitful for the assessment of unknown oyster diversity in understudied regions such as in Kuwait (Al-Kandari et al., 2021), see also the case study II of chapter four (Salvi et al., 2022).

In conclusion, our study represents the first assessment on oyster biodiversity at a global scale and demonstrated that we still have a very limited knowledge on oysters' diversity and distribution. Molecular data available in BOLD and GB repositories are not sufficient for barcoding applications. In this respect, sequences associated to museum vouchers represent an unvaluable source for the implementation of a comprehensive and reliable reference library for oyster identification. Further integrative taxonomic studies are particularly needed for Ostreinae and in tropical regions. Building on the results from this study, further studies will lead to a large amount of species discoveries and new species descriptions. Moving towards a global barcoding reference library for oysters studies such this one will give new life to old museum collections and will contribute to our understanding of the *true* diversity of *true* oysters.

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Table S1. List of all the geographical coordinates of the sampling sites reported in Table 2. The localities are named according to the quadrants with the following order: NE, NW, SE and SO. When coordinates were absent, we retrieved the latitude and longitude from Country/State/Locality information of the museum databases, and we put them in grey.

Locality code	Latitude	Longitude	Museum	N/S	W/E
1	49.582	-0.374	MNHN	N	E
2	42.125	9.519	MNHN	N	E
3	41.621	9.311	MNHN	N	E
4	41.476	9.069	MNHN	N	E
5	40.144	11.619	MNHN	N	E
6	33.895	10.885	MNHN	N	E
7	28.404	34.741	MNHN	N	E
8	26.406	127.850	MNHN	N	E
9	26.296	127.906	MNHN	N	E
10	26.295	127.906	MNHN	N	E
11	26.293	127.917	MNHN	N	E
12	26.212	127.664	MNHN	N	E
13	26.212	127.664	MNHN	N	E
14	25.018	121.990	MNHN	N	E
15	23.848	57.952	MNHN	N	E
16	23.625	58.479	MNHN	N	E
17	23.623	58.472	MNHN	N	E
18	23.595	58.602	MNHN	N	E
19	23.586	58.610	MNHN	N	E
20	23.557	58.651	FLMNH	N	E
21	22.569	59.540	FLMNH	N	E
22	22.543	114.439	FLMNH	N	E
23	22.501	114.356	FLMNH	N	E
24	22.437	114.221	FLMNH	N	E
25	22.282	39.086	FLMNH	N	E
26	21.943	120.792	FLMNH	N	E
27	20.610	58.925	FLMNH	N	E
28	20.588	58.828	FLMNH	N	E
29	20.399	58.490	FLMNH	N	E
30	20.351	58.451	FLMNH	N	E
31	20.338	58.388	FLMNH	N	E
32	20.254	58.624	FLMNH	N	E
33	20.011	58.633	FLMNH	N	E
34	18.506	40.670	FLMNH	N	E
35	18.073	40.886	FLMNH	N	E
36	17.607	41.670	FLMNH	N	E
37	16.969	54.688	FLMNH	N	E
38	16.966	54.708	FLMNH	N	E
39	16.836	42.584	FLMNH	N	E

40	16.684	53.189	FLMNH	N	E
41	16.266	122.083	FLMNH	N	E
42	14.401	120.840	FLMNH	N	E
43	13.525	120.946	FLMNH	N	E
44	13.513	120.917	FLMNH	N	E
45	13.460	144.663	FLMNH	N	E
46	13.446	144.659	FLMNH	N	E
47	13.445	144.659	FLMNH	N	E
48	13.444	144.658	FLMNH	N	E
49	13.439	144.664	FLMNH	N	E
50	13.430	144.663	FLMNH	N	E
51	12.266	109.207	FLMNH	N	E
52	11.773	42.926	FLMNH	N	E
53	11.517	42.666	FLMNH	N	E
54	10.388	106.932	FLMNH	N	E
55	9.682	123.852	FLMNH	N	E
56	9.618	123.768	FLMNH	N	E
57	7.297	134.447	FLMNH	N	E
58	7.254	134.344	FLMNH	N	E
59	1.215	103.852	FLMNH	N	E
60	1.215	103.851	FLMNH	N	E
61	1.215	103.851	FLMNH	N	E
62	1.160	103.741	FLMNH	N	E
63	0.450	124.481	FLMNH	N	E
64	30.382	-86.507	FLMNH	N	W
65	29.477	-83.713	FLMNH	N	W
66	28.920	-83.505	FLMNH	N	W
67	28.207	-80.662	FLMNH	N	W
68	28.052	-82.801	FLMNH	N	W
69	27.818	-83.094	FLMNH	N	W
70	26.289	-82.369	FLMNH	N	W
71	26.234	-82.376	FLMNH	N	W
72	25.925	-81.736	FLMNH	N	W
73	25.653	-81.834	FLMNH	N	W
74	25.178	-81.536	FLMNH	N	W
75	24.913	-80.937	FLMNH	N	W
76	24.840	-80.783	FLMNH	N	W
77	24.777	-80.924	FLMNH	N	W
78	24.765	-80.754	FLMNH	N	W
79	24.655	-81.303	FLMNH	N	W
80	24.596	-82.675	FLMNH	N	W
81	24.000	-166.000	MNHN	N	W
82	23.898	-166.220	MNHN	N	W
83	23.873	-166.234	MNHN	N	W
84	23.637	-166.187	FLMNH	N	W
85	21.467	-157.818	FLMNH	N	W
86	21.450	-157.796	FLMNH	N	W
87	21.415	-157.779	FLMNH	N	W

88	21.090	-157.251	FLMNH	N	W
89	21.082	-157.028	FLMNH	N	W
90	19.200	-104.683	FLMNH	N	W
91	18.102	-62.983	FLMNH	N	W
92	16.393	-61.523	FLMNH	N	W
93	16.341	-61.526	FLMNH	N	W
94	16.269	-61.585	FLMNH	N	W
95	16.212	-61.505	MNHN	N	W
96	14.764	-60.882	MNHN	N	W
97	14.686	-60.856	MNHN	N	W
98	14.672	-60.892	MNHN	N	W
99	14.671	-60.880	FLMNH	N	W
100	14.665	-60.898	FLMNH	N	W
101	14.601	-61.066	MNHN	N	W
102	14.465	-61.019	MNHN	N	W
103	14.446	-60.892	MNHN	N	W
104	13.967	-16.537	MNHN	N	W
105	12.375	-69.159	MNHN	N	W
106	9.352	-82.257	FLMNH	N	W
107	9.334	-82.248	FLMNH	N	W
108	9.301	-82.294	FLMNH	N	W
109	9.290	-82.343	FLMNH	N	W
110	9.234	-82.345	FLMNH	N	W
111	9.231	-82.121	FLMNH	N	W
112	8.388	-79.093	FLMNH	N	W
113	8.333	-79.117	FLMNH	N	W
114	5.849	-51.493	FLMNH	N	W
115	5.280	-52.585	FLMNH	N	W
116	5.150	-52.672	FLMNH	N	W
117	4.916	-52.356	FLMNH	N	W
118	4.888	-52.167	FLMNH	N	W
119	-0.422	121.682	FLMNH	S	E
120	-0.425	122.003	FLMNH	S	E
121	-0.438	122.266	FLMNH	S	E
122	-0.442	130.686	FLMNH	S	E
123	-0.449	130.501	FLMNH	S	E
124	-0.450	130.486	FLMNH	S	E
125	-0.455	130.493	FLMNH	S	E
126	-0.473	122.139	FLMNH	S	E
127	-2.604	150.527	FLMNH	S	E
128	-2.610	118.896	FLMNH	S	E
129	-2.685	150.577	FLMNH	S	E
130	-2.687	150.687	FLMNH	S	E
131	-2.691	150.836	FLMNH	S	E
132	-2.699	150.861	FLMNH	S	E
133	-2.699	150.683	FLMNH	S	E
134	-2.716	150.719	FLMNH	S	E
135	-2.719	150.630	FLMNH	S	E

136	-2.744	150.718	FLMNH	S	E
137	-2.844	150.648	FLMNH	S	E
138	-3.591	148.032	FLMNH	S	E
139	-5.075	145.791	FLMNH	S	E
140	-5.079	145.815	FLMNH	S	E
141	-5.118	145.823	FLMNH	S	E
142	-5.142	145.808	FLMNH	S	E
143	-5.147	145.798	FLMNH	S	E
144	-5.150	145.801	FLMNH	S	E
145	-5.156	145.805	FLMNH	S	E
146	-5.180	145.830	FLMNH	S	E
147	-5.188	145.807	FLMNH	S	E
148	-5.188	145.818	FLMNH	S	E
149	-5.193	145.823	FLMNH	S	E
150	-5.195	145.824	FLMNH	S	E
151	-5.197	145.807	FLMNH	S	E
152	-5.197	145.822	FLMNH	S	E
153	-5.198	145.789	FLMNH	S	E
154	-5.201	145.802	FLMNH	S	E
155	-5.203	145.791	FLMNH	S	E
156	-5.206	145.813	MNHN	S	E
157	-5.255	145.777	MNHN	S	E
158	-5.257	145.781	MNHN	S	E
159	-5.300	145.779	MNHN	S	E
160	-5.435	150.354	MNHN	S	E
161	-5.487	150.160	MNHN	S	E
162	-5.612	150.186	MNHN	S	E
163	-8.526	179.192	MNHN	S	E
164	-10.418	105.676	MNHN	S	E
165	-11.042	152.479	MNHN	S	E
166	-13.416	48.246	MNHN	S	E
167	-13.479	48.237	MNHN	S	E
168	-14.390	145.274	MNHN	S	E
169	-14.878	46.934	MNHN	S	E
170	-14.911	46.926	MNHN	S	E
171	-15.496	46.092	MNHN	S	E
172	-15.559	167.141	MNHN	S	E
173	-15.779	44.759	MNHN	S	E
174	-15.806	44.740	MNHN	S	E
175	-20.266	164.296	MNHN	S	E
176	-20.324	164.320	MNHN	S	E
177	-20.334	164.342	MNHN	S	E
178	-20.477	164.002	MNHN	S	E
179	-20.569	164.277	MNHN	S	E
180	-20.582	164.276	MNHN	S	E
181	-20.656	164.297	MNHN	S	E
182	-20.682	164.360	MNHN	S	E
183	-20.704	164.304	MNHN	S	E

184	-20.937	164.650	MNHN	S	E
185	-24.785	47.198	MNHN	S	E
186	-24.923	47.111	MNHN	S	E
187	-24.937	47.112	MNHN	S	E
188	-24.942	47.118	MNHN	S	E
189	-24.949	47.106	FLMNH	S	E
190	-25.021	47.008	FLMNH	S	E
191	-25.025	46.997	FLMNH	S	E
192	-25.037	47.000	FLMNH	S	E
193	-25.053	46.918	FLMNH	S	E
194	-25.064	46.935	FLMNH	S	E
195	-25.074	46.963	FLMNH	S	E
196	-25.158	46.756	FLMNH	S	E
197	-25.212	44.142	FLMNH	S	E
198	-25.420	44.938	FLMNH	S	E
199	-25.426	44.937	FLMNH	S	E
200	-25.457	44.957	MNHN	S	E
201	-25.465	44.961	MNHN	S	E
202	-25.619	46.160	MNHN	S	E
203	-26.023	32.912	MNHN	S	E
204	-26.038	32.902	MNHN	S	E
205	-26.042	32.892	MNHN	S	E
206	-26.199	35.048	MNHN	S	E
207	-33.670	114.943	MNHN	S	E
208	-33.831	121.933	MNHN	S	E
209	-42.973	147.431	MNHN	S	E
210	-43.060	147.336	FLMNH	S	E
211	-8.935	-140.168	FLMNH	S	W
212	-8.942	-140.161	FLMNH	S	W
213	-17.484	-149.824	FLMNH	S	W
214	-17.517	-149.853	FLMNH	S	W
215	-17.558	-149.873	FLMNH	S	W
216	-18.304	-140.864	FLMNH	S	W
217	-22.018	-136.201	FLMNH	S	W

CHAPTER 4

Integrative taxonomy applications to oyster systematics

Case study I

Are morphological diagnoses applicable to oysters? DNA sequence data from holotype and topotype specimens reveal two new synonyms among *Magallana* cupped oysters (Ostreidae: Crassostreinae)

Introduction

True oysters are well-known molluscs, with a relevant ecological importance (Giglio et al., 2023), and with a long and commercially significant association with humans as a source of food (Botta et al., 2020). Many oysters are harvested at local scales since centuries and a few species dominate shellfish aquaculture with a global industry which annually produce million tonnes for billions US dollars (FAO, 2022; van der Schatte Olivier et al., 2020). A correct species identification is therefore critical both for aquaculture industry and for understanding the biodiversity of coastal ecosystems. The family Ostreidae Rafinesque, 1815 includes 78 species found in tropical and temperate waters worldwide (MolluscaBase, 2024). Oyster systematic treatment continues to evolve, with new, mostly molecular, data revealing unexpected relationships and previously undetected new species that challenge earlier classifications (Cui et al., 2021; Lam & Morton, 2003). Recently, systematic revision on this family assigned to the new genus *Magallana* Salvi & Mariottini, 2016 several Asian species that are important for aquaculture (Salvi & Mariottini, 2017, 2021)

Deep changes in the systematic and taxonomy made in the last two decades are due to the application of molecular approaches to the assessment of oyster diversity. It is now well-established the need for molecular tools from barcoding to phylogenetic systematics of oysters (Al-Kandari et al., 2021; Salvi et al., 2021, 2022; Wu et al., 2013). Nevertheless, molecular data are not always available for species description and recently several species have been described based on morphology alone. This is the case of cupped oysters (subfamily Crassostreinae) of the genus

Crasssostrea from Brazil (Amaral & Simone, 2014) and of the genus *Magallana* from Asia (Thach, 2018).

In this study, we focused on Indo-Pacific cupped oysters of the genus *Magallana*: *Magallana markushuberi* (Thach, 2018) and *M. valentichscotti* (Thach, 2018) described from Vietnam. These two new species have striking morphological similarities with congeneric species some of which are syntropic with them (Thach, 2018). In this study, we generated DNA sequence data from both holotype specimens hosted at the Muséum national d'Histoire naturelle (MNHN) and from fresh specimens collected in the type locality of both species with the main aim to perform a molecular assessment of the phylogenetic and taxonomic relationships of these taxa.

Material and Methods

Sampling

Samples for molecular analyses were collected either from dry shells of the holotype specimens deposited at the MNHN or from freshly collected specimens at the type locality of *Magallana markuschumberi* and *M. valentichscotti* (Figure 1).

Dry tissues of holotypes were taken from the inner shells using a scalpel and a thin tweezer and stored in pure ethanol until the DNA extraction. The quantity of available tissues varied between the two holotypes: a small piece of dry mantle was taken from the *Magallana markuschumberi* holotype MNHN-IM-2000-34035, while a small quantity of encrusted tissues (dust) was taken from the *Magallana valentichscotti* holotype MNHN-IM-2000-34036.

Fresh specimens were collected and identified by one of the authors, Nguyên Ngoc Thach, in 2022 at the type locality of each species, at the same sites where holotype specimens were collected by the author a few years before. Three specimens of *M. markuschumberi* were collected from Nha Trang area (Khánh Hoa Province, Central Vietnam) and three specimens of *M. valentichscotti* from Duyên Hài (formerly called Can Giò; Hochiminh City, South Vietnam). Specimens were levered open, and part of the adductor muscle and mantle were excised and fixed in pure ethanol. All specimens used for the taxonomic assessment are deposited in the Museum National d'Histoire Naturelle of Paris (MNHN) and voucher numbers are reported in Table 1.

DNA extraction, amplification and sequencing: new COI primers for Ostreidae

Total genomic DNA was extracted from alcohol-preserved tissues of the fresh specimens following standard high-salt protocols (Evans, 1990). For tissue gathered from dry holotype shells we used the E.Z.N.A.® Mollusc DNA Kit (OMEGA Bio-tech), following the manufacturer recommendations. DNA was diluted in 80 µL of DNA-free water in the final step. Final DNA extracts from dry holotype shells were subjected to quantification using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) with the Qubit dsDNA HS Assay Kit.

Two mitochondrial gene fragments were amplified by polymerase chain reaction (PCR), the barcode fragment of the cytochrome oxidase subunit I (COI) and the 16S rRNA (16S). For the COI fragment we designed new primers specific to Ostreidae based on 3,844 aligned sequences from GenBank and mapped to the complete *cox1* sequence of mitochondrial genome of *Magallana ariakensis* - HQ700934 in Geneious Prime 11.0.18+10 (Biomatters Ltd., Auckland, New Zealand): osLCO1490 (5' – TIT CIA CIA AYC AYY TRG AYA TTG G - 3') and osHCO998-R (5' – ACR GTI GCI GCI CTR AAR TAA GCI CG - 3').

The forward primer osLCO1490 is a modification of the degenerated primer jgLCO1490 proposed by (Geller et al., 2013) and target the same site as the universal barcode primer LCO1490 (Folmer et al., 1994). The reverse primer osHCO998-R binds a region placed downstream the target region of the universal barcode primer HCO2198 (Folmer et al., 1994). In addition, to increase the sensitivity and specificity of COI amplifications of holotypes, for these specimens we applied a nested protocol with two sequential amplification reactions. The first one using primers as above, the second one using the product of the first amplification reaction (1 ul of product diluted 1:100) as the template for the second PCR, and the primers osLCO139-F (5' – ATT GGI AGI TTW TAY ATR GTI TTY GG - 3') and osHCO2198 (5' – TAI ACY TCI GGR TGI CCR CAA RAA ICA - 3'). The forward primer osLCO139-F binds a region placed downstream the Folmer primer LCO1490. The reverse primer osHCO2198 is a modification of the degenerated primer jgLHCO2198 proposed by Geller et al., (2013) and binds in the same position as the Folmer primer HCO2198. PCR conditions were as follow: 94°C (3 min); [94°C (60 s), Ta (annealing temperature) (60 s), 72°C (60 s)] x 35 cycles; 72°C (10 min). Ta for the first primer pair (osLCO1490/osHCO998-R) was 51°C and for the second primer pair (osLCO139-F/osHCO2198) was 48°C. The 16S sequences were amplified using the universal primers 16Sar-L and 16Sbr-H (Palumbi et al., 1991) with same cycling protocol as for COI but with the Ta set at 52°C.

Sequencing of PCR products was carried out by the company GENEWIZ (https://www.genewiz.com), using the forward and reverse primers employed for amplification. The chromatograms of each sequence were manually edited and assembled into a consensus sequence using Geneious. The COI chromatograms did not show any double peak and the translated aminoacidic sequence did not have any stop codon, thus confirming that nuclear copies (pseudogenes) were not occurring in our mitochondrial COI sequence dataset. All COI sequences were trimmed to the Folmer's fragment length.

Molecular species-level identification and phylogenetic analyses

Newly generated COI and 16S sequences were compared against the NCBI nucleotide database using NCBI BLASTn (Altschul et al., 1990) to determine the sequence identity. For each sequence the best BLASTn matches (sequence identity ≥ 99.5%) were retained. Uncorrected genetic distance between DNA sequences (*p*-distance) were calculated in MEGA X (Kumar et al., 2018) using the pairwise deletion option.

The newly generated sequences of both gene fragments were aligned with sequences of four individuals for each of ten Indo-Pacific species of the genus *Magallana* plus the outgroup species *Talonostrea salpinx* (Al-Kandari et al., 2021), obtained from GenBank. GenBank accession numbers and voucher references for the DNA sequences used for phylogenetic analyses are reported in Figure 2. Multiple sequence alignments were performed with MAFFT v.7 (Katoh & Standley, 2013) using the E-INS-i iterative refinement algorithm.

Phylogenetic relationships between *Magallana markuschumberi* and *M. valentichscotti* and other Crassostreinae were inferred based on the Maximum Likelihood (ML) and Bayesian inference methods using both single-gene alignments and the concatenated COI+16S alignment. ML analyses were performed with IQ-TREE on the web server v.1.6.12 (http://iqtree.cibiv.univie.ac.at/ ; Trifinopoulos et al., 2016) based on the best substitution model determined by the ModelFinder module (Kalyaanamoorthy et al., 2017) using edge-linked model and the cAIC criterion. For the concatenated alignment we used a partitioned model and implemented the best model for each gene partition. ML tree searches were based on the 20 best initial trees (out of 100 parsimony trees and the BIONJ generated) and until IQ-TREE cannot find a new best tree in the last 100 iterations (nstop=100). Node support was assessed with 1,000 pseudoreplicates of ultrafast bootstrapping (uBS).

Bayesian analyses were carried out with MrBayes v.3.2.7 (Ronquist et al., 2012), using for each gene partition the same substitution models selected by ModelFinder. We ran two Markov chains of two million generations each, sampled every 200 generations. Consensus trees (50% majority rule) and Bayesian posterior probability values (BPP) were calculated on trees sampled after a burn-in of 25%. Tracer v.1.7 (Rambaut et al., 2018) was used to check for run convergence and ESS values > 200. FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) was used to visualize the trees.

Results

We obtained sequences of the entire barcoding fragment of the COI (650 base pair, bp) and 16S (437 bp) from all fresh specimens. Specimens of *Magallana markuschumberi* show a single haplotype for both markers; specimens of *M. valentichscotti* show two haplotypes (with a single nucleotide substitution) at both markers.

From dry holotype shells, the DNA purification yield measured by fluorometric quantification was 2.62 ng/µL for *M. markuschumberi* and 0,14 ng/µL for *M. valentichscotti*. We obtained COI and 16S sequence of the holotype of *M. markuschumberi* that were identical to the haplotypes found in fresh specimens. Whereas for the holotype of *M. valentichscotti*, despite multiple attempts, we obtained COI and 16S sequences with high background noise along the primary trace, most likely because of the limited amount and quality of DNA available as template. However, for both markers the holotype sequences obtained by automated base-calling were very similar (identity >99%) to the sequences obtained from fresh *M. valentichscotti* specimens (data available upon request), further corroborating the taxonomic identity of the latter. Details on sample data and GenBank accession numbers of sequences generated in this study are provided in Table 1 where we also indicated the GenSeq nomenclature for genetic sequences based on the taxonomic reliability of the source specimens following Chakrabarty et al. (2013).

Results of BLASTn searches using as query the *cox1* and the 16S sequences of topotype specimens and the 16S sequence of the holotype specimen of *M. markuschumberi* retrieved as best hit sequences assigned to *M. angulata* with a sequence identity of 100% both for *cox1* and 16S (Table 2; see Supplementary Table S1 for the full list of hits with sequence identity ≥ 99.5%). The *cox1* and the 16S sequences of topotype specimens of *M. valentichscotti* retrieved as best hit sequences assigned to *M. belcheri* with a sequence identity of 100% both for *cox1* and 16S (Table 3; Supplementary Table S1). One of the best five hits for *cox1* of *M. valentichscotti*, with accession number FJ262985 is reported in GenBank as *Crassostrea* (=*Magallana*) *gryphoides*. This sequence has not been published and was likely mislabelled since it is identical to other sequences annotated as *Magallana belcheri* (MW349636 and GU591436).

Table 1. Voucher catalogue number and sampling locality for holotype and topotype specimens of *Magallana markuschumberi* and *M. valentichscotti* analysed in this study. GenBank accession number and GenSeq nomenclature (Chakrabarty et al., 2013) for genetic sequences obtained are reported.

Species	Type	Voucher Catalogue #	Locality	GenSeq nomenclature
Magallana markuschumberi	**Holotype**	**IM-2000-34035**	**Nha Trang area, Khánh Hoa Province, Central Vietnam**	**genseq-1 COI, 16S**
Magallana markuschumberi	Topotype	A	Nha Trang area, Khánh Hoa Province, Central Vietnam	genseq-3 COI, 16S
Magallana markuschumberi	Topotype	B	Nha Trang area, Khánh Hoa Province, Central Vietnam	genseq-3 COI, 16S
Magallana markuschumberi	Topotype	C	Nha Trang area, Khánh Hoa Province, Central Vietnam	genseq-3 COI, 16S
Magallana valentichscotti	**Holotype**	**IM-2000-34036**	**Duyên Hài (formerly called Can Giò), Hochiminh City, South Vietnam**	**genseq-1 COI**
Magallana valentichscotti	Topotype	IM-2019-16315	Duyên Hài (formerly called Can Giò), Hochiminh City, South Vietnam.	genseq-3 COI, 16S
Magallana valentichscotti	Topotype	IM-2019-16316	Duyên Hài (formerly called Can Giò), Hochiminh City, South Vietnam.	genseq-3 COI, 16S
Magallana valentichscotti	Topotype	IM-2019-16317	Duyên Hài (formerly called Can Giò), Hochiminh City, South Vietnam.	genseq-3 COI, 16S

Table 2. Top 5 best hits of BLASTn results using as query the sequences of the barcoding fragments COI and 16S rRNA of *Magallana markuschumberi* (holotype).

Query sequence	Accession	Reported scientific name	Current scientific name[1]	Isolate / Voucher	Country	% Pairwise Identity	Query coverage	Sequence lenght	Grade
***cox1* sequence** *Magallana markuschumberi*	LC383462	*Crassostrea angulata*	*Magallana angulata*	KU-14	Japan: Wakayama	100.00%	100.00%	650	100.00%
	KP216776	*Crassostrea angulata*	*Magallana angulata*	n.a.	n.a.	100.00%	100.00%	650	100.00%
	NC012648	*Crassostrea angulata*	*Magallana angulata*	Cangtaiwh-9	China: Taiwan	99.80%	100.00%	650	99.90%
	LC383458	*Crassostrea angulata*	*Magallana angulata*	KU-6	Japan: Wakayama	99.80%	100.00%	650	99.90%
	KP216771	*Crassostrea angulata*	*Magallana angulata*	n.a.	n.a.	99.80%	100.00%	650	99.90%
16S sequence *Magallana markuschumberi*	MH997922	*Crassostrea angulata*	*Magallana angulata*	n.a.	China: Wenzhou, Zhejiang	100.00%	100.00%	437	100.00%
	KY446769	*Crassostrea angulata*	*Magallana angulata*	n.a.	n.a.	100.00%	100.00%	437	100.00%
	KY081322	*Crassostrea angulata*	*Magallana angulata*	MIEE2015-15CA1	n.a.	100.00%	98.17%	429	99.10%
	KX345694	*Crassostrea angulata*	*Magallana angulata*	CAN_1	n.a.	100.00%	99.54%	435	99.80%
	KJ855249	*Crassostrea angulata*	*Magallana angulata*	DA01	China: Dong'an, Xiamen, Fujian	100.00%	100.00%	437	100.00%

[1]: Taxonomic names according to MolluscaBase eds. (2021).

Table 3. Top 5 best hits of BLASTn results using as query the sequences of the barcoding fragments COI and 16S rRNA of *Magallana valentichscotti* (voucher OS501).

Query sequence	Accession	Reported scientific name	Current scientific name[1]	Isolate / Voucher	Country	% Pairwise Identity	Query coverage	Sequence lenght	Grade
***cox1* sequence** *Magallana valentichscotti*	MW349636	*Crassostrea belcheri*	*Magallana belcheri*	UPMMC0008	Malaysia	100.00%	90.92%	591	95.50%
	GU591436	*Crassostrea belcheri*	*Magallana belcheri*	TW01	Malaysia	100.00%	89.38%	581	94.70%
	FJ262985	*Crassostrea gryphoides*	*Magallana gryphoides*	WBO 3705	India: Sunderbans	100.00%	96.00%	624	98.00%
	MH051332	*Crassostrea belcheri*	*Magallana belcheri*	n.a.	Vietnam: Can Gio	99.80%	100.00%	650	99.90%
	GU591467	*Crassostrea belcheri*	*Magallana belcheri*	K14	Malaysia	99.80%	89.38%	581	94.60%
16S sequence *Magallana valentichscotti*	MW354039	*Crassostrea belcheri*	*Magallana belcheri*	UPMMC0008	Malaysia	100.00%	99.54%	435	99.80%
	MH051332	*Crassostrea belcheri*	*Magallana belcheri*	n.a.	Vietnam: Can Gio	100.00%	100.00%	437	100.00%
	KX961679	*Crassostrea belcheri*	*Magallana belcheri*	C	Myanmar	99.80%	100.00%	437	99.90%
	KX961674	*Crassostrea belcheri*	*Magallana belcheri*	Z	Myanmar	99.80%	100.00%	437	99.90%
	MW354042	*Crassostrea belcheri*	*Magallana belcheri*	UPMMC0011	Malaysia	99.80%	99.54%	435	99.70%

[1]: Taxonomic names according to MolluscaBase eds. (2021).

Phylogenetic analyses based on single gene alignments or on the concatenated alignment and on different methods (ML and BA) gave congruent results (Figure 2). In all phylogenetic trees, *Magallana markuschumberi* specimens are nested within the *M. angulata* clade and *M. valentichscotti* specimens are nested within the *M. belcheri* clade with maximum node support (uBS=100; BPP =1.0). Phylogenetic divergence between *M. markuschumberi* and *M. angulata* specimens and between *M. valentichscotti* and *M. belcheri* specimens is not noticeable with length of terminal branches within these clades close to zero.

Figure 1. The holotypes of *Magallana markuschumberi* and *M. valentichscotti* preserved in the type collection of the MNHN.

Figure 2. Maximum-Likelihood tree of *Magallana* cupped oysters based on concatenated mitochondrial DNA sequences (*cox1* + 16S rRNA). *Talonostrea salpinx* was used as an outgroup. The Bayesian tree shows an identical topology. Bootstrap values (uBS) are reported above main nodes, Bayesian support (BPP) below nodes. Phylogenetic distance between *Magallana markuschumberi* and *M. angulata* and between *M valentichscotti* and *M. belcheri* is close to zero (mean genetic *p*-distance ≤ 0.5%).

Discussion

Taxonomic identification of oysters based on morphological characters is challenging because of the lack of reliable diagnostic characters. In the last two decades, molecular studies have disclosed several species misidentifications both in aquaculture species harvested since centuries (Lam & Morton, 2003; Sigwart et al., 2021) and in wild species (Al-Kandari et al., 2021; Pagenkopp Lohan et al., 2015; Reece et al., 2008; Salvi et al., 2021; Sekino et al., 2015). On the other hand, in several cases molecular data provided evidence for synonimization of oysters species or genera (e.g., Salvi & Mariottini, 2017; Wu et al., 2013). However, it remains difficult to validate the status of many species described without molecular data associated to type specimens, such as in the case of Atlantic oysters of genus *Crassostrea* (Ferreira et al., 2023).

Our study represents the first attempt made in the Ostreidae family of an *a posteriori* molecular validation of species described based on morphology alone and based on molecular data generated using holotypes and topotypes. Our results corroborated the distinction between *Magallana markuschumberi* and *M. valentichscotti*, recently described for Vietnam. However, the molecular assessment provides compelling evidence that these two species are not new to science, but indeed represent two well-known and common species from this area: *M. angulata* and *M. belcheri* that are therefore junior synonyms of *M. markuschumberi* and *M. valentichscotti*, respectively.

This study corroborates the notion that morphology alone is not enough for taxonomic diagnosis of oysters and that the integration of morphological and molecular data is necessary for a robust identification and systematic assessment. In this respect, it is crucial link information on molecular and morphological variation within and between species. Most importantly, vouchers specimens associated to sequence data should be deposited and maintained in public repositories to allow *a posteriori* systematic assessment and comparisons between molecularly identified specimens and museum vouchers and type material.

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Case study II

Molecular characterization and phylogenetic position of the giant deep-sea oyster *Neopycnodonte zibrowii* Gofas, Salas and Taviani, 2009

Introduction

Deep-sea is the Earth's largest biome but it is still one of the most underexplored region (Ramirez-Llodra et al., 2010). Deep-sea biodiversity is mostly unknown due to the extreme environmental conditions that limits sampling capabilities (Rogers et al. 2015; Sinniger et al. 2016; Woodall et al. 2018). Along with advances in exploration technologies (Feng et al., 2022), new molecular technologies such as high-throughput sequencing and the molecular identification of multiple species in environmental DNA (eDNA metabarcoding; Taberlet et al. 2012) have strongly boosted deep-sea biodiversity assessments (Everett & Park, 2018; Guardiola et al., 2016). However, the low number of reference sequences taxonomically validated in online repository databases (e.g. GenBank) limits the identifications of MOTUs (Molecular Operational Taxonomic Unit), thus reducing the taxonomic resolution of eDNA studies (Ruppert et al., 2019). Studies using an integrative taxonomic approach − combining molecular, morphological and environmental data − on new deep-sea taxa have been carried out in several groups of organisms, such as Anthozoa (López-González et al., 2022), Mollusca (Xu et al., 2019), and some others (Błażewicz et al., 2019; Silva et al., 2016). However, while molecular data are still not available for a great portion of known deep-sea biodiversity, new deep-sea explorations have also continued contributing the discovery of new benthic ecosystems and associated communities. Therefore, there is a tremendous need to constantly improve with reliable reference sequences the taxonomic coverage of deep-sea taxa in repository databases.

In this study, we focused on a keystone deep-sea habitat builder species discovered about fifteen years ago in the Azores Archipelago: the giant deep-sea oyster *Neopycnodonte zibrowii* Gofas, C. Salas & Taviani, 2009 (Gryphaeidae Vialov, 1936) (Wisshak, López Correa, et al., 2009). This reef forming oyster was first observed during a submersible dive along the Faial Channel (480-500 meter depth) (Wisshak, López Correa, et al., 2009). Deep-sea reefs of *N. zibrowii* are built by both stacked alive and death specimens on vertical rocky substrate of seamounts, escarpments and in canyons (Beuck et al., 2016), and host peculiar deep-sea communities. Benthic associations between *N. zibrowii* and the cyrtocrinid *Cyathidium foresti* have been documented in the Atlantic Ocean (Wisshak, Neumann, et al., 2009), and between *N. zibrowii* and cold-water corals in both the Atlantic Ocean (Van Rooij et al., 2010) and the Mediterranean Sea (Taviani et al., 2017, 2019). Recently, new records and observations on *N. zibrowii* in the Atlantic Ocean allowed updating its ecology and distribution (Beuck et al., 2016). The giant deep-sea oyster has been meticulously described in terms of external morphology, microstructures of shell and anatomy (Wisshak, López Correa, et al., 2009). The systematic placement of this species in the genus *Neopycnodonte* was based on morphological characteristics such as the circular muscle scar, the enlarged vermiculate chomata (see 'neopycnodontine chomata' in Harry, 1985) and the vesicular structures in the inner shell layer. *N. zibrowii* is morphologically different from the only extant congeneric species *N. cochlear* at several characters such as the shell architecture and outline, the absence of the resilifer bulge in the latter species and the shape and thickness of the vesicular microstructures. On the other hand, at 15 years since its discovery, molecular data are still not available for this species, thus limiting the assessment of its phylogenetic position and systematic placement.

Taxonomic assessment of oysters based on morphology can be challenging due to a high shell variability and a low number of diagnostic characters (Lam & Morton, 2006; Raith et al., 2015; Salvi et al., 2021). Molecular data have a key role in species delimitation and taxonomic identification of oyster species (Lam and Morton 2003, Bieler et al. 2004, Kirkendale et al. 2004, Al-Kandari et al. 2021, Salvi et al. 2022) and would provide a compelling evidence that the giant deep-sea oyster *Neopycnodonte zibrowiii* is a distinct species rather than a deepwater ecophenotype of *Neopycnodonte cochlear* (Wisshak, López Correa, et al., 2009).

In this study, we generated DNA sequence data of the giant deep-sea oyster *N. zibrowii* for both mitochondrial and nuclear markers based on the holotype specimen and performed a multilocus phylogenetic analyses to establish its relationships with other gryphaeids. The main aims of this study are to provide (i) a molecular phylogenetic framework for the systematic assessment of the giant deep-sea oyster, and (ii) a reliable (i.e., holotype-based) reference sequence set for multilocus DNA barcoding approaches.

Materials and methods

Specimens and sequence data gathering

We gathered tissue samples for molecular analyses from museum collections and by field collection. The holotype of *Neopycnodonte zibrowii* (MNHN-IM-2000-20888) and the specimen of *Hyotissa numisma* (MNHN-IM-2013-13700) are deposited at the National Museum of Natural History (MNHN) of Paris, while the specimen of *Pycnodonte taniguchii* (UF 280382) is preserved in the collection of Florida Museum of Natural History (FLMNH). *Neopycnodonte cochlear* (OS239) was collected during scuba diving off the coast of Civitavecchia (nearby Rome, Italy) and stored in pure ethanol. Total genomic DNA was extracted from abductor muscles following standard high-salt protocols (Sambrook et al., 1989). We amplified two mitochondrial − cytochrome oxidase subunit I (COI) and 16S rRNA (16S) − and two nuclear − 28S rRNA (28S) and ITS2 rRNA (ITS2) − gene fragments by polymerase chain reaction (PCR). Primers and conditions used for the amplification are reported in Table 1. Sequencing of PCR products were carried out by the company Genewiz® (https://www.genewiz.com), using the same primers employed for amplification. Sequences generated from these specimens were complemented with sequences obtained from GenBank for additional gryphaeid species. Localities and GenBank accession numbers of sequences used for molecular analyses are showed in Table 2. GenBank sequences were selected in order to minimise the use of chimeric sequences in concatenated alignments (i.e., sequences of different gene fragments obtained from different voucher specimens), therefore whenever possible for each species we selected mitochondrial (COI and 16S) and nuclear (28S and ITS2) sequences from the same voucher. Three specimens (*Hyotissa hyotis* #2, *Hyotissa numisma* and *Neopycnodonte cochlear* #1) have GenBank sequences from different vouchers (chimeric concatenated sequences). We validated the taxonomic identification of each of these vouchers based on single-gene NJ trees. First, we built four single-gene datasets (COI, 16S, 28S and ITS2) including all the sequences of Gryphaeidae species in GenBank and museum sequences. Then for each marker we selected GenBank sequences that clustered within the same clade of museum sequences of these three species (results not shown).

Table 1. Primers used in this study: forward primers are listed above and reverse primers below. For the COI and ITS2 gene fragments we designed new primers specific to Ostreoidea Rafinesque, 1815, and we used the following PCR cycling conditions: denaturation step: 94 °C / 3 min; 35 cycles of: 94 °C / 60 s, T° annealing (COI: 49°C; ITS2: 50°C) / 60 s, 72 °C / 60 s; final extension: 10 min at 72°C.

Gene	Primer	Sequence	Reference	Notes
COI	Moll-F	5’ – ATAATYGGNGGNTTTGGNAAYTG – 3’	This study	Dr Zuccon D. (MNHN), pers. comm.
	osHCO998-R	5’ – ACRGTIGCIGCICTRAARTAAGCICG – 3’		Salvi et al., in press?
16S	16Sar-L	5’ – CGCCTGTTTATCAAAAACAT – 3’	(Salvi et al. 2010)	
	16Sbr-H	5’ – CCGGTCTGAACTCAGATCAC – 3’		
28S	D1F-OS	5’ – GAGACTACGCCCTGAACTTAAGCAT – 3’	This study (Salvi et al. 2022)	
	D6R-OS	5’ – GCTATCCTGAGGGAAACYTCAGAGG – 3’		
ITS2	its3d-OS	5’ – GGGTCGATGAAGARCGCAGC – 3’	This study	Modified from Oliverio and Mariottini (2001)
	its4r-OS	5’ – CCTAGTTAGTTTCTTTTCCTGC – 3’		

Table 2. Details on the species and DNA sequence data used in this study. Asterisks indicate specimens sequenced in this study. GenBank data are as follows: [1]: Matsumoto 2003; [2]: Matsumoto and Hashimoto unpublished; [3]: Kirkendale et al. 2004; [4]: Plazzi and Passamonti 2010; [5]: Kim et al. 2009; [6]: Plazzi et al. 2011; [7]: Li et al. unpublished; [8]: Ren et al. 2016; [9]: Salvi et al. 2014; [10]: Ip et al. 2022.

Specimen	**Locality**	**Genbank accession number**			
		COI	**16S**	**28S**	**ITS2**
Hyotissa hyotis #1	Madagascar	GQ166583[6]	GQ166564[6]	-	-
Hyotissa hyotis #2	Singapore (COI); Maldives (16S and ITS2)	OM946450[10]	LM993886[8]	-	LM993876[9]
Hyotissa imbricata	Japan: Okinawa (COI and ITS2); China: Beibu Bay (16S and 28S)	AB076917[1]	KC847136[7]	KC847157[7]	AB102758[2]
Hyotissa numisma #1	Guam	-	AY376598[4]	AF137035[3]	-
Hyotissa numisma #2 *	Papua New Guinea: Rempi Area	-	requested	requested	-
Neopycnodonte cochlear #1	Italy: Mediterranean Sea (COI, 16S and ITS2)	JF496772[6]	JF496758[6]	-	LM993878[9]
Neopycnodonte cochlear #2 *	Italy: Civitavecchia	requested	requested	requested	requested
Neopycnodonte zibrowii *	Azores: Faial Channel	requested	requested	requested	requested
Pycnodonte taniguchii #1	Japan: Okinawa	AB076916[1]	-	AB102759[2]	-
Pycnodonte taniguchii #2 *	Indonesia: Sulawesi Island	requested	requested	requested	requested
Magallana gigas (outgroup)	Japan (COI, 16S and 28S); South Korea (ITS2)	KJ855241[8]	KJ855241[8]	AB102757[2]	EU072458[5]

Phylogenetic analyses

Newly generated sequences for each marker were used as query in BLAST searches (*blastn* algorithm) using default settings to evaluate contaminants and to confirm the identifications of the specimens from family to species level. Multiple sequence alignments of each marker were performed with MAFFT v.7 (Katoh et al., 2019) using the G-INS-I iterative refinement algorithm for the COI and the E-INS-i iterative refinement algorithm for the rRNA markers. GBlocks (Castresana, 2000) was used to remove poorly aligned and ambiguous position of the hypervariable regions of the rRNA alignments using a relaxed selection of blocks (Talavera & Castresana, 2007). Single-gene alignments were concatenated using the software SequenceMatrix (Vaidya et al., 2011).

Phylogenetic relationships were inferred using Maximum Likelihood (ML) and Bayesian Inference (BI) methods. We used the true oyster *Magallana gigas* (Thunberg, 1793) as outgroup based on previous phylogenetic studies (Plazzi et al., 2011; Tëmkin, 2010). ML analyses were performed in the W-IQ-TREE web server v.1.6.12 [http://iqtree.cibiv.univie.ac.at/; (Trifinopoulos et al., 2016)] based on a partitioned substitution model. For each gene partition, the best substitution model was calculated by the ModelFinder module (Kalyaanamoorthy et al., 2017) using an edge-linked model and the BIC criterion (COI: TPM2u+F+G4; 16S: HKY+F+G4; 28S: TN+F+G4; ITS2: K2P+G4). ML analysis was performed with 1,000 pseudoreplicates of ultrafast bootstrapping [uBS; (Minh et al., 2013)]. Bayesian analyses (BA) were carried out with MrBayes v.3.2.7 (Ronquist et al., 2012), using the substitution models selected by ModelFinder for each gene partition. We ran two Markov chains of two million generations each, with a sample frequency of 200 generations. Convergence of the runs (ESS values > 200) were checked with Tracer 1.7 (Rambaut et al., 2018) after a burn-in of 25%. Nodal support was estimated as Bayesian posterior probability (BPP). FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/ figtree/) was used to visualize both ML and BI trees.

Genetic divergence between species at each marker (COI, 16S, 28S and ITS2) were calculated using both uncorrected genetic distance (*p*-distance) and genetic distance corrected under the Kimura 2-paramer model (K2P-distance) using the software Mega11 and the option "*Compute Between Groups Mean Distance*" (Tamura et al., 2021).

Results

BLAST searches using mitochondrial sequences (COI and 16S) of the newly sequenced specimens of *H. numisma*, *N. cochlear* and *P. taniguchii* confirmed the taxonomic identifications of these species (sequence identity of 99-100%). BLAST searches using the mitochondrial sequences generated from the holotype of *N. zibrowii* recovered as best hits sequences belonging to Gryphaeidae species (COI: sequence identity of 73.2%/72.5%/73.1% with GenBank sequences of *Hyotissa* sp./*Neopycnodonte* sp./*Pycnodonte* sp. respectively; 16S: sequence identity of 87.3%/87.5% with GenBank sequences of *Hyotissa* sp./*Neopycnodonte* sp. respectively). This confirms the lack of contamination during the amplification and the affiliation of this species to Gryphaeidae.

The concatenated dataset included 2409 positions (COI: 455, 16S: 449, 28S: 1078, ITS2: 427 positions) and among the 828 variable positions 436 were phylogenetically informative (i.e., parsimony informative). Maximum likelihood and Bayesian trees show two main clades: one including *Hyotissa* species (uBS = 95; BPP = 1), and the other one including *Pycnodonte* and *Neopycnodonte* species (uBS = 83; BPP = 0.94) (Fig. 1). *Neopycnodonte zibowii* is nested within the second clade with a sister relationship with *N. cochlear* (uBS = 56; BPP = 0.72), whereas *P. taniguchii* is sister to *Neopycnodonte* species.

The COI genetic distance (K2P/*p*-distance) between *N. zibrowii* and *N. cochlear* and between *N. zibrowii* and *P. taniguchii* are respectively 35.8%/28.2% and 35%/27.6% (Table 3). The 16S genetic distance (K2P/*p*-distance) between *N. zibrowii* and either *N. cochlear* or *P. taniguchii* are 13.5%/12.1% (Table 3). The mean interspecific genetic distance (K2P/*p*-distance) among the six gryphaeid species are 33.7% ± 4.6% / 26.8% ± 3% at the COI and 15.5% ± 4.6%/13.7% ± 3.7% at the 16S. The 28S genetic distance (K2P/*p*-distance) between *N. zibrowii* and *N. cochlear* and between *N. zibrowii* and *P. taniguchii* are respectively 2.5%/2.4% and 9%/8.4% (Table 4). The ITS2 genetic distance (K2P/*p*-distance) between *N. cochlear* and between *N. zibrowii* and *P. taniguchii* are respectively 15.8%/14.9% and 38.2%/29.6% (Table 4). The mean interspecific genetic distance (K2P/*p*-distance) among the six gryphaeid species are 5.5% ± 2.5% / 5.1% ± 2.2% at the 28S and 27.4% ± 16.8% / 22.0% ± 11.9% at the ITS2.

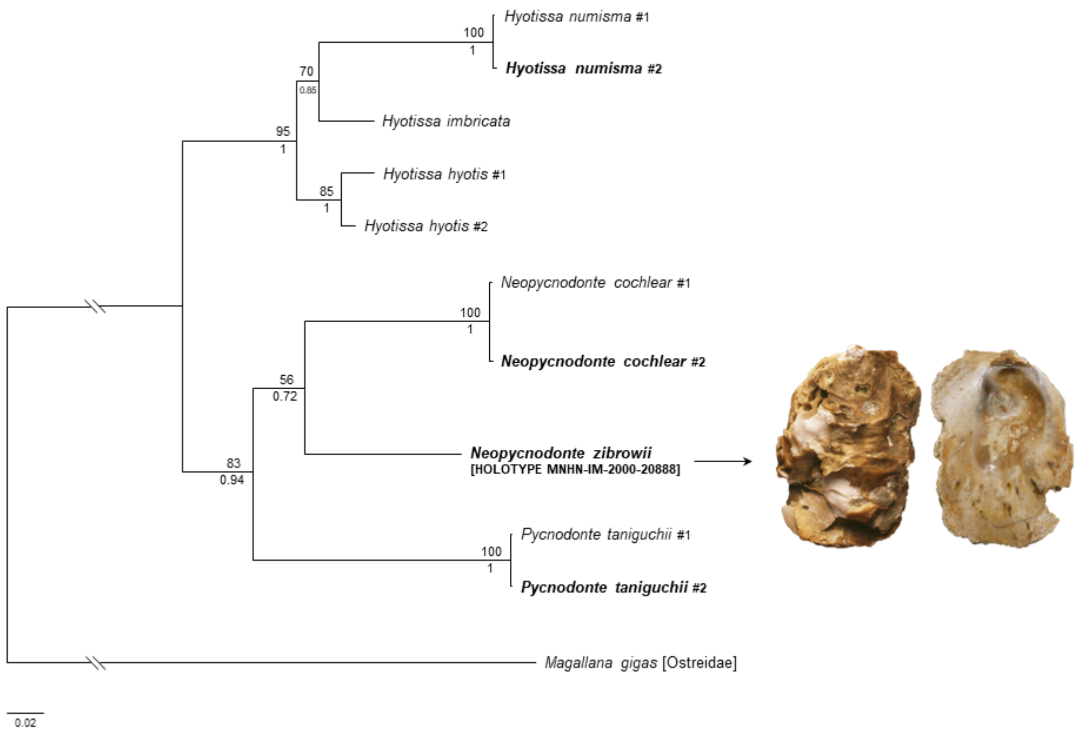

Figure 1. Bayesian phylogenetic tree of six Gryphaeidae species based on COI, 16S, 28S and ITS2 markers. Nodal supports indicate the values of uBS (upper) and the BPP (lower). The tree is rooted with *Magallana gigas* which belongs to the sister family Ostreidae Rafinesque, 1815. The new sequenced specimens from this work are showed in bold.

Table 3. Mean genetic distance based on COI (lower triangular matrix) and 16S (upper triangular matrix) DNA sequences, calculated using the K2P model (first value) and uncorrected (p-distance: value inside brackets). The COI and 16S dataset are composed by 2 sequences for each species, except for *N. zibrowii* (one sequence for each marker), *H. imbricata* (one COI and one 16S sequence) and *P. taniguchii* (one 16S sequence), see Table 1. (n. a.: not available).

	Neopycnodonte zibrowii	*Neopycnodonte cochlear*	*Pycnodonte taniguchii*	*Hyotissa hyotis*	*Hyotissa numisma*	*Hyotissa imbricata*
Neopycnodonte zibrowii	-	13.5% (12.1%)	13.5% (12.1%)	15.1% (13.4%)	23.5% (19.9%)	14.9% (13.3%)
Neopycnodonte cochlear	35.8% (28.2%)	-	11.2% (10.3%)	15.5% (13.8%)	22.9% (19.5%)	14.9% (13.4%)
Pycnodonte taniguchii	35.0% (27.6%)	35.4% (28.1%)	-	14.9% (13.3%)	22.3% (19.0%)	14.2% (12.7%)
Hyotissa hyotis	33.3% (26.7%)	39.6% (30.5%)	32.7% (26.5%)	-	15.6% (13.9%)	5.3% (5.1%)
Hyotissa numisma	n. a.	n. a.	n. a.	n. a.	-	15.1% (13.6%)
Hyotissa imbricata	34.0% (27.2%)	37.0% (28.8%)	31.8% (25.8%)	22.4% (19.1%)	n. a.	-

Table 4. Mean genetic distance based on 28S (lower triangular matrix) and ITS2 (upper triangular matrix) DNA sequences, calculated using the K2P model (first value) and uncorrected (p-distance: value inside brackets). The 28S and ITS2 dataset are composed by 2 sequences for each species, except for *N. zibrowii* (one sequence for each marker), *H. hyotis* (one ITS2 sequence), *N. cochlear* (one 28S sequence) and *P. taniguchii* (one ITS2 sequence), see Table 1. Any 28S sequences of *H. hyotis* were available. The ITS2 sequence of *H. imbricata* was excluded because too short. (n. a.: not available).

	Neopycnodonte zibrowii	*Neopycnodonte cochlear*	*Pycnodonte taniguchii*	*Hyotissa hyotis*	*Hyotissa numisma*	*Hyotissa imbricata*
Neopycnodonte zibrowii	-	15.8% (14.9%)	38.2% (29.6%)	23.8% (20.4%)	n. a.	n. a.
Neopycnodonte cochlear	2.5% (2.4%)	-	43.5% (33.0%)	20.9% (19.4%)	n. a.	n. a.
Pycnodonte taniguchii	9.0% (8.4%)	4.4% (4.2%)	-	48.4% (35.4%)	n. a.	n. a.
Hyotissa hyotis	n. a.	n. a.	n. a.	-	n. a.	n. a.
Hyotissa numisma	6.8% (6.5%)	4.4% (4.2%)	7.3% (6.9%)	n. a.	-	n. a.
Hyotissa imbricata	6.8% (6.5%)	4.6% (4.4%)	7.7% (6.3%)	n. a.	1.2% (1.2%)	-

Discussion

Benthic organisms such as oysters, with extensive phenotypic variation and few diagnostic characters, are prone to misidentification in morphological assessments. The utility of molecular characters for taxonomic identification and systematic assessment of these organisms cannot be overstated and has been proven over and over by studies on true oysters (Lam & Morton, 2006; Raith et al., 2015; Salvi et al., 2021), pearl oysters (Cunha et al., 2011), tree oysters (Garzia et al., 2022) as well as gryphaeid oysters (Li et al., 2023). Conchological convergence, phenotypic plasticity, and the occurrence of cryptic species make molecular taxonomic validation of new oyster species necessary to accurately estimate the diversity of these taxa.

Our molecular phylogenetic results clearly demonstrate that *N. zibrowii* represent a distinct species with extremely high genetic divergence from any other gryphaeid at all the markers analysed (Table 3 and Table 4). *Neopycnodonte zibrowii* is nested within the clade "*Neopycnodonte*/*Pycnodonte*" with closer affinity to *N. cochlear* rather than *P. taniguchii* (Fig. 1), and thus supporting its assignment to the genus *Neopycnodonte* based on morphological features (Wisshak, López Correa, et al., 2009). Phylogenetic relationships within this clade are not well supported, like in a previous phylogenetic study including *N. cochlear* and *P. taniguchii* and based on COI and 28S markers (. However, the extended dataset of our study improved nodal support and allowed clarifying the source of phylogenetic uncertainty. Indeed, at both mitochondrial markers, values of pairwise genetic distance between *N. cochlear* / *N. zibrowii* / *P. taniguchii* are similar and remarkably high (COI: 35.0-35.8%; 16S: 11.2-13.5%); whereas at nuclear markers the genetic distance between *P. taniguchii* and *N. zibrowii* is two-three times higher than between the latter and *N. cochlear* (Table 4). Such pattern suggests that mitochondrial variation is inflated by saturation that eroded phylogenetic signal, implying an old split between taxa within this clade. Wisshak et al. (2009b) highlighted a low number of morphological and ecological differences between the genus *Neopycnodonte* and *Pycnodonte* and pointed out the need of a systematic revision of the genera. Our results highlight that nuclear data will have a key role in further systematic assessment of these genera.

The availability of taxonomically validated reference sequence is a premise for DNA barcoding and metabarcoding approaches for large-scale, fast, and cost-effective molecular taxonomic identification (Hebert et al., 2003; Moritz & Cicero, 2004; Salvi et al., 2020; Schindel & Miller, 2005). The mitochondrial COI and 16S are the most common markers in DNA barcoding studies on Ostreidae and Gryphaeidae (Hsiao et al., 2016; Kirkendale et al., 2004; Lam & Morton, 2004, 2006, p. 200; Liu et al., 2011; Salvi et al., 2021). However, also nuclear rRNA markers such as

28S (Mazón-Suástegui et al., 2016) and ITS2 (Salvi et al., 2014; Salvi & Mariottini, 2017) revealed useful for molecular taxonomic identification of oysters. Moreover, rRNA markers are frequently selected as target genes in eDNA metabarcoding projects (Ruppert et al., 2019). In this respect, the set of four reference (holotype-based) barcode sequences of *N. zibrowii* provided in this study will be useful for a wide plethora of barcoding applications in deep-sea biodiversity surveys. During the last decade, deep-sea oysters from a mounting number of areas across the Atlantic Ocean and the Mediterranean Sea have been morphologically identified as *N. zibrowii*: from Bay of Biscay (Van Rooij et al., 2010), Gulf of Cadiz (Gofas et al., 2010), Celtic Sea (Johnson et al., 2013), Angola and Mauritania (Beuck et al., 2016), southern Sardinia (Taviani et al., 2017), Sicilian Channel (Rueda et al., 2019) and Gulf of Naples (Taviani et al., 2019). Molecular validation of these records will be crucial to clarify the distribution of *N. zibrowii* and assess the phenotypic variation and ecology of this enigmatic species. Finally, given the high prevalence of cryptic species in oysters, it is not unlikely that future molecular assessments of deep-sea oysters will disclose new species.

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Case study III

Cryptic marine diversity in the northern Arabian Gulf: an integrative approach uncovers a new species of oyster (Bivalvia: Ostreidae), *Ostrea oleomargarita*

Introduction

Marine animal biodiversity is greatly underestimated, especially outside temperate regions (Appeltans et al., 2012) This lack of knowledge is due to different sources of taxonomic impediment, including the difficulty of sampling in marine ecosystems and the lower availability of systematists and support infrastructures for documenting biodiversity in tropical and sub-tropical regions (Duarte, 2001). In addition, many intertidal benthic organisms, such as bivalve molluscs, show limited morphological complexity and high phenotypic plasticity driven by different environmental factors, making traditional taxonomic assessment based on morphology inherently challenging (Clark et al., 2020). This is particularly true for oysters (Ostreidae Rafinesque, 1815) a relatively small family of bivalves with 74 currently accepted species (MolluscaBase, 2021) widely distributed in world oceans and estuaries.

Despite the relatively low species diversity oysters play an important role in marine ecosystems and are important fishery resources that support aquaculture industries worldwide, however, the diversity and distribution of living oysters is far from being fully understood (e.g., Guo et al., 2018; Li et al., 2017; Sekino & Yamashita, 2016). The assessment of species diversity in oysters is hampered by the lack of reliable morphological characters for species identification as well as for their assignment to higher taxa (Salvi & Mariottini, 2021). Historically, oysters have been described based on few shell characters, however these characters are phenotypically plastic and phylogenetically unreliable (Littlewood, 1994; Raith et al., 2015; Salvi et al., 2014). Molecular studies have provided several examples of how shell morphology is unreliable and misleading for oyster classification (Salvi et al., 2014; Wang et al., 2004), even between different subfamilies (Salvi et al., 2021). Particularly emblematic in this respect are those cases where allopatric ecomorphs of a single species have been classified as distinct species in different subfamilies (Raith et al., 2015), or where syntopic populations of distinct species have long been considered as a single species due to their phenotypic convergence (e.g., Ignacio et al., 2000; Wang et al., 2004). Not surprisingly, in the last decades an integrative molecular and morphological approach has allowed a great leap forward in our knowledge of oyster diversity and evolution and in the characterization of local and endemic faunas (e.g. Al-Kandari et al., 2021; Wu et al., 2013). However, while a molecular approach has great potential in the discovery of new species of oysters, these studies have been biased toward the clarification of the taxonomic status of cultured species and have been focused especially on the Eastern Indo-Pacific (e.g., Lam & Morton, 2003; Wang et al., 2004). Far less is known on the oyster diversity of the Western Indo-Pacific, despite this region represent a centre of origin for shallow water marine biota (Obura, 2016).

Recent surveys along Kuwaiti coastal areas in the northern Arabian Gulf suggests that in these shallow waters there are a number of unidentified oyster species including some that that form prominent ecological feature of shores. This is perhaps not surprising since serious biological investigation, in Kuwait, only dates from the 1980s (Al-Bakri et al., 1985). A recent survey of the intertidal macrofauna of Kuwait, based on a morphological assessment, reported five species of Ostreidae (Al-Kandari et al., 2020). These authors listed the ostreids *Alectryonella* cf. *crenulifera* (Sowerby, 1871) = *Dendostrea sandvichensis* (Sowerby, 1871), cf. *Booneostrea subucula* (Jousseaume in Lamy, 1925), cf. *Nanostrea deformis* (Lamarck, 1819), *Crassostrea* sp., and *Saccostrea cuccullata* (Born, 1778). It is evident from this listing that there was considerable doubt with many of the morphological identifications and that many of these oysters might represent undescribed species. One oyster listed by Al-Kandari et al. (2020) as *Crassostrea* sp. has now been described as a new species *Talonostrea salpinx* Oliver Salvi Al-Kandari 2021 based on molecular and morphological data (Al-Kandari et al., 2021). That was the first record of the genus *Talonostrea* X.-X. Li & Z.-Y. Qi, 1994 in the Arabian region and the Indian Ocean as a whole.

In this study, we performed an integrative molecular and morphological assessment of specimens of the small oyster (here referred to as "the Kuwait oyster") that was tentatively referred to the ostreine taxon *Nanostrea deformis* by Al-Kandari et al. (2020) but showing a type of small pustular (chomata) that are characteristic of lophine genera (Harry, 1985; Stenzel, 1977). The subfamily Lophinae Vialov, 1936 is no longer accepted and lophine genera are now included in the subfamily Ostreinae Rafinesque, 1815 because they form a sub-clade that is nested within the Ostreinae clade (Raith et al., 2015; Salvi et al., 2014; Salvi & Mariottini, 2017). However, it is still unclear whether their shared morphological characteristics do represent synapomorphies of this sub-clade or instead lack any phylogenetic and systematic value within Ostreidae (Harry, 1985; Littlewood, 1994; Malchus, 1998; Stenzel, 1977). In this respect, the taxonomic and phylogenetic assessment of this small oyster from Kuwait might provide some insights on the phylogenetic significance of lophine chomata. Here, we used mitochondrial and nuclear markers to estimate phylogenetic relationships of this taxon within Ostreidae and we used cytochrome oxidase I data to assess species delimitation, intraspecific variation and phylogeographic relationships with its closest relative. Combined with a morphological assessment these results support the recognition of Kuwaiti specimens as a new species, that is described herein. The aim of this study is to identify independent evolutionary lineages or cryptic taxonomic units within northern Arabian Gulf flat oysters and to contribute to our understanding of the biogeographic and biodiversity patterns of intertidal fauna of this region.

Materials and Methods

Study area

Details of the oceanography and marine biology of Kuwait can be found in Al-Yamani et al. (2004) and more recently revised by Al-Yamani, (2021). These works contain an extensive synthesis of Kuwait's marine environments and we have extracted some pertinent characteristics relevant to the subject of this paper.

The intertidal of Kuwait features hard and soft substrate shores. In the inner Kuwait Bay and the northern regions around Boubyan soft sediment consisting of fine muds dominate. On the outer shores of Kuwait Bay the soft sediments become more sandy and this trend continues along the south coast. Hard substrate shores are present throughout the country and can consist of bedrock reefs or boulder and cobble shores intermixed with soft sediments. A similar assortment of substrates is also found on the outer islands of Failaka and Miskan.

The waters of Kuwait are shallow, much of the region being a flooded estuary rarely exceeding a few meters depth and with a maximum of 35 m offshore. The tidal range on average is 2 m but can reach 3.5 to 4 m during equinoxes. This in combination with the shallow topography results in extensive intertidal flats exposed for many hours. While sea water temperatures vary little air temperature range from a possible 0° C on winter nights to 51°C at the height of summer. Consequently, the intertidal fauna is subjected to extreme variations when exposed at low tide. The shallowness of the water and predominance of fine muds offshore results in a high level of sediment resuspension especially in Kuwait Bay and around Boubyan. This is exacerbated around Boubyan through the input of riverine suspended solids from the Shatt el-Arab such that the concentration can reach 510 mg/l. In Kuwait Bay concentrations may reach 40 mg/l but drop off along the south coast to a recorded minimum of 0.3 mg/l.

The extremes and variation in temperature, salinity and turbidity make the rocky intertidal a rather inhospitable environment for unprotected animals. On exposed surfaces the molluscan fauna is limited to a small variety of gastropods, mainly thick shelled ceriths and muricids, and cemented bivalves such as larger oysters, spondylids and plicatulids. However, in cryptic environments such as rock crevices and beneath rocks there is a diverse fauna of smaller species Al-Kandari et al. (2020).

Sampling

Sample collection was performed during the KISR intertidal survey of 2014–2017 Al-Kandari et al. (2020), and in 2019 further samples were collected specifically for tissue extraction for the molecular study. We collected 56 specimens of the morphotype listed as in cf. *Nanostrea deformis* by Al-Kandari et al. (2020) from two localities (Figure 1; Table 1). Twenty-three specimens selected for molecular analyses were levered open and the adductor muscle and mantle were excised whole or in part and fixed in 100% ethanol.

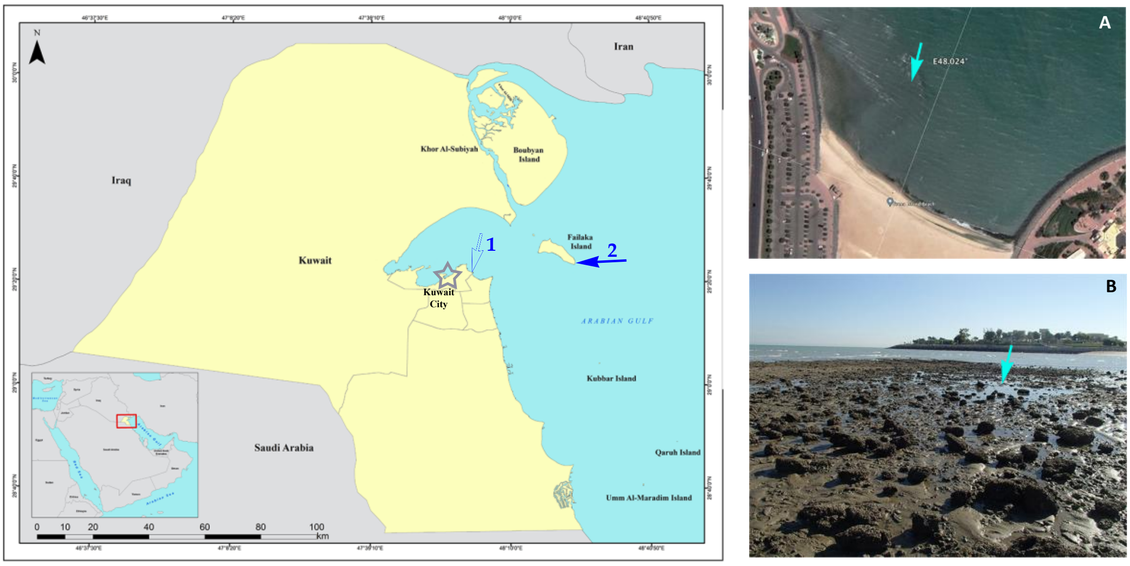

Figure 1. Map of Kuwait indicating known distribution of the Kuwait oyster under study. Blue arrows indicate collecting sites near Kuwait City (1: Al Sha'ab) and Failaka Island (2: Al Liwan). **A**, satellite image of the type locality, Al Sha'ab; **B**, photograph of the beach at low tide at Al Sha'ab.

Morphological assessment

Morphometric assessments of oyster taxa are of little taxonomic utility because of the wide and overlapping intra-specific and inter-specific variability in shell size and shape that are strongly determined by habitat type, growth stage and settlement condition. Therefore, a morphological assessment of collected specimens was performed based on qualitative characters traditionally used to discriminate oyster taxa. Harry (1985) and Littlewood (1994) gave lists of characters used to define five groups of oysters: Saccostreinae; Striostreini; Crassostreinae (Crassostreini sensu Harry, 1985); Ostreinae; 'lophinae' (Lophinae sensu Harry, 1985; now considered as part of Ostreinae; Raith et al., 2015; Salvi et al., 2014; MolluscaBase, 2021) and Pycnodonteinae (Gryphaeidae Vialov, 1936) The shell characters listed were as follows: plication of upper valve; hyote spines; lamellation of upper valve; chalky deposits; commissural shelf; shape of adductor muscle; colour of adductor scar. These characters were derived from Harry (1985)and in that paper the characters were further refined especially the form of the chomata, which are listed as vermicular, pustular and ostreine (anachomata on the right valve and catachomata on the left valve). Vermicular chomata take the form of dense transverse ridges either side of the ligament; ostreine take the form of single tubercles along the outer margin and where the anachomata and catachomata interlock; lophine or pustular chomata take the form of small raised pustules on the inner part of the margin and have no corresponding pits on the opposing valve (Fig. 2). These characters will be used to compare species under consideration here.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from alcohol-preserved tissues of the 23 specimens following standard high-salt protocols (Evans, 1990). Two mitochondrial gene fragments were amplified by polymerase chain reaction (PCR), the barcode fragment of the cytochrome oxidase subunit I (COI) and the 16S rRNA (16S), using primers and PCR protocols described in Salvi et al., (2010) and Crocetta et al. (2015). Additionally, for selected specimens we amplified a fragment of the nuclear 28S rRNA (28S) using published primers slightly modified to better match the available 28S sequences of oysters: the primer F4ostrd-OS [5'-TCCGATAGCAAACAAGTACCGTG-3'] modified from 28S-F4-OSTRD by Mazón-Suástegui et al. (2016) and the primer D6R-OS [5'-GCTATCCTGAGGGAAACYTCAGAGG-3'] modified from D6R by Park & Foighil (2000). Sequencing of PCR products was carried out by the company GENEWIZ (https://www.genewiz.com), using the same primers employed for amplification. Details on sample data and GenBank accession numbers of sequences generated in this study are provided in Table 1 where we also indicated the GenSeq nomenclature for genetic sequences based on the taxonomic reliability of the source specimens following (Chakrabarty et al., 2013).

DNA-barcoding and phylogenetic analyses

After verification that all the sequenced specimens belong to the same species (COI sequence identity ≥ 99%), we used one sequence as a query to perform a preliminary molecular species-level identification with the DNA-barcoding approach implemented in the BOLD Identification System (IDS; Ratnasingham & Hebert, 2007). IDS analyses based on all COI barcode records with a minimum sequence length of 500bp did not find either a species-level (less than 1% divergence) or a genus-level (less than 3% divergence) match for the query sequence. The top 20 matches were sequences of *Ostrea futamiensis* from Japan with a similarity score of 90.8 - 91.2%. Therefore, we performed phylogenetic analyses to assess the systematic relationships of the Kuwait oyster.

The phylogenetic assessment was performed in two steps. First, to establish the affiliation of the Kuwait oyster with extant subfamilies of Ostreidae, newly generated sequences of the three genes of two specimens were aligned with homologous sequences of 20 oyster species obtained from Genbank representing all currently accepted genera within the subfamily Striostreinae, Saccostreinae and Crassostreinae, and six out of nine currently accepted genera of Ostreinae (MolluscaBase, 2021). For this analysis we used two species of Gryphaeidae as the outgroup. Second, once established the affiliation of the Kuwait oyster to the subfamily Ostreinae, we built another dataset using 24 species of Ostreinae and two species of the Saccostreinae subfamily as outgroup. Genbank accession number and reference for the DNA sequences used for phylogenetic analyses are reported in Table 2.

Multiple sequence alignments were performed with MAFFT web server (Katoh et al., 2019) using the E-INS-i iterative refinement algorithm. Single gene alignments were concatenated in a single matrix that was used for downstream phylogenetic analyses using Maximum Likelihood (ML) and Bayesian Inference (BI) methods. ML analyses were performed in the W-IQ-TREE web server (Trifinopoulos et al., 2016) using for each gene partition the best substitution model determined by the ModelFinder module (Kalyaanamoorthy et al., 2017) and 1000 replicates of ultrafast bootstrapping (uBS). Bayesian analyses were carried out with MrBayes 3.2.7 (Ronquist et al., 2012), using for each gene partition the same substitution models selected by ModelFinder. We run two Markov chains of five million generations each, sampled every 1000 generations. Consensus trees and posterior probability values (PP) were calculated on trees sampled after a burn-in of 25%. FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) was used to visualize the tree.

The close phylogenetic relationship between the Kuwait oyster and *Ostrea futamiensis* recovered in ML and BI analyses was further investigated using a phylogenetic network approach based on COI sequence data. We used POPART v1.7 (Leigh & Bryant, 2015) to construct phylogenetic networks based on the median-joining network (Bandelt et al., 1999) and the statistical parsimony (Clement et al., 2000) methods.

Species delimitation

The IDS uses a standard conservative threshold of < 1% sequence divergence for animal species identification as this method is designed to defend against 'overdiagnosis' (Ratnasingham & Hebert, 2007). Therefore, a lack of species-level match does not guarantee an inter-specific divergence for the COI sequence of our unidentified oyster. To aid discrimination between intraspecific and interspecific variation we applied species delimitation methods. For this purpose, we built a non-redundant database of COI of Ostreinae mining sequences from the public repositories of GenBank and BOLD, with accepted and synonymized genus names as search queries (data updated to 23/11/2021). Retrieved sequences not identified to species level or shorter than 300 base pairs were eliminated. Duplicated () function (Becker et al., 1988) of R studio was used to dereplicate the dataset by removing sequences having identical GenBank accession number (see Salvi et al 2021 for more details). This dataset was aligned with the newly generated COI sequences of oysters from Kuwait using the G-INS-i progressive method algorithm implemented in MAFFT web server (Katoh et al., 2019). The alignment was trimmed to the standard COI barcoding fragment (5' portion of the gene) using the Folmer's primers as reference (Folmer et al., 1994).

We applied species delimitation on the generated dataset to infer species clusters using both distance-based and tree-based approaches. Assemble Species by Automatic Partitioning (ASAP) analyses were run using on the ASAP web-interface (https://bioinfo.mnhn.fr/abi/public/asap) with the Kimura-two parameters (K2P; Kimura, 1980) substitution model. ASAP delimitation was defined evaluating both the partitions with first and the second best asap-score according to Puillandre et al. (2021). As a tree-based method we used the multi-rate Poisson Tree Processes model (mPTP) applied on the maximum likelihood tree obtained using the W-IQ-TREE web interface (http://iqtree.cibiv.univie.ac.at/; Trifinopoulos et al. (2016). *Saccostrea cuccullata* (EU816078) was used as outgroup and then removed from the SD analysis. mPTP analyses were performed using the version 0.2.4 (https://github.com/Pas-Kapli/mptp) with 10 runs of 100 million MCMC generations each, sampling every 10,000 (burn-in=10%). Results from SD analyses were compared with the software LIMES (Ducasse et al., 2020) using the Taxonomic index of congruence C_{tax}, which quantifies the congruence between two taxonomies, the Relative taxonomic resolving power index R_{tax}, which quantifies the potential of an approach to capture a high number of species boundaries (Miralles & Vences, 2013), and the Match ratios (Ahrens et al., 2016).

Results

Morphology and patterns of chomata

Using the characters listed above the morphology of the Kuwait oyster is compared with that of the five major groups of extant Ostreoidea (Table 3).

Considering all characters, the Kuwait oyster has most characters in common with the Ostreinae and only in the presence of lophine chomata does it differ (Fig.2). Of the 56 specimens collected for this study including the 23 used for the molecular study in only 7 shells was the for the presence of lophine-like pustules expressed. There was no difference in numbers between Failaka and Al Sha'ab material. Given the confined sampling sites no association between habitats can be inferred. Pustules are most often seen in thinner (perhaps less mature) shells and in some thicker shells they can be seen below the nacreous layer suggesting that they can be overgrown as the shell thickens.

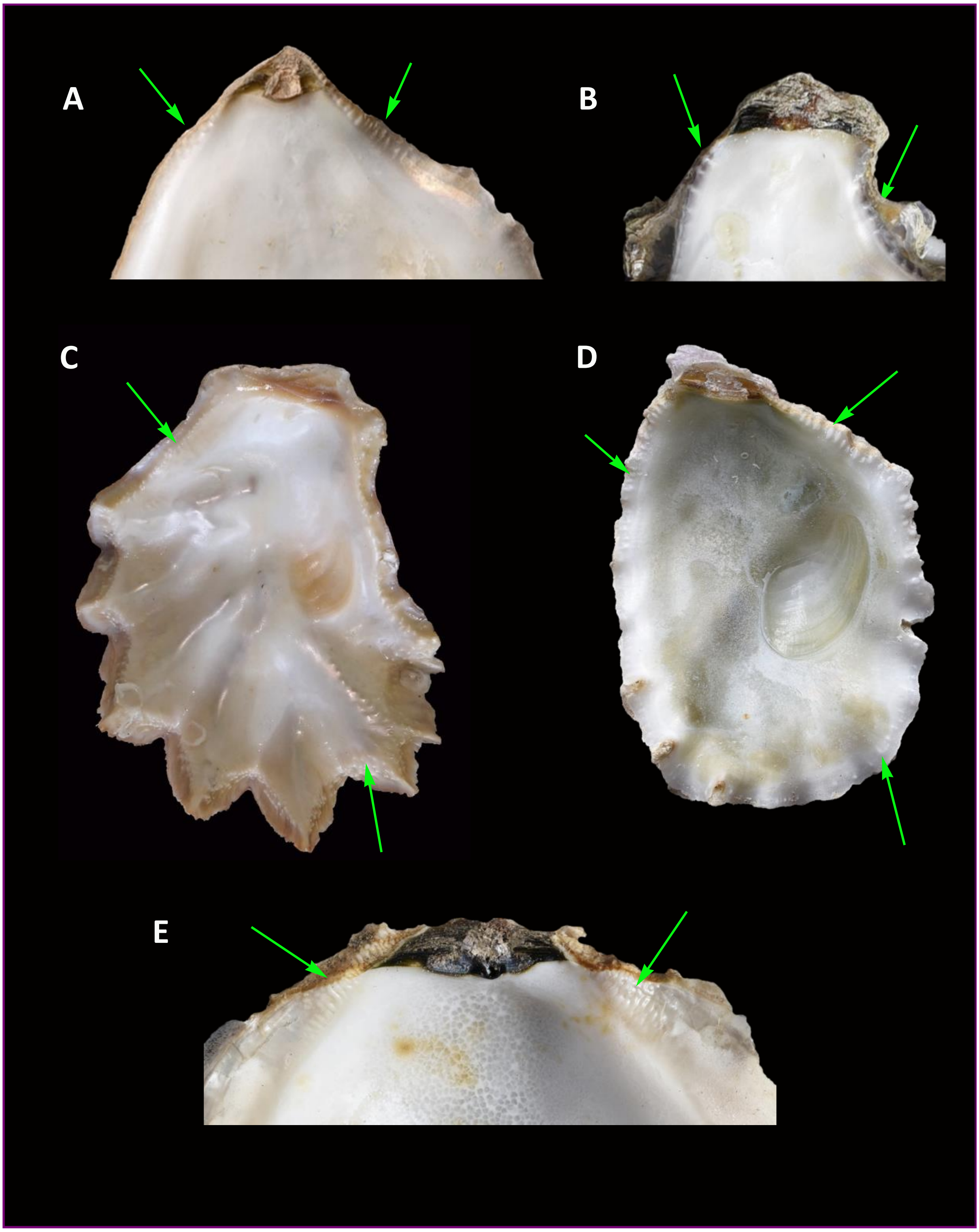

Figure 2. Types of chomata as defined by Stenzel (1971) and Harry (1985). **A**, small ostreine chomata in *Ostrea edulis*. **B**, large tuberculous ostreine chomata in *Saccostrea cuccullata*. **C**, dense pustular lophine chomata in *Lopha cristagalli*. **D**, irregular ostreine chomata close to the hinge and sparse lophine pustules around the margin in *Dendostrea sandvichensis*. **E**, vermiculate chomata in *Hyotissa inermis* (Gryphaeidae).

Table 1. GenBank accession number, COI haplotype, and GenSeq nomenclature (after Chakrabarty et al. 2013) for genetic sequences obtained from voucher specimens of *Ostrea oleomargarita* sp. nov. analysed in this study.

Species	**Specimen Catalog #**	**Locality**	**GenBank accession number**			**COI haplotype**	**GenSeq Nomenclature**
			COI	**16S**	**28S**		
Ostrea oleomargarita	NMW.Z.2021.009.009/1 (paratype)	Kuwait City, Al Sha'ab	-	ON614105	-	-	genseq-2 COI, 16S
Ostrea oleomargarita	NMW.Z.2021.009.009/2 (paratype)	Kuwait City, Al Sha'ab	ON614732	ON614106	-	h8	genseq-2 COI, 16S
Ostrea oleomargarita	NMW.Z.2021.009.009/3 (paratype)	Kuwait City, Al Sha'ab	ON614733	ON614107	-	h1	genseq-2 COI, 16S
Ostrea oleomargarita	NMW.Z.2021.009.009/4 (paratype)	Kuwait City, Al Sha'ab	ON614734	ON614108	-	h9	genseq-2 COI, 16S
Ostrea oleomargarita	NMW.Z.2021.009.009/5 (paratype)	Kuwait City, Al Sha'ab	ON614735	ON614109	-	h1	genseq-2 COI, 16S
Ostrea oleomargarita	NMW.Z.2021.009.009/6 (paratype)	Kuwait City, Al Sha'ab	ON614736	ON614111	-	h1	genseq-2 COI, 16S
Ostrea oleomargarita	NMW.Z.2021.009.009/7 (paratype)	Kuwait City, Al Sha'ab	ON614737	ON614112	-	h1	genseq-2 COI, 16S
Ostrea oleomargarita	NMW.Z.2021.009.009/8 (paratype)	Kuwait City, Al Sha'ab	ON614738	ON614113	-	h1	genseq-2 COI, 16S
Ostrea oleomargarita	NMW.Z.2021.009.009/9 (paratype)	Kuwait City, Al Sha'ab	ON614739	ON614114	-	h1	genseq-2 COI, 16S
Ostrea oleomargarita	NMW.Z.2021.009.009/10 (paratype)	Kuwait City, Al Sha'ab	ON614740	ON614116	-	h1	genseq-2 COI, 16S
Ostrea oleomargarita	NMW.Z.2021.009.009/11 (paratype)	Kuwait City, Al Sha'ab	ON614741	ON614117	ON614128	h1	genseq-2 COI, 16S, 28S
Ostrea oleomargarita	NMW.Z.2021.009.011/1 (paratype)	Ras Al Liwan, Failaka Island	ON614721	ON614100	ON614127	h4	genseq-2 COI, 16S, 28S
Ostrea oleomargarita	NMW.Z.2021.009.011/2 (paratype)	Ras Al Liwan, Failaka Island	ON614722	ON614104	-	h5	genseq-2 COI, 16S

Ostrea oleomargarita	NMW.Z.2021.009.011/3 (paratype)	Ras Al Liwan, Failaka Island	ON614723	ON614110	-	h1	genseq-2 COI, 16S
Ostrea oleomargarita	NMW.Z.2021.009.011/4 (paratype)	Ras Al Liwan, Failaka Island	ON614724	ON614115	-	h1	genseq-2 COI, 16S
Ostrea oleomargarita	NMW.Z.2021.009.011/5 (paratype)	Ras Al Liwan, Failaka Island	ON614725	ON614118	-	h1	genseq-2 COI, 16S
Ostrea oleomargarita	NMW.Z.2021.009.011/6 (paratype)	Ras Al Liwan, Failaka Island	ON614726	ON614119	-	h1	genseq-2 COI, 16S
Ostrea oleomargarita	NMW.Z.2021.009.011/7 (paratype)	Ras Al Liwan, Failaka Island	ON614727	ON614120	-	h2	genseq-2 COI, 16S
Ostrea oleomargarita	NMW.Z.2021.009.011/8 (paratype)	Ras Al Liwan, Failaka Island	ON614728	ON614121	-	h3	genseq-2 COI, 16S
Ostrea oleomargarita	NMW.Z.2021.009.011/9 (paratype)	Ras Al Liwan, Failaka Island	ON614729	ON614122	-	h6	genseq-2 COI, 16S
Ostrea oleomargarita	NMW.Z.2021.009.011/10 (paratype)	Ras Al Liwan, Failaka Island	ON614730	ON614101	-	h7	genseq-2 COI, 16S
Ostrea oleomargarita	NMW.Z.2021.009.011/11 (paratype)	Ras Al Liwan, Failaka Island	ON614731	ON614102	-	h1	genseq-2 COI, 16S
Ostrea oleomargarita	NMW.Z.2021.009.011/12 (paratype)	Ras Al Liwan, Failaka Island	-	ON614103	-	-	genseq-2 COI, 16S

Phylogenetic analyses

Maximum Likelihood and Bayesian analyses resolve the phylogenetic position of the Kuwait oyster within the subfamily Ostreinae with high statistical support (uBS = 90; PP = 1.0; Fig. 3). Phylogenetic trees of Ostreinae show three main clades (Fig. 3): the *edulis* group including *Ostrea edulis*, *O. angasi* and *O. chilensis* (uBS = 100; PP = 1.0) which is siter to *O. denselamellosa*; the *stentina* group including *Ostrea stentina* and 9 allied *Ostrea* species (uBS = 100; PP = 1.0); and the *algoensis* group including the Kuwait oyster, 4 remaining *Ostrea* species, and species of the genera *Nanostrea*, *Planostrea*, *Alectryonella*, *Dendostrea*, and *Lopha* (uBS = 74; PP = 0.99). The Kuwait oyster has a sister relationship with *O. futamiensis* that is well supported (uBS = 100; PP = 1.0), and this sister-pair form a clade with *O. algoensis* (uBS = 84; PP = 1.0). Species previously included in the lophine form a clade, nested within the *algoensis* lineage, that received low support (uBS = 84; PP = 0.59).

The Median joining network show a clear phylogeographic separation between haplotypes of the Kuwait oyster and haplotypes of *O. futamiensis* (Fig. 4). The haplogroups corresponding to these two species are recovered as two separate networks in TCS analyses (result not shown). Both species show a lack of geographic structure at the investigated geographical scale.

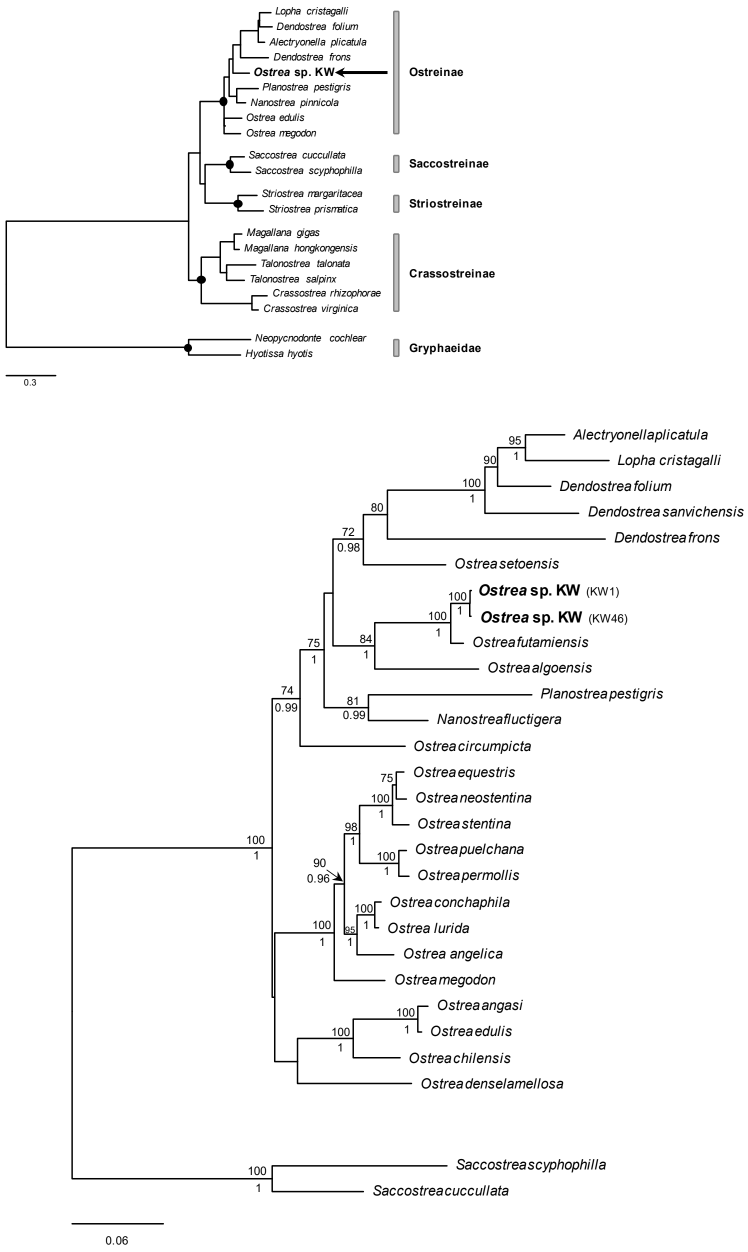

Figure 3. Maximum Likelihood (ML) phylogenetic tree of Ostreidae (above) and Ostreinae (below) based on concatenated COI, 16S and 28S sequences. The Ostreidae tree is rooted with Gryphaeidae species and represents the phylogenetic position of the Kuwait oyster relative to current subfamilies; black circles represent nodal support at main nodes (ultrafast bootstrap of Maximum-Likelihood analysis, uBS > 90; posterior probability of Bayesian analysis, PP > 0.95). The Ostreinae tree is rooted with *Saccostrea* species and represents the phylogenetic relationships between the Kuwait oyster (*Ostrea* sp. KW) and Ostreinae species; nodal support is reported at nodes (uBS/PP).

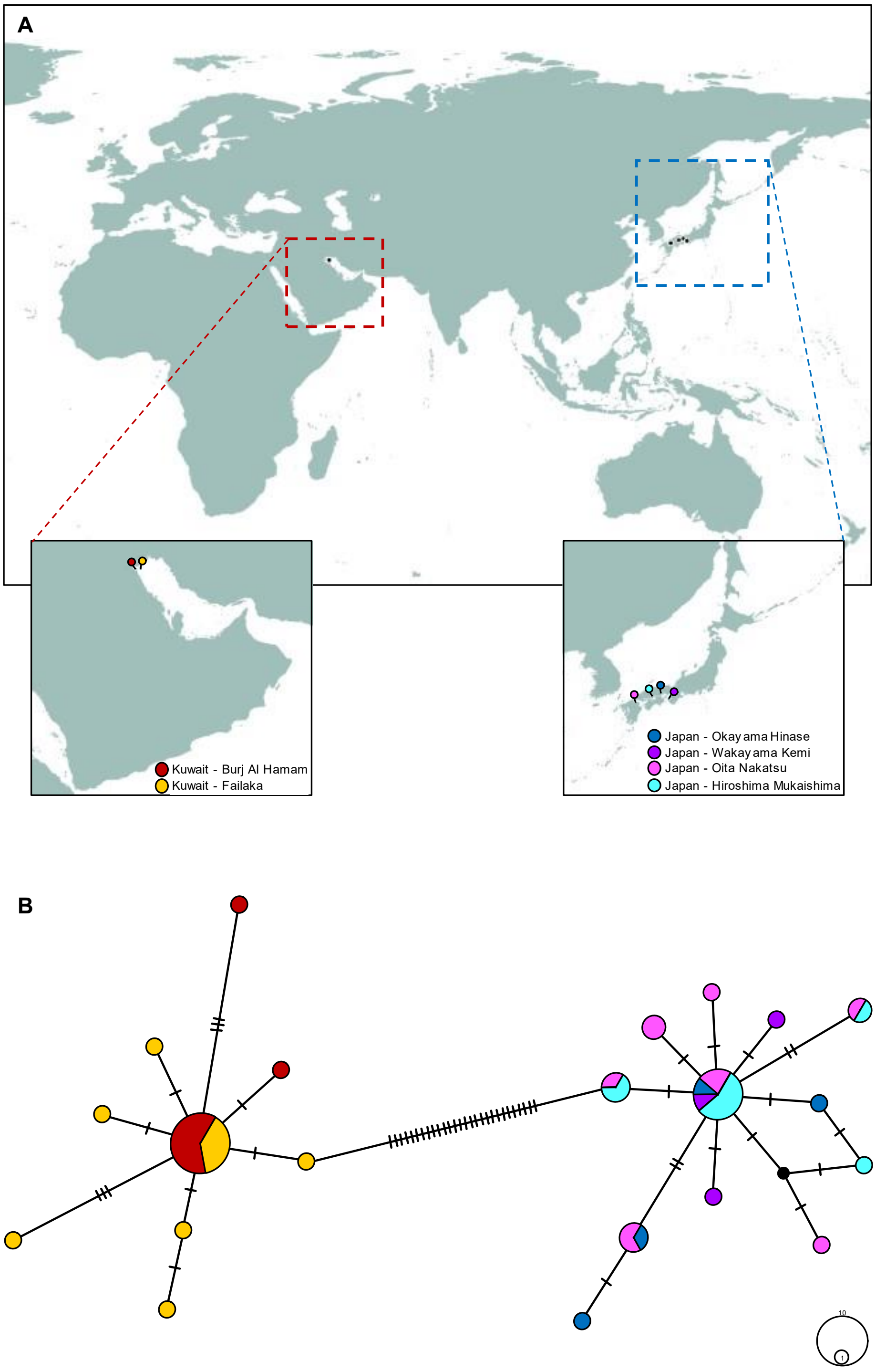

Figure 4. Phylogeography of *Ostrea* sp. KW from Kuwait and *Ostrea futamiensis* from Japan. **A**, Maps represent collection localities. **B**, Statistical parsimony phylogenetic network based on COI sequences generated in this study (Kuwait samples) and Hamaguchi et al., (2014) (Japan samples). Haplotypes in the network are represented by circles with size proportional to their frequencies and coloured according to locality of origin; small vertical bars represent nucleotide substitutions.

Molecular species delimitation

Results of species delimitation analyses based on ASAP and mPTP recovered the oyster form Kuwait as a species cluster distinct from the cluster of the closely related *O. futamiensis* (Fig. 5). This result is confirmed in both partitions with the first (1.50) and second (4.50) best ASAP-score and received maximum mPTP support (1.0). Overall, SD results by ASAP and the mPTP were highly congruent, with a C_{tax} value of 0.84 and a Match ratio of 0.81. ASAP recovered 17 species groups, whereas mPTP recovered 20 groups because further splits two of the groups recovered by ASAP: the group of the *O. stentina* complex sequences into three clusters and the group of *O. lurida* / *O. concaphila* into two clusters. Both methods lumped *O. permollis/ O. puelcana* and *O. edulis/ O. angasi* sequences into a single cluster. Relative to the current taxonomy, as represented in MolluscaBase (2021), the resolving power (R_{tax}) of ASAP and mPTP was 0.84 and 1.0, respectively. Based on morphological and molecular assessments we assign the Kuwait oyster to a new species that is described in the following section.

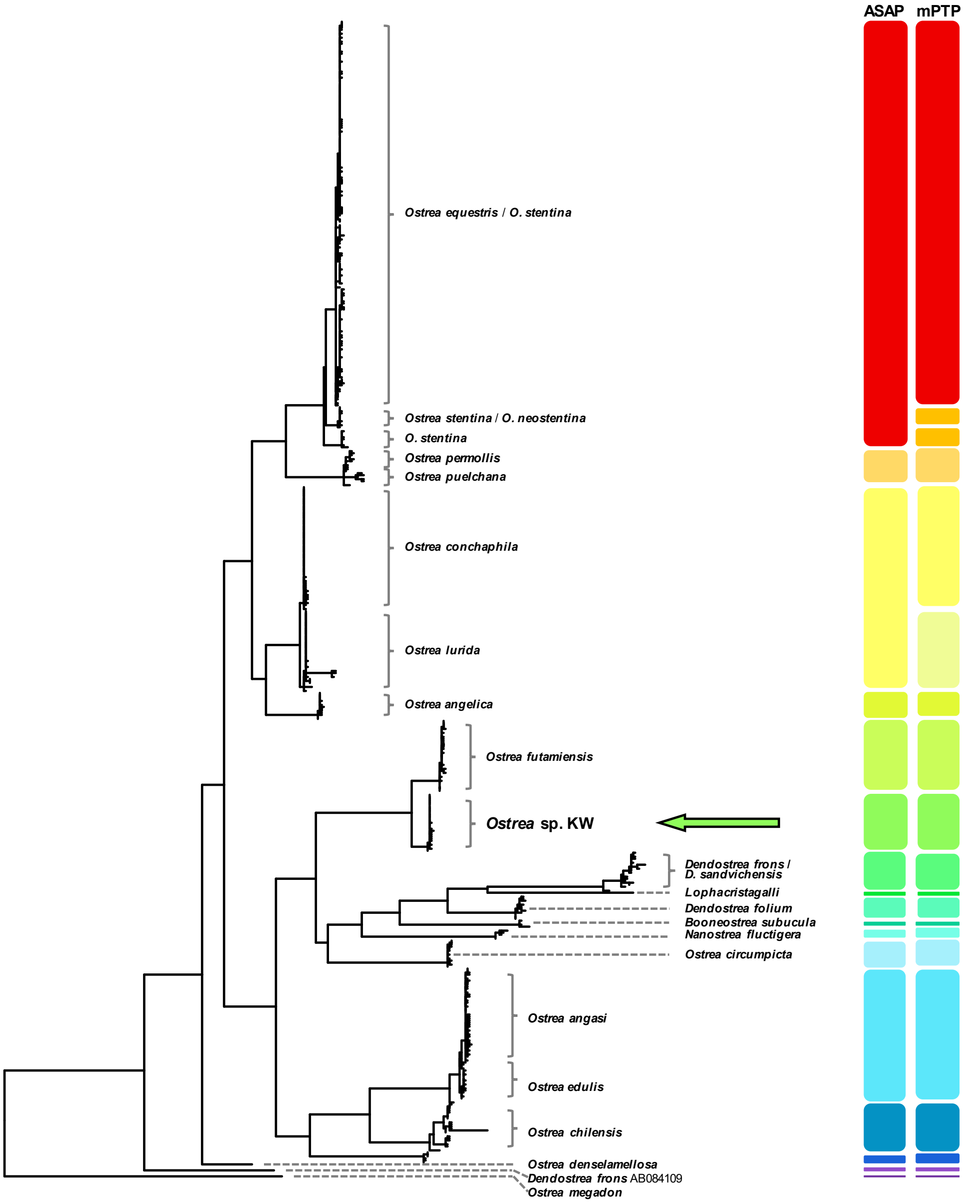

Figure 5. Rooted maximum likelihood tree based on the COI gene fragment (456 sequences) with information about taxonomic assignment of sequences as in GenBank and BOLD records (morphospecies assignments). On the right, results of species delimitations based on Assemble Species by Automatic Partitioning (ASAP) and multi-rate Poisson Tree Process (mPTP) methods. Candidate species obtained with each analysis are depicted as coloured boxes arranged in columns. The green arrow highlights the clade representing sequences of the Kuwait oyster.

Systematics

Ostreoidea Rafinesque, 1815

Ostreidae Rafinesque, 1815

Ostreinae Rafinesque, 1815

Ostrea Linnaeus, 1758

Generic definition

It is commonplace to give a generic definition when describing a new species. However, the molecular data presented here suggests that *Ostrea* is polyphyletic. and that the new species described here and its sister taxon *O. futamiensis* do not cluster within the clade that includes *Ostrea edulis*, the type species of the genus *Ostrea* (see the Discussion section). While a systematic revision of the genus *Ostrea* is clearly needed, we have resorted to use *Ostrea* in its common understanding until a much more detailed phylogeny of the Ostreinae can be presented.

Ostrea oleomargarita Oliver, Salvi & Al-Kandari, sp. nov. (Figs 6–7)

Type Material

Kuwait • **Holotype,** 1 complete shell attached to rock. Kuwait City, Al Sha'ab, 29.3675°N 48.0244°E. Low intertidal, attached under rock on gravel. Coll. PG. Oliver, December 2019, not sequenced. NMW.Z.2021.009.008 (Fig. 6A-B; 7A). **Paratypes,** 11 spec. used for sequencing NMW.Z.2021.009.009/#1 –#11. 4 figured shells, data as holotype. NMW.Z.2021.009.010. (Figs 6c,d,e,f, Figs 7b &d))

Kuwait • **Paratypes,** 12 spec. used for sequencing, NMW.Z.2021.009.011/#1–#12; #7 figured, Figs 6g & 7c). Ras Al Liwan, Failaka Island, 29.3902°N, 48.3988°E. Low intertidal, attached under rocks. Coll. PG Oliver, December 2019. 5 shells + 3 upper valves, not sequenced, NMW.Z.2021.009.012., as NMW.Z.2021.009.011.

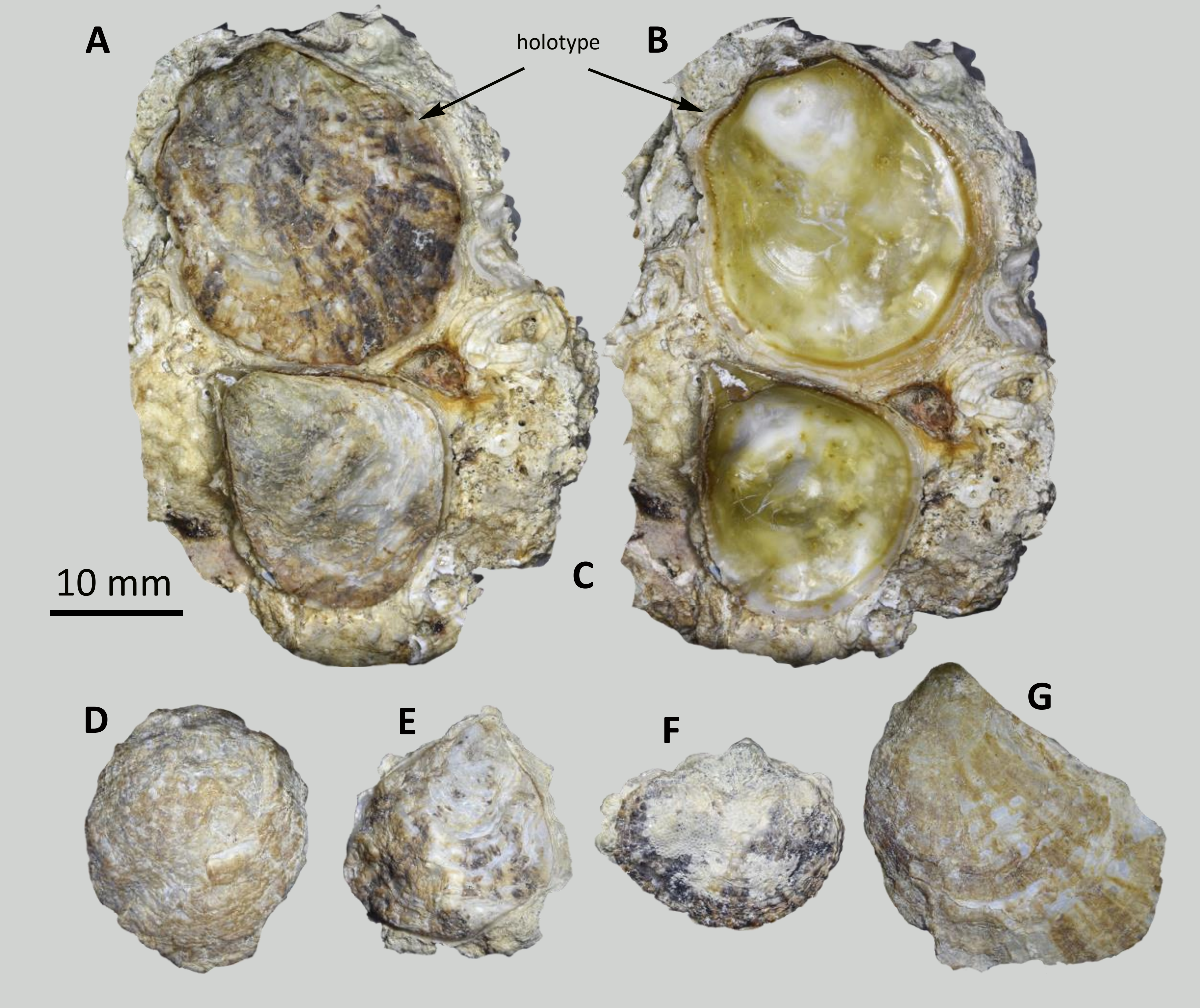

Figure 6. *Ostrea oleomargarita* sp. nov. **A**, Holotype upper shell, Al Sha'ab specimens in situ **B**, Holotype upper shell, internal view of attached valves showing catachomata, greenish interior and brown margin [NMW.Z.2021.009.008]. **C–F** Paratypes, variations in external sculpture and colouration, all Al Sha'ab; **D**, brown tubercular; **E**, black and beige, foliar; F, Al Sha'ab typically encrusted with bryozoa; [NMW.Z.2021.009.010.] G, Failaka, rayed foliar. [NMW.Z.2021.009.011 #7]

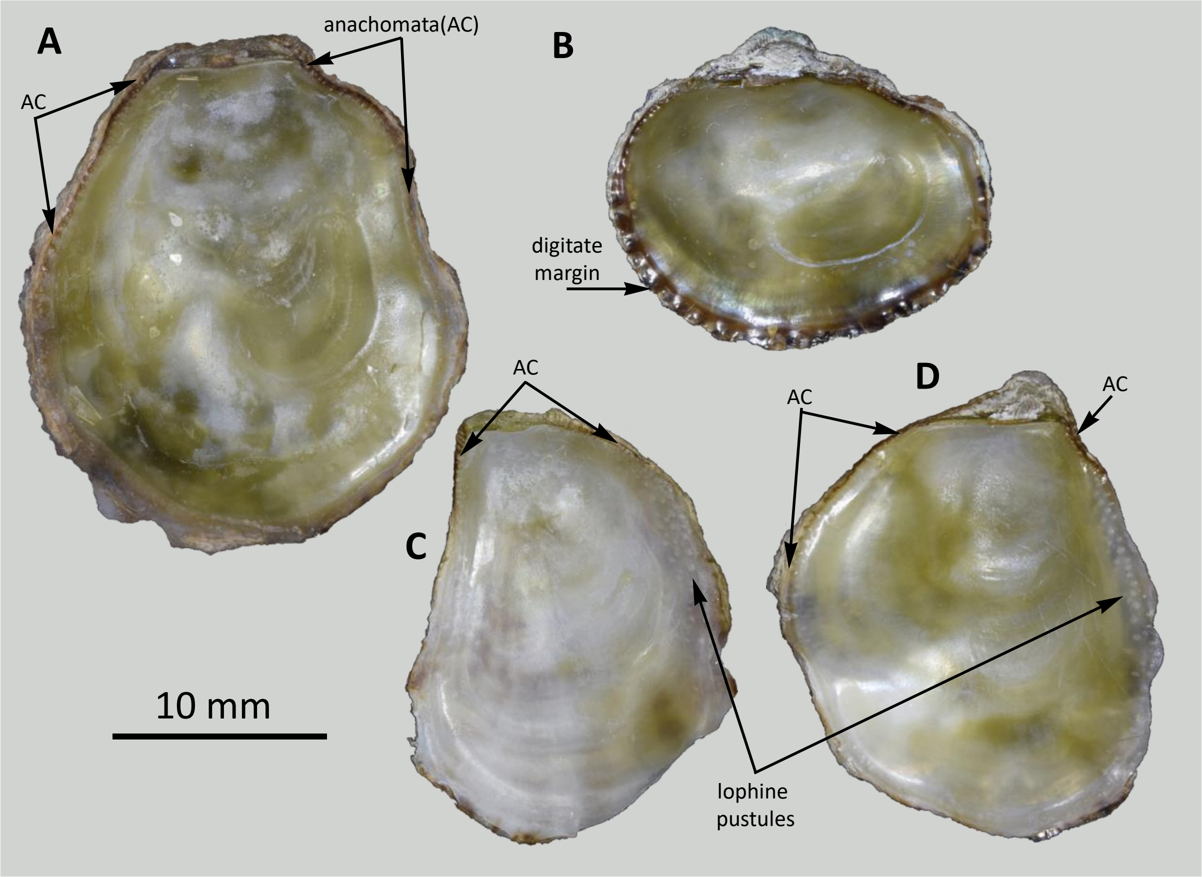

Figure 7. *Ostrea oleomargarita* sp. nov. Internal views of upper valves to show differences in marginal form and chomata. **A**, Holotype Al Sha'ab, anachomata and ventral smooth margin [NMW.Z.2021.009.008]. **B**, Al Sha'ab, digitate margin [NMW.Z.2021.009.010]. **C**, Failaka, weak anachomata and lophine pustules. **D**, Al Sha'ab anachomata and lophine pustules [NMW.Z.2021.009.010].

Other material examined

Kuwait • 20 shells attached to rock. Kuwait City, Al Sha'ab, 29.3675°N 48.0244°E. Low intertidal, attached under rock on gravel. Coll. PG. Oliver, December 2019. NMW.Z.2021.009.013.

India • 1 shell + 1v. Bombay (Mumbai), Bandra (approx. 19.06°N, 72.82°E). 5 April 1946. Annotated "Ranson 26". 3v. Bombay (Mumbai), Juhu (approx. 19.10°N, 72.8°E). Coll. Winckworth, 23 October 1936. Annotated "Ranson 27".

Shell description

Small shells to 20 mm in diameter. Thin but robust. Roughly circular, oval to pyriform. Lower valve shallowly cupped to irregular; cemented for most or all of its attachment, margin smooth or if free then finely plicate. Non-nacreous margin very narrow. Ligament area narrow, elongate in some. Prominent catachomata on both anterior and posterior dorsal margins. Adductor muscle large, reniform. Interior colour flushed olive-green, paler in some smaller shells, most with a distinct brown, narrow marginal band. Upper valve flat, irregular to domed. Anachomata corresponding to catachomata. Sparse elongate tubercles (lophine pustules) on posterior ventral edge in some. Internal colour as lower valve. Outer surface usually obscured by epifaunal growths, typically bryozoa, calcareous algae, worm tubes or other oysters. Sculpture mostly of flattened foliar scales, some areas finely tubercular, colour of radial bands of rust brown to black on a grey-beige ground, some almost uniform ground colour. Thin chalky layers present most visible in attached valves.

Derivation of name

Derived from the combination *oleo* from *olivarius* (Latin) referring to the colour olive-green and *margarita* from *margarites* (Latin) referring to the pearly lustre of the inner surfaces of the valves. Thus, to the typical olive-green lustre of the nacreous inner surface of the valves.

Type locality

Kuwait, Kuwait City, Al Sha'ab, 29.3675°N 48.0244°E. Intertidal, under rocks.

Distribution

In Kuwait specimens from only two sites were verified as *O. oleomargarita* by molecular data. Similar shells were recorded from a number of other sites (Al-Kandari et al., 2020) under the name of *Nanostrea deformis*) ranging from Khor Al-Subaya in the north of the country to other sites in Kuwait Bay and on Failaka. This species is probably widely distributed in the Northern Arabian Gulf but given the morphological plasticity all records need confirmation by molecular data; West coast of India (confirmed by shell morphology only).

Discussion

The northern Arabian Gulf is confirmed as a key region for the discovering of marine animal diversity. This study documented a new overlooked oyster species with phylogenetic affinities to an Indo-West Pacific species and a peculiar combination of morphological characters among oysters. This result echoes the recent discovery of another enigmatic oyster species in the same waters, *Talonostrea salpinx*, also related to Indo-West Pacific taxa (Al-Kandari et al., 2021). The results are relevant to our comprehension of oysters' diversity and distribution and the biogeography of the marine biota of the Pacific Ocean, as discussed below., as discussed below.

The new ostreine from Kuwait: insights into the classification of Ostreinae and on the taxonomic value of chomata

The Kuwait oyster, *Ostrea oleomargarita* sp. nov., shows an unprecedented combination of morphological characters typical of different groups of Ostreinae. The shell morphology is most similar to *O. futamiensis* but in none of the descriptions or figures of that species is there mention of the presence of lophine pustules on the inner margin (Hamaguchi et al., 2017; Inaba & Torigoe, 2004; Seki, 1929). Although, these pustules are not seen on all specimens from Kuwait and in itself this character may not be enough to justify the raising of the Kuwait populations to new species level. However, the molecular data presented here provides compelling evidence that the Japanese and Kuwaiti populations do represent distinct species.

Combination a phylogeographic approach with species delimitation methods strongly supports an inter-specific divergence between *O. oleomargarita* sp. nov. and O. *futamiensis* (Fig. 4 and 5). The genetic divergence between these two species at the COI is similar to, or higher than, values observed between closely related oysters [e.g. *O. conchaphila*/*O. lurida*, *O. angasi*/*O. edulis*, *O. equestris*/*O. stentina*; (Guo et al., 2018; Hu et al., 2019; Raith et al., 2015)]. Remarkably, species delimitation methods, applied to oysters for the first time in this study, revealed a close match between current ostreine taxonomy (MolluscaBase, 2021) and species clusters inferred based on the barcoding marker COI (Fig. 5). The only differences in species delimitations produced by distance-based and tree-based methods concern two well-known species complexes: the *O. stentina* complex and the *O. lurida*/*O. concaphila* pair are lumped into two species clusters by the former method, whereas they are split into more species by the tree-based approach. These complexes have undergone several taxonomic changes following various phylogenetic assessments (Guo et al., 2018; Harry, 1985; Hu et al., 2019; Morton et al., 2003) and are currently considered as composed of distinct species of recent diversification (Hu et al., 2019; Polson et al., 2009). On the other hand, both SD methods lumped *O. permollis*/*O. puelcana* and *O. edulis*/*O. angasi* sequences into two clusters, an issue that will require further taxonomic assessments.

From a morphological point of view, features of *Ostrea oleomargarita* have most in common with the Ostreinae and only in the presence of lophine-like pustules is there affinity with lophine genera (e.g. *Lopha* and *Dendostrea*). The flattened form with only weak plications, lack of hyote spines, appressed lamella and presence of chalky deposits are ostreine not lophine characters. The description and distribution of lophine pustular chomata are more variable than suggested by Stenzel, (1971) and Harry, (1985) who described them as "minute and numerous, they are located along margins and form from one to several lines". This description fits well with *Lopha cristagalli* (Linnaeus, 1758) but not with many species assigned to the genus *Dendostrea* where the pustules are larger and variably present (see Fig. 2 above). Interestingly Harry (1985) in his Table 2 indicates that the ostreine genera *Ostrea* and *Ostreola* have lophine chomata but no ostreine chomata but this must surely be an error and should be reversed. In that table Harry also states that his ostreine *Booneostrea* lacks any chomata but this is in error as in some shells ostreine chomata are present on the posterior dorsal margin (pers. obs. PG Oliver). Therefore, there is an indication that the form and distribution of chomata are not as definitive as suggested in previous studies. The variable expression of pustules in *O. oleomargarita* appears to have no pattern of occurrence, although they are most often present in thinner (perhaps less mature) shells and in some thicker shells they can be seen below the surface suggesting they have been overgrown by later shell growth. The pattern and the dimension of these structures might drastically change within the life of an individual in lophine species too. The pustules as exhibited in *O. oleomargarita* are low and sparse unlike the dense pustules seen in *L. cristagalli* or some species of *Dendostrea* suggesting that they may not be analogous and a more considered review of ostreiod chomata is warranted.

Ambiguous classification based on morphological characters is commonplace in oysters (e.g. Lam & Morton, 2003, 2006; Polson et al., 2009; Tureck et al., 2020; Wu et al., 2013), and in most cases a molecular approach allowed a straightforward systematic assessment of debated species (Raith et al., 2015; Salvi et al., 2014). Phylogenetic results unequivocally place *O. oleomargarita* within the subfamily Ostreinae. Ironically, also in the molecular phylogeny of Ostreinae the position of *O. oleomargarita* and *O. futamiensis* is somehow in between the *Nanostrea*/*Planostrea* clade and the lophine clade. The phylogenetic trees based on ML and BI analyses of multilocus data (Fig. 3), support three major clades within the Ostreinae, two of these consisting entirely of species currently recognised within the genus *Ostrea* (the *edulis* group and the *stentina* group), whereas in the third clade (the *algoensis* group) the lophine genera *Alectryonella*, *Lopha*, and *Dendostrea* cluster with *Nanostrea*, *Planostrea*, and with four species of *Ostrea* (*O. circumpicta*, *O. algoensis*, *O.futamiensis* and *O. oleomargarita*). The non-monophyly of the genus *Ostrea*, as currently conceived, is not surprising and has been recovered in many molecular phylogenetic studies (Al-Kandari et al., 2021; D. Ó Foighil et al., 1998; Guo et al., 2018; Hamaguchi et al., 2017; Hu et al., 2019; Jozefowicz & Foighil, 1998; Li et al., 2021; Morton et al., 2003; Ó Foighil & Taylor, 2000; Salvi et al., 2014; Salvi & Mariottini, 2017; Shilts et al., 2007). However, unlike other subfamilies such as the Crassostreinae for which the same main lineages have recovered with high support in all phylogenetic studies (see the review by Salvi & Mariottini, 2021), relationships among Ostreinae are poorly resolved and unstable across studies, especially among those using single genes. A better definition of the main lineages within Ostreinae is apparent in recent multi-locus assessments based on mitochondrial and nuclear data and wider taxon sets (Li et al., 2021; Salvi et al., 2014; Salvi & Mariottini, 2017; this study), suggesting that more data are needed to robustly resolve the phylogeny and systematics of this subfamily.

Phylogenetic trees estimated in this study and previous studies suggest that careful consideration of the criteria used to define Ostreinae genera is particularly needed. Many taxonomic incongruences observed are perhaps only so because a number of genera based on morphology have been retained (for example in the lophine sub-clade) contrasting with the other clades where at least five other available generic names have been regarded as junior synonyms of *Ostrea* (e.g. *Cryptostrea* Harry, 1985, *Myrakeena* Harry, 1985, *Ostreola* Monterosato, 1884 and *Undulostrea* Harry, 1985; indicated in Fig. 3). Indeed, some of these available generic names might be useful to resolve the extensive polyphyly of *Ostrea* as currently conceived. This implies that *O. oleomargarita, O. futamiensis* and *O. algoensis* could be assigned to a different genus in the future. However, the molecular data set of Ostreinae is far from complete and certainly not sufficient to begin to alter their taxonomy and nomenclature. A further example of this complexity is the situation with species previously assigned to the genus *Ostreola* (the *O. stentina* complex) but where the available data was not used to modify the nomenclature and bring the genus *Ostreola* back into acceptance (Hu et al., 2019). This study and others such as Hu et al., (2019) highlight the continuing difficulty of resolving the taxonomy of the Ostreidae, especially using morphological characters. Despite the increasing number of molecular studies, the current taxonomy remains based on morphological decisions. Firstly, the introduction of genera by Harry, (1985) was based entirely on morphology, and secondly, the current nomenclature used in MolluscaBase resulted from the decisions made by Huber, (2010) again based on morphology. It is unfortunate that the decisions made by Huber are not supported by evidence so for example why have *Nanostrea* and *Booneostrea* been retained while other distinctive oysters such *Undulostrea* been synonymised. Of note here is that many of these more obscure genera, following synonymies by Huber, (2010) are now monospecific. It will probably become increasingly accepted to prioritise molecular data for oyster systematics, paradigmatic in this respect is the situation with *Crassostrea* and *Magallana* a recent example where shell morphology is of no value in distinguishing these genera despite their remarkable evolutionary distinctiveness (Salvi & Mariottini, 2021).

Potential geographic distribution and focus on underestimated biodiversity of Persian Gulf and East/West Indian Ocean

The close phylogenetic relationship between *O. oleomargarita* and *O. futamiensis* and available sequences of these two species discloses a remarkably wide biogeographic gap. To date, we only have molecular data evidence for the presence of *O. oleomargarita* along the Kuwait coasts in the northern Persian Gulf. Shells in the NHMUK from the west coast of India agree morphologically with those from the Arabian Gulf but were identified as *O. futamiensis* by Ranson. (Ranson, 1967: 236) does not cite these specimens of *O. futamiensis*; instead, he lists material from the Bay of Bengal, Malaya, Singapore, Thailand, Philippines and Japan. Ramakrishna and Dey, (2010) cite *Saccostrea futamiensis* from Gujerat (WIO) and Orissa (Bay of Bengal) and Subba Rao, (2017) does mention the species. The shell from Orissa is described by Subba Rao et al, (1991) and in that it states the interior is white, thus making the identification suspect. The distribution *O. futamiensis* extends in a few scattered regions of the West Pacific Ocean (Bishop et al., 2017)such as Hong Kong (Lam & Morton, 2004) and Korea (Hongying, Q. and Byung, 2000). Therefore, it may be inferred that *O. futamiensis* is an East Indo-West Pacific species, whereas *O. oleomargarita* is a West Indian Ocean and Arabian species.

This distribution pattern could be supported by the fact that currents usually form an effective barrier for larval dispersal (Coleman et al., 2013). In particular, Sivadas & Ingole, (2016) studied the biodiversity pattern of Indo-Pacific benthic organisms and found that the low similarity between the diversity patterns along the Western and Eastern Indian coasts is due to environmental factors such as habitat heterogeneity and spatiotemporal variability of coastal currents. Unfortunately, biogeographic patterns and genetic structures of marine species in the Western Indian Ocean are scarce, but studies start to highlight an effective allopatric distribution and cryptic diversity of benthic species between W and E Indian coasts (Alam et al., 2015; Trivedi et al., 2021; Tsang et al., 2012). This suggests that there may be many such examples to be discovered, especially if we consider that the biodiversity of the Western Indian Ocean is limited to some species or groups (Wafar et al., 2011). The underestimated number of marine species of the Arabian/Persian Gulf is also based on a low current understanding of the species diversity of some countries (Wafar et al., 2011). In addition, many intertidal benthonic organisms, such as bivalve molluscs, show high levels of phenotypic plasticity, challenging traditional taxonomic assessment based on morphology (Clark et al., 2020). Currently, two of the authors here are preparing an Atlas to the Bivalvia of Kuwait, and within the recognised 210 species, 23 are currently without verified species names (Oliver PG, pers. comm). Oysters, in particular, are engendered with very wide geographical ranges throughout the Indo-West Pacific and it is likely that none of the species cited by Al-Kandari et al. (2020) were correctly identified or have been properly described. The work of Taylor and Glover, on the Lucinidae, over the period 1997–2021 revealed many examples where assumed pan Indo-Pacific species were complexes of species with more restricted ranges (Taylor & Glover, 2021). Al-Kandari et al. (2020) cited examples where some bivalve species (e.g. *Congetia chesneyi* Oliver & Chesney, 1994) had disjunct distributions occurring in the northern Gulf and then on the Pakistan/ North-West Indian coast, such species were also absent from Oman. Tsang et al., (2012) using *Tetraclita* barnacles showed a pattern of regional species within the Western Ocean and this pattern may be expected from a more detailed study of oysters. New efforts using a molecular approach are uncovering new marine groups and species in this region (Al-Kandari et al., 2021; Gharahkhani et al., 2020; Khatami et al., 2018).

Given the inferred complexity of the malacofauna of the Western Indian Ocean fauna (Oliver, 2019), a wide-ranging study of the Bivalvia is warranted. The Red Sea and Arabian upwelling regions have high levels of endemicity but also share species with the Gulf, Indo-Pakistan and wider north WIO area (Oliver, 2019). Not only are the relationships between East and West Indian Oceans to be further elucidated but also biodiversity patterns within the Arabian region itself remains to be documented.

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GENERAL CONCLUSIONS

DNA barcoding approach is a fast automated method that can be performed in preliminary taxonomic workflows to speed up specimen identification and sorting, instead of time consuming morphological assessments (Hubert & Hanner, 2015). Taxonomically reliable barcoding libraries as references for specimen identifications remain a crucial key to the success of DNA barcoding studies (Floyd et al., 2010). However, the production of reference library remains the main challenge for DNA barcoding (Collins & Cruickshank, 2013; Keck et al., 2023). This is often due to the shortage of taxonomists, or to weak taxonomy in understudied animal taxa (Will et al., 2005), or in groups showing extensive cryptic diversity (Bickford et al., 2007). This thesis tackles a DNA barcoding study in two animal groups for which morphological identification is challenging but with different taxonomic backgrounds that uncover two opposite situations. The first model study, the tribe Alticini, is characterized by high species diversity that is accompanied with a shared morphology with subtle differences differentiating several sympatric species a high number of species. Despite morphological identifications require a high level of expertise, often with deep expertise on regional faunas (Salvi et al., 2020), the taxonomy of European species is well-defined (Biondi, 2006). The second model study, the family Ostreidae, includes 78 species distributed worldwide (MolluscaBase, 2024). Morphological identifications of oysters are unreliable due to the limited availability of diagnostic characters and the extensive phenotypic plasticity of shells, resulting in a weak and unresolved taxonomic framework (Salvi et al., 2021; Wang et al., 2004).

We demonstrated a relatively high reliability of BOLD and GenBank (GB) repositories as reference for specimen identification of Italian Alticini using a DNA barcoding approach, with correct identifications of 86 species within 19 genera (88.1%). This result can be explained by the robust taxonomy of this group (Biondi, 1991; Biondi et al., 2013) combined with the availability of several molecular studies at the European scale (Magoga et al., 2018; Montagna et al., 2016; Salvi et al., 2020). This thesis generated the first reliable barcoding library for Alticini from Apennines based on the accurate identification of 99 species in 21 genera made by the taxonomic specialist of this group. This library allowed the molecular identification of 252 specimens from Apennines and the a posteriori re-assessment and correction of 684 wrong identifications occurring in the BOLD-GB library. This study corroborated the crucial role of expert taxonomists throughout the DNA barcoding pipeline from the library construction to the a-posteriori library validation. The uncovered biodiversity of Italian Alticini consists of 85% of the European species (Jong et al., 2014). In this respect, the barcoding library generated in this thesis will play a key role as reference for future biodiversity assessments at regional, national, and European scale.

DNA barcoding results for oysters showed a very different pattern. The BOLD-GB repositories allowed the identification of only 17 species within 8 genera (28.1%), among 424 museum specimens covering 217 sites in all world's ocean, thus revealing a low reliability of public libraries for molecular identification of oysters. Most of these species belong to temperate ecosystems, underlining a severe gap of knowledge in tropical regions. While some of the 38 unassigned MOTUs found might represent known species that have not yet been sequenced (and therefore are not represented in the BOLD-GB library), most of them likely represent new species to science. To clarify the taxonomic status of all these unassigned MOTUs it will be necessary an integrative taxonomic approach (Dayrat, 2005; Puillandre, Bouchet, et al., 2012; Will et al., 2005) that we have successfully applied for some taxa in this thesis project. Results of this global DNA barcoding study on oysters will guide, and boost, future systematic and taxonomic studies followed by several species' discoveries and descriptions.

The uncertain taxonomy of oysters and their highly underestimated diversity implied that for most museum specimens the integrative taxonomy step of the thesis pipeline (Step B, Figure 1, in "General Introduction") is necessary to reach a species identification. Whereas, in the case of Alticini, the DNA barcoding approach (step A, Figure 1 in "General introduction") allowed to successfully identify the great majority of specimens. As a consequence, most integrative taxonomy efforts were conducted on oysters with three case studies that allowed the description of a new species of oyster from Kuwait, and the use of holotype specimens for the molecular validation and systematic assessment of a deep-sea oyster, and for the synonymization of two recently species described from Vietnam on a morphological basis. These studies indicated that morphology alone can be misleading for oysters' identification, and further corroborated the importance of integrating molecular analyses in taxonomic studies. In the case of Alticini we applied an integrative approach to validate the species status of three morphologically similar species within the *Longitarsus candidulus* species-group. Based on a multi-locus phylogenetic approach we explained the morphological homogeneity and biogeographic pattern of this group with a scenario o recent (Pleistocene) allopatric divergence.

In conclusion, this thesis substantiated the relevance of integrating molecular data for taxonomic and biodiversity assessments and provides a pipeline for tackling the study of morphologically challenging animal groups. Hot areas for further taxonomic research are identified in oysters both in terms of taxa and regions, while a solid reference library is provided for flea beetles. This knowledge will guide and facilitate biodiversity assessments in both groups.

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