Molecular Genetics of Aliens in Maltese Waters

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Declaration of Authenticity

I, the undersigned, declare that this dissertation is my own original work, except as acknowledged in the text, and that it was carried out under the supervision of Prof. Adriana Vella. I have clearly indicated where I have used published or unpublishedwork of others and I have provided the source of such work. I have acknowledged all main sources of help, and where the work was done jointly with others, I have specified what I have contributed and what has been contributed by others. Any conclusions, suggestions or assumptions are mine unless otherwise stated or attributed.

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May 2023

Declaration of Ethics Compliance

I declare that I have abided by the University's Research Ethics Review Procedures. My URECA form has been submitted under ID SCI-2022-00055.

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May 2023

Declaration of Supervision

The undersigned confirms that this dissertation has been undertaken under their supervision and that they approve of its submission for final assessment by the Board of Examiners.

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Declaration of Correction

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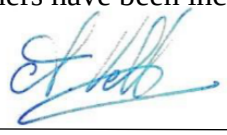

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Abstract

The Mediterranean Sea is a hotspot of marine biodiversity, characterised by endemism and host to emblematic species of concern for conservation. However, its habitats and ecosystems face many threats of anthropogenic origin, among the most prominent of which are biological invasions. Their initial introduction facilitated primarily by the opening of the Suez Canal and by the fouling of hulls and the transport of ballast water along shipping routes, invasive alien species have on several occasions caused rapid population declines, range shifts, and even local extirpations. Such ecological declines have in turn incurred the states that border the Mediterranean Sea costs amounting to billions of euros, ultimately prompting the enactment of Union-level regulations that demand the early detection and rapid eradication of the alien species invading the basin.

The capacity to identify alien species is fundamental to their effective management. Their monitoring necessitates the use of diagnostic tools that are accurate, readily deployable, cost-effective, and applicable across a range of taxa. Traditional approaches to identification that are reliant on morphological characteristics fall short of these criteria, prompting investigations into novel molecular approaches. This provided, the scope of the present project was to investigate the applicability of the cytochrome c oxidase subunit I (COI) gene in identifying newcomers to Maltese coastal waters and to contrast the genetic data it yields with morphological lines of evidence as part of an integrative approach towards correct species identification.

Specimens from diverse metazoan taxa suspected to be of alien origin were collected and their morphologies documented. Tissue samples were excised from each specimen and treated with proteinase K for DNA extraction. Segments of the COI gene were then amplified, sequenced, and compared with the genetic data available in the international repositories of GenBank and BOLD. The genetic species identifications thus derived were complemented with morphological species identifications. Phylogenetic trees of maximum likelihood were also constructed.

The molecular approach described above allowed for the species identification of 57 specimens. This enabled in turn the reliable distinction of alien specimens from native specimens. Morphological identifications corroborating the genetic identifications were possible for 50 specimens. Moreover, the single specimen of *Siganus rivulatus* presented here constitutes the first record of the species for the Maltese Islands. 12 of the haplotypes sequenced in the process of completing this project constitute newly discovered genetic variants for 7 species.

The results of the present project emphasise the need for a molecular approach to species identification, especially in scenarios where: specimen morphologies are largely lost or deteriorated; morphological keys are not sufficiently informative; and specimen morphologies are cryptic or at least highly similar. Ultimately, the integration of genetic and morphological lines of evidence throughout this project produced a more robust approach that consistently guided the research undertaken towards accurate specimen identification. This approach's application in regimes tasked with detecting, managing, and preventing marine invasions is thus recommended.

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1. Introduction

1.1 Marine aliens and range expanders

1.1.1 Defining origins

Indigenous species (also referred to as ‘native’ species) are organisms that are within their historically native ranges. Alien species (also referred to as ‘non-native’, ‘non-indigenous’, or ‘exotic’ species) and range expanders (also referred to as ‘neo-natives’), on the other hand, are organisms that are found to occur outside their historically native ranges. While alien species occur outside their native ranges as a direct result of human-mediated introductions, accidental or otherwise, range expanders shift their natural distributions as dictated by their own powers of dispersal and with only indirect human assistance, mainly in the form of human-induced climate change (Carlton, 1996; Gilroy, Avery & Lockwood, 2016; Russell & Blackburn, 2017; Essl *et al.*, 2019). Functionally speaking, however, aliens and range expanders are broadly similar. Both represent new additions to the local ecosystems that receive them and both act as possible agents of biotic change. The term ‘newcomers’ may hence be used to refer to aliens and range expanders collectively (Evans, Barbara & Schembri, 2015; Naselli-Flores & Marrone, 2019). In this context, cryptogenic species are organisms that may be either alien or indigenous in origin but that cannot be reliably assigned to either category, largely on account of deficiencies in the available records (Carlton, 1996). Further to this, Essl *et al.* (2018) propose that ‘data deficient’ be used to refer to that subset of cryptogenic species for which an assessment of biogeographic status is most unfeasible on account of the deficiencies in the available records being the most severe.

1.1.2 Defining statuses

When an alien species is first introduced into a new region, its presence there may first be defined as ‘questionable’ or ‘casual’. Questionable status is assigned to those alien species on which the existing information is insufficient and, therefore, the records of which outside their native ranges are doubtful. Casual status is assigned to those alien species that are known not to have yet formed reproductive populations within the foreign region. In practice, the term ‘casual’ is routinely applied to those alien species that have

only been recorded within a specified area less than three times. Alien species are only considered ‘established’ when they are able to form and sustain reproductive populations within the foreign region, and this status can then be changed to ‘invasive’ if and when the population growth within the foreign region becomes exponential. Attaining invasive status is also usually associated with impairing ecosystem functioning, causing local biodiversity losses, and possibly incurring net socioeconomic losses (Walther *et al.*, 2009; Evans *et al.*, 2015; Iglésias & Frotté, 2015).

1.1.3 Consequences thereof

Biological invaders are among the most prominent global threats to biodiversity and the livelihoods that depend on ecosystem goods and services (Pyšek *et al.*, 2020). A report published by the Convention on Biological Diversity (Ainsworth, 2006) claims that invasive alien species (IAS) have since the 1600s contributed to just under 40% of all the animal extinctions that have taken place and for which the cause is known. It further emphasises that IAS are most threatening to isolated ecosystems like islands, where natural predators and competitors that are able to control IAS populations are most scarce. Moreover, IAS are held to be capable of exacerbating poverty and ultimately even impeding sustainable development. Supporting this claim are Haubrock *et al.* (2021), who estimate that IAS cost the European continent alone €117 billion between 1960 and 2020.

Marine invaders are similarly acknowledged to endanger biodiversity, ecosystem functioning, marine industries, and human health at the global scale. It is known that they threaten marine biodiversity at the genetic, species and ecosystem level and are among the largest causes of its loss (IPBES, 2019; Pyšek *et al.*, 2020).

1.2 The case of the Mediterranean Sea

1.2.1 Extent of invasion

Records suggest that among the European seas, the Mediterranean Sea has suffered the highest number of invasions (Nunes, Katsanevakis, Zenetos & Cardoso, 2014). By 2012, 986 marine alien species had been reported from the Mediterranean, 775 of which were from the East Basin, 308 from the West Basin, 249 from the Central Mediterranean, and 190 from the Adriatic Sea (Zenetos *et al.*, 2012). About half of these species were already thought to be spreading and by the end of 2019, at least 666 (excluding

foraminiferans) were assumed to have established reproductive populations (Zenetos & Galanidi, 2020). The rate at which the Mediterranean is being invaded has also been at an increase. Less than 200 alien species are known to have been introduced into the Mediterranean before 1950, but over 800 species are known to have been introduced since then (Zenetos *et al.*, 2012). Moreover, between 2017 and 2019, new introductions were being reported at an average rate of 8 species per year when one excludes from consideration casual records and instances of potential reporting lags (Zenetos & Galanidi, 2020). Still, these records likely understate the true extent to which the Mediterranean has been and is still being invaded, considering the knowledge gaps on the marine biota that still exist, the size bias evident in the published literature, and the tendency for observations beyond first records to remain unpublished and retained only in private databases (Carlton, 2000; Galil, 2007; Ragkousis *et al.*, 2023)

1.2.2 Vectors enabling introduction and dispersal

The opening of the Suez Canal in 1869, the fouling of hulls and the transportation of ballast water along shipping routes, the aquarium trade, and the aquaculture business all facilitate the introduction of alien species into the Mediterranean Sea (Zenetos *et al.*, 2012; Katsanevakis, Zenetos, Belchior & Cardoso, 2013). Approximately 40% of the alien species now present in the Mediterranean Sea are thought to have been introduced by means of direct passage through the Suez Canal, these species being referred to as 'Lessepsian' or ‘Erythrean’ immigrants. Approxiamtely 50% are thought to have probably (although not certainly) been introduced via shipping, its associated ballast water, and hull fouling. The aquarium trade and aquaculture only contribute to a small proportion of the total introductions (Katsanevakis, Coll, *et al.*, 2014; Bereza, Rosen & Shenkar, 2020).

The recent acceleration in the rate with which aliens are being introduced into the Mediterranean may be partly explained by the rising trend in surface water temperatures congruent with climate change (Raitsos *et al.*, 2010; Mannino, Balistreri & Deidun, 2017), the recent increase in the seaborne trade (Katsanevakis *et al.*, 2013; Bereza *et al.*, 2020), and the continuous development surrounding the Suez Canal (Arndt & Schembri, 2015; Galil *et al.*, 2015; Bereza *et al.*, 2020). Rising temperatures render the sea more amenable to invasion by aliens that are thermophilic, such as those of Indo-Pacific affinity entering as Lessepsian immigrants (Raitsos *et al.*, 2010). Similarly, it enables thermophilic species

of Eastern Atlantic affinity to extend their distribution into the Mediterranean Sea (Mannino *et al.*, 2017) and allows for thermophilic newcomers in general to disperse well into the cooler waters of the Aegean and Adriatic Sea (Zenetos & Galanidi, 2020). The result of this is the so-called 'tropicalisation' of the Mediterranean Sea (Katsanevakis, Coll, *et al.*, 2014). With regards to the seaborne trade, the size of the average ship passing through the Suez Canal has reportedly been at an increase. This is in turn associated with longer turnover times spent in ports, larger wetted surface areas that are susceptible to fouling, and wider opportunities for fouling organisms to propagate and invade novel habitats (Bereza *et al.*, 2020). The Suez Canal has been deepened repeatedly ever since its opening in 1869 and with the construction of dams across the Bitter Lakes, the then extant salinity barrier at the Gulf of Suez that restricted Lessepsian immigration into the Mediterranean was gradually destroyed (Galil 2006; Belmaker, Parravicini & Kublicki, 2013). More recent modifications to the architecture of the Suez Canal (in its depth, width, and profile) in the past decades are thought to have also altered the pattern of the currents within the canal, which may in turn be enabling additional species to overcome previously extant dispersal barriers (Arndt & Schembri, 2015). However, though the canal's capacity was effectively doubled in 2015 (Galil *et al.*, 2015) the rate of invasion by direct passage through the Suez Canal two years later was not observed to increase, as originally anticipated, but to decrease (Zenetos, 2017).

1.2.3 Ecosystem impacts

The Mediterranean Sea is a hotspot of marine biodiversity that boasts over 17,000 marine species, approximately 20% of which are considered endemic (Coll *et al.*, 2010). It is home to emblematic species of concern for conservation like the bluefin tuna *Thunnus thynnus* and the Mediterranean monk seal *Monachus monachus* and boasts unique habitats like seagrass meadows of the endemic *Posidonia oceanica* (Coll *et al.*, 2011).

Human-induced climate change and anthropogenic activities such as overexploitation, habitat degradation, and pollution threaten this biodiversity in a synergistic fashion (Coll *et al.*, 2010; Lotze, Coll & Dunne, 2011). Marine invasions pose additional threats to this already-stressed biodiversity; although there are as yet no records of marine alien species causing basin-wide extinctions of native Mediterranean biota, sudden declines in abundances, range shifts, and even local extirpations concurrent with

alien invasions have been observed on numerous occasions (Galil, 2007; Katsanevakis, Wallentinus, *et al.*, 2014). Such invasions impair native ecosystem functioning on multiple fronts, including through the displacement of native species, the loss of native genotypes, the modification of habitats, the alteration of community structures, the disruption of food webs, and the hindrance of ecosystem services (Katsanevakis, Coll, *et al.*, 2014).

The herbivorous rabbitfish *Siganus luridus* and *S. rivulatus*, for instance, two Lessepsian immigrants, have had a significant adverse impact on the food web and community structure of the Eastern Mediterranean's infralittoral zone. By way of overgrazing, they maintain barren areas nearly devoid of any erect algae and contribute to the transformation of the ecosystem from one characterised by diverse brown algal forests to a degraded one characterised solely by bare rock and scarce patches of crustose coralline algae (Giakoumi, 2013; Vergés *et al.*, 2014).

The invasive green alga *Caulerpa cylindracea*, native to Australia, forms compact multilayered mats reaching up to 15 cm in thickness that trap sediment and create anoxic conditions underneath them. Several biotopes native to the Mediterranean, such as coralligenous communities and communities of sublittoral algae, are negatively impacted by *C. cylindracea*. The alga smothers local populations of invertebrates and other macroalgae and diminishes native ecosystems' structural complexities and species richness (Klein & Verlaque, 2008; Piazzi *et al.*, 2016).

Large beds of the mytilid mussel *Brachidontes pharaonis*, another Lessepsian immigrant, have proved detrimental to the biota of the Eastern Mediterranean's mediolittoral zone. The species displaces entire beds of the native mussel *Mytilaster minimus* and in turn changes local predation patterns; namely, it has lead to increases in the population of the native whelk *Stramonita haemastoma* that preferentially preys on *B. pharaonis* (Rilov, Gasith & Benayahu, 2002; Sará, Romano & Mazzola, 2008).

However, it is important to note that alien species may also have a few positive effects on Mediterranean ecosystems. They can create novel habitats, control other invasive species, provide nutrition, and enhance ecosystem functioning in stressed or degraded environments (Katsanevakis, Coll, *et al.*, 2014). The serpulid *Ficopomatus enigmaticus*, for instance, builds reefs that provide a novel habitat for a number of native

Mediterranean species (Charles *et al.*, 2018); the cornetfish *Fistularia commersonii* can prey on *Pterois miles*, another alien invading the Mediterranean (Ulman *et al.*, 2021); and despite its aforementioned negative impacts, *B. pharaonis* is still attributed to reduce water turbidity and increase light penetration through filter-feeding, as well as act as a food source (Katsanevakis, Wallentinus, *et al.*, 2014). Regardless of this potential for benefits, however, the negative impacts alien species have had and continue to have on the Mediterranean's marine biodiversity outweigh the positive impacts in both prevalence and magnitude (Tsirintanis *et al.*, 2022).

1.2.4 Socioeconomic impacts

Alien species can to some extent have positive impacts on Mediterranean socioeconomics. For instance, the invasive blue crabs *Callinectes sapidus* and *Portunus segnis* are exploited by a number of Mediterranean fisheries, to which the two crabs have now come to represent a source of income (Marchessaux *et al.*, 2023). Still, such benefits are minimal relative to the losses these blue crabs and other alien species can incur. Indeed, Kourantidou *et al.* (2021) estimate that the countries bordering the Mediterranean Sea collectively suffered the loss of €3.3 billion on account of the invasions that occurred within the basin between 1990 and 2017.

For instance, the goldband goatfish *Upeneus moluccensis*, a Lessepsian immigrant, has replaced the native red mullet *Mullus barbatus* within Levantine fisheries (Galil *et al.*, 2014). The bluespotted cornetfish *Fistularia commersonii*, also a Lessepsian immigrant, specialises in consuming the juveniles of the native bogue *Boops boops* and the picarel *Spicara smaris*, both of which are of commercial importance within the Mediterranean (Bariche, Alwan, El-Assi & Zurayk, 2009). The silver-cheeked toadfish *Lagocephalus sceleratus*, another Lessepsian immigrant, eats two cephalopods also of commercial importance, namely the common cuttlefish *Sepia officinalis* and the common octopus *Octopus vulgaris* (Kalogirou, 2013). Moreover, it proves a nuisance to fishermen as it damages their fishing gear, thereby decreasing their catch sizes and incurring them the costs of gear repairs (Ünal *et al.*, 2015). It also poses serious health risks since tetrodotoxin, a potent paralytic neurotoxin, is present in its internal organs. Symptoms of tetrodotoxin include vomiting, respiratory arrest, convulsions, coma, and even death. Between 2005 and 2008, 13 people had to be treated for tetrodotoxin poisoning in Israel

alone (Bentur *et al.*, 2008; Galil *et al.*, 2014). Since the early 1980s, the nomad jellyfish *Rhopilema nomadica*, another Lessepsian immigrant, has each summer generated vast swarms along the Levantine coast that negatively impact local fisheries, coastal infrastructure, and tourism. Namely, the annual swarming has impeded purse-seining because it clogs nets and makes sorting catches impossible, and has disrupted desalination and power-plant operations because it blocks water-intake pipes. Moreover, the envenomation consequent to *R. nomadica* stings can continue for weeks or even months (Benmeir *et al.* 1990; Galil *et al.*, 2014).

1.3 The case of the Maltese Islands

By 2016, a total of 72 aliens and 8 range expanders were recorded in Maltese waters. Half of these newcomers had by then established viable populations and 8 had already reached invasive status. Namely, these invaders are *Caulerpa cylindracea*, *Lophocladia lallemandi*, *Womersleyella setacea*, *Brachidontes pharaonis*, *Percnon gibbesi*, *Fistularia commersonii*, *Siganus luridus*, and *Sphoeroides pachygaster* (Evans *et al.*, 2015; Evans & Schembri, 2016). More newcomers to local shores have been reported since, including *Abudefduf vaigiensis* (Vella, Agius Darmanin & Vella, 2016); *Kyphosus vaigiensis* (Vella, Vella & Agius Darmanin, 2016a); *Cephalopholis nigri* (Vella, Vella & Agius Darmanin, 2016b); *Acanthurus chirurgus* (Evans, Tonna & Schembri, 2017); *Apolemia uvaria*, *Phacellophora camtschatica*, and *Physophora hydrostatica* (Dragičević *et al.*, 2019); and *Naso annulatus* (Nour *et al.*, 2022).

The most common pathways introducing alien species to Maltese waters are reported to be shipping and secondary dispersal from other regions of the Mediterranean Sea. More than half of the newcomer species occurring in Maltese waters have been recorded since the year 2000, this being indicative of an accelerated rate of introductions in the more recent decades or at least of an increase in the efforts to detect and identify said newcomers. Furthermore, when considering both casual and established species, molluscs, fish, macrophytes, and crustaceans appear to be the most common alien taxa present in Maltese waters. However, these taxa are also the ones to have received the most attention in local studies, and thus this statistic may be biased (Evans *et al.*, 2015; Evans & Schembri, 2016).

1.4 Implementing biosecurity measures

Given the severe ecological impacts marine invasions have on the Mediterranean region, Descriptor D2 of the European Commission's Marine Strategy Framework Directive lists "non-indigenous species introduced by human activities [being] at levels that do not adversely alter the ecosystems" as one of the 11 qualitative descriptors necessary for claiming Good Environmental Status (Tsiamis *et al.*, 2021). Moreover, EU Regulation 1143/2014 (otherwise referred to as the IAS Regulation), which came into force on 1st January 2015, calls for the prevention of the intentional or unintentional introduction of IAS into the European Union, their early detection and rapid eradication, and concerted management in order that their spread and the harm they inflict are kept to a minimum (European Commission, 2014).

The effective management of invasive species necessitates, at the minimum, the capacity to identify such species. Their early detection and monitoring necessitate especially the utilisation of diagnostic tools that can be rapidly deployed and that are cost-effective, technically accessible, accurate, and applicable across a broad range of taxa (Darling & Blum, 2007).

1.5 Species identification

1.5.1 Using morphological characters

Traditional taxonomy is morphology-based and depends on the observation and measurement of diagnostic phenotypic traits in combination with the use of morphological keys. However, such a morphological approach is limited on several fronts: genetic variability (e.g. polymorphisms) and phenotypic plasticity in the morphological characters used for species recognition can lead to incorrect identifications (Karahan *et al.*, 2017); morphological keys are largely incapable of distinguishing between cryptic species (i.e., genetically distinct species that are morphologically nearly identical), which are prevalent in many groups (Jörger & Schrödl, 2013); many morphological keys are also life-stage-specific, often making the identification of eggs and juvenile individuals challenging; and finally, specimens cannot be correctly identified if the anatomical structures that morphological keys depend upon are deteriorated or lost (Valdez-Moreno *et al.*, 2010; Trivedi, Aloufi, Ansari & Ghosh, 2016). These limitations and the decrease in taxonomic

knowledge highlight the need for a novel approach to species identification (Hebert, Cywinska, Ball & deWaard, 2003; Vella, Vella, Karakulak & Oray, 2017).

For much the same reasons, traditional taxonomic techniques are ineffective at detecting and monitoring marine invasions, often costing researchers valuable time and money (Armstrong & Ball, 2005; Valdez-Moreno, Quintal-Lizama, Gómez-Lozano & García-Rivas, 2012). Traditional taxonomy is especially inadequate in detecting invasions on account of the fact that invaded regions are unlikely to have the taxonomic experts that are familiar with and therefore able to recognise the newcomer species (Vella, Vella, Karakulak, *et al.*, 2017). This often leads researchers to make their identifications solely to the family level or to what is known as a 'morphological species' or 'morphospecies' (Caesar, Sorensson & Cognato, 2006; Kress *et al.*, 2015).

1.5.2 Using genetic markers

DNA barcoding is based on the idea that sequences of DNA can serve as unique identifiers, similar to how the barcodes on retail products distinguish between the different items on the market. To this extent, a DNA 'barcode' is defined as one or few short sequences of nucleotides within standardised segments of the genome that can be used for species identification, or more broadly for identification at any taxonomic level (Kress *et al.*, 2015). It was two decades ago that Hebert *et al.* (2003) first proposed the barcoding of all extant lifeforms as a more reliable alternative to morphological species identification. Indeed, none of the limitations discussed hereabove for morphological species identification hold true for genetic species identification.

For a genetic sequence to be practical as a DNA barcode, it must meet three criteria: it must have significant genetic variability and divergence at the species level; it must possess conserved flanking sites to allow for the development of universal PCR primers; and it must be relatively short in length in order to facilitate DNA extraction and amplification. The power of the DNA barcode is also closely tied to the availability and completeness of the barcode library stored in the online bioinformatic repositories. Also of particular note is the inherent trade-off that exists between the ability to amplify a DNA barcode using a universal primer and the rate of divergence that locus exhibits across a range of different taxa; therefore, selecting a barcode with improved amplificability

automatically comes at a cost of it having reduced divergence across different taxa, and vice versa (Ekrem, Willassen & Stur, 2007; Kress & Erickson, 2008).

One of the key advantages of using DNA barcoding for species identification is the large amount of information that can be derived from small segments of DNA (Hebert *et al.*, 2003). Moreover, DNA barcoding has significant advantages over older molecular approaches that, for example, analyse restriction fragment length polymorphisms or allozymes; most importantly, it represents a more accurate and robust approach to species identification since it enables the use of all the genetic data that is obtained (Armstrong & Ball, 2005).

Local studies that have successfully employed DNA barcoding in species identification include, among others, Vella, Agius Darmanin, *et al.* (2016) and Vella, Vella, *et al.* (2016a) reporting the arrival of the aliens *Abudefduf vaigiensis* and *Kyphosus vaigiensis* in Maltese waters and Vella, Vella & Acosta-Diaz (2021) resurrecting the cryptic *Serranus papilionaceus*.

Regardless, though the use of genetic barcodes presents a promising approach towards the identification of and differentiation between the extant biota, there are a number of limitations that merit consideration, namely: instances of mitochondrial or plastid introgression, including current or previous hybridisation events, are difficult to detect using the majority of DNA barcodes (Ward, Hanner & Hebert, 2009); accurate sequence recovery may be impeded by heteroplasmy, this being the coexistence of two or more mitochondrial haplotypes within the same individual (Magnacca & Brown, 2010); the nuclear genome frequently contains non-functional organellar DNA segments, which can lead to uncertainties when analysing data because these extranuclear sequences may resemble and thus be mistaken for the actual barcode sequences (Anderson, 2012); across several taxa, the 'universal' primers may occasionally fail to amplify the targetted region; DNA degradation, such as is found in aging museum specimens and tissue samples exposed to high temperatures, decreases the success of sequence recovery (Centre for Biodiversity Genomics, University of Guelph, 2021); and, most importantly, the technique of DNA barcoding relies heavily on the availability of appropriate voucher specimens that serve as references, yet these are not always available (Kress *et al.*, 2015).

1.5.3 Using an integrated approach

DNA barcoding, therefore, promises a considerable improvement in accuracy and reliability when compared to the traditional morphology-based taxonomy. As of yet, however, given its limitations, DNA barcoding cannot wholly replace the morphological approach and the two should instead be used jointly to complement one another (Fujita *et al.*, 2012). The use of such an integrated taxonomic approach is exemplified by, among others, Vella, Agius Darmanin & Vella (2015) reporting the first record of *Stegastes variablis* in the Mediterranean Sea; Vella, Vella, *et al*. (2016b) reporting the first record of *Cephalopholis nigri* in Maltese waters; and Vella, Vella, Karakulak, *et al*. (2017) reducing knowledge gaps on the members of Family Tetraodontidae that are present within the Mediterranean Sea.

1.6 DNA barcoding

1.6.1 Using the cytochrome c oxidase subunit I gene

Hebert *et al.* (2003) were the researchers to have originally proposed the use of the sequence diversity extant in part of the mitochondrial cytochrome c oxidase subunit I (COI) gene as the basis of the DNA barcoding system applied to the animal taxa. In their original paper on the subject, they found that the sequence diversity in this same gene was 100% successful in discriminating between 150 closely related species of lepidopterans. Subsequent studies on marine taxa obtained similar results. Ward *et al.* (2005) found the COI gene to be fully capable of differentiating between 207 species of fish native to Australia. Radulovici, Sainte-Marie & Dufresne (2009) found the COI gene to be capable of differentiating between 460 specimens belonging to 80 species of crab in 95% of cases and, moreover, that genetic divergences between species were on average 25 times larger than genetic divergences within species, this number being exceptionally high relative to the findings for other taxa. Nowadays, the COI gene is universally recognised to be a practical and standardised DNA barcode applicable to the majority of the extant animal taxa, some notable exceptions being a few groups of invertebrates (Evans & Paulay, 2012) and cnidarians (Vella, Falzon & Vella, 2021). More suitable genetic markers exist for these exceptional animal taxa, as well as for plants and fungi in general (Kress *et al.*, 2015).

The advantage to obtaining genetic barcode data from the mitochondrial genome rather than from the nuclear genome lies in the fact that the mitochondrial genome lacks introns, is minimally exposed to recombination, and displays a haploid mode of inheritance (Saccone *et al.*, 1999). The fact that the COI gene encodes proteins as opposed to ribosomal DNA is also advantageous as the former class of genes tend to have less prevalent insertions and deletions, collectively referred to as 'indels', that make sequence alignment a more difficult task (Doyle & Gaut, 2000). The COI gene is particularly practical because the primers that have been developed for its amplification are considerably robust and enable the gene's recovery from the vast majority of the animal taxa (Folmer *et al.* 1994). Moreover, the gene appears to carry a stronger phylogenetic signal than any of the other mitochondrial genes and evolves substitutions at a rate approximately 3 times faster than that of mitochondrial markers 12S and 16S rDNA (Knowlton & Weigt, 1998).

1.6.2 Using the Barcode of Life Data System

The Barcode of Life Data System (BOLD) is an informatics workbench that aims to assist in the acquisition, storage, analysis, and publication of DNA barcode records. It contains barcode data for millions of specimens, representing over 200,000 species from around the world, and is maintained by the Biodiversity Institute of Ontario at the University of Guelph, Canada. By incorporating molecular, morphological, and geographical data, it effectively bridges the gap between traditional bioinformatic approaches and assembles a centralised and more convenient repository for use by researchers and conservationists (Ratnasingham & Hebert, 2007; Trivedi *et al.*, 2016).

1.7 Aim and objectives

In light of the above, the aim of this study was to investigate the applicability of the cytochrome c oxidase subunit I (COI) gene in identifying metazoan newcomers to Maltese coastal waters and to contrast the genetic data it yields with morphological lines of evidence. The objectives of this work included:

- To sample metazoan specimens suspected to be newcomers to the Maltese Islands;
- To gather diagnostic morphological data on the specimens collected, with special focus on the specimens belonging to classes Actinopterygii and Malacostraca;

- To extract, amplify and sequence part of the cytochrome c oxidase subunit I (COI) gene present in the specimens collected and employ it in species identification; and
- To construct phylogenetic trees based on the divergences in the COI sequences obtained and thereby analyse phylogenetic relationships.

2. Material and Methods

2.1 Specimen collection and processing

The scientific literature was searched for records of newcomer metazoan species that occur in Maltese waters and a list of said organisms and their known whereabouts compiled. Keeping these records in mind, dead specimens suspected to be newcomers to the Maltese Islands were collected from the Marsaxlokk fish market and from fishermen's landings observed first-hand. Further to this, in order to proactively aid the search effort, a number of fishermen with whom contact was established were shown photographs of type newcomer specimens and supplied with a relevant poster that was produced in connection with the Interreg Italia-Malta SEA MARVEL project. Supplementary specimens were provided by the University of Malta's Conservation Biology Research Group.

Detailed photography of the freshly procured specimens was taken (**Figure 1**) prior to preservative storage on ice or in absolute ethanol. A record of each specimen's date and location of collection was taken.

Fig. 1. Example specimen photography taken by author. Rulers for scale. **Left**, dorsal view of a *Portunus segnis*. **Right**, lateral view of a *Siganus luridus*.

2.2 Morphological analysis

Tentative species identification of the specimens collected was made by thorough visual inspection of the specimens' external morphologies and by following the keys and descriptions indicated in **Table 3**.

Further to this, morphometric measurements were obtained for the collected fish and crab specimens following the protocol designed for SEA MARVEL. Lengths smaller than 140 mm were measured using a Vernier calliper to the nearest 0.1 mm; lengths greater than 140 mm were measured using a ruler to the nearest 0.5 mm. Wet weights up to 15 g were measured on an analytical balance to the nearest 0.1 g; wet weights greater than 15 g were measured on a gross weighing scale to the nearest 1 g. Meristic counts were obtained for the fish specimens and the crab specimens were sexed by inspection of their abdomen-to-body size ratios. Crab egg broods, when present, were also recorded.

2.3 Genetic analysis

2.3.1 Tissue sampling

A small sample of tissue was excised from each specimen in a sterile working environment using sterile blades, scissors and/or forceps and subsequently stored in absolute ethanol. Excisions were preferentially performed on soft, internal tissues that were less likely to be contaminated with exogenous genetic material. In order to preserve external morphologies and with the exception of cases in which open wounds were already present, fish specimens were sampled solely from their gill filaments. Said gill filaments were also sampled unilaterally so as to always leave one set of gill arches intact. Crab specimens were exclusively sampled from the musculature of their walking appendages. When specimens where expected to yield poorer amounts of DNA product, multiple tissue samples were taken from different anatomical regions.

2.3.2 DNA extraction and purification

Each tissue sample was dried of ethanol on filter paper and finely cut with a sterile blade. DNA extraction was carried out using a GF-1 Tissue DNA Extraction Kit (Vivantis), following the manufacturer's protocol. Approximately 3 mg of each tissue were added to a microcentrifuge tube and mixed with 20 µL of proteinase K, 250 µL of lysis buffer, and 12 µL of lysis enhancer. Controls were prepared at this stage (in order to

flag exposure of the tissue samples to contaminations, if any) by adding the same mixture of solutions to empty microcentrifuge tubes. The tissues and controls were then left in a water bath set at 65°C overnight in order to allow for protein digestion.

The following day, 560 µL of tissue binding buffer were mixed with each sample, each solution was vortexed, and the microcentrifuge tubes were returned to the water bath still set at 65°C. The solutions were removed from the water bath after 10-15 min and each mixed with 200 µL of absolute ethanol, followed by immediate vortexing. 570 µL of each solution were transferred to an extraction column and centrifuged at 5,000 x *g* for 1 min. The resulting filtrates were discarded, and the step repeated with a second volume of 570 µL. 610 µL of wash buffer were subsequently added to each extraction column and centrifuged at 5,000 x *g* for 1 min. The resulting filtrates were again discarded, and the step repeated with another 610 µL of wash buffer. The solutions were then centrifuged at 10,000 x *g* for 1 min. Next, the tubes at the bottom of the extraction columns were replaced with new, clean microcentrifuge tubes and 200 µL of elution buffer were added to each extraction column. After 2 min had elapsed, the solutions were centrifuged at 5,000 x *g* for 1 min and the upper tubes of the extraction columns were discarded. The resultant filtrates that contained the purified DNA extracts were then stored at -20°C until further processing.

2.3.3 Marker amplification

2.3.3.1 For ichthyofauna

Two primers designed by Ward *et al.* (2005) were used to amplify a fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene of 655 bp length, namely: FishF1, 5'-TCA ACC AAC CAC AAA GAC ATT GGC AC-3'; and FishR2, 5'-ACT TCA GGG TGA CCG AAG AAT CAG AA-3'. Polymerase chain reactions were performed in 25 µL aliquots, each containing: 2 µL of a fish-derived DNA template; 0.1 µL of each of the two 100 nM primers; 5 µL of PCR buffer (FIREPol Master Mix, Solis BioDyne) containing 200 µM of each dNTP, 1 unit of *Taq* polymerase, and 10 mM Tris-HCl; 1 µL of 2.5 mM $MgCl_2$; and 16.8 µL of ultrapure water. The thermal profile used consisted of 1 cycle at 95°C for 15 min; 35 cycles at 95°C for 45 s, then at 52°C for 45 s, and then at 72°C for 45s; and 1 cycle at 72°C for 10 min.

2.3.3.2 For invertebrate fauna

Two primers designed by Folmer *et al.* (1994) were used to amplify a fragment of the COI gene of 658 bp length, namely: LCO1490, 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3'; and HC02198, 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3'. Polymerase chain reactions were performed in 25 µL aliquots, each containing: 1.5 µL of an invertebrate-derived DNA template; 0.1 µL of each of the two 100 nM primers; 5 µL of PCR buffer (FIREPol Master Mix, Solis BioDyne) containing 200 µM of each dNTP, 1 unit of *Taq* polymerase, and 10 mM Tris-HCl; 1 µL of 2.5 mM $MgCl_2$; and 17.3 µL of ultrapure water. The thermal profile used consisted of 1 cycle at 95°C for 15 min; 7 cycles at 95°C for 1 min, 44°C for 1 min 15 s, and 72°C for 1 min 15 s; 37 cycles at 95°C for 1 min, then at 48°C for 1 min 15 s, and then at 72°C for 1 min 15 s; and 1 cycle at 72°C for 10 min.

2.3.4 Product visualisation

A 1.5% agarose gel was prepared using Tris/Borate/EDTA buffer and stained with ethidium bromide. 1 µL of each PCR product was mixed with 6 µL of blue loading dye and the resulting mixture loaded into each respective well. 100 bp DNA ladders were also loaded into each row, and then the gel was run at 80V for 30 min. The resultant bands were visualised under UV light and inspected to confirm that the PCR reaction worked and produced sufficient quantities of the amplicons, that the amplicons produced were of the expected size (confirmed by way of comparison with the 100 bp ladders), and that non-specific bands were absent (**Figure 2**).

Fig. 2. Example results of gel electrophoresis used to confirm marker amplification. As seen under UV. **Left**, full view of a gel loaded with 4 rows of PCR products and controls (the latter indicated by arrowheads). **Right**, close-up view of 6 amplicons against a DNA ladder that confirms the expected amplicon size of approximately 700 bp (including primers).

2.3.5 Sequencing and analysis

The amplicons confirmed by gel electrophoresis were purified and analysed through Sanger sequencing. The raw sequence data files that were returned were processed using Geneious v10 (www.geneious.com; Kearse *et al.*, 2012). Following the manual trimming and removal of the flanking primer sequences, the forward and reverse sequences of each specimen were aligned, inspected for any inconsistencies, and corrected as necessary. The COI data were then translated using the vertebrate mitochondrial genetic code for the fish specimens and the invertebrate mitochondrial genetic code for the rest of the specimens. These translations were used to check for the absence of stop codons and thereby verify the quality of the barcode sequences obtained (provided that the COI gene is protein-coding and thus lacks exonal stop codons) (**Figure 3**). In instances where species were represented by more than one specimen, the corresponding barcodes were trimmed down to the smallest homologous sequence to allow for comparability within the species-level data.

BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare the final barcode sequence of each specimen with the closest matching sequence in GenBank. Similarly, the BOLD Species Level Barcode Records Identification Engine (http://www.boldsystems.org) was used to determine which Barcode Index Number (BIN) in BOLD each specimen's barcode sequence clusters most with. In addition, a number of sequences belonging to a number of related specimens were aligned within Geneious and

then transferred to MEGA v7 (Kumar, Stecher & Tamura, 2016) for the construction of maximum-likelihood phylogenetic trees with 1000 bootstraps. The *p*-distance model was used following Vella, Vella & Schembri (2017).

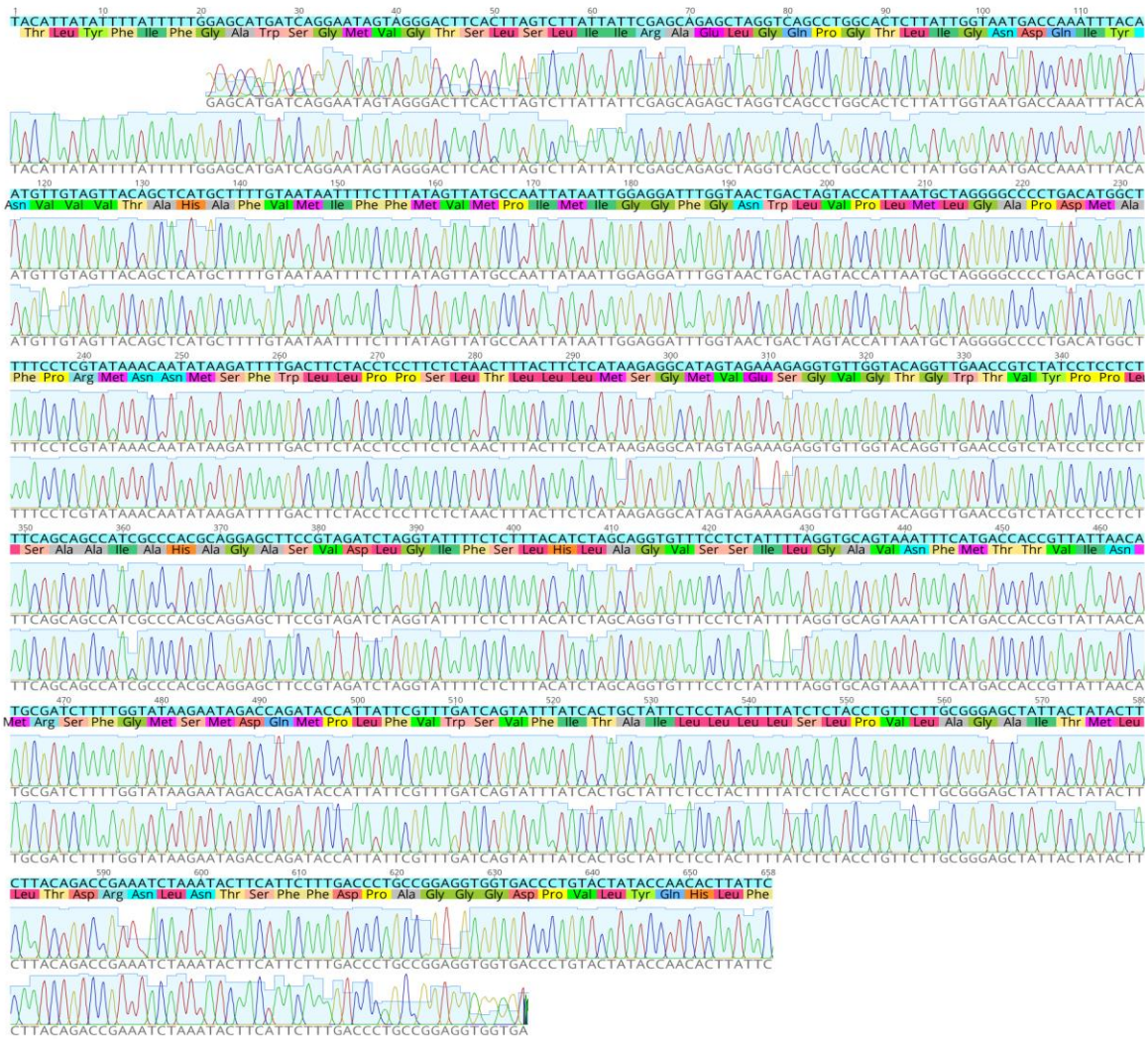

Fig. 3. Chromatograms of the forward and reverse barcode sequences obtained for one of the collected *Portunus segnis* specimens following sequence trimming, editing, and alignment. Also shown are the derived consensus nucleotides and the amino acids they translate to. The numerical values at the top reflect base pair positions. Image screenshotted within Geneious v10.

3. Results

79 specimens suspected to be newcomers to Maltese waters were collected between July and October of 2022. Amplification was achieved for 72 specimens (91.1% of 79). Amplicons from 59 specimens (74.7 %) returned good-quality sequence data, of

which 57 (72.2%) enabled identification down to species level and 2 (2.5%) to only genus level (see **Appendix 1** for the collection details of the identified specimens).

Morphological analyses allowed for the corroboration of these genetic identifications in 50 (84.7% of 59) instances. Specimen morphologies were not sufficiently intact in the remaining 9 (15.3%) instances to allow for any analysis.

Ultimately, 15 specimens belonging to 8 species were confirmed by the sequence and morphological data to be native to Maltese waters. Their identities and associated genetic data are listed in full in **Table 1**. 42 specimens were confirmed to be newcomers belonging to 13 different species, and the 2 remaining specimens were confirmed to be of cryptogenic origin and to belong to 1 species. Their identities and genetic data are listed in full in **Table 2**. Phylogenetic analyses of *Portunus segnis* and *Percnon gibbesi* (**Figure 4)** revealed that the specimens from this study grouped well with conspecifics from the native ranges, further confirming these specimens' identifications.

The diagnostic characters that were observed and employed in the morphological identification of these specimens are detailed in full in **Table 3**. Also indicated in this table are the papers that were consulted for the morphological keys and descriptions they provided. In summary, the trochid top shell *Steromphala rarilineata* was identified by the rows of red-purple dots on its shell, the strong tilt of its lip aperture, and its lack of suturing. The pteriid pearl oyster *Pinctada radiata* was identified by its pearly interior, its curved hinge line, and the rows of flattened spines on its exterior. Portunid swimmer crabs were identified by the paddle-shaped dactyli of their posteriormost pereiopods: *Callinectes sapidus* was then distinguished by the absence of medial spines on its cheliped carpora; *Portunus segnis* was distinguished by the prominent medial spines on its cheliped carpora and its triangle-shaped male abdomen; and *Achelous hastatus* was distinguished by its small carapace length-to-width ratio and ensiform-to-cultriform anterior pereiopods. The grapsoid Sally Lightfoot crab *Percnon gibbesi* was identified by its discoid carapace that bears 4 acute anterolateral spines, as well as by the yellow markings and anterior rows of spines on its pereiopods. The cidarid pencil urchin *Stylocidaris affinis* was identified by its cylindrical-to-subulate spines, its subglobose test lined with 18 longitudinal ridges, and its wide intertubercular margins.

The doctorfish *Acanthurus monroviae* was uniquely identified by the spine that emerges out of its caudal peduncle and that is surrounded by a bright orange-yellow spot. The sea chub *Kyphosus vaigiensis* was identified by the 11 hard spines of its dorsal fin and the 3 hard spines of its anal fin, its blue-to-white dorsoventral colour gradient, its alternately-coloured horizontal scale rows, and its moderately emarginated caudal fin. The haemulid grunt *Pomadasys incisus* was identified by its yellow fins, its operculum that is black at the margin, its third anal fin spine that exceeds in length the second anal fin spine, and its forked caudal fin. Siganid rabbitfish were identified by the 13 hard spines of their dorsal fins that are preceded by procumbent spines: *Siganus luridus* was then distinguished by its truncated caudal fin and dark brown colouration and *S. rivulatus* by its forked caudal fin and olive-green colouration. Tetraodontid pufferfish were identified by their inflatable bellies, four strong teeth, and the absence of pelvic fins: *Lagocephalus sceleratus* was then distinguished by its dorsal black markings and its lunate caudal fin, and *Sphoeroides pachygaster* by its lack of markings and its truncated caudal fin. Regarding carangids: the runner *Caranx crysos* was identified by its upper jaw ending below the mideye, the scutes on the posterior end of its lateral line, and the 2 hard spines of its anal fin; and the amberjack *Seriola fasciata* was identified by the slender end of its upper jaw, the 9 hard spines of its dorsal fin, the absence of scutes on its lateral line, and the furrow on the dorsum of its caudal peduncle. Regarding blenniiformes: the clinid blenny *Clinitrachus argentatus* was identified by its cycloid scales, supraorbital tentacle, its anterior dorsal fin that is inserted immediately posterior to the eye, and its convex caudal fin; the combtooth blenny *Scartella cristata* was identified by the 12 hard spines of its dorsal fin, the cirri that line its nape, and the dark bars lining its body that extend into the lower dorsal fin; and the triplefin *Tripterygion tripteronotum* was identified by the obtuse profile of its head, its non-protruding lips, its head mask that extends to the tip of the pectoral fin, and the dark bars lining its body that do not extend onto the base of the caudal fin.

Among the confirmed newcomer and cryptogenic species were 22 specimens belonging to 8 species of fish and 16 specimens belonging to 3 species of crab. Their summarised meristics and morphometrics are presented in **Table 4** and **Table 5**, respectively (see **Appendix 2** for the raw data).

The single specimen of *Siganus rivulatus* presented here constitutes the first confirmed record of the species for the Maltese Islands (Vella *et al.*, 2023). Vella *et al.* (2023) discuss this specimen in more detail and illustrate its phylogenetic relationships to other conspecifics and congenerics from both the native range and the Mediterranean Sea.

Lastly, 12 of the haplotypes sequenced in the process of completing this project were found to have less than 100% identity with both the closest matching BIN in BOLD and the closest matching sequence in GenBank (**Table 1**, **Table 2**). These haplotypes, coming from 7 different species, constitute newly discovered genetic variants for the respective species.

Table 1. The genetic and morphological identifications of the confirmed native specimens. Also indicated are the number of specimens each species is represented by; the length of the trimmed barcode sequences that were analysed; the haplotypes recorded per species; and each haplotype's percent identity with the closest matching BIN in BOLD and sequence in GenBank. Reference numbers for the closest matches in BOLD and GenBank are indicated where the percent identity is ≥ 96%.
n = sample size; H = haplotype; * = haplotype is newly discovered.

Genetic identity	*n*	Analyte sequence length (bp)	Haplotype	Identity (%) with closest match in BOLD per haplotype	Identity (%) with closest match in GenBank per haplotype	Morphological identity
Polychaeta						
Hermodice carunculata (Pallas, 1766)	4	658	H1* (25% *n*) H2 (25% *n*) H3* (50% *n*)	99.8% BOLD:AAB3315 100% BOLD:AAB3315 99.7% BOLD:AAB3315	99.7% KC017536 100% KC017555 99.7% KC017554	Morphology not intact (100% *n*)
Gastropoda						
Steromphala rarilineata (Michaud, 1829)	1	658	H1	100% BOLD:AAO7212	99.8% JQ839341	*Steromphala rarilineata*
Brachyura						
Achelous hastatus (Linnaeus, 1767)	2	658	H1* (100% *n*)	99.7% BOLD:AEU0773	99.7% KT365747	*Achelous hastatus* (50% *n*) Morphology not intact (50% *n*)
Echinoidea						
Stylocidaris Mortensen, 1909	1	658	H1	100% BOLD:ABA4413	88.9%	*Stylocidaris affinis*
Actinopterygii						
Caranx crysos (Mitchill, 1815)	3	655	H1 (100% *n*)	100% BOLD:AAC4853	100% MN869864	*Caranx crysos* (100% *n*)
Clinitrachus Swainson, 1839	1	655	H1	95.8%	96% KY176435	*Clinitrachus argentatus*
Scartella cristata (Linnaeus, 1758)	2	655	H1 (100% *n*)	100% BOLD:ABA7013	100% MG837119	*Scartella cristata* (100% *n*)
Tripterygion tripteronotum (Risso, 1810)	1	655	H1*	99.6% BOLD:AAB2566	99.6% AJ937864	*Tripterygion tripteronotum*

Table 2. The genetic and morphological identifications of the confirmed newcomer specimens. Also indicated are the number of specimens each species is represented by; the length of the trimmed barcode sequences that were analysed; the haplotypes recorded per species; and each haplotype's percent identity with the closest matching BIN in BOLD and sequence in GenBank. Reference numbers for the closest matches in BOLD and GenBank are indicated where the percent identity is ≥ 96%.
n = sample size; H = haplotype; * = haplotype is newly discovered; Cryp. = species is cryptogenic.

Genetic identity	*n*	Analyte sequence length (bp)	Haplotype	Identity (%) with closest match in BOLD per haplotype	Identity (%) with closest match in GenBank per haplotype	Morphological identity
Scyphozoa						
Rhopilema nomadica Galil, Spanier & Ferguson, 1990	1	658	H1	99.8% BOLD:ACH6588	100% MW427690	Morphology not intact
Bivalvia						
Pinctada radiata (Leach, 1814)	4	649	H1 (50% *n*) H2 (50% *n*)	94.3% 94.3%	100% KT768195 100% KT768195	*Pinctada radiata* (100% *n*)
Brachyura						
Callinectes sapidus Rathbun, 1896	2	658	H1 (100% *n*)	100% BOLD:AAB6460	100% OQ108525	*Callinectes sapidus* (100% *n*)
Percnon gibbesi (H. Milne-Edwards, 1853)	7	658	H1* (14.3% *n*) H2* (14.3% *n*) H3* (14.3% *n*) H4* (14.3% *n*) H5 (14.3% *n*) H6 (14.3% *n*) H7* (14.3% *n*)	99.8% BOLD:AAC3991 99.8% BOLD:AAC3991 99.4% BOLD:AAC3991 99.4% BOLD:AAC3991 100% BOLD:AAC3991 100% BOLD:AAC3991 99.2% BOLD:AAC3991	99.8% JQ306098 99.8% JQ306098 99.4% JQ306098 99.7% JQ306096 100% JQ306098 100% JQ306096 99.2% JQ306101	*Percnon gibbesi* (100% *n*)
Portunus segnis (Forsskål, 1775)	7	658	H1 (85.7% *n*) H2* (14.3% *n*)	100% BOLD:ACD1567 99.9% BOLD:ACD1567	99.5% KF793331 99.8% KF793331	*Portunus segnis* (71.4% *n*) Morphology not intact (28.6% *n*)
Ascidiacea						
Herdmania momus (Savignyi, 1816)	1	481	H1	100% BOLD:ACD2563	100% MH383135	Morphology not intact
Actinopterygii						
Acanthurus monroviae Steindachner, 1876	1	655	H1	100% BOLD:ABX8395	99.8% KT283583	*Acanthurus monroviae*
Kyphosus vaigiensis (Quoy & Gaimard, 1825)	1	655	H1	100% BOLD:AAC3456	100% MN870142	*Kyphosus vaigiensis*
Lagocephalus sceleratus (Gmelin, 1789)	1	655	H1	100% BOLD:AAC5565	100% KX017816	*Lagocephalus sceleratus*
Pomadasys incisus (Bowdich, 1825)	1	655	H1*	99.9% BOLD:AAD1379	99.8% KJ768283	*Pomadays incisus*
Seriola fasciata (Bloch, 1793)	2	655	H1 (50% *n*) H2 (50% *n*)	100% BOLD:AAF1011 100% BOLD:AAF1011	100% MZ436498 100% MZ436497	*Seriola fasciata* (100% *n*)
Siganus luridus (Rüppell, 1829)	13	655	H1 (15.4% *n*) H2 (61.6% *n*) H3 (15.4% *n*) H4* (7.7% *n*)	100% BOLD:AAL9467 100% BOLD:AAL9467 100% BOLD:AAL9467 99.9% BOLD:AAL9467	99.8% KY176633 100% MH331866 100% MW376909 99.8% KY675486	*Siganus luridus* (100% *n*)
Siganus rivulatus Forsskål & Niebuhr, 1775 (Cryp.)	1	614	H1	100% BOLD:ABY0829	100% KF434772	*Siganus rivulatus*
Sphoeroides pachygaster (CR) (Müller & Troschel, 1848)	2	655	H1 (100% *n*)	100% BOLD:AAB7651 100% BOLD:AAB7651	100% OP430519 100% OP430519	*Sphoeroides pachygaster* (100% *n*)

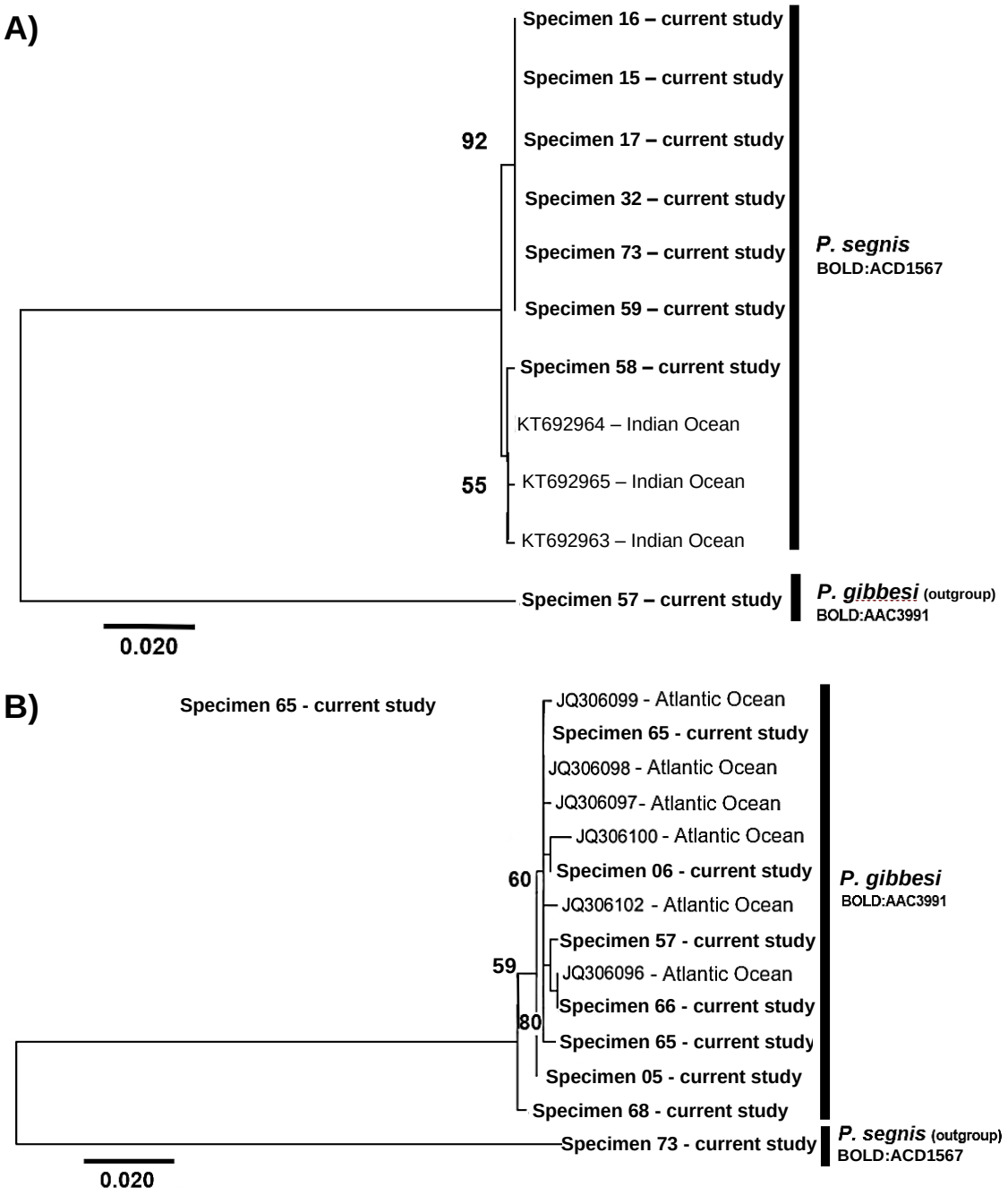

Fig. 4. The phylogenetic relationships between *Portunus segnis* (**A**) and *Percnon gibbesi* (**B**) specimens collected from local waters as part of this project (noted in bold), other specimens from each species' respective native range, and a single outgroup specimen also collected as part of this project (noted in bold and situated at the base of each tree). Each phylogram was constructed using the maximum-likelihood estimation method and the *p*-distance model. The values at the nodes indicate bootstrap values >60. The codes starting with 'KT' and 'JQ' are GenBank accession numbers. The codes to their right indicate BOLD BINs.

Table 3. The specimens whose identities were confirmed by genetic analysis and the corroborating diagnostic characters that were observed in their morphologies. The sources of the morphological descriptions and keys that were consulted are also indicated (*continues on the next page*).

Species identification	Observed diagnostic morphological characters	References
Mollusca		
Steromphala rarilineata	The shell consists of angular whorls that are adorned with multiple rows of red-purple dots or squares; the overall shape of the snail is triangular, and its diameter and height are approximately equal; the base is slightly concave; the lip of the aperture is strongly tilted and attaches to the lower edge of the last whorl such that the shell is left with no distinct suture.	Affenzeller, Haar & Steiner (2017)
Pinctada radiata	The valve exterior is lined with concentric lamellae that bear numerous rows of flattened spines; the shell is round in outline, as opposed to ovate; the hinge line is curved rather than straight; the valve interior is very pearly; the dorsal margin of the shell is extended both anteriorly and posteriorly.	Scuderi, Balistreri & Germanà (2019)
Brachyura		
Achelous hastatus	The carapace is flattened, much shorter than it is wide, and grey-brown in colour; the anterolateral margin of the carapace is lined with 9 spines, the last of which is the longest and directed laterally; the chelipeds are long relative to the rest of the body; the dactyli of the anterior pereiopods are ensiform or cultriform and distinctly ribbed; the dactyli of the posteriormost pereiopods are modified into paddles.	Zariquiey Álvarez (1968); Rodrigues, Cardoso & Serejo (2017)
Callinectes sapidus	The cheliped carpus does not bear a prominent spine medially; the anterior margin of the carapace is lined with 2 prominent spines; the anterolateral margin of the carapace is lined with 9 spines, the last of which is the longest and directed laterally; the dactyli of the posteriormost pereiopods are modified into paddles.	Holthuis (1987); Bariche (2012)
Percnon gibbesi	The carapace is discoid, flattened, and pubescent except in symmetrical raised areas that are glabrous; the anterolateral margin of the carapace is lined with 4 acute spines, the first of which is the largest; the left cheliped is larger than the right, especially in the palm; the pereiopods are adorned with yellow rings at their joints and lined with rows of spines anteriorly.	Relini, Orsi, Puccio & Azzurro (2000)
Portunus segnis	The cheliped carpus bears a prominent spine medially; male abdomens are triangular in shape as opposed to resembling an 'inverted T'; the anterior margin of the carapace is lined with 4 prominent spines; the anterolateral margin of the carapace is lined with 9 spines, the last of which is the longest and directed laterally; the dactyli of the posteriormost pereiopods are modified into paddles.	Holthuis (1987); Bariche (2012)
Echinoidea		
Stylocidaris affinis	The test is subglobose and depressed on both sides; the spines are cylindrical-subulate in shape, approximately half the diameter of the test in length, and arranged in a series of 18 longitudinal ridges; the larger tubercles are surrounded by deep grooves and by wide margins of smaller tubercles.	Philippi (1845)

Table 3 ***cont.*** The specimens whose identities were confirmed by genetic analysis and the corroborating diagnostic characters that were observed in their morphologies. The sources of the morphological descriptions and keys that were consulted are also indicated.

Species identification	Observed diagnostic morphological characters	References
Actinopterygii		
Acanthurus monroviae	A single spine emerges out of the caudal peduncle, itself adorned with a single bright orange-yellow spot; the caudal fin is largely truncated but deeply emarginated at the tips.	Bariche (2012); Agius Darmanin, Vella & Vella (2016)
Caranx crysos	The upper jaw ends below the mideye as opposed to below the eye's anterior margin; the top corner of the operculum is adorned with a single black spot rather than a black blotch that is itself surrounded by a white spot; the eye is not covered by a posterior adipose eyelid; the lateral line is not strongly curved; scutes are only present posteriorly; the caudal fins are dark at the tips; the anal fin consists of 2 hard spines.	Bariche (2012); Devine & Fisher (2014)
Clinitrachus argentatus	The body is flattened laterally, covered in cycloid scales, dark green or brown in colour, and adorned with a marbled pattern; the head is distinctly pointed and a small tentacle protrudes supraorbitally; the anterior dorsal fin is inserted immediately posterior to the eyes and is comprised of 3 hard spines; the anal and posterior dorsal fin increase in height posteriorly; the caudal peduncle is narrow and the caudal fin small and convex.	Orlando-Bonaca & Trkov (2016)
Kyphosus vaigiensis	The body is elongate, oval-shaped, metallic blue dorsally, and white ventrally; the horizontal scale rows that run along the body from the operculum to the caudal fin are alternately golden and blueish; the dorsal and anal fins are very low; the dorsal fin bears 10 or 11 hard spines and 13 or 14 soft rays; the anal fin bears 3 hard spines and 11 to 14 soft rays; the pectoral fin bears 17 to 20 soft rays; the caudal fin is moderately emarginated.	Knudsen (2013)
Lagocephalus sceleratus	The pelvic fin is absent and the dorsal and anal fins are comprised of 11 to 19 soft rays; the body bears no typical scales and is adorned with black dots dorsally; the jaws are each equipped with 2 strong teeth; the belly is inflatable and is lined with small spinules; the caudal fin is lunate in shape.	Bariche (2012); Farrag *et al* . (2015)
Pomadasys incisus	The anal fin is comprised of 12 or 13 soft rays; the third anal fin spine is at least equal in length to the second anal fin spine; the operculum is black at its margin; the fins are yellow; the upper jaw does not extend to the eye's anterior margin; the body is not lined with dark bands anteroposteriorly; the caudal fin is forked.	Kapiris, Kallias & Conides (2008); Bariche (2012)
Scartella cristata	The dorsal fin is comprised of 12 hard spines and 14 or 15 soft rays; the anal fin is comprised of 2 hard spines and 14 to 17 soft rays; the nape is lined by a median series of cirri and another short cirrus is present in the supraorbital region; the body is dull green or olive and lined with dark bars that extend onto the lower dorsal fin.	Amaoka, Araga, Uyeno & Yoshino (1984); Randall (1996)
Seriola fasciata	The end of the upper jaw is relatively slender; the dorsal fin is comprised of a total of 9 hard spines; the eye is not covered by a posterior adipose eyelid; the lateral line is not strongly curved; scutes are entirely absent; the caudal peduncle is furrowed dorsally; the anal fin consists of 3 hard spines.	Fischer & Schneider (1987); Bariche (2012)
Siganus luridus	The dorsal fin is comprised of 13 hard spines and is preceded by a procumbent spine; the caudal fin is truncated as opposed to forked; the anterior nostril bears a long flap that covers the posterior nostril when depressed; the body is dark brown in colour as opposed to olive-green.	Schembri, Deidun & Falzon (2012); Bariche (2012)
Siganus rivulatus	The dorsal fin is comprised of 13 hard spines and is preceded by a procumbent spine; the caudal fin is forked as opposed to truncated; the anterior nostril bears a flap that does not reach the posterior nostril when depressed; the body is olive-green in colour as opposed to dark brown; the body is adorned with golden undulating lines ventrally.	Schembri, Deidun & Falzon (2012); Bariche (2012)
Sphoeroides pachygaster	The pelvic fin is absent, and the dorsal and anal fins are comprised of 8 or 9 soft rays; the body bears no typical scales and is not adorned with any markings; the jaws are each equipped with 2 strong teeth; the belly is inflatable; the caudal fin is truncated.	Bariche (2012); Giordano *et al* . (2012)
Tripterygion tripteronotum	The profile of the head is obtuse rather than acute; the lips do not protrude; the head mask extends to the tip of the pectoral fins; the eyes are moderately large; the head length is more than 2.5 times the orbital diameter; the posteriormost dark bar on the body does not extend onto the base of the caudal fin.	Carreras-Carbonell, Pascual & Macpherson (2007)

Table 4. The meristics and morphometrics of the newcomer and cryptogenic fish specimens whose identities were confirmed by genetic analysis (*continues on the next page*).
n = sample size; m.a. = morphology absent; SL = standard length.

	***Acanthurus monroviae* (*n* =1)**		***Kyphosus vaigiensis* (*n* =1)**		***Lagocephalus scleratus* (*n* =1)**		***Pomadasys incisus* (*n* =1)**		***Siganus rivulatus* (*n* =1)**	
Meristic counts:										
Procumbent spines	m.a.		m.a.		m.a.		m.a.		I	
Dorsal fin hard spines	IX		XI		m.a.		XII		XIII	
Dorsal fin soft rays	25		13		11		17		10	
Pectoral fin hard spines	m.a.		m.a.		m.a.		m.a.		m.a.	
Pectoral fin soft rays	16		20		17		17		16	
Pelvic fin hard spines	I		I		m.a.		I		I + I	
Pelvic fin soft rays	5		5		m.a.		5		3	
Anal fin hard spines	II		III		m.a.		III		VII	
Anal fin soft rays	23		12		11		12		9	
Caudal fin soft rays	22		18		15		17		20	
Morphometrics:										
Wet weight (g)	200.0		850.0		115.0		80.0		195.0	
Total length (mm)	229.0		362.0		201.0		177.0		240.0	
Fork length (mm)	216.5		330.0		193.0		166.5		226.0	
Standard length (mm)	184.0		295.0		177.0		146.5		203.0	
Maximum body depth (mm)	77.5	42.1% SL	126.5	42.9% SL	36.5	20.6% SL	54.4	37.1% SL	71.0	35.0% SL
Dorsal fin length (mm)	117.2	63.7% SL	147.2	49.9% SL	13.5	7.6% SL	68.8	47.0% SL	144.2	71.0% SL
Pectoral fin length (mm)	35.1	19.1% SL	52.6	17.8% SL	26.0	14.7% SL	42.4	28.9% SL	21.6	10.6% SL
Anal-fin base length (mm)	81.8	44.5% SL	73.1	24.8% SL	8.2	4.6% SL	21.4	14.6% SL	82.5	40.6% SL
Pre-pelvic length (mm)	46.8	25.4% SL	84.0	28.5% SL	m.a.		48.3	33.0% SL	50.2	24.7% SL
Pre-pectoral length (mm)	43.0	23.4% SL	63.8	21.6% SL	52.4	29.6% SL	46.9	32.0% SL	38.7	19.1% SL
Pre-anal length (mm)	77.4	42.1% SL	173.0	58.6% SL	110.5	62.4% SL	95.6	65.3% SL	101.1	49.8% SL
Head length (mm)	45.3	24.6% SL	68.5	23.2% SL	59.7	33.7% SL	44.3	30.2% SL	39.8	19.6% SL
Pre-orbital length (mm)	25.3	13.8% SL	18.9	6.4% SL	23.5	13.3% SL	11.1	7.6% SL	15.5	7.6% SL
Eye diameter (mm)	11.6	6.3% SL	13.5	4.6% SL	14.9	8.4% SL	12.4	8.5% SL	9.7	4.8% SL
Inter-orbital distance (mm)	20.8	11.3% SL	34.1	11.6% SL	23.3	13.2% SL	18.5	12.6% SL	13.0	6.4% SL
Caudal peduncle depth (mm)	17.4	9.5% SL	42.4	14.4% SL	7.1	4.0% SL	16.1	11.0% SL	10.1	5.0% SL

Table 4 *cont.* The meristics and morphometrics of the newcomer and cryptogenic fish specimens whose identities were confirmed by genetic analysis.
n = sample size; SD = standard deviation; m.a. = morphology absent; SL = standard length.

	Seriola fasciata **(*n* =2)**		***Siganus luridus*** **(*n* =13)**		***Sphoeroides pachygaster*** **(*n* =2)**	
Meristic counts:		mean ± SD		mean ± SD		mean ± SD
Procumbent spines	m.a.		I		m.a.	
Dorsal fin hard spines	VIII + I		XIII		m.a.	
Dorsal fin soft rays	29-30	29.5 ± 0.7	10		9	
Pectoral fin hard spines	I		m.a.		m.a.	
Pectoral fin soft rays	19		15-17	16.2 ± 0.8	14-15	14.5 ± 0.7
Pelvic fin hard spines	I		I + I		m.a.	
Pelvic fin soft rays	5		3		m.a.	
Anal fin hard spines	II + I		VII		m.a.	
Anal fin soft rays	17-18	17.5 ± 0.7	9		9	
Caudal fin soft rays	21-23	22.0 ± 1.4	16-20	18.2 ± 1.1	9	
Morphometrics:						
Wet weight (g)	95.0-230.0		165.0-460.0		530.0-935.0	
Total length (mm)	180.0-239.0		209.0-279.0		275.0-326.0	
Fork length (mm)	158.5-213.0		m.a.		m.a.	
Standard length (mm)	142.0-193.0		19.6-232.0		254.0-278.0	
Maximum body depth (mm)	53.8-72.6	37.8% ± 0.1% SL	74.1-104.1	44.0% ± 2.2% SL	101.1-124.5	42.3% ± 3.5% SL
Dorsal fin length (mm)	18.5-23.6 + 61.4-86.7	12.6% ± 0.3% SL 44.1% ± 0.6% SL	125.4-160.0	70.4% ± 1.3% SL	13.2-13.5	5.0% ± 0.4% SL
Pectoral fin length (mm)	23.4-26.7	15.2% ± 0.9% SL	29.8-41.6	16.8% ± 1.3% SL	40.3-45.2	16.1% ± 2.3% SL
Anal-fin base length (mm)	37.5-54.4	27.3% ± 0.6% SL	69.1-87.3	38.6% ± 1.4% SL	10.2-12.2	4.2% ± 0.3% SL
Pre-pelvic length (mm)	49.6-62.2	33.6% ± 1.0% SL	38.8-57.3	23.9% ± 2.2% SL	m.a.	
Pre-pectoral length (mm)	47.2-56.7	31.3% ± 1.4% SL	34.2-48.3	19.3% ± 1.4% SL	104.5-117.7	41.7% ± 0.8% SL
Pre-anal length (mm)	88.4-122.5	62.9% ± 0.4% SL	89.5-123.0	50.1% ± 2.1% SL	199.5-229.0	80.5% ± 2.7% SL
Head length (mm)	44.3-54.5	29.7% ± 1.0% SL	36.2-50.8	20.9% ± 1.3% SL	101.2-122.1	41.9% ± 2.9% SL
Pre-orbital length (mm)	15.0-15.6	9.4% ± 1.1% SL	10.0-19.1	7.0% ± 1.2% SL	40.1-50.8	17.0% ± 1.8% SL
Eye diameter (mm)	9.2-11.1	6.1% ± 0.3% SL	10.6-14.3	6.1% ± 0.4% SL	16.2-26.3	8.1% ± 3.2% SL
Inter-orbital distance (mm)	19.3-20.8	12.2% ± 1.0% SL	14.3-21.2	8.9% ± 0.9% SL	47.6-48.6	18.1% ± 0.9% SL
Caudal peduncle depth (mm)	9.3-12.6	6.5% ± 0.0% SL	12.1-23.1	8.5% ± 1.4% SL	23.1-28.6	9.7% ± 0.8% SL

Table 5. The morphometrics of the alien crab specimens whose morphologies were entirely available and whose identities were confirmed by genetic analysis. Sexes and the presence of egg broods are both indicated as percentage proportions of *n*. Wet weights are inclusive of any egg broods that were present.
n = sample size; SD = standard deviation; F = female.

	Callinectes sapidus (***n*** **=2**)		***Percnon gibbesi*** (***n*** **=7**)		***Portunus segnis*** (***n*** **=5**)	
		mean ± SD		mean ± SD		mean ± SD
Wet weight (g)	157.9-164.7	161.3 ± 4.8	6.3-19.0	11.2 ± 4.3	70.0-200.0	152.0 ± 51.8
Maximum carapace width (mm)	134.3-149.2	141.8 ± 10.5	23.5-33.6	29.4 ± 4.0	108.7-137.4	122.6 ± 11.0
Maximum carapace length (mm)	57.1-60.5	58.8 ± 2.4	26.6-34.1	29.9 ± 2.7	48.0-65.7	56.6 ± 7.0
Abdomen width (mm)	45.1-49.0	47.1 ± 2.8	11.6-26.6	20.6 ± 5.3	37.8-49.0	44.3 ± 5.0
Right dactyl length (mm)	27.8-31.5	29.7 ± 2.6	3.4-9.1	6.0 ± 2.1	30.6-54.5	40.5 ± 9.3
Left dactyl length (mm)	30.6-36.9	33.8 ± 4.5	5.0-7.0	5.8 ± 0.7	34.8-53.3	42.7 ± 7.2
Right pollex length (mm)	56.2-62.3	59.3 ± 4.3	6.5-17.6	10.7 ± 4.2	67.0-125.4	94.2 ± 26.0
Left pollex length (mm)	59.4-69.4	64.4 ± 7.1	9.2-14.6	10.8 ± 2.0	67.3-125.4	90.8 ± 23.5
Right pollex width (mm)	10.3-14.7	12.5 ± 3.1	2.3-3.6	3.0 ± 0.5	12.2-15.8	14.0 ± 1.6
Left pollex width (mm)	11.9-14.8	13.4 ± 2.1	2.6-3.4	3.1 ± 0.3	10.6-17.3	13.9 ± 2.8
Right pollex depth (mm)	14.5-18.6	16.6 ± 2.9	4.0-10.3	6.2 ± 2.4	17.1-23.2	19.2 ± 2.5
Left pollex depth (mm)	15.5-17.6	16.6 ± 1.5	4.8-9.3	6.0 ± 1.7	13.3-21.0	17.3 ± 3.2
Right merus length (mm)	35.3-44.8	40.1 ± 6.7	7.1-19.6	11.8 ± 4.7	42.8-83.5	59.1 ± 16.6
Left merus length (mm)	43.5-43.8	43.7 ± 0.2	9.7-18.3	12.2 ± 3.1	42.4-81.1	58.5 ± 15.7
Sex	100% F 100% egg-bearing		57.1% F 42.9% egg-bearing		40% F 40% egg-bearing	

4. Discussion

4.1 Inferences on the genetic and morphological identifications

In the present study, the COI genetic marker proved capable of both recognising species belonging to vastly different phyla (from Cnidaria and Annelida to Arthropoda and Chordata) and of differentiating between closely related non-native species belonging to the same family (for instance, Portunidae) and even the same genus (for instance, *Siganus*). Ward *et al*. (2005), Radulovici *et al*. (2009), and Bariche *et al*. (2015) obtained comparable results when investigating the broad application of the COI gene in barcoding fish, crustaceans, and Lessepsian immigrants, respectively. Vella, Vella, Karakulak, *et al*. (2017) and Vella, Vella & Schembri (2017) obtained comparable results when investigating the more focused application of the COI gene in barcoding tetraodontids and elasmobranchs, respectively.

Use of this genetic approach was of particular importance to this project in specific instances where morphologies were either unavailable, unpreserved, uninformative, or too ambiguous. For instance, 3 crab specimens suspected to be of alien

origin were only known from single pereiopods and DNA barcoding was able to recognise that the pereiopods belonged to the alien *Portunus segnis* and the native *Achelous hastatus* despite the fact that traditional morphological identification of these species is heavily dependent on the observation of other anatomical structures like the carapace, the cheliped and the abdomen (Bariche, 2012; Rodrigues *et al.*, 2017). Single specimens of jellyfish, fireworms and sea squirts, all of which are soft-bodied, quickly lost much of their original shape once taken out of the water and this effectively prevented the thorough inspections that were required for their morphological identification. Still, DNA barcoding was able to identify these specimens as the alien *Rhopilema nomadica*, the native *Hermodice caranculata*, and the alien *Herdmania momus*, respectively. Furthermore, the fish specimens that were collected lost some of their original colourations after death and more so after freezing and thawing. This is problematic because colouration is important for the morphological identification of many fish species (Vella *et al.*, 2017), including *Pomadasys incisus*, *Kyphosus vaigiensis*, and especially *Siganus luridus* and *S. rivulatus* (Bariche, 2012; Vella *et al.*, 2023). DNA barcoding was thus necessary in this regard in order to overcome the ambiguity that is associated with the interpretation of such diagnostic characters that are so difficult to preserve.

The genetic approach was also important in other instances where morphological keys and descriptions proved either ineffective or unreliable. For example, morphological identification of the alien crab *Callinectes sapidus* is in part dependent on the observation of the male abdomen that is characteristically in the shape of an 'inverted T' (Bariche, 2012). The 2 specimens of this species that were collected were both females, and as such their identities could only be fully confirmed by way of genetic analyses. Furthermore, one of the main morphological characteristics that distinguishes the swimmer crab genera *Achelous* and *Portunus* is the carapace length-to-width ratio, and yet this difference is reportedly not fully consistent (Rodrigues *et al.*, 2017). Therefore, genetic analysis was also imperative to distinguish the single specimen of *Achelous hastatus* from the rest of the swimmer crab specimens that were collected.

Lastly, use of the genetic approach was of importance to this project in several instances where the morphological differences between closely related species were

small and not so apparent. In fact, all the specimens that were ultimately confirmed to be native to Maltese waters were investigated on account of their close resemblances to species of alien origin known to occur within the Mediterranean Sea. For example, the native sea urchin *Stylocidaris affinis* that was collected resembles the confamilial alien *Eucidaris tribuloides*, and the native top shell *Steromphala rarilineata* that was collected resembles the congeneric alien *Steromphala cineraria*. Therefore, the identification of these specimens was facilitated by and largely dependent on the use of a genetic approach. This same approach was of even greater importance in extreme scenarios where specimen morphologies were homogenous to such an extent that they qualify as cryptic. For instance, the confamilial alien swimmer crabs *Portunus segnis* and *Callinectes sapidus* are morphologically distinguished by the presence or absence of medial spines on their chelipeds. Similarly, the congeneric alien rabbitfish *Siganus luridus* and *S. rivulatus* are distinguished by whether their tails are truncated or forked (Bariche, 2012). In fact, a number of local records of *Portunus segnis* were at first erroneously attributed to *Callinectes sapidus* (Crocetta *et al.*, 2015) and a number of records of *Siganus luridus* were at first erroneously attributed to *Siganus rivulatus* (Schembri *et al.*, 2012). These errors were in part facilitated by a reliance on only one method of species identification, and highlight in turn the need for scientific studies to employ more diverse toolboxes in order to improve the accuracy of their identifications.

Regardless of how practical DNA barcoding can be, however, it remains user dependent. As became evident throughout this project, the technique is highly sensitive to contaminations. Unless sterilisation protocols are strictly adhered to and opportunities for cross-contamination are heavily diminished, contaminant DNA can easily be sequenced (despite the use of controls), resulting in turn in the interpretation of misleading genetic data. It is for this reason that voucher specimens and their associated morphological data are needed for the corroboration of genetic identifications and why the importance of employing an integrated approach is emphasised. In the words of Schander & Willassen (2005), "barcodes are the 'essence' of species identities no more than taxonomic holotypes are 'the species'. It makes no sense to think that morphology and other biological information about organisms can be made obsolete by barcode systems." Moreover, the integration of morphological data with genetic data has the

potential to reveal and corroborate novel morphological patterns that can be employed in distinguishing between species.

4.2 Inferences on population structures and invasion histories

DNA barcoding also allows for inferences on population structures to be made in scenarios where the species that are sequenced are represented by numerous specimens. In scenarios involving alien species, further inferences relating to invasion histories can be made.

The 7 *Portunus segnis* specimens presented here shared 2 haplotypes, 1 of which was more dominant. This low haplotype diversity is in agreement with the findings of Lai, Ng & Davie (2010) from Israel. It may be indicative of the genetic bottleneck effect that is observed in many alien species that establish new invasive populations from much smaller founder populations and that rapidly expand their distributions (Fratini *et al.*, 2016). A narrower gene pool is typically associated with poorer population health, provided that it renders populations more vulnerable to possible environmental changes, yet *Portunus segnis* still managed to establish itself in abundance within local waters (Katsanevakis *et al*., 2020).

In contrast, the 7 *Percnon gibbesi* specimens presented here each had their own unique haplotype. This remarkably high genetic diversity indicates that the species' local population is probably not suffering from a genetic bottleneck and is well equipped to cope with possible environmental changes. Confirmation of such an inference requires, however, further testing with microsatellite data (Zachariah *et al.*, 2012) and comparison with other populations of the species. Alternatively, the high genetic diversity could suggest that the species was introduced to Maltese waters on more than one occasion, or even that multiple vectors were responsible for its introduction into the Mediterranean. In fact, multiple vectors of introduction have been speculated for the species, namely shipping, larval drift by the Atlantic surface current (in which case the species would qualify as a range expander rather than an alien), and the aquarium trade (Katsanevakis *et al*., 2011). The high haplotype diversity observed in this project could very well suggest that *Percnon gibbesi* found itself in the Mediterranean through a combination of these vectors.

The 14 *Siganus luridus* specimens presented here shared 4 haplotypes, 1 of which was more dominant. A high haplotype diversity was anticipated on account of the findings of Vella *et al*. (2023) from Maltese waters and of Hassan, Harmelin-Vivien & Bonhomme (2003) and Azzurro, Golani, Bucciarelli & Bernardi (2006) from other parts of the Mediterranean. These authors suggested in turn that the Mediterranean invasion of the species could have involved multiple founder individuals. This genetic diversity could also indicate that *Siganus luridus*' local population is, much like that of *Percnon gibbesi*, probably well equipped to cope with future environmental changes.

It must be acknowledged, however, that the results obtained in this project only provide the first insights into these species's genetic diversities. The use of larger sample sizes and of other genetic markers may indeed reveal differing levels of genetic diversities (Fratini *et al.*, 2016), thereby suggesting population structures and invasion histories that differ from what has been inferred here.

4.3 Further considerations

DNA barcodes can highlight valuable information below the species level when geographical variation is present. In the context of managing marine invasions, this data can help to pinpoint both the exact source of an invasive species' introduction into a new region and the pathway it subsequently used to disperse itself in it. This information can, in turn, inform important management decisions and priorities, and possibly even aid to circumvent invasions altogether (Gozlan *et al.*, 2010; Bariche *et al.*, 2015). However, markers that mutate at a faster rate than the COI gene are better suited to track species' phylogeographic connections as these display greater degrees of intraspecific divergences. One such marker that carries a stronger phylogeographic signal is the mtDNA control region which has already been widely used in population studies of various fish species (Vella *et al.*, 2017).

eDNA metabarcoding is a novel molecular approach that involves the sequencing of DNA present in environmental samples like water and sediment. It is capable of identifying multiple species at once without requiring the organisms in question to be physically encountered. Thus, it is not difficult to envision this revolutionary technique's strong implementation in future efforts tasked with monitoring marine invasions. In this regard, metabarcoding has the potential to allow for detections to be made at even earlier

stages, in turn allowing for preventative and management actions to be more rapid and effective. However, the technique is still in development and concerns about its applicability have been raised (Kress *et al.*, 2015; Ruppert, Kline & Rahman, 2019).

4.4 Concluding remarks

Ultimately, this project has demonstrated the capacity of the COI gene to identify the extant metazoan diversity and has thereby suggested its prospective application in regimes aimed at monitoring and preventing marine invasions. The integration of genetic and morphological lines of evidence throughout this project produced a more robust approach that consistently guided the research undertaken towards accurate specimen identification. Genetic analysis was especially valuable for species identification in instances where specimen morphologies were lost, deteriorated, uninformative, or cryptic. Thus, genetics truly promises to become a valuable expertise in the field of monitoring marine invasions, especially considering how the local taxonomic expertise may alone be insufficient to ascertain the morphological identification of certain non-native species (Vella *et al.*, 2017).

Despite the integrated approach's improved accuracy, informative nature and broad applicability, its benefits come at the cost of: the longer processing times needed to extract, amplify, sequence and process the genetic data; the need for highly expensive equipment, namely ultracentrifuges and thermocyclers; and the need for technical skill in performing the requisite laboratory work. Still, the importance of correct species identifications in monitoring efforts, as well as the need to avert the propagation of identification errors in subsequent research, cannot be understated. It is for these reasons that it is strongly recommended that both morphological and genetic data be taken into consideration when making species identifications.

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6. Appendices

Appendix 1 - The collection details of the identified specimens

Reference Number	*Specimen Description**	*Confirmed Specimen Identity*	*Source and Date of Collection or Provision*
YS70	doctorfish	*Acanthurus monroviae*	Provided by CBRG-UM on 21-10-2022
YS01	crab pereiopod	*Achelous hastatus*	Collected from Imgiebah Bay on 17-09-2022
YS12	crab	*Achelous hastatus*	Collected from Delimara on 21-07-2022
YS54	crab	*Callinectes sapidus*	Provided by CBRG-UM on 19-10-2022
YS55	crab	*Callinectes sapidus*	Provided by CBRG-UM on 19-10-2022
YS38	scad	*Caranx crysos*	Collected from Marsaxlokk on 01-10-2022
YS39	scad	*Caranx crysos*	Collected from Marsaxlokk on 01-10-2022
YS40	scad	*Caranx crysos*	Collected from Marsaxlokk on 01-10-2022
YS44	blenny	*Clinitrachus* sp.	Collected from Birzebbuga on 01-10-2022
YS22	sea squirt	*Herdmania momus*	Collected from Marsaxlokk on 10-09-2022
YS13	fireworm	*Hermodice carunculata*	Collected from Munxar, Zejtun on 12-07-2022
YS24	fireworm	*Hermodice carunculata*	Collected from Marsaxlokk on 09-08-2022
YS25	fireworm	*Hermodice carunculata*	Collected from Marsaxlokk on 07-07-2022
YS26	fireworm	*Hermodice carunculata*	Collected from Marsaxlokk on 07-07-2022
YS69	chub	*Kyphosus vaigiensis*	Provided by CBRG-UM on 21-10-2022
YS74	pufferfish	*Lagocephalus scleratus*	Provided by CBRG-UM on 25-10-2022
YS05	crab	*Percnon gibbesi*	Collected from Saint George's Bay, Birzebbuga on 18-09-2022
YS06	crab	*Percnon gibbesi*	Collected from Saint George's Bay, Birzebbuga on 18-09-2022
YS56	crab	*Percnon gibbesi*	Provided by CBRG-UM on 19-10-2022
YS57	crab	*Percnon gibbesi*	Provided by CBRG-UM on 19-10-2022
YS65	crab	*Percnon gibbesi*	Provided by CBRG-UM on 20-10-2022
YS66	crab	*Percnon gibbesi*	Provided by CBRG-UM on 20-10-2022
YS68	crab	*Percnon gibbesi*	Provided by CBRG-UM on 20-10-2022
YS07	bivalve	*Pinctada radiata*	Collected from Saint George's Bay, Birzebbuga on 18-09-2022
YS21	bivalve	*Pinctada radiata*	Collected from Sliema Creek, Manoel Island on 13-09-2022
YS47	bivalve	*Pinctada radiata*	Provided by CBRG-UM on 19-10-2022
YS49	bivalve	*Pinctada radiata*	Provided by CBRG-UM on 19-10-2022
YS75	grunt	*Pomadasys incisus*	Provided by CBRG-UM on 26-10-2022
YS15	crab	*Portunus segnis*	Collected from Marsaxlokk on 17-07-2022
YS16	crab	*Portunus segnis*	Collected from Marsaxlokk on 18-07-2022
YS17	crab	*Portunus segnis*	Collected from Marsaxlokk on 19-07-2022
YS32	crab	*Portunus segnis*	Collected from Delimara on 29-09-2022
YS73	crab	*Portunus segnis*	Provided by CBRG-UM on 25-10-2022
YS58	crab pereiopod	*Portunus segnis*	Provided by CBRG-UM on 20-10-2022
YS59	crab pereiopod	*Portunus segnis*	Provided by CBRG-UM on 20-10-2022
YS85	jellyfish	*Rhopilema nomadica*	Provided by CBRG-UM on 01-11-2022
YS10	blenny	*Scartella cristata*	Collected from Exiles Beach, Saint Julian's Bay on 21-09-2022
YS11	blenny	*Scartella cristata*	Collected from Exiles Beach, Saint Julian's Bay on 22-09-2022
YS71	amberjack	*Seriola fasciata*	Provided by CBRG-UM on 21-10-2022
YS72	amberjack	*Seriola fasciata*	Provided by CBRG-UM on 25-10-2022
YS28	rabbitfish	*Siganus luridus*	Collected from Delimara on 23-07-2022
YS29	rabbitfish	*Siganus luridus*	Collected from Gnejna on 29-07-2022
YS30	rabbitfish	*Siganus luridus*	Collected from Gnejna on 29-07-2022
YS31	rabbitfish	*Siganus luridus*	Collected from Gnejna on 29-07-2022
YS60	rabbitfish	*Siganus luridus*	Provided by CBRG-UM on 20-10-2022
YS61	rabbitfish	*Siganus luridus*	Provided by CBRG-UM on 20-10-2022
YS62	rabbitfish	*Siganus luridus*	Provided by CBRG-UM on 20-10-2022
YS63	rabbitfish	*Siganus luridus*	Provided by CBRG-UM on 20-10-2022
YS64	rabbitfish	*Siganus luridus*	Provided by CBRG-UM on 20-10-2022
YS80	rabbitfish	*Siganus luridus*	Provided by CBRG-UM on 26-10-2022
YS81	rabbitfish	*Siganus luridus*	Provided by CBRG-UM on 26-10-2022
YS82	rabbitfish	*Siganus luridus*	Provided by CBRG-UM on 26-10-2022
YS83	rabbitfish	*Siganus luridus*	Provided by CBRG-UM on 26-10-2022
YS27	rabbitfish	*Siganus rivulatus*	Collected from Delimara on 23-07-2022
YS77	pufferfish	*Sphoeroides pachygaster*	Provided by CBRG-UM on 26-10-2022
YS84	pufferfish	*Sphoeroides pachygaster*	Collected from 70 nm SE off Malta on 12-10-2022
YS19	gastropod	*Steromphala rarilineata*	Collected from Rinella Bay on 15-09-2022
YS53	sea urchin	*Stylocidaris* sp.	Provided by CBRG-UM on 19-10-2022
YS34	blenny	*Tripterygion tripteronotum*	Collected from Birzebbuga on 01-10-2022

* Unless otherwise indicated, the specimens were whole.

Appendix 2.1 - The raw morphometric data of the identified newcomer crab specimens

Reference Number	*Specimen identity*	*Wet Weight (g)* *	*Maximum Carapace Width (mm)*	*Maximum Carapace Length (mm)*	*Abdomen Width (mm)*	*Dactyl Length (R) (mm)*	*Dactyl Length (L) (mm)*	*Pollex Length (R) (mm)*	*Pollex Length (L) (mm)*	*Pollex Width (R) (mm)*	*Pollex Width (L) (mm)*	*Pollex Depth (R) (mm)*	*Pollex Depth (L) (mm)*	*Merus Length (R) (mm)*	*Merus Length (L) (mm)*	*Sex*
YS54	*Callinectes sapidus*	164.7	149.2	60.5	49.0	27.8	36.9	56.2	69.4	10.3	14.8	14.5	17.6	35.3	43.8	F (egg-bearing)
YS55	*Callinectes sapidus*	157.9	134.3	57.1	45.1	31.5	30.6	62.3	59.4	14.7	11.9	18.6	15.5	44.8	43.5	F (egg-bearing)
YS05	*Percnon gibbesi*	19.0	33.0	28.7	17.2	9.1	7.0	17.6	14.6	3.3	3.3	10.3	9.3	19.6	18.3	M
YS06	*Percnon gibbesi*	10.2	23.5	29.8	11.6	3.4	5.0	6.5	9.8	2.3	3.3	4.0	5.6	7.1	11.7	M
YS56	*Percnon gibbesi*	10.2	31.1	31.1	25.6	6.5	5.6	10.2	10.6	3.6	3.4	5.8	5.6	10.2	11.4	F (egg-bearing)
YS57	*Percnon gibbesi*	10.8	32.0	31.6	26.6	5.5	5.7	10.9	11.3	3.3	3.1	5.8	5.8	12.3	11.8	F (egg-bearing)
YS65	*Percnon gibbesi*	7.5	26.3	27.1	23.0	5.3	5.4	8.5	9.3	2.6	2.9	4.9	4.8	9.8	9.7	F
YS66	*Percnon gibbesi*	6.3	26.4	26.6	22.4	m.l.	6.1	m.l.	9.2	m.l.	2.6	m.l.	4.8	m.l.	10.2	F (egg-bearing)
YS67	*Percnon gibbesi*	14.5	33.4	34.7	17.7	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	M
YS68	*Percnon gibbesi*	14.6	33.6	34.1	18.1	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	M
YS15	*Portunus segnis*	170.0	120.4	59.0	45.1	44.4	45.6	104.5	104.0	14.5	16.1	17.1	19.1	68.1	67.6	M
YS16	*Portunus segnis*	185.0	137.4	65.7	49.0	54.5	53.3	125.4	125.4	15.0	17.3	20.0	21.0	83.5	81.1	M
YS17	*Portunus segnis*	200.0	129.0	59.1	48.9	35.4	34.8	79:6	79.0	15.8	13.0	23.2	18.5	47.3	48.4	F (egg-bearing)
YS32	*Portunus segnis*	135.0	117.4	51.4	40.6	30.6	42.0	67.0	67.3	12.4	12.4	18.5	14.8	42.8	42.4	F (egg-bearing)
YS73	*Portunus segnis*	70.0	108.7	48.0	37.8	37.8	37.9	80.0	78.4	12.2	10.6	17.4	13.3	54.0	53.1	M
YS58	*Portunus segnis*	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	?
YS59	*Portunus segnis*	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	m.l.	?

* Where present, wet weights are inclusive of egg broods.
R = right; L = left; m.l. = specimen's morphology was lost; M = male specimen; F = female specimen; ? = specimen sex unknown.

Appendix 2.2 - The raw meristic data of the identified newcomer fish specimens

Reference Number	*Specimen Identity*	*Procumbent Spines*	*Dorsal Fin Hard Spines*	*Dorsal Fin Soft Rays*	*Pectoral Fin Hard Spines*	*Pectoral Fin Soft Rays*	*Pelvic Fin Hard Spines*	*Pelvic Fin Soft Rays*	*Anal Fin Hard Spines*	*Anal Fin Soft Rays*	*Caudal Fin Soft Rays*
YS70	*Acanthurus monroviae*	N/A	IX	25	N/A	16	I	5	II	23	22
YS69	*Kyphosus vaigiensis*	N/A	XI	13	N/A	20	I	5	III	12	18
YS74	*Lagocephalus sceleratus*	N/A	m.a.	11	N/A	17	N/A	0	N/A	11	15
YS75	*Pomadasys incisus*	N/A	XII	17	N/A	17	I	5	III	12	17
YS71	*Seriola fasciata*	N/A	VIII + I	30	I	19	I	5	II + I	18	23
YS72	*Seriola fasciata*	N/A	VIII + I	29	I	19	I	5	II + I	17	21
YS28	*Siganus luridus*	I	XIII	10	N/A	15	I + I	3	VII	9	16
YS29	*Siganus luridus*	I	XIII	10	N/A	15	I + I	3	VII	9	18
YS30	*Siganus luridus*	I	XIII	10	N/A	16	I + I	3	VII	9	17
YS31	*Siganus luridus*	I	XIII	10	N/A	15	I + I	3	VII	9	17
YS60	*Siganus luridus*	I	XIII	10	N/A	17	I + I	3	VII	9	19
YS61	*Siganus luridus*	I	XIII	10	N/A	17	I + I	3	VII	9	19
YS62	*Siganus luridus*	I	XIII	10	N/A	16	I + I	3	VII	9	19
YS63	*Siganus luridus*	I	XIII	10	N/A	16	I + I	3	VII	9	19
YS64	*Siganus luridus*	I	XIII	10	N/A	17	I + I	3	VII	9	20
YS80	*Siganus luridus*	I	XIII	10	N/A	17	I + I	3	VII	9	19
YS81	*Siganus luridus*	I	XIII	10	N/A	17	I + I	3	VII	9	18
YS82	*Siganus luridus*	I	XIII	10	N/A	16	I + I	3	VII	9	18
YS83	*Siganus luridus*	I	XIII	10	N/A	17	I + I	3	VII	9	18
YS27	*Siganus rivulatus*	I	XIII	10	N/A	16	I + I	3	VII	9	20
YS77	*Sphoeroides pachygaster*	N/A	N/A	9	N/A	14	N/A	0	N/A	9	9
YS84	*Sphoeroides pachygaster*	N/A	N/A	9	N/A	15	N/A	0	N/A	N/A	N/A

N/A = the field is not applicable to the specimen.

Appendix 2.3: The raw morphometric data of the identified newcomer fish specimens

Reference Number	*Specimen Identity*	*Wet Weight (g)*	*Total Length (mm)*	*Fork Length (mm)*	*Standard Length (mm)*	*Maximum Body Depth (mm)*	*Dorsal Fin Length (mm)*	*Pectoral Fin Length (mm)*	*Anal-Fin Base Length (mm)*	*Pre-pelvic Length (mm)*	*Pre-pectoral Length (mm)*	*Pre-anal Length (mm)*	*Head Length (mm)*	*Pre-orbital Length (mm)*	*Eye Diameter (mm)*	*Inter-orbital Distance (mm)*	*Caudal Peduncle Depth (mm)*
YS70	*Acanthurus monroviae*	200.0	229.0	216.5	184.0	77.5	117.2	35.1	81.8	46.8	43.0	77.4	45.3	25.3	11.6	20.8	17.4
YS69	*Kyphosus vaigiensis*	850.0	362.0	330.0	295.0	126.5	147.2	52.6	73.1	84.0	63.8	173.0	68.5	18.9	13.5	34.1	42.4
YS74	*Lagocephalus sceleratus*	115.0	201.0	193.0	177.0	36.5	13.5	26.0	8.2	N/A	52.4	110.5	59.7	23.5	14.9	23.3	7.1
YS75	*Pomadasys incisus*	80.0	177.0	166.5	146.5	54.4	68.8	42.4	21.4	48.3	46.9	95.6	44.3	11.1	12.4	18.5	16.1
YS71	*Seriola fasciata*	230.0	239.0	213.0	193.0	72.6	23.6 + 86.7	26.7	54.4	62.2	56.7	122.5	54.5	15.0	11.1	20.8	12.6
YS72	*Seriola fasciata*	95.0	180.0	158.5	142.0	53.8	18.5 + 61.4	23.4	37.5	49.6	47.2	88.4	44.3	15.6	9.2	19.3	9.3
YS28	*Siganus luridus*	190.0	221.0	N/A	182.0	76.4	131.4	32.8	70.7	47.2	35.9	93.7	37.8	13.9	12.0	14.3	12.1
YS29	*Siganus luridus*	285.0	256.0	N/A	213.0	90.5	148.1	32.1	83.2	44.1	36.0	102.6	39.2	10.0	13.5	16.9	14.3
YS30	*Siganus luridus*	260.0	242.0	N/A	202.0	90.0	146.5	33.2	77.7	43.5	37.8	95.1	41.0	11.6	13.3	17.8	14.5
YS31	*Siganus luridus*	375.0	270.0	N/A	226.0	104.1	160.0	37.2	86.7	55.6	44.2	115.2	50.8	16.7	13.9	19.8	15.3
YS60	*Siganus luridus*	270.0	225.0	N/A	185.0	86.9	127.7	32.3	70.3	38.8	34.2	90.0	37.4	11.8	11.4	19.5	17.4
YS61	*Siganus luridus*	460.0	279.0	N/A	232.0	104.1	160.0	34.6	83.6	55.5	48.3	123.0	49.6	19.1	14.3	20.7	23.1
YS62	*Siganus luridus*	425.0	259.0	N/A	217.0	101.2	146.7	34.5	86.1	47.3	41.9	113.5	48.2	18.3	12.7	21.2	17.0
YS63	*Siganus luridus*	165.0	209.0	N/A	177.0	74.1	125.4	29.8	69.1	46.5	37.4	89.5	39.3	15.5	11.4	18.0	18.5
YS64	*Siganus luridus*	320.0	252.0	N/A	210.0	92.0	149.2	34.9	79.3	55.0	36.6	110.0	46.4	15.5	12.3	17.9	17.6
YS80	*Siganus luridus*	245.0	235.0	N/A	196.0	77.5	139.4	31.1	79.4	45.1	34.5	91.1	36.2	12.6	10.6	16.5	17.2
YS81	*Siganus luridus*	385.0	270.0	N/A	223.0	95.2	157.0	41.6	86.6	55.2	44.4	112.7	46.2	13.4	12.3	16.6	21.5
YS82	*Siganus luridus*	330.0	255.0	N/A	210.5	92.6	148.0	34.2	77.2	57.3	44.0	106.6	45.3	15.5	12.9	19.0	20.7
YS83	*Siganus luridus*	325.0	253.0	N/A	213.0	98.8	150.0	41.2	87.3	51.4	44.3	105.2	45.5	14.0	12.6	19.0	20.0
YS27	*Siganus rivulatus*	195.0	240.0	226.0	203.0	71.0	144.2	21.6	82.5	50.2	38.7	101.1	39.8	15.5	9.7	13.0	10.1
YS77	*Sphoeroides pachygaster*	935.0	326.0	N/A	278.0	124.5	13.2	40.3	12.2	N/A	117.7	229.0	122.1	50.8	16.2	48.6	28.6
YS84	*Sphoeroides pachygaster*	530.0	275.0	N/A	254.0	101.1	13.5	45.2	10.2	N/A	104.5	199.5	101.2	40.1	26.3	47.6	23.1

N/A = the field is not applicable to the specimen.