Project Completion Report

Development of DNA mini-barcodes for forensic identification of molluscs species

2026

Tamil Nadu Forest Department
Advanced Institute for Wildlife Conservation
(Research, Training & Education)
Vandalur, Chennai

Funded by the State Planning Commission
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TAMIL NADU INNOVATION INITIATIVE (TANII) SCHEME OF STATE PLANNING COMMISSION (SPC)

Development of DNA mini-barcodes for Forensic identification of Molluscs species

Sanctioned in G.O.No.273 Environment, Climate Change and Forests (FR.5) Department dated 01.12.2022

Project duration: February 2023 – March 2025

Final report

SUBMITTED BY

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1. Project Completion Report

Government Order No	G.O.No.273 Environment, Climate Change and Forests (FR.5) Department dated 01.12.2022
Project Title	Development of DNA mini-barcodes for forensic identification of molluscs species
Duration	February 2023 to March 2025
Funded by	Tamil Nadu Innovation Initiatives, State Planning Commission
Project Budget	Rs. 33,00,000 /-
Implementing Centre	Centre for Wildlife Forensic Sciences, AIWC
Research Personnel	Ms. Madhumita Rajkumar, Senior Research Fellow
Principal Investigator	Principal Chief Conservator of Forest and Director Advanced Institute for Wildlife Conservation (Research, Training & Education), Tamil Nadu Forest Department, Vandalur – Kelambakkam road, Vandalur, Chennai – 600 048
Co-Principal Investigator	Nil
Collaborating organisation	Nil

2. Summary Report

Project objectives

S. No.	**Objectives**	**Fully Achieved / Partially achieved (indicate shortfall)**	**Reason for partial achievement**
1	Development of DNA mini-barcodes for forensic identification of marine molluscs species	Fully achieved	Nil
2	Creation of reference genetic database for molluscs	Fully achieved	Nil
3	Capacity building of enforcement department staff in molluscs identification to strengthen wildlife	Partially achieved	The fund for capacity building in the second year was not allotted.

Deliverables

A total of 289 sequences have been barcoded and submitted in National Center for Biotechnology Information (NCBI).

Specific Benefits/outcomes

i) Patent, if any: Nil

ii) **Product/Process developed/Technology transferred:** Two new species discovered through this project.

iii) Publications Under process.

iv) Linkages developed NIL

v) Manpower trained 1 No. of Senior Research Fellow

Summary of significant S&T achievements

Three sets of primers were designed and validated for mollusc species identification using nanopore sequencing.

Suggestion and utilisation of the project outcome

The project has significantly strengthened the Department’s capacity to monitor, investigate, and prosecute cases involving protected marine molluscs. The development of reference sequences, novel mini-barcodes, and a portable Nanopore-based sequencing workflow has enhanced the Department’s forensic capabilities, enabling on-site and rapid species verification. Additionally, targeted training and awareness programs for field officers, legal practitioners, and forensic personnel have improved interdepartmental coordination and responsiveness.

Financial utilisation

Component	**Amount Sanctioned for 1st year (Rs.)**	**Amount Sanctioned for 2nd year (Rs.)**	**Total Amount sanctioned (Rs.)**	**Amount spent up to March 2023 (Rs.)**	**Amount spent up to March 2024 (Rs.)**	**Amount spent up to March 2025 (Rs.)**	**Total amount spent (Rs.)**
Senior Research Fellow (1 Person)	4,20,000/-	4,20,000/-	8,40,000/-	61,000	4,18,793/-	3,59,000/-	8,38,793/-
Equipment - Mini ION Mk1C portable sequencer (1 No.)	3,00,000/-	0	3,00,000/-	0	0	3,00,000/-	3,00,000/-
Consumables for molecular analysis	7,50,000/-	7,50,000/-	15,00,000/-	0	7,22,307/-	7,50,000/-	14,72,307/-
Consultation/Expert fee	50,000/-	50,000/-	1,00,000/-	0	0	50,000/-	50,000/-
Travel	1,50,000/-	1,50,000/-	3,00,000/-	0	1,50,000/-	1,49,906/-	2,99,906/-
Training	0	3,00,000/-	3,00,000/-	0	2,71,901/-	0	2,71,901/-
Miscellaneous costs	0	50,000/-	50,000/-	0	32,281/-	0	32,281/-
Total	**16,70,000/-**	**17,20,000/-**	**33,90,000/-**	**61,000/-**	**15,95,282/-**	**16,08,906/-**	**32,65,188/-**

3. Introduction

Tamil Nadu harbours a huge diversity of marine fauna and flora with a coastline of about 1,076 km. The Government of Tamil Nadu took pioneering steps to declare the Gulf of Mannar as the first Marine National Park in 1986 and later as the Gulf of Mannar Biosphere Reserve (GOMBR), Asia's first marine biosphere reserve in 1989, for sustainable development with an integrated approach of conserving the unique marine biodiversity

Phylum Mollusca (including marine shells, octopus, sepia, limpets, and elephant tusk shells) represent the largest marine category with about 23 per cent of all named marine organisms and the most diverse species inhabiting coastal waters. Because of their diversity, molluscs are better indicators of marine biodiversity and ecosystem health. The marine molluscan fauna of India includes about 3,370 species, of which 9 are included in Schedule I, and 15 are included in Schedule II of the Indian Wildlife (Protection) Act, 1972. The protected species include the horned helmet shell (*Cassis cornuta*), Trumpet triton (*Charonia tritonis*), "Glory of India" cone shell (*Conus millnedwardsii*), Red Helmet (*Cypraecassis rufa*), Emperor nautilus *(Nautilus pompilus*), Cowries and spider conches **(Table 1)**. As many as eight varieties of oyster, two species of mussel, 17 species of clam, six species of pearl oyster, four species of giant clam and one species of window-pane oyster, as well as gastropods such as *Turbinella pyrum*, *Trochus* spp., *Turbo* spp., and *Babylonia* spp., and 15 species of cephalopods are among the commercially exploited species of molluscs in India.

Table 1: Protected Molluscs of India

Schedule-I of the Indian Wildlife (Protection) Act, 1972

S.No	**Scientific Name**	**தமிழ் பெயர்**	**Common Name**	**Class**	**Sub-Class**	**Order**	**Family**	**Maximum Depth (m) at which present**
1	*Cassis cornuta*	*மாட்டுத்தலை சங்கு*	Horned Helmet	Gastropoda	Caenogastropoda	Littorinimorpha	Cassidae	100
2	*Cypraecassis rufa*	*அன்னாசிபழ சங்கு*	Bull Mouth Helmet	Gastropoda	Caenogastropoda	Littorinimorpha	Cassidae	100
3	*Charonia tritonis*	*தவளை சங்கு*	Triton's Trumpet Shell	Gastropoda	Caenogastropoda	Littorinimorpha	Charoniidae	50
4	*Tudicla spirillus*	*வெள்ளை பூண்டு சங்கு*	Spiral Tudicla	Gastropoda	Caenogastropoda	Neogastropoda	Tudiclidae	30
5	*Conus milneedwardsi*	*வாழைப்பூ சங்கு*	Glory of India	Gastropoda	Caenogastropoda	Neogastropoda	Conidae	200
6	*Nautilus pompilius*	*யானைக்கை கணவாய்*	Emperor Nautilus	Cephalopoda	Nautiloidea	Nautilida	Nautilidae	500
7	*Tridacna maxima*	*பெரிய மட்டி*	Small Giant Clam	Bivalvia	Autobranchia	Cardiida	Cardiidae	30
8	*Tridacna squamosa*	*குழிவான பெரிய சிப்பி*	Fluted Giant Clam	Bivalvia	Autobranchia	Cardiida	Cardiidae	30
9	*Hippopus hippopus*	*பெரிய பால் சிப்பி*	Horse's Hoof clam	Bivalvia	Autobranchia	Cardiida	Cardiidae	50

Schedule-II of the Indian Wildlife (Protection) Act, 1972

S. No	**Scientific Name**	**தமிழ் பெயர்**	**Common Name**	**Class**	**Sub-Class**	**Order**	**Family**	**Maximum Depth (m) at which present**
10	*Pleuroploca trapezium*	*குதிரை முள்ளி சங்கு*	Trapezium Horse conch	*Gastropoda*	*Caenogastropoda*	Neogastropoda	Fasciolariidae	100
11	*Harpulina arausiaca*	*சிவப்பு வரி சங்கு*	Gold Banded Volute	Gastropoda	Caenogastropoda	Neogastropoda	Volutidae	40
12	*Lambis crocata*	*எட்டுவிரல் சங்கு*	Orange spider conch	Gastropoda	Caenogastropoda	Littorinimorpha	Strombidae	20
13	*Lambis millepeda*	*பூரான் சங்கு*	Millepede Spider Conch	Gastropoda	Caenogastropoda	Littorinimorpha	Strombidae	30
14	*Lambis indomaris*	*தேள் சங்கு*	Scorpio Spider Conch	Gastropoda	Caenogastropoda	Littorinimorpha	Strombidae	30
15	*Lambis truncata*	*வெள்ளை விரிஞ்சான் சங்கு*	Truncate Spider Conch	Gastropoda	Caenogastropoda	Littorinimorpha	Strombidae	15
16	*Placuna placenta*	*வெண் பதக்க சிப்பி*	Windowpane Oyster	Bivalvia	Autobranchia	Pectinida	Placunidae	30

S. No	**Scientific Name**	**தமிழ் பெயர்**	**Common Name**	**Class**	**Sub-Class**	**Order**	**Family**	**Maximum Depth (m) at which present**
17	*Turbo marmoratus*	*பாகை சங்கு*	Green Turban	Gastropoda	Vetigastropoda	Trochida	Turbinidae	30
18	*Harpago arthriticus*	*சிலந்தி சங்கு*	Arthritic spider conch	Gastropoda	Caenogastropoda	Littorinimorpha	Strombidae	15
19	*Harpago chiragra*	*ஐவிரல் சங்கு*	Chiragra spider conch	Gastropoda	Caenogastropoda	Littorinimorpha	Strombidae	20
20	*Dolomena sibbaldii*	*விரிஞ்சான் சங்கு*	Sibald's conch	Gastropoda	Caenogastropoda	Littorinimorpha	Strombidae	100
21	*Staphylaea limacina*	*பழுப்பு சோவி*	Limacina Cowrie	Gastropoda	Caenogastropoda	Littorinimorpha	Cypraeidae	20
22	*Leporicypraea mappa*	*செம்பட சோவி*	Map Cowrie	Gastropoda	Caenogastropoda	Littorinimorpha	Cypraeidae	30
23	*Talparia talpa*	*மூவரி சோவி*	Mole Cowrie	Gastropoda	Caenogastropoda	Littorinimorpha	Cypraeidae	30
24	*Tectus niloticus*	-	Commercial Top Shell	Gastropoda	Vetigastropoda	Trochida	Tegulidae	30

Huge quantities of shells are harvested as bycatch by fishing trawlers, which most often throng illegal trade markets to meet national and international demands for use as food, lampshades, cutlery, jewellery, furniture, decorative items and souvenirs (Nijman, 2019). The Gulf of Mannar and Palk Bay are the major areas for the collection and supply of such seashells in Tamil Nadu and the proximity of seashell processing industries is assumed to be a major reason for this occurrence. Apart from local procurement, seashells were also imported from Sri Lanka, Mexico, China and Tanzania using air and sea routes through Chennai, Kochi and Trivandrum. Kanyakumari, Rameswaram, Mahabalipuram, Pondicherry and Chennai being the important tourist spots, are identified as major marine curio markets (John, 2012). Due to a lack of awareness among the fishing community about the protection & conservation of marine molluscs, and a lack of an organized cum licensed system for shell trade and huge demand in the pilgrimage and tourist hotspots, their unsustainable and frequent illegal procurement continues. In 2019, India ranked as the second largest exporter, accounting for 6.36% of mollusc trade in the world with a value of $690 million, which marks the volume of harvest in India as a trader of marine molluscs. It is, therefore, very crucial to monitor the trade of protected species, which could ultimately help curb illicit trade and aid in the conservation of the species.

DNA barcoding is an accurate, rapid, cost-effective and universally accessible DNA-based species identification tool, and is also authenticated by the United Nations Convention on Biological Diversity (CBD) as an efficient method of documenting the diversity of species in ecosystems. However, DNA barcoding studies on marine molluscs, either in its broad sense or aimed at attesting specimen identification success, are relatively few. The DNA barcoding technique is very competent in the assessment of cryptic mollusc species that are morphologically identical but genetically dissimilar (Ali et al. 2014), and there have been several reported studies of the application of DNA barcoding for marine mollusc species identification (Barco et al., 2016, Bursic et al., 2021). As commercially traded seashells are commonly treated with mild acids and oils, DNA extraction using conventional barcodes, as demonstrated in the literature, becomes a challenging task. This problem can be overcome by the development of mini-barcodes, typically amplifying a shorter region of gene in size, informative and capable of distinguishing the species from one another.

The project aims to: a) develop primers, which will help in the standardisation of DNA isolation protocol from processed seashells, b) amplify the mini-barcode region, and c) determine the DNA sequence using a portable nano-pore sequencer. Apart from providing precise information on the taxonomy of molluscs, the data would act as important tool in marine wildlife forensics with the created genetic referral library.

3.1 Objectives

1. Development of DNA mini-barcodes for forensic identification of marine molluscs species
2. Creation of a reference genetic database of marine molluscs species
3. Capacity building of enforcement department staff in molluscs identification to strengthen wildlife crime investigations.

4. Review of literature

4.1. DNA barcoding of molluscs

DNA barcoding for species identification in forensic science plays a pivotal role where traditional morphological methods fall short (Zhang *et al*., 2016). DNA barcoding utilizes short, standardized gene regions as unique molecular markers that facilitate rapid and accurate identification of species, even from degraded or fragmented samples. (Aquino *et al.*, 2011). Phylum Mollusca is the second largest phylum in kingdom Animalia with over 85,000 recognized species.

DNA barcoding has emerged as a valuable tool in molluscan research, impacting fields like biodiversity assessment, evolutionary studies, and forensic science (Debenedetti *et al*., 2014; Puillandre *et al*., 2009; Zou & Li, 2016). Studies have utilized DNA barcoding to reveal patterns of molluscan diversity, gastropod diversity, and even to identify gastropod during developmental stages (Barco *et al.,* 2014; Borges *et al*., 2016; Debenedetti *et al.*, 2014; Layton *et al*., 2014; Puillandre *et al*., 2009a, 2009b; Radulovici *et al.*, 2009; Sun *et al.*, 2016; Zou & Li, 2016). DNA barcoding extends to molecular phylogeny and evolutionary studies, offering insights into complex molluscan groups (Borges *et al*., 2016; Layton *et al*., 2014; Lin *et al*., 2015; Ran *et al*., 2020). Furthermore, in forensics, DNA barcoding plays a crucial role, aiding in the identification of illegally harvested or endangered species (Debenedetti *et al*., 2014). However, the efficacy of molluscan barcoding is often limited by incomplete reference libraries and the inherent complexities of molluscan taxonomy (Radulovici *et al.*, 2021). This hinders accurate species identification, as samples may not match existing database entries.

Furthermore, the presence of cryptic species, morphologically similar but genetically distinct, can lead to misidentification if DNA barcode gap variation within and between species is not well-characterized (Aliabadian *et al.*, 2009; Magnacca & Brown, 2010; Moura *et al.*, 2018; Wiemers & Fiedler, 2007; Zou & Li, 2016), Also, the presence of nuclear mitochondrial pseudogenes (NUMTs), being nuclear copies of mitochondrial genes, can be mistakenly amplified and sequenced, leading to inaccurate identifications (Radulovici *et al.*, 2021; Zou & Li, 2016).

These challenges impact the reliability and accuracy of species identification in forensic investigations. Addressing these challenges will require a concerted effort to expand and curate barcode databases, improve taxonomic understanding, and refine analytical methods to account for the complexities of molluscan genomes.

4.2. DNA isolation from molluscs

Isolating DNA from molluscs shells, rather than soft tissues comes with unique challenge due to the low quantity of DNA and highly degraded DNA (Martin *et al.*, 2020). Molluscs' shells comprise multiple layers, whose nature depends upon the composition and microstructure. The outermost layer, the periostracum, is organic and often contributes to shell coloration (Marin *et al.*, 2012). Beneath outer layer lies the prismatic layer, followed by the thickest, innermost nacre layer (Fig 1).

These layers are composed of calcium carbonate in the form of aragonite or calcite, which are arranged in various microstructures. Even though the shell does not contain any living cells, during its formation, cells from the mantle epithelium or hemocytes get trapped within these layers of shell (Martin et al., 2020). These entrapped cells leave behind DNA remnants, which become the source of DNA from the shell. In order to release DNA from the shell matrix, the shells are broken into fine particles either by grinding or drilling and then decalcified using EDTA along with or without mild detergent like SDS or Triton-100 and Proteinase K to help to expose DNA trapped within the calcium (Coutellec, 2017; Ferreira et al., 2020) or carbonate matrix (Ferreira et al., 2020; Martin et al., 2020).

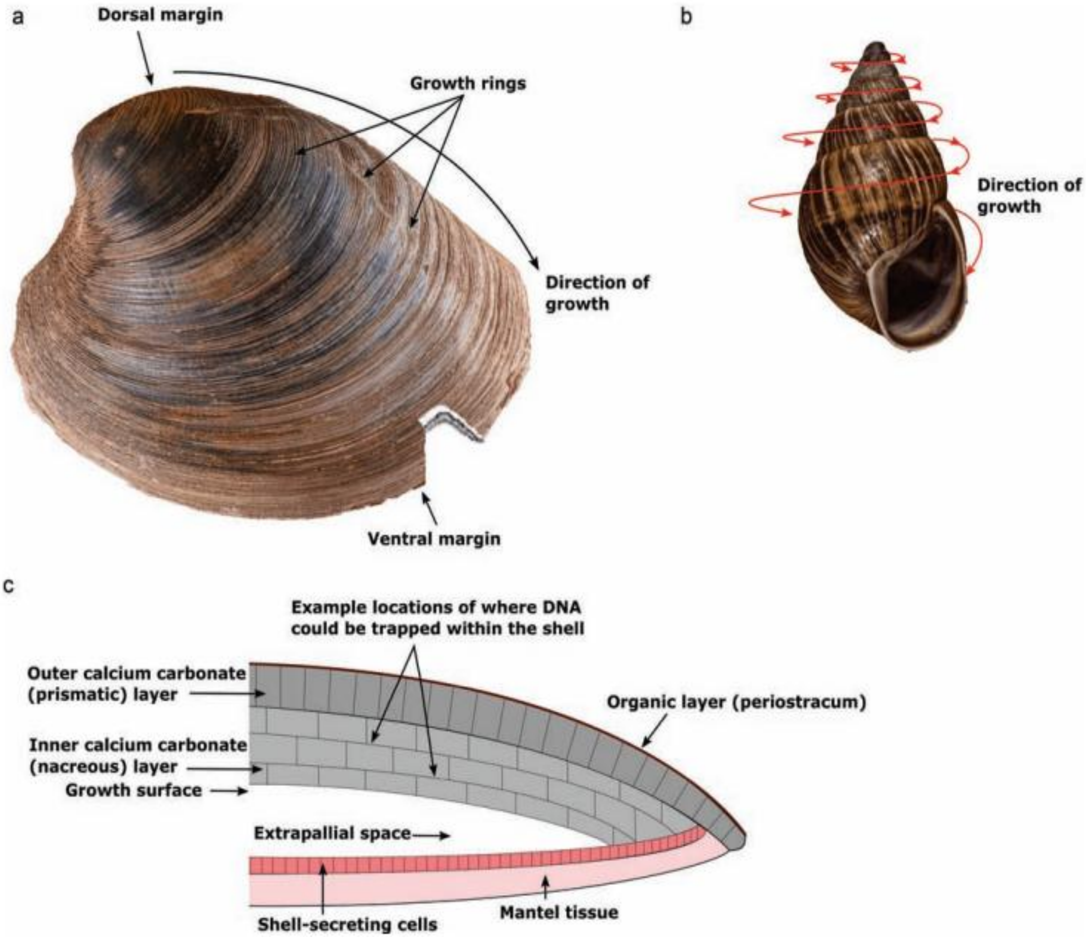


Figure 1: Different Layers of Molluscs shell. Adapted from Martin *et al.* (2020)

However, excessive grinding or harsh decalcification have been reported to cause DNA damage (Martin et al., 2020). Silica-based DNA binding and salting-out purification, or column-based purification methods have proven effective for isolating DNA from shell material (Höss & Pääbo, 1993), although phenol-chloroform extraction has also been used (Martin et al., 2020). Martin et al. (2020) emphasizing the importance of considering the shell's condition, whether fresh, aged, or ancient, when choosing an extraction method. The success of PCR amplification from shell-derived DNA depends on several factors, including DNA preservation, extraction method, and target sequence length (Sarkissian et al., 2020; Walton et al., 2022).

In addition, extracting DNA from molluscan tissues poses unique challenges due to the biological properties of these organisms. According to Adema (2021), molluscan bio mineralization, adhesive structures, and mucus involve polyphenolic proteins and mucopolysaccharides that can hinder DNA extraction or PCR amplification. Conventional DNA extraction procedures have often been reported to be ineffective or isolate poor quality DNA samples that are not readily suitable for down-streaming process. Further, pigments which are present in molluscs outer tissue may also interfere with spectrophotometry hindering accurate quantification.

4.3. Choice of gene selection

The selection of a suitable gene for DNA barcoding in molluscs is crucial. The choice of gene is significantly influenced by the presence of a distinct "barcoding gap" – the difference between intra- and interspecific genetic variation (Meier et al., 2008). An ideal barcode gene exhibits high interspecific and low intraspecific variation, allowing for unambiguous species delineation. (Wiemers & Fiedler, 2007). While cytochrome c oxidase subunit I is the standard barcode for many animals (Hebert et al., 2003), its universality in molluscs has been questioned due to factors such as primer universality and variation in amplification success across taxa (Deagle et al., 2014; Lobo et al., 2013; Marshall et al., 2006; Waugh, 2007).

Yu et al., (2014) directly addressed the COI limitations by evaluating the efficacy of twelve mitochondrial protein-coding genes as potential barcodes in molluscs. Their analysis revealed that all twelve genes performed similarly well in species discrimination, with none significantly outperforming the others. Although COI performed adequately, the study demonstrated that other mitochondrial Protein Coding Genes (PCGs), such as NADH dehydrogenase (ND) - ND1, ND2, ND4 and ND5, also exhibited comparable success in species identification, indicating their potential as viable alternatives. Furthermore, the research showed that combining multiple genes did not improve species resolution, suggesting that a single, well-chosen mitochondrial gene is sufficient for effective molluscan barcoding.

Utilising alternative genetic markers can be crucial for accurately identifying cryptic species and assessing molluscan biodiversity, as demonstrated in previous studies (Layton et al., 2014; Zou & Li, 2016). This approach can resolve cases where the commonly used cytochrome c oxidase subunit I gene has proven problematic. Furthermore, as phylo-genomic research continues to elucidate molluscan evolutionary relationships (Smith *et al.,* 2011), incorporating additional markers can further refine species identification, particularly within taxonomically complex groups (Puillandre *et al.*, 2017).

4.4. Primers available for species identification of molluscs

Before designing primers, an intensive search for existing primer sets for molluscan species identification was conducted, with a focus on those available for DNA barcoding using BOLD database (BOLD Systems, 2016; BOLD Systems: Taxonomy Browser, 2014) and list of primers that were available for identification is tabulated in table 2.

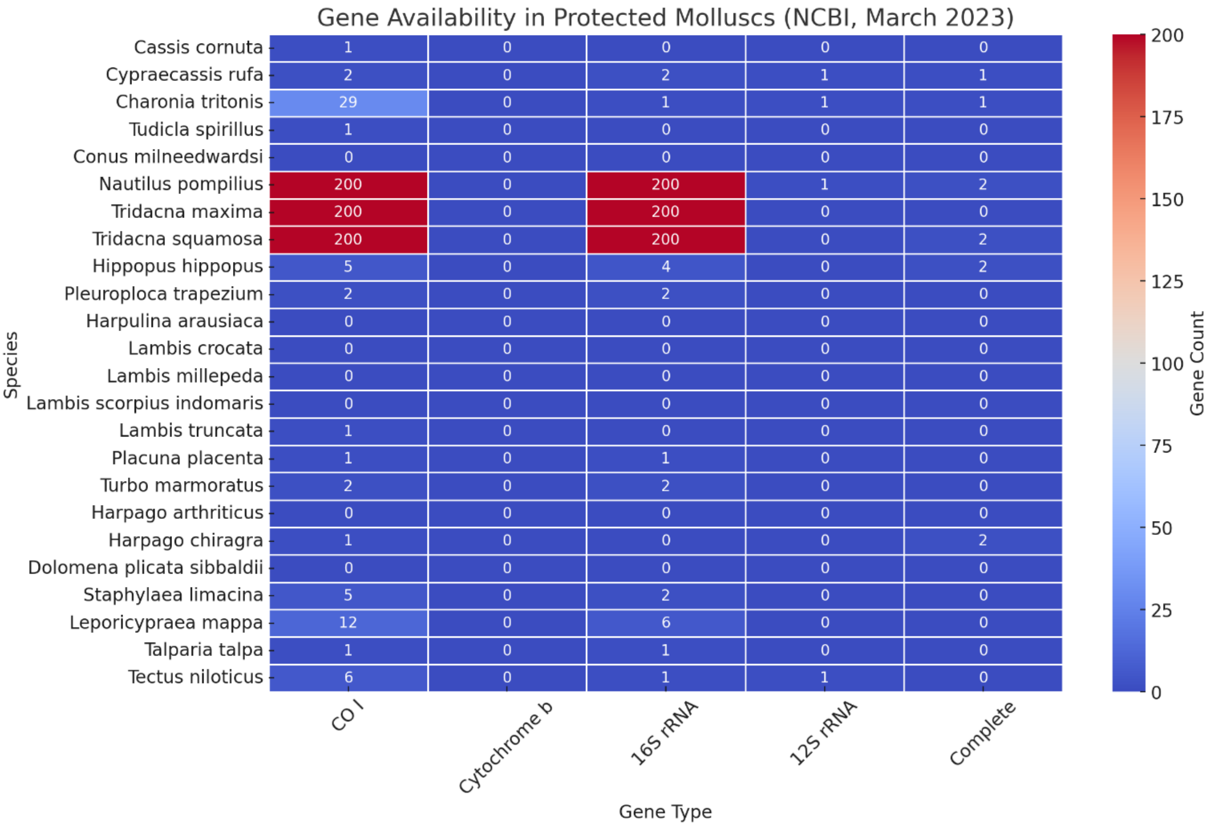
Table 2: Available primers for species identification of molluscs

Primer name	**Sequence name**	**Expected base pairs**	**Annealing temperature**	**Reference**
16Sar	CGCCTGTTTATCAAAAACAT	454 -602	49	Palumbi, 1996
16Sbr	CCGGTCTGAACTCAGATCACGT			
16Sar-ALT	GCCTGTTTATCAAAAACATSG	454 -602	48-50	Mikkelsen *et.al* (2006)
16Sbr-ALT	CCGGTCTGAACTCAGATCATGT			
16SL3-Ven	GCAAYGGAGTTGTRCTAAGGTAGC	303	58-61	Kappner & Bieler, (2006)
16SH1-Ven	ATAATCCAACATCGAGGTCGCAA			
LCO1490	GGTCAACAAATCATAAAGATATTGG	658-661	48	Folmer *et al.*(1994)
HC02198	TAAACTTCAGGGTGACCAAAAAATCA			
COIF-ALT	ACAAATCAYAARGAYATYGG	658 - 661	48	Mikkelsen *et.al* (2006)
COIR-ALT	TTCAGGRTGNCCRAARAAYCA			
COIMIDF	ATRMTNGGNGGDTTYGGNAAYTG	658 - 661	48-50	Mikkelsen *et.al* (2006)
COIMIDR	GGRTANABDGTYCANCCNGTNCC			
28SD1F	GGGACTACCCCCTGAATTTAAGCAT	1200	55-60	Park & O Folghil, (2000)
28SD6R	CCAGCTATCCTGAGGGAAACTTCG			
28D1F-ALT	AACCAGGATTCCCTCAGTAA	270	50-55	Mikkelsen *et al* (2006)
28SMIDF	CTTGAAACACGGAGCAAGG			
H3F	ATGGCTCGTACCAAGCAGACVGC	328	50-55	Colgan *et al.*, 2000
H3R	ATATCCTTRGGCATRATRGTGAC			
COIfolmerfwd	GGTCAACAATCATAAA-	705	50	Duda *et al*(2011)

Primer name	**Sequence name**	**Expected base pairs**	**Annealing temperature**	**Reference**
	GATATTGG			
CO-Ischneckrev	TATACTTCTGGATGAC-CAAAAAATCA			
16Sfwd	CGCAG-TACTCTGACTGTGC	395	55	Duda *et al*(2011)
16S_sch_rev	CGCCGGTCTGAACTCAGATC			
16Sar	CGCCTGTTTATCAAAAACAT	603	60	Xiong, B. & T. D. Kocher (1991)
16Sb	CTCCGGTTT-GAACTCAGATCA			
LCO1490_CephF	TTTCAACAAATCATAAA-GATATTGG	652	45	. Braid *et al* (2014).
HCO2198_CephR	ACTTCTGGGTGAC-CAAAAAATCA			
dgLCO1490	GGTCAACAAATCATAAAGAYATYGG	600	40 - 44	Meyer CP.(2003).
dgHCO2198	TAAACTTCAGGGTGAC-CAAARAAYCA			
jgLCO	TITCIACIAAYCAYAAR-GAYATTGG	650	48	Geller et al (2013)
jgHCO	TAIACYTCIGGRT-GICCRAARAAYCA			
Uni-MinibarR1	GAAAATCATAATGAAGGCAT GAGC	100 – 150	46	Meusnier *et al* (2008)
Uni-MinibarF1	TCCACTAATCACAAR-GATATTGGTAC			
mlCOIintF	GGWACWGG-WTGAACWGTW-TAYCCYCC	313	62	Leray *et al* (2013)
mlCOIintR	GGRGGRTASAC-SGTTCASCCSGTSCC			
matFwd	ATYGGNGGNTTYGG-NAAYTG	903	52	Matsumoto, M. & Hayami, I. (2000).
matRev	ATNGCRAAYTTYGGNTC			
UCYTB151F	TGTGGRGC-NACYGTWATYACTAA	430	48	Merritt *et al* (1998).
UCYTB270R	AANAGGAARTAYCAY-TCNGGYTG			
AGM18SF	GCCAGTAGTCAT-ATGCTTGTCTC	450	55	Harasewych and McArthur (2000)
AGM18SRev	AGACTT-GCCCTCCAAT(A/G)GATCC			

4.5. Status of DNA barcoding of protected molluscs

Before designing primers for protected molluscs, a survey of relevant mitochondrial sequences was conducted, and sequences were retrieved from the NCBI Nucleotide database (2023). While whole mitochondrial genomes were scarce, the mitochondrial genes COXI, 16SrRNA, 12SrRNA, and CYTB were present, with their abundance decreasing in the same order, respectively. The Heat map showing the number of sequences in NCBI is shown in figure 2. Among the protected species, *Nautilus pompilius*, *Tridacna squamosa*, and *Tridacna maxima* had higher abundance of sequences compared to other species. However, COXI's universality as a barcode in molluscs has been questioned (Yu et al., 2014), with some studies suggesting other mitochondrial protein-coding genes may perform better (Yu et al., 2014).



Species	CO I	Cytochrome b	16S rRNA	12S rRNA	Complete
Cassis cornuta	1	0	0	0	0
Cypraecassis rufa	2	0	2	1	1
Charonia tritonis	29	0	1	1	1
Tudicla spirillus	1	0	0	0	0
Conus milneedwardsi	0	0	0	0	0
Nautilus pompilius	200	0	200	1	2
Tridacna maxima	200	0	200	0	0
Tridacna squamosa	200	0	200	0	2
Hippopus hippopus	5	0	4	0	2
Pleuroploca trapezium	2	0	2	0	0
Harpulina arausiaca	0	0	0	0	0
Lambis crocata	0	0	0	0	0
Lambis millepeda	0	0	0	0	0
Lambis scorpius indomaris	0	0	0	0	0
Lambis truncata	1	0	0	0	0
Placuna placenta	1	0	1	0	0
Turbo marmoratus	2	0	2	0	0
Harpago arthriticus	0	0	0	0	0
Harpago chiragra	1	0	0	0	2
Dolomena plicata sibbaldii	0	0	0	0	0
Staphylaea limacina	5	0	2	0	0
Leporicypraea mappa	12	0	6	0	0
Talparia talpa	1	0	1	0	0
Tectus niloticus	6	0	1	1	0

Figure 2: Heatmap showing the number of mitochondrial sequence retrieved from NCBI as on March, 2023

4.6. Mini-barcode and its application

Conventional DNA barcoding fails in highly degraded samples like that of the shell, archival specimens, and highly processed biological materials. To overcome this issue, (Meusnier et al., 2008) proposed the use of mini-barcodes. Mini-barcodes are short, amplifiable DNA sequences (typically less than 300 base pairs) designed for species-level identification when DNA is degraded or fragmented (Kress & Erickson, 2012), such as in forensic samples.

While DNA mini-barcodes offer advantages for molluscan species identification, particularly when dealing with degraded DNA commonly found in shells, archival specimens, and processed samples, they also have inherent limitations. Gao et al., (2019) noted that the shorter length of mini-barcodes results in fewer variable sites compared to full-length barcodes. This can be especially problematic for molluscs, where cryptic species are common and require careful analysis of barcode gap variation.

5. Work Plan

The overall work plan of the project is given in Figure 3

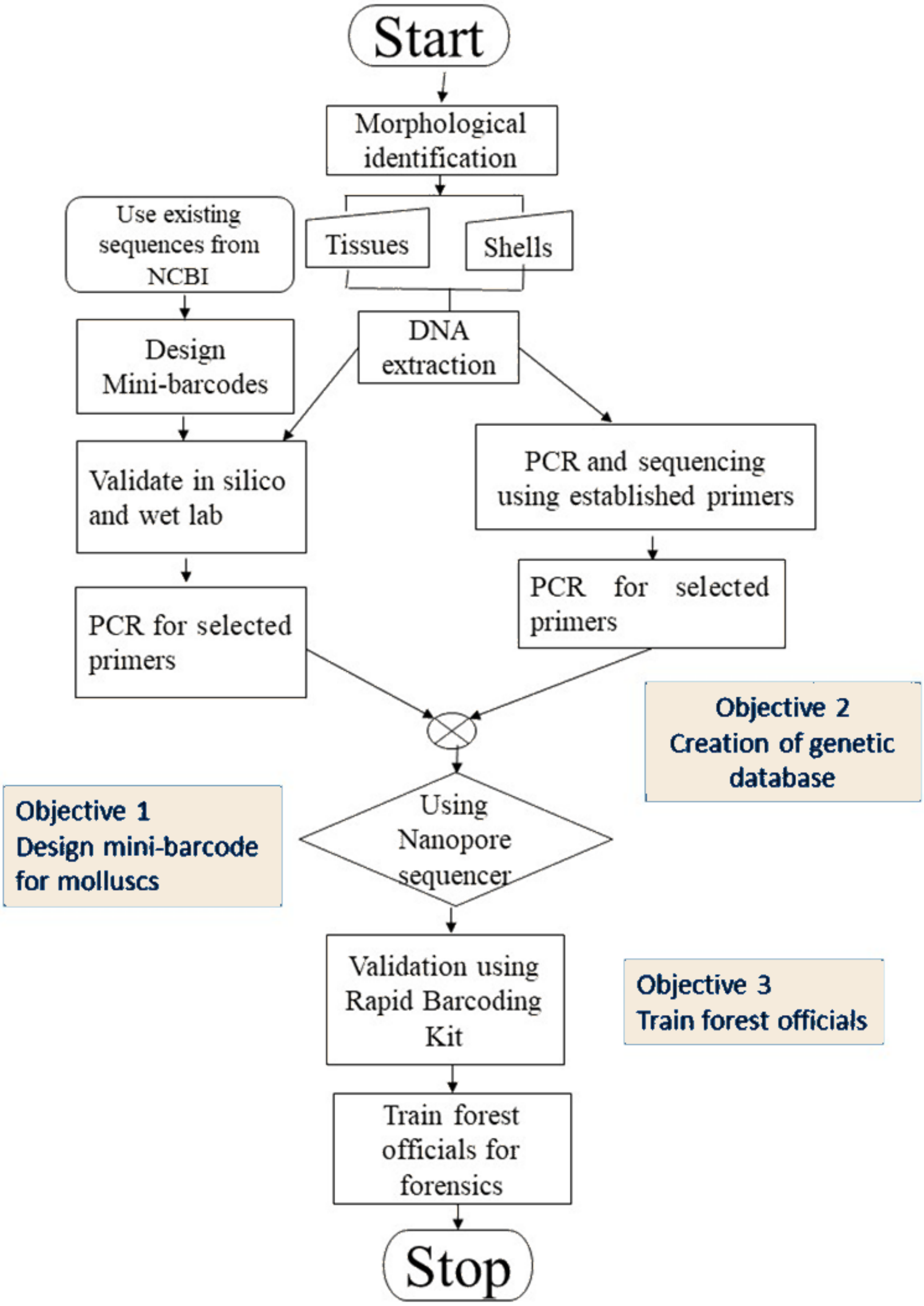


Figure 3: Overall work plan of the project

6. Methodology

6.1. In-silico validation

A total of 96 mitochondrial genomes were downloaded from the Genus / Family of protected species. Since COX1 and 16S genes had more species in NCBI database and havebeen used conventionally for species identification, these two genes were selected for primer designing. From the genome, using customised bash script, the sequences were retrieved and aligned using Multiple Alignment using Fast Fourier Transform (MAFFT). Potential Mini-barcode fragments were identified using Sliding Window ANalysis (SWAN) in the SPecies IDentity and Evolution package in R (SPIDER) (http://www.r-project.org). The SWAN function was used to generate windows varying in size from 100 to 500 base pairs (bp). Windows were shifted along the length of gene alignment using 10 bp intervals. The top three mini-barcode fragments for each window length were selected for further analyses based on: (1) high mean Kimura 2-Parameter (K2P) distance; (2) few zero pairwise non-conspecific distances; and (3) high proportion of clades shared between the neighbour-joining tree from the full-length

DNA sequence alignment and the tree constructed using only data from selected windows. From this analysis, a total of 28 potential mini-barcode alignments were created. Maximum likelihood analysis was conducted on the 29 datasets (1 full-length reference dataset and 28 SWAN mini-barcodes) using IQTREE. In all analyses, the K2P model of sequence evolution was implemented as this is the model implemented on BOLD. The 28 mini-barcode maximum likelihood trees were then compared to the full-length reference tree using TreeDist in R. The TreeDist then calculated the K-scores (topology and branch length differences).

For both methods, lower values indicated a high degree of similarity between the reference tree and the mini-barcode tree. A DNA barcode gap analysis was conducted on the top-scoring mini-barcode dataset. The number of variable sites (V) and parsimony informative characters (Pi), and the average nucleotide composition were estimated for the data (full-length and mini-barcode alignments) using MEGA 6.0 [31]. Primers were designed flanking the top-scoring mini-barcode region using PrimerMiner.

6.2. Sample collection

Dead mollusc samples, along with intact shells, were collected from various locations across Tamil Nadu to validate the designed primers (Fig. 4). Most of these samples were obtained as bycatch from landing harbour (Plate 2 & 3). Of the 24 protected mollusc species in

India, only seven are known to be distributed along the Tamil Nadu coast. Efforts were made to collect samples of these species from the region. Additionally, shells were collected from Range offices after the completion of offence cases for this research after obtaining special permission.

6.3. Species Identification

The specimens were carefully identified based on morphological characteristics using standard taxonomic literature (Abbott & Dance, 1990; Subba Rao, 2003, 2017), the Marine Species Identification Portal website, and expert consultation for molluscan species identification.

Figure 4 : Sample collection sites across Tamil Nadu

6.4. DNA isolation

DNA isolation from tissue

Following morphological identification, tissue samples were collected for DNA isolation: >10 mg of foot muscle for gastropods, mantle tissue for bivalves, and arm tissue for cephalopods. Since molluscs' tissue contains more inhibitors, the tissue was washed twice in 200 µl of PBS buffer for 20 mins before performing the DNA extraction. Even after washing the sample twice for 20 min with PBS, if the supernatant remains was not clear, another 200 µl of PBS buffer was added and kept for overnight before DNA extraction. This step was performed to remove the mucosaccharaides layer present in mollusc's tissue. The DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions and eluted in 40 µl of ATE buffer. After that, the quality of the extracted DNA was examined using 1% agarose gel electrophoresis and the DNA concentration was estimated using NanoDrop (Thermo Fisher, Boston, MA, United States).

DNA isolation from shell

For DNA extraction, molluscs shells were thoroughly cleaned to avoid contamination. Shells were wiped to remove debris and soaked with 10% bleach for half an hour. After rinsing with distilled water, shells were air-dried and powdered using mortar and pestle for DNA isolation (Fig 5a). The powdered samples are then transferred into labeled, sealed 5 ml tubes for future analysis (Fig 5b).

(a) (b)

Figure 5: DNA isolation from shells. (a) Grinding the shells using the Mortar & pestle (b) Powdered shell sample is stored in 5 ml tubes for future purpose

Standardisation of DNA extraction from shell

Currently, there are no specific protocols recommended for isolation of DNA from the shell, therefore three different methods were tested for isolating DNA from the shell.

Shell decalcification

To the 100 mg of powdered shell, 500µl of 0.5 M EDTA was added and incubated in thermomixer for at 56°C for 1000 rpm. Based on the shell type, the incubation period varies. Once the sample showed mild discolouration, it was then centrifuged for 10,000 rpm for 5 mins. The supernatant was discarded and to the pellet, 500 µl of 0.5 M EDTA was added. The sample was incubated in the thermomixer for the incubation for 3 hours at 56 °C at 1000 rpm. The supernatant was discarded and washed once with 500 µl PBS.

Method 1

To the pellet, DNA was isolated as per the manufacture's instruction using Qiagen investigator kit and eluted in 20 µl of ATE buffer. After that, the quality of the extracted DNA was examined using 1% agarose gel electrophoresis and the DNA concentration was estimated using NanoDrop (Thermo Fisher, Boston, MA, United States).

Method 2

To the pellet, DNA was isolated as per the manufacture's instruction using Qiagen Blood and Tissue kit and eluted in 20 µl of ATE buffer. After that, the quality of the extracted DNA was examined using 1% agarose gel electrophoresis and the DNA concentration was estimated using NanoDrop (Thermo Fisher, Boston, MA, United States).

Method 3

The pellet was resuspended in 300 µL of Triton X-100 lysis buffer (10% Triton X-100, 0.5 M EDTA, 10 mg/mL Proteinase K) and incubated at 56 °C overnight. The sample was centrifuged at 14,000 rpm for 10 minutes, and the supernatant was transferred to a new tube while the pellet was discarded. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, PCI) was added to the supernatant, followed by centrifugation at 14,000 rpm for 5 minutes. The clear supernatant was collected in a fresh tube, and the PCI extraction was repeated if the supernatant appeared dark. To the colourless supernatant, 70 µL of 3 M sodium acetate, 500 µL ice-cold isopropanol, and 350 µL of 6 M NaCl were added, mixed, and incubated at −20 °C for 30 minutes. The mixture was centrifuged at 14,000 rpm for 20 minutes at

4 °C, and the resulting pellet was washed with 200 µL of 80% ethanol, centrifuged again at 14,000 rpm for 20 minutes, and air-dried until no ethanol smell remained. The DNA pellet was resuspended in 30 µL of AE buffer, and DNA concentration was quantified using a microplate reader.

6.5. PCR amplification

To 30 ng of genomic DNA was isolated from foot muscle tissue/ shell , PCR amplifications were performed in 30 µL volumes containing final concentrations of 1× PCR buffer. Polymerase chain reactions (PCRs) were carried out in a total volume of 30 µL including 15 µL of Amplicon Red dye Master mix or Qiagen multiplex master mix, 0.1 pM of Forward and Reverse primers along with 30 ng of genomic DNA. The details of the PCR condition is given in Table 3.

Table 3: PCR conditions for molluscs primers used in this project

PCR Steps	**Fwd 464 + HCOI/ Folmer primers/ Palumbi 16S primers**	**Rev 461 + LCOI**	**16S bar1F+ 16S bar1R**
Initial Denaturation	95°C &15 mins for multiplex master mix or 95°C & 5 mins for Amplicon red dye master mix		
Denaturation	95°C& 30 sec		
Annealing	49°C &40 sec	46°C &40 sec	44°C& 40 sec
Extension	72°C & 40 sec		
Final Extension	72°C &7 mins		
Cycles	40 cycles		

6.6. Statistical analysis

Statistical analysis was performed using R. Kruskal-Wallis test was used with Dunn’s test with Bonferroni correction as Post-hoc test for checking the quantity and quality DNA extracted among three different protocol used for extraction from the shells statistically varied. Chi-Square test was performed to know whether is significant difference in success rates among primers. Phylogenetic Tree was constructed using MEGA for Maximum Likelihood Analysis, and distance matrix was calculated to determine whether amplified segment of the gene has sufficient the interspecies and intra-species distance for species identification. Even though the threshold percentage between the species differs between the taxa, a threshold of greater than 3% between the species is the used cut-off value for species delineation for marine molluscs.

7. Results and discussion

7.1. Primer designing

The 24 protected molluscs belonging to major Families namely Cardiidea, Ranellidae, *Strombidae, Nautilidae, Conidae, Turbinidae, Fasciolariidae and Trochidae* were taken for designing primers.

Table 4: Species taken for primer designing

Family	**Genus**	**Species**	**Individuals**
Cardiidae	*Hippopus*	*porcellanus*	*1*
	Tridacna	*crocea*	*3*
		derasa	*3*
		gigas	*2*
		squamosa	*2*
Cypraeidae	*Erronea*	*caurica*	*1*
		errones	*1*
		onyx	*1*
Naticidae	*Neverita*	*didyma*	*1*
	Paratectonatica	*tigrina*	*3*
Ranellidae	*Charonia*	*lampas*	*3*
		tritonis	*3*
Rostellariidae	*Tibia*	*fusus*	*1*
Strombidae	*Harpago*	*chiragra*	*2*
	Laevistrombus	*canarium*	*1*
	Lambis	*lambis*	*4*
Tonnidae	*Tonna*	*galea*	*1*
Nautilidae	*Nautilus*	*macromphalus*	*2*
		pompilius	*2*
Buccinidae	*Babylonia*	*areolata*	*1*
		lutosa	*1*
Conidae	*Conasprella*	*wakayamaensis*	*1*
	Conus	*betulinus*	*2*
		borgesi	*1*
		capitaneus	*2*
		consors	*2*
		ebraeus	*2*
		gloriamaris	*2*
		imperialis	*1*
		marimaris	*1*
		marmoreus	*1*
		miles	*2*
		quercinus	*3*

		striatus	*2*
		tessulatus	*2*
		textile	*2*
		tribblei	*1*
		tulipa	*1*
		ventricosus	*15*
		virgo	*1*
Fasciolariidae	*Fusinus*	*longicaudus*	*1*
Muricidae	*Chicoreus*	*torrefactus*	*1*
	Rapana	*venosa*	*2*
Syngnathidae	*Hippocampus*	*kuda*	*1*
Tegulidae	*Tectus*	*pyramis*	*1*
Trochidae	*Trochus*	*sacellum*	*2*
Turbinidae	*Turbo*	*cornutus*	*1*
Mactridae	*Mactra*	*alta*	*1*
		antiquata	*1*
		chinensis	*1*
		cumingii	*1*
		quadrangularis	*1*

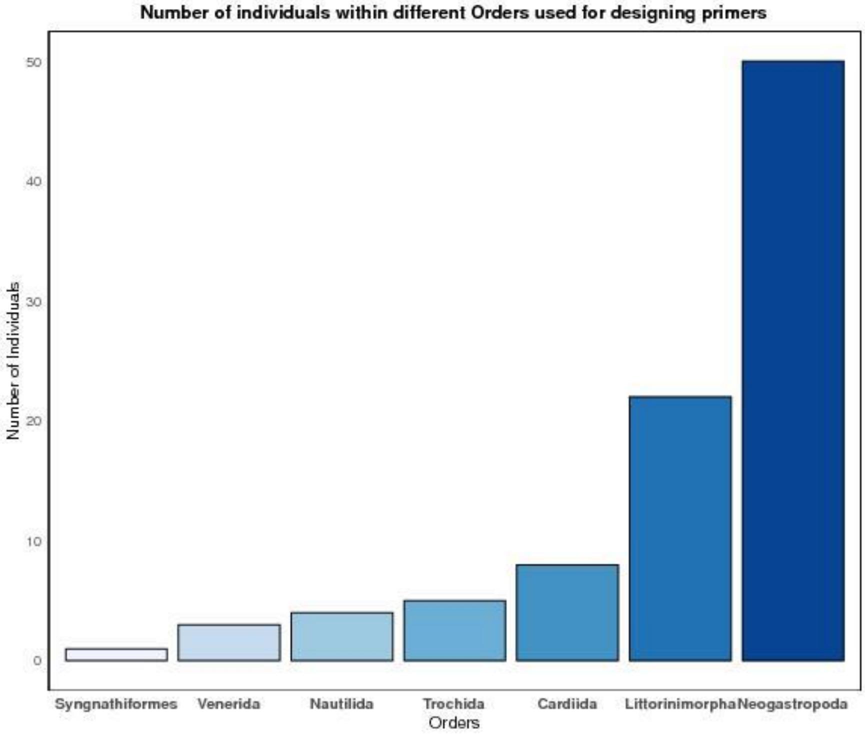
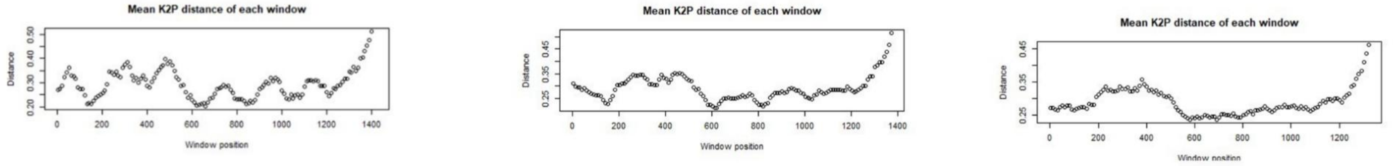


Figure 6: Number of individuals used for designing primers

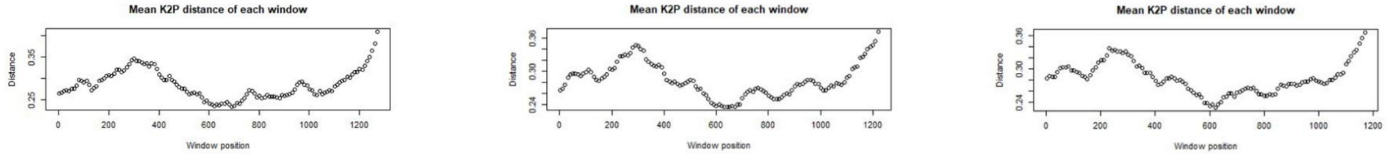
7.2. Sliding Window Analysis

Sliding window analysis in SPIDER package (Brown *et. al*, 2012) was performed to identify regions of high or low divergence in a sequence alignment and to determine the shortest informative window best discriminating the species. Fig 6 and Fig 7 shows a sliding window analysis of mean K2P (Kimura 2-Parameter) genetic distance across a gene or alignment, using different window sizes (from 100 to 500 base pairs). In each panel, genetic divergence along with changes along the sequence (window position) at different resolutions was plotted (Fig 7 & 8).

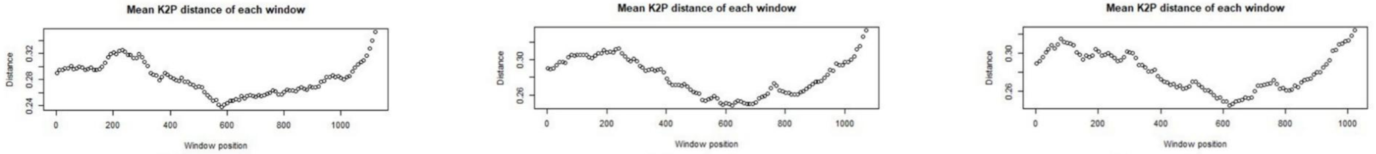
Using the various functions in SPIDER package, for each window, the proportion of zero non-conspecific distances, the number of diagnostic nucleotides, the number of zero-length distances and overall mean distance, tree-based measures including the proportion of species that are monophyletic and the proportion of clades that are identical between the neighbour-joining tree were calculated. Using the R package PrimerMiner, the primers were then manually designed (Fig 9 & Fig 10).



(a)100 bps (b) 150 bps (C) 200 bps

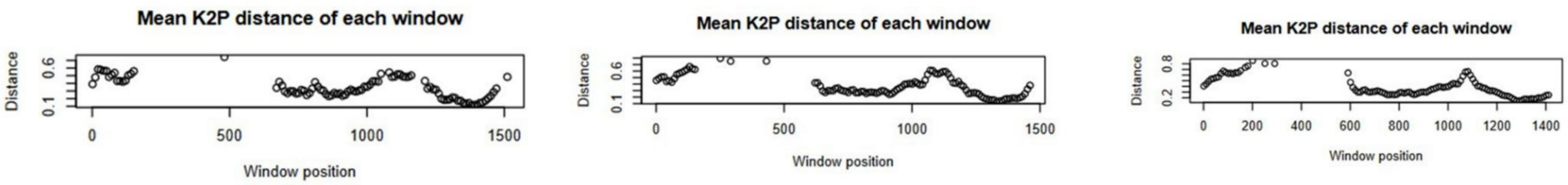


(d) 250 bps (e) 300 bps (f) 350 bps



(g) 400 bps (h) 450 bps (i) 500 bps

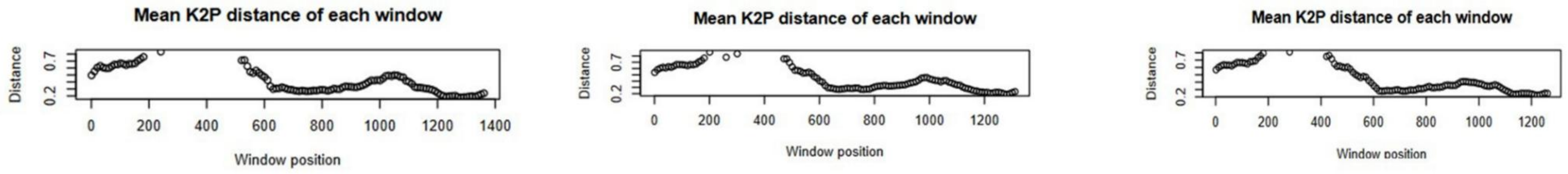
Figure 7 Sliding Window Analysis for Cytochrome Oxidase I (COX I)



(a)100 bps

(b) 150 bps

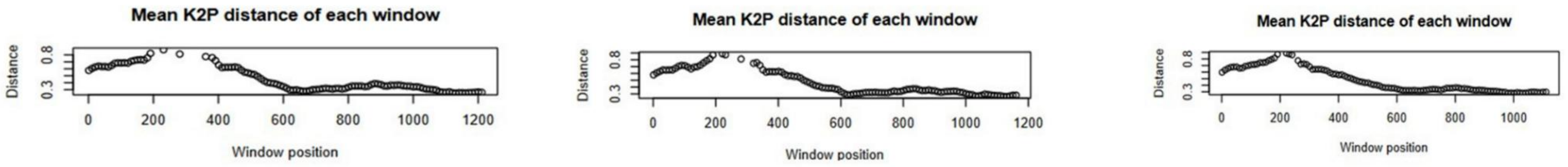
(C) 200 bps



(d) 250 bps

(e) 300 bps

(f) 350 bps



(g) 400 bps

(h) 450 bps

(i) 500 bps

Figure 8 Sliding window Analysis for 16S RNA

Table 5: Sliding Windows Analysis for Cytochrome Oxidase I (COX1)

Base pair Size	Window Number	Mean Distance	Zero Non-Con Distances	Zero Diagonal Nucleotide Distances	Diagonal Nucleotide Distance	Monophyly	Clade Comparison	Clade Comparison Shallow	Conserved	Variable	Pi	Singleton
100	141	0.51	0.60	0.01	3838.00	0.55	0.54	0.78	10	91	90	1
100	140	0.48	0.59	0.01	3755.00	0.55	0.59	0.80	12	89	88	1
100	139	0.45	0.56	0.01	3711.00	0.55	0.60	0.76	13	88	87	1
150	138	0.51	0.49	0.01	5732.00	0.55	0.56	0.76	15	136	133	3
150	137	0.47	0.52	0.01	5606.00	0.55	0.58	0.78	18	133	130	3
150	136	0.44	0.54	0.01	5479.00	0.55	0.58	0.80	21	130	127	3
200	133	0.46	0.48	0.01	7330.00	0.55	0.59	0.80	27	174	170	4
200	132	0.43	0.51	0.01	7203.00	0.55	0.59	0.78	30	171	161	4
200	131	0.41	0.51	0.01	7077.00	0.55	0.63	0.80	33	168	164	4
250	128	0.41	0.46	0.01	8549.00	0.55	0.69	0.89	48	203	199	4
250	127	0.38	0.46	0.01	8296.00	0.55	0.68	0.91	54	197	193	4
250	126	0.36	0.46	0.01	8085.00	0.55	0.66	0.85	59	192	188	4
300	123	0.37	0.44	0.01	9729.00	0.55	0.67	0.85	70	231	224	7

Base pair Size	Window Number	Mean Distance	Zero Non-Con Distances	Zero Diagonal Nucleotide Distances	Diagonal Nucleotide Distance	Monophyly	Clade Comparison	Clade Comparison Shallow	Conserved	Variable	Pi	Singleton
300	122	0.36	0.44	0.01	9561.00	0.55	0.65	0.85	74	227	219	8
300	30	0.35	0.49	0.01	8756.00	0.55	0.67	0.87	93	208	200	8
350	118	0.37	0.42	0.00	11160.00	0.55	0.70	0.89	86	265	265	9
350	117	0.36	0.42	0.00	11037.00	0.55	0.74	0.93	89	262	252	10
350	116	0.35	0.41	0.00	10912.00	0.55	0.73	0.91	92	259	248	11
400	113	0.35	0.41	0.00	12342.00	0.55	0.67	0.87	108	293	283	11
400	112	0.34	0.41	0.00	12047.00	0.55	0.69	0.89	115	289	275	11
400	111	0.33	0.41	0.00	11794.00	0.55	0.69	0.87	121	280	269	11
450	108	0.33	0.41	0.00	13225.00	0.55	0.71	0.89	137	314	303	11
450	107	0.33	0.41	0.00	13057.00	0.55	0.73	0.91	141	310	299	11
450	106	0.32	0.41	0.00	12888.00	0.55	0.70	0.87	145	306	295	11
500	103	0.32	0.41	0.00	14402.00	0.55	0.71	0.91	159	342	331	11
500	102	0.32	0.41	0.00	14233.00	0.55	0.71	0.91	163	338	327	11
500	9	0.31	0.43	0.00	13462.00	0.55	0.66	0.89	211	363	351	12

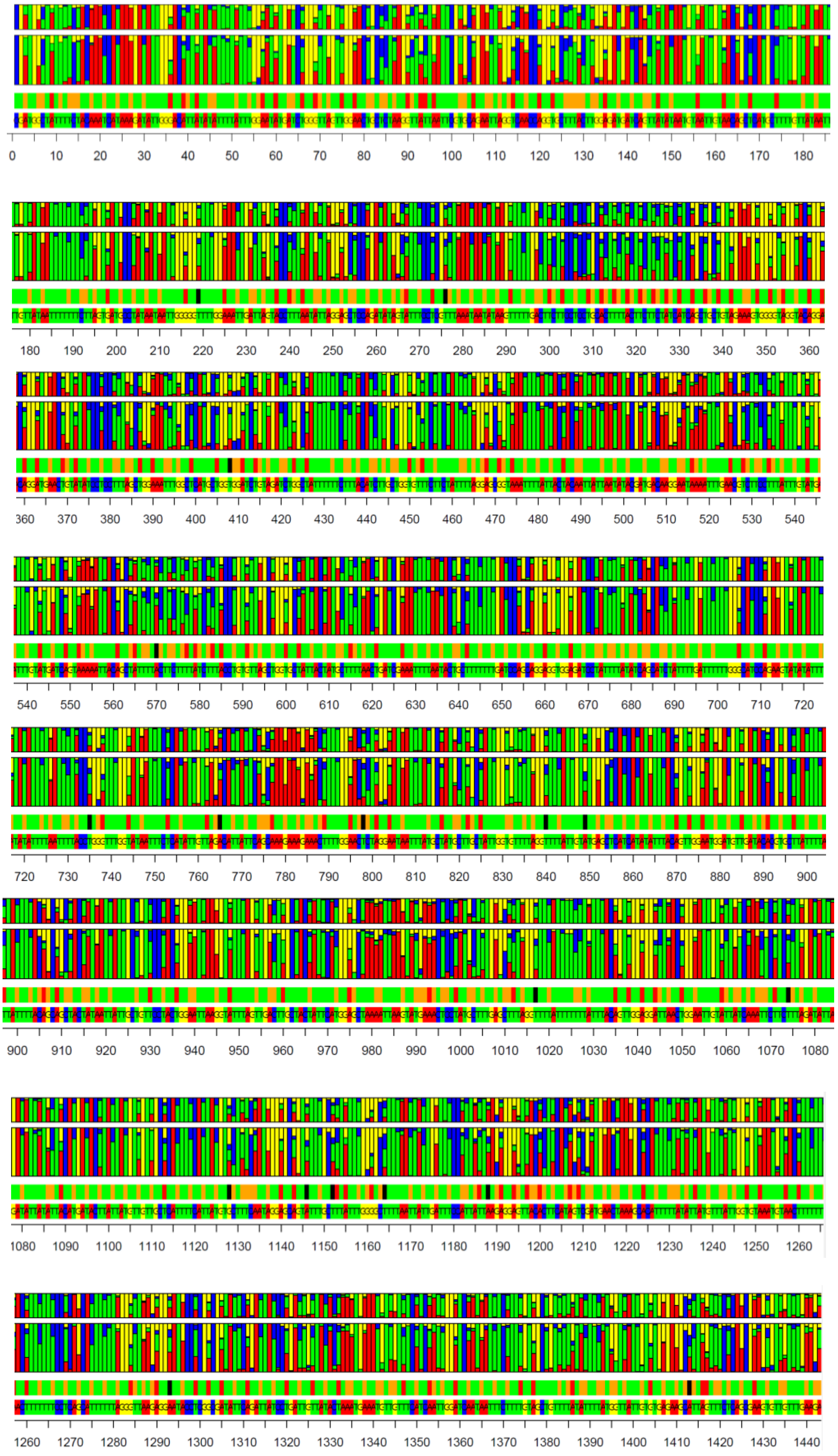
Table 6 Sliding window Analysis for 16S RNA

Basepair Size	Window Number	Position	Zero Non-Con Distances	Zero Diagonal Nucleotide Distances	Diagonal Nucleotide Distance	Monophyly	Clade Comparison	Clade Comparison Shallow	Conserved
100	141	1401	0.51	0.60	0.01	26.00	1.00	0.54	0.78
100	140	1391	0.48	0.59	0.01	27.00	1.00	0.59	0.80
100	139	1381	0.45	0.56	0.01	25.00	1.00	0.60	0.76
150	138	1371	0.51	0.49	0.01	36.00	1.00	0.56	0.76
150	137	1361	0.47	0.52	0.01	35.00	1.00	0.58	0.78
150	136	1351	0.44	0.54	0.01	33.00	1.00	0.58	0.80
200	133	1321	0.46	0.48	0.01	42.00	1.00	0.59	0.80
200	132	1311	0.43	0.51	0.01	39.00	1.00	0.59	0.78
200	131	1301	0.41	0.51	0.01	37.00	1.00	0.63	0.80
250	128	1271	0.41	0.46	0.01	44.00	1.00	0.69	0.89
250	127	1261	0.38	0.46	0.01	41.00	1.00	0.68	0.91
250	126	1251	0.36	0.46	0.01	38.00	1.00	0.66	0.85
300	123	1221	0.37	0.44	0.01	49.00	1.00	0.67	0.85

Basepair Size	**Window Number**	**Position**	**Zero Non-Con Distances**	**Zero Diagonal Nucleotide Distances**	**Diagonal Nucleotide Distance**	**Monophyly**	**Clade Comparison**	**Clade Comparison Shallow**	**Conserved**
300	122	1211	0.36	0.44	0.01	47.00	1.00	0.65	0.85
300	30	291	0.35	0.49	0.01	35.00	1.00	0.67	0.87
350	117	1161	0.36	0.42	0.00	54.00	1.00	0.74	0.93
350	116	1151	0.35	0.41	0.00	53.00	1.00	0.73	0.91
350	24	231	0.34	0.44	0.01	37.00	1.00	0.65	0.87
400	113	1121	0.35	0.41	0.00	59.00	1.00	0.67	0.87
400	112	1111	0.34	0.41	0.00	56.00	1.00	0.69	0.89
400	111	1101	0.33	0.41	0.00	54.00	1.00	0.69	0.87
450	108	1071	0.33	0.41	0.00	63.00	1.00	0.71	0.89
450	107	1061	0.33	0.41	0.00	61.00	1.00	0.73	0.91
450	106	1051	0.32	0.41	0.00	58.00	1.00	0.70	0.87
500	103	1021	0.32	0.41	0.00	65.00	1.00	0.71	0.91
500	102	1011	0.32	0.41	0.00	62.00	1.00	0.71	0.91
500	9	81	0.31	0.43	0.00	46.00	1.00	0.66	0.89

7.3. Primer designing using PrimerMiner

Figure 9: Alignment for COX1 gene



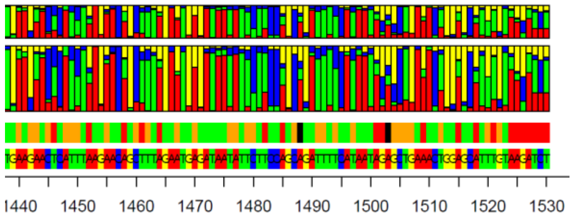
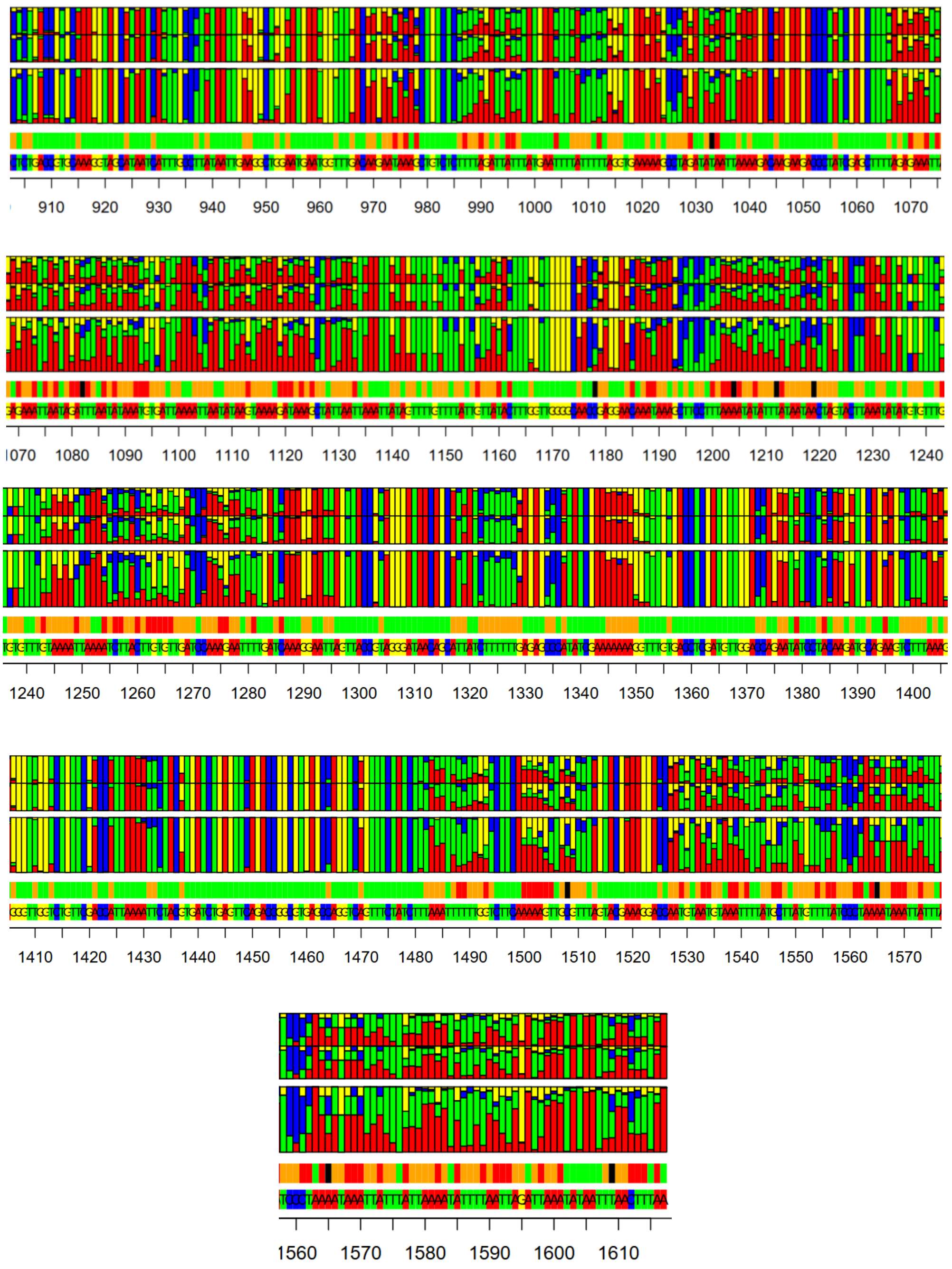


Figure 10: Alignment of 16 S gene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1570
1580
1590
1600
1610

7.4. In silico PCR validation

Insilico PCR was performed for the primers, and only those primers which bound above 95% were considered for wet lab analysis. (Fig 11-Fig 13).

Fwd464

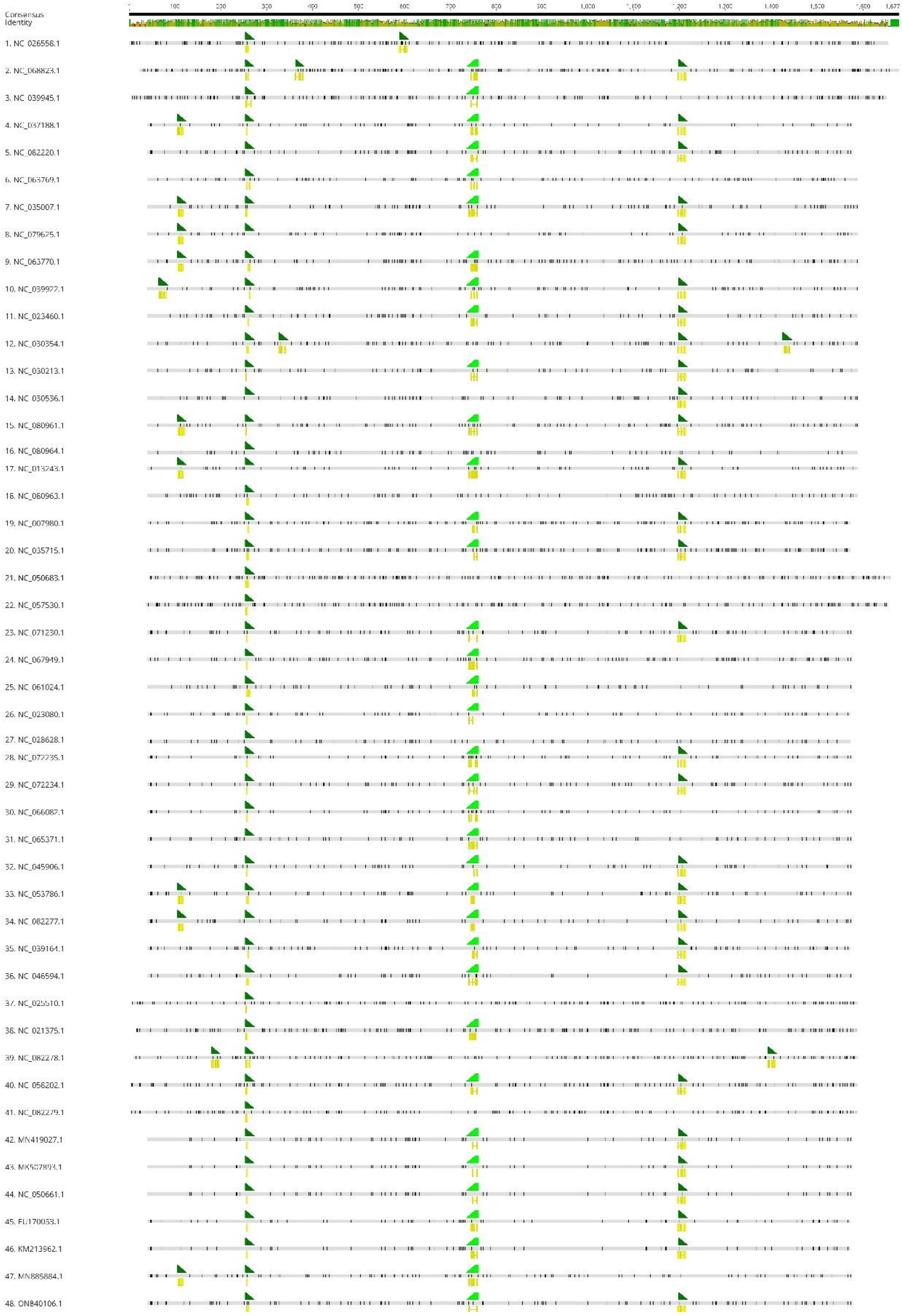


Figure 11 In-silico analysis of Fwd464 using Geneious prime

Rev464

Figure 12 In-silico analysis of Rev461 using Geneious prime

16Sbar-1

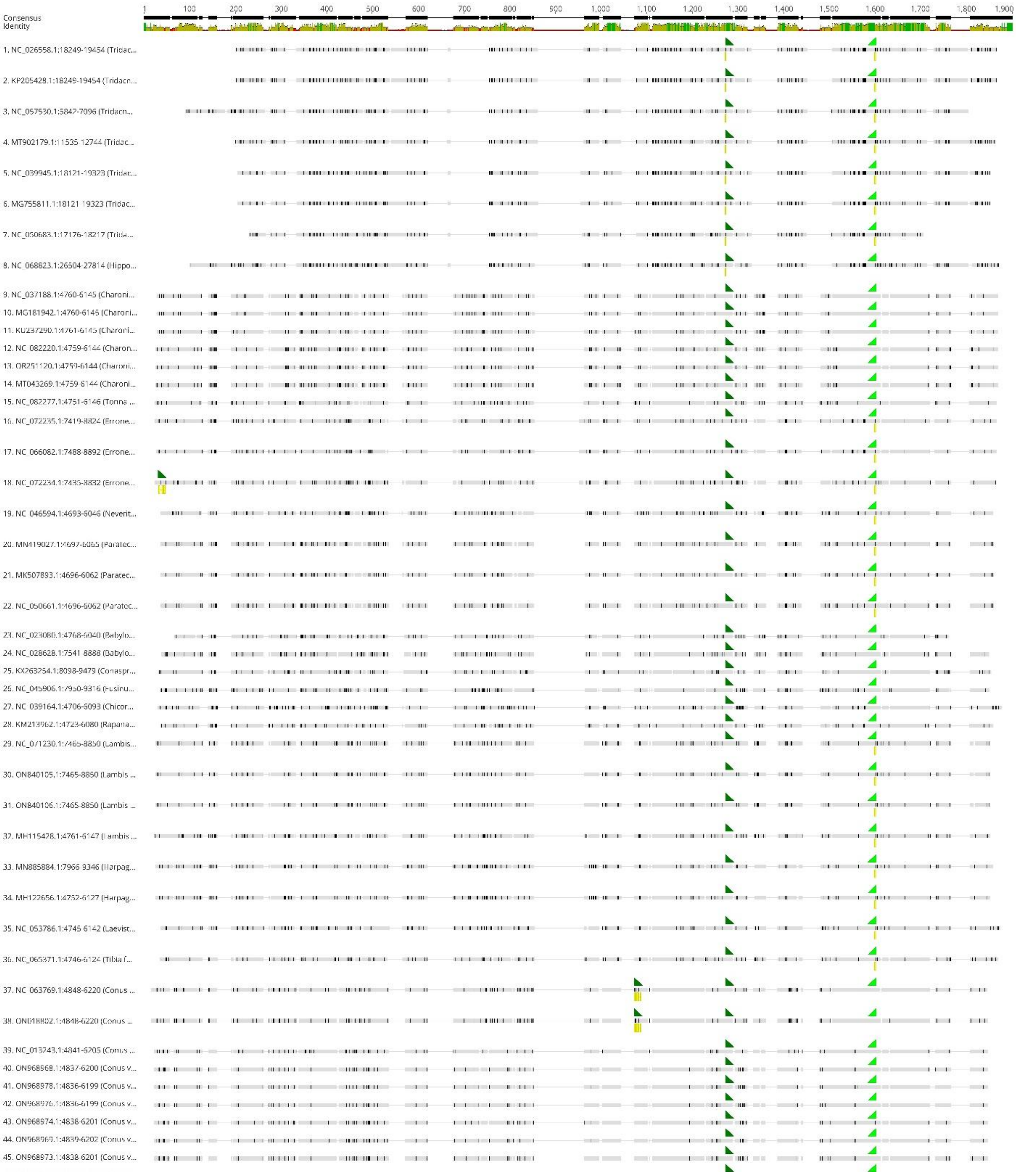


Figure 13 In-silico analysis of 16Sbar using Geneious prime

7.5. Sample Collection

The following samples were collected for wet lab analysis. Samples were collected representing three classes, 10 orders, 24 families and 40 species (plate 4-8).

Table 7: List of samples collected

Class	**Order**	**Family**	**S. No**	**Species name**
Gastropoda	Littorinimorpha	Naticidae	*1*	*Paratectonica tigrina*
			2	*Neverita* sp.*1*
			3	*Cryptonatica sp.1*
			4	*Natica sp.1*
		Tonnidae	*5*	*Tonna tessellata*
		Bursidae	*6*	*Bufonaria crumena*
		Strombidae	*7*	*Lambis crocata*
			8	*Lambis lambis*
			9	*Laevistrombus canarium*
			10	*Harpago chiragra*
		Cassidae	*11*	*Cassia coronata*
	Neogastropoda	Babyloniidae	*12*	*Babylonia spirata*
		Fasciolariidae	*13*	*Fusinus sp. 1*
			14	*Pleuroploca trapezium*
			15	*Marmorofusus nicobaricus*
		Muricidae	*16*	*Chicoreus ramosus*
			17	*Purpura bufo*
			18	*Indothais lacera*
			19	*Rapana rapiformis*
			20	*Murex sp.1*
		Cypraeidae	*21*	*Erronea onyx*
		Nassariidae	*22*	*Nassarius sp. 1*
			23	*Nassarius sp. 2*
		Melongenidae	*24*	*Pugilina pugilina*
			25	*Volegalea cochlidium*
		Cymatiidae	*26*	*Lotoria triangularis*
			27	*Ranularia oboesa*
		Conidae	*28*	*Conus amadis*
			29	*Conus araneosus*
		Volutidae	*30*	*Harpulina lapponica*
		Olividae	*31*	*Agaronia gibbosa*
		Harpidae	*32*	*Harpa sp. 1*
			33	*Harpa sp. 2*
	Nudibranchia	Glaucidae	*34*	*Glaucus atlanticus*

	Patellogastropoda	Nacellidae	*35*	*Cellana sp. 1*
Cephalopoda	Octopoda	Octopodidae	*36*	*Amphioctopus aegina*
	Sepiida	Sepiidae	*37*	*Sepiella inermis*
	Myopsida	Loliginidae	*38*	*Uroteuthis duvaucelii*
Bivalvia	Anomiida	Placunidae	*39*	*Placuna.sp*
	Venerida	Veneridae	*40*	*Protapes gallus*
	Mytiloida	Mytilidae	*41*	*Perna viridis*

7.6. DNA isolation from tissues

The tissue from the following research samples were used, and DNA was isolated using DNA Blood and Tissue kit. The quality and quantity of the DNA isolated from the kit are tabulated in the table below.

Table 8: Species List - DNA Isolation from tissues

ID	**Research ID**	***Scientific name***	**DNA concentration (ng/µl)**	**$A_{230/280}$**	**$A_{260/280}$**
Moll 1	AIWC/RES/03/23/0025(1)	*Perna viridis*	785.1	2.14	1.23
Moll 2	AIWC/RES/03/23/0026(1)	*Cellana sp.*	285.1	2.21	2.19
Moll 3	AIWC/RES/03/23/0028	*Babylonia spirata*	47.1	1.78	0.59
Moll 4	AIWC/RES/03/23/0027	*Fusinus.sp*	560.2	2.08	2.28
Moll 5	AIWC/RES/12/23/0322(1)	*Pleuroploca trapezium*	81.1	2.17	1.35
Moll 6	AIWC/RES/12/23/0356	*Turbinella pyrum*	168.6	2.11	2.04
Moll 7	AIWC/RES/12/23/0358(1)	*Chicoreus ramosus*	152.4	2.09	1.46
Moll 8	AIWC/RES/12/23/0359	*Purpura bufo*	619.5	2.05	1.71
Moll 9	AIWC/RES/12/23/0334	*Erronea onyx*	122.7	2.25	2.29
Moll 10	AIWC/RES/12/23/0372	*Paratectonica tigrina*	306.1	0.83	0.28
Moll 11	AIWC/RES/12/23/0330	*Volegalea cochlidium*	121.1	2.04	1.52
Moll 12	AIWC/RES/12/23/0331	*Vepricardium coronatum*	197.8	2.25	1.84
Moll 13	AIWC/RES/12/23/0354(1)	*Nassarius.sp1*	700.3	1.97	2.16
Moll 15	AIWC/RES/12/23/0358(2)	*Chicoreus ramosus*	81.4	2.09	2.12
Moll 16	AIWC/RES/12/23/0372	*Paratectonica tigrina*	61.4	2.12	2.99
Moll 17	AIWC/RES/12/23/0354	*Nassarius bicallosus*	161.7	1.97	2.34
Moll 18	AIWC/RES/12/23/0365(1)	*Cryptonatic andoi*	101.6	1.98	1.97

ID	**Research ID**	***Scientific name***	**DNA concentration (ng/µl)**	**$A_{230/280}$**	**$A_{260/280}$**
Moll 19	AIWC/RES/12/23/0355	*Nassarius sp.*	338.8	2.93	2.08
Moll 20	AIWC/RES/12/23/0355	*Nassarius sp.*	121.3	1.98	2.51
Moll 21	AIWC/RES/12/23/0355	*Nassarius sp.*	163.2	2.09	1.76
Moll 22	AIWC/RES/12/23/0358(3)	*Chicoreus ramosus*	38.7	2.05	2.79
Moll 23	AIWC/RES/12/23/0358(4)	*Chicoreus ramosus*	65.7	2.11	0.96
Moll 24	AIWC/RES/12/23/0336	*Indothais lacera*	6.1	2.11	0.48
Moll 25	AIWC/RES/12/23/0365(2)	*Cryptonatic andoi*	59	1.86	0.55
Moll 26	AIWC/RES/12/23/0330	*Pugilina pugilina*	184.9	2.2	1.69
Moll 27	AIWC/RES/12/23/0358(5)	*Chicoreus ramosus*	514.7	2.13	2.42
Moll 28	AIWC/RES/12/23/0326	*Lotoria triangularis*	86.3	2.14	1.7
Moll 29	AIWC/RES/12/23/0349	*Tonna tessellata*	186.7	2.26	2.33
Moll 30	AIWC/RES/12/23/0338(1)	*Marmorofuscus nicobaricus*	350.4	2.12	2.35
Moll 31	AIWC/RES/12/23/0338(2)	*Marmorofuscus nicobaricus*	295.6	2.08	1..97
Moll 32	AIWC/RES/12/23/0348(1)	*Conus amadis*	1376.7	2.32	2.45
Moll 33	AIWC/RES/12/23/0348(2)	*Conus amadis*	140.7	2.16	2.02
Moll 34	AIWC/RES/12/23/0344	*Conus araneosus*	408.1	2.06	1.95
Moll 35	AIWC/RES/12/23/0342	*Glaucus atlanticus*	1919.5	2.2	2.3
Moll 36	AIWC/RES/12/23/0354(1)	*Nassarius.sp.*	254	2.04	2.48
Moll 37	AIWC/RES/12/23/0353	*Agaronia gibbosa*	46.3	2.12	1.56
Moll 38	AIWC/RES/10/23/0290	*Mimachlamys senotoria*	23	2.23	1.24
Moll 39	AIWC/RES/12/23/0360	*Harpulina lapponica*	49.3	2.28	1.88
Moll 40	AIWC/RES/12/23/0322	*Pleuroploca trapezium*	62.2	1.97	1.24
Moll 41	AIWC/RES/12/23/0329	*Harpa sp.*	153.3	2.28	2.36
Moll 42	AIWC/RES/12/23/0329	*Harpa sp.*	167.7	2.28	2.67
Moll 43	AIWC/RES/12/23/0375	*Rapana rapiformis*	160.3	2.27	1.92
Moll 44	AIWC/RES/12/23/0351	*Bufornaria Crumena*	190.5	2.11	2.39
Moll 45	AIWC/RES/12/23/0351	*Bufornaria Crumena*	337.4	2.17	2.47
Moll 46	AIWC/RES/12/23/0361	*Architectonica perspectiva*	247.3	2.13	2.08
Moll 47	AIWC/RES/12/23/0361	*Architectonica perspectiva*	531.8	2.19	2.2

ID	**Research ID**	***Scientific name***	**DNA concentration (ng/µl)**	**$A_{230/280}$**	**$A_{260/280}$**
Moll 48	AIWC/RES/01/24/0008(1)	*Provanna sp.*	119.3	2.12	1.98
Moll 49	AIWC/RES/01/24/0008(2)	*Provanna sp.*	43.6	2.02	1.26
Moll 50	AIWC/RES/01/24/0008(3)	*Provanna sp.*	26.2	1.99	0.87
Moll 51	AIWC/RES/12/24/0479	*Cassia cornonata*	5.96	1.8	0.288
Moll 52	AIWC/RES/12/23/0480(1)	*Lambis croatea*	23.64	0.9	0.117
Moll 53	AIWC/RES/12/24/0490	*Erronea onyx*	*15.3*	1.75	0.88
Moll 54	AIWC/RES/12/23/0323(1)	*Lambis lambis*	580.766	1.956	1.916
Moll 55	AIWC/RES/12/23/0323(2)	*Lambis lambis*	506.24	2.051	2.137
Moll 56	AIWC/RES/12/23/0341	*Laevistrombus canarium*	549.424	1.96	1.77
Moll 57	AIWC/RES/09/24/0399	*Anadara antiquata*	10.87	2.108	0.87
Moll 60	AIWC/RES/12/23/0356	*Turbinella pyrum*	185	2.02	1.9
Moll 61	AIWC/RES/03/23/0025(1)	*Perna viridis*	109.4	1.88	2.02
Moll 62	AIWC/RES/12/23/0354	*Nassarius sp.*	334.6	1.87	1.57
Moll 63	AIWC/RES/03/23/0026(2)	*Cellana sp.*	430.3	2.05	2.27
Moll 64	AIWC/RES/03/23/0026(3)	*Cellana sp.*	539.4	2.1	2.33
Moll 65	AIWC/RES/03/23/0025(3)	*Perna viridis*	1568.8	2.17	2.22
Moll 66	AIWC/RES/03/23/0025(4)	*Perna viridis*	1438.8	2.17	2.23
Moll 67	AIWC/RES/09/24/0400(1)	*Placuna placenta*	1346.8	2.12	2.35
Moll 68	AIWC/RES/09/24/0400(1)	*Placuna placenta*	882.7	2.2	2.3
Moll 69	AIWC/RES/09/24/0399	*Anadara sp.*	199.1	2.01	1.25
Moll 70	AIWC/RES/09/24/0398	*Paphia malabarica*	211.1	2.04	1.74
Moll 71	AIWC/RES/09/24/0396	*Pinctada fucata*	509.9	2.09	1.82
Moll 72	AIWC/RES/09/24/0397	*Nevida sp.*	622.6	2.14	1.91
Moll 73	AIWC/RES/09/24/0399	*Anadara sp.*	1619	2.22	2.38
Moll 74	AIWC/RES/09/24/0395	*Murex sp.*	253.1	2.11	1.79
Moll 75	AIWC/RES/03/23/0034(3)	*Lambis lambis*	481.5	2.03	1.97
Moll 76	AIWC/RES/12/24/0490	*Erronea onyx*	5.4	1.61	1.03
Moll 77	AIWC/RES/12/24/0489	*Lambis croatea*	60.0	2.03	1.17
Moll 78	AIWC/RES/12/24/0491	*Harpago chiragra*	*1004.4*	0.41	0.40

ID	**Research ID**	***Scientific name***	**DNA concentration (ng/µl)**	**$A_{230/280}$**	**$A_{260/280}$**
Moll 79	AIWC/RES/12/23/0480(2)	*Lambis croatea*	260.17	1.93	2.27
Moll 80	AIWC/RES/12/24/0484	*Amphioctopus aegina*	582.79	2.04	1.413
Moll 81	AIWC/RES/12/24/0482	*Sepiella inermis*	146.34	1.62	0.44
Moll 82	AIWC/RES/12/24/0483	*Uroteuthis duvaucelii*	23.64	0.9	0.117
Moll 83	AIWC/RES/12/24/0481	*Ranularia oboesa*	14.69	1.99	1.028

7.7. DNA isolation from shell

Table 9: Species List - DNA isolation from shell

Species	**260/280**	**260/230**	**ng/µl**	**260/280**	**260/230**	**ng/µl**	**260/280**	**260/230**	**ng/µl**
	Method 1			**Method 2**			**Method 3**		
Perna viridis	1.51	0.16	17.42	1.33	0.31	13.7	1.21	0.82	1988.51
Babylonia spirata	2.18	0.15	11.61	1.65	0.86	3.72	1.23	0.95	2762.06
Anadara antiquata	1.74	0.75	12.15	1.90	1.12	3.45	1.05	1.07	1848.02
Lambis lambis	2.16	0.11	8.9	1.51	0.75	3.92	1.49	1.49	846.
Harpago arthriticus	2.17	0.12	12.18	1.4	0.20	2.12	0.87	1.34	891.09
Cassis cornuta	2.06	0.12	7.70	1.77	0.37	2.73	1.26	1.71	903.37

DNA concentration among different protocols

The Kruskal-Wallis test revealed a statistically significant difference in DNA concentration among the protocols (Kruskal-Wallis chi-squared = 42.25, df = 2, $p < 0.001$). This result indicates that at least one protocol differs significantly from the others in terms of DNA concentration. Post-hoc analyses using Dunn’s test with Bonferroni correction showed that manual method resulted in higher DNA concentrations compared to the other two methods.

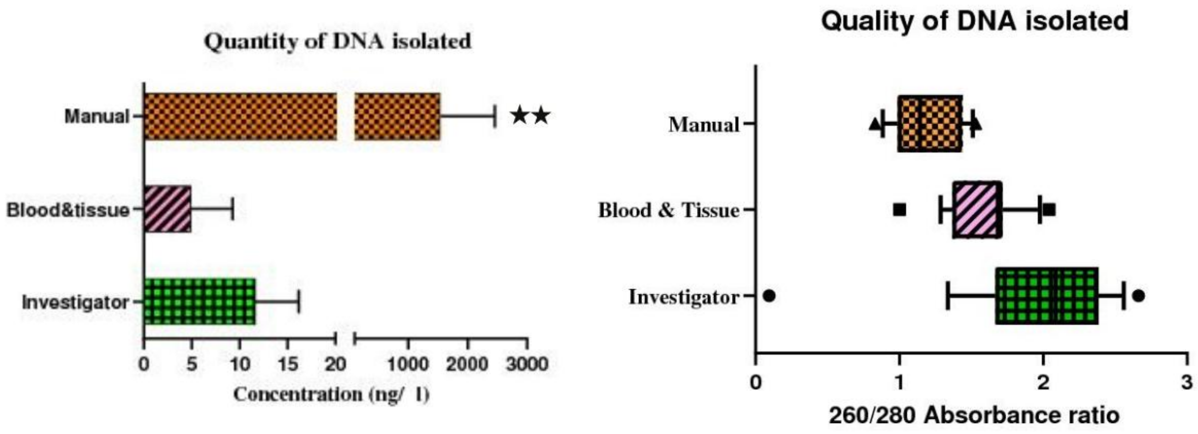


Figure 14 DNA concentration among different protocols

7.8. PCR amplification

PCR amplification was carried out for both Cytochrome Oxidase I (COX1) and 16S rRNA genes using the following primers. Folmer *et al.*(1994) and Palumbi *et al.* (2002) were used as the reference set. The details of the primers are given in table 10.

Table: 10 Primers used in this study

Primer Name	**Sequence**	**Reference**
Folmer Forward	GGTCAACAAATCATAAAGATATTGG	Folmer *et al.*(1994)
Folmer Reverse	TAAACTTCAGGGTGACCAAAAAATC	Folmer *et al.*(1994)
Paul_16S_Forward	CGCCTGTTTATCAAAAACAT	Palumbi *et al.* (2002)
Paul_16S_Reverse	CCGGTCTGAACTCAGATCACGT	Palumbi *et al.* (2002)
Fwd 461	ATTGGSGGWTTTGGDAAYTG	current study
Rev 464	CARTTRCCAAANCCWCCAAT	current study
16Sbar1_fwd1	AAGAAGRCCCTRTYGAGC	current study
16Sbar1_rev4	TGRTCCAACATCGAGGTC	current study

7.9. Agarose gel visualization

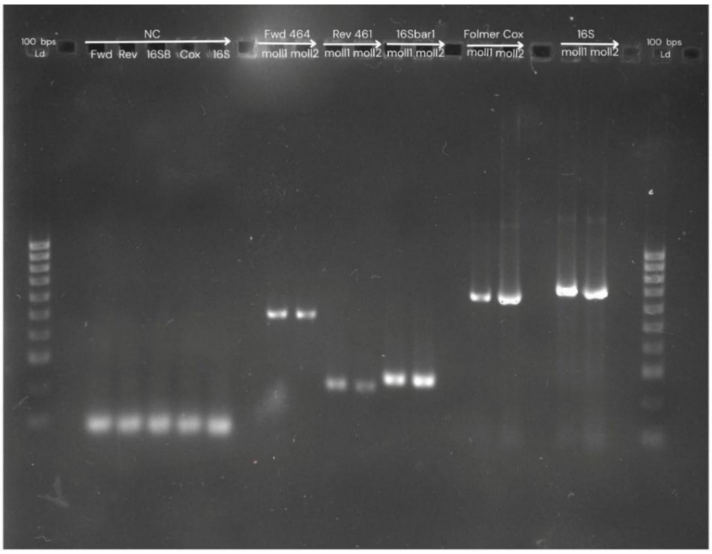


Figure 15 Gel visualization of Products amplified
Lane 1: 100 bps ladder, Lane 3: Negative control_Fwd464, Lane 4: Negative control_Rev461, Lane 5: Negative control_16Sbar1, Lane 6: Negative control_Folmer_Cox, Lane 7: Negative Paul16S, Lane 9: Fwd464_Moll1, Lane 10: Fwd464_Moll2, Lane 11: Rev461_Moll1, Lane 12: Rev461_Moll2, Lane 13: 16Sbar_Moll1, Lane 14: 16Sbar_Moll2, Lane 16: Folmer_Moll1, Lane 17: Folmer_Moll2, Lane 19: 16S_Moll1, Lane 20: 16S_Moll2

A total of 289 sequences were sequenced and uploaded in NCBI (Table 8). For the species *Vepricardium coronatum, Mimachlamys senotoria, Architectonica perspectiva and Turbinella pyrum,* only the reference primer set got amplified and therefore, these four species were excluded for comparing designed primer's efficacy to delimit a species. The amplification success rates of the five primers ranged from 78.26% to 92.75%. A Chi-square test ($\chi^2 = 7.33$, df = 4, p = 0.12) showed no significant difference in success rates among primers.

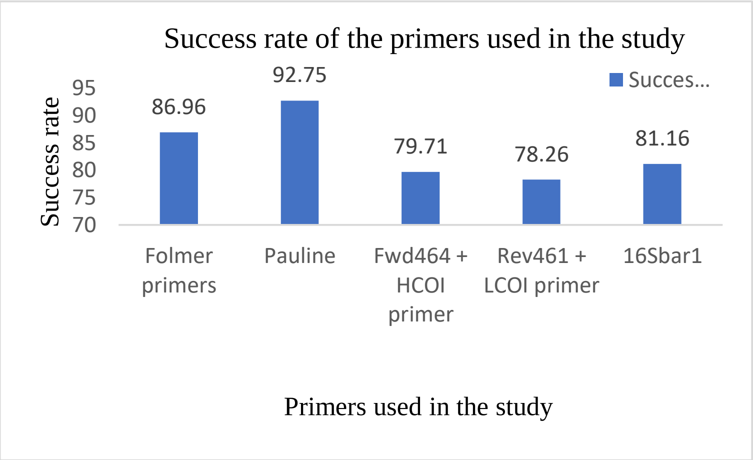


Figure 16: Success rate of the primers used in the study

7.10. Creation of reference genetic database of marine molluscs species

A total of 289 sequences were sequenced and uploaded in NCBI (Table 8). The genetic information of several species – *Lotoria triangularis, Marmorofuscus nicobaricus, Murex sp., Harpa davidis, Harpa major, Ranularia oboesa, Cellana sp., Provanna sp., Nassarius sp., and Neverida sp.* – were submitted to National Center for Biotechnology Information (NCBI) GenBank for the first time as part of this initiative (Plate 9).

Table 11: List of accession ID generated and uploaded in NCBI. Red indicate No Amplification.

Sample Id	Folmer COX	Paul 16S	Fwd464	Rev461	16Sbar-1
Moll1	PV798916	PV727002	PV335986	PV344635	
Moll2	PV714751	PV738174	PV340232	PV436948	PV388526
Moll3	PV799304	PV793681	PV335998	PV344718	PV367241
Moll4	PV449482	PV738177	PV340233	PV355411	PV436854
Moll5	PV282423	PV770192	PV872896	PV883011	PV367232
Moll6	PV639327	PV282424			
Moll7	PV952730	PV282425	PV336037	PV355671	PV392653
Moll8	PV639643	PV793669	PV336010	PV342508	PV367235
Moll9				PV345588	
Moll10					PV355661
Moll 11	PV639663	PV282426	PV363390	PV355677	PV383263
Moll12	PV643980	PV282427			PV390824
Moll13	PV715108	PV282428	PV340234	PV359244	PV843550
Moll15	PV871994	PV282429	PV523544	PV359267	PV404209
Moll16	PV715899	PV282430	PV336093	PV342511	PV382324
Moll17			PV339412	PV437162	PV404210
Moll18	PV875915	PV282431	PV875906	PV839854	PV842274
Moll19	PV871895	PV282433	PV339765	PV437260	PV392652
Moll20	PV871502	PV282434	PV339538	PV437583	PV412626

Sample Id	Folmer COX	Paul 16S	Fwd464	Rev461	16Sbar-1
Moll22	PV973314	PV282435	PV339616	PV533986	PV412767
Moll23	PV973163	PV282436	PV617358	PV839831	PV840023
Moll24		PV282437	PV618250		PV412605
Moll25	PV875930	PV282438	PV625056	PV839813	PV840024
Moll26	PV804121	PV282439	PV523572	PV362282	PV426257
Moll27	PV804124	PV282440	PV523569	PV595855	PV840021
Moll28	PV804128	PV282441		PV362509	PV423072
Moll29	PV810778	PV282442	PV341495		PV423074
Moll30	PV812495	PV282443	PV362829	PV595850	PV839929
Moll31	PV819304	PV282444	PV523582	PV841837	PV839910
Moll32	PV819348	PV282445	PV366942	PV595843	PV422863
Moll33	PV361749	PV282446	PV344187	PV595851	PV422872
Moll34	PV819380	PV282447	PV362829	PV839827	PV426262
Moll35	PV793619	PV729984	PV523586	PV342599	
Moll36	PV834037	PV282448	PV871462	PV839841	PV426264
Moll37				PV342527	
Moll38		PV282449			
Moll39	PV362633	PV282450		PV961628	PV524734
Moll40	PV826619	PV282451	PV366914	PV362622	PV942239
Moll41	PV960913	PV282452	PV523727		PV416837
Moll42	PV975992	PV282453	PV362829		PV405788
Moll43	PV571731	PV282454	PV422815	PV342553	PV942269
Moll44	PV571751	PV282455	PV523733		PV388527
Moll45	PV571692	PV282456	PV366860		PV390839
Moll46		PV282457			
Moll47		PV282458			
Moll51	PV799788	PV730100	PV366721		PV395534

Sample Id	Folmer COX	Paul 16S	Fwd464	Rev461	16Sbar-1
Moll52	PV803114	PV730109	PV335952	PV362307	PV395535
Moll54	PV361758	PV730134	PV555054	PV871497	PV390973
Moll55	PV361755	PV366633	PV555092	PV362316	PV361768
Moll56	PV826713	PV730156	PV366676	PV362319	PV390988
Moll60	PV826767	PV849389	PV363581	PV952832	
Moll61	PV827103	PV849398		PV362324	PV390994
Moll62				PV342580	PV395537
Moll63	PV656052	PV738175	PV554967	PV437073	PV395536
Moll64	PV719643	PV738176	PV584113	PV362626	PV600641
Moll65	PV834416	PV849723	PV533910	PV362623	
Moll66	PV827209	PV770175	PV533814	PV362423	
Moll67	PV834417	PV800131			
Moll68	PV834418	PV800131	PV563500		
Moll70	PV833931	PV793489	PV843153	PV362356	PV395538
Moll72	PV765454	PV793454	PV563085	PV839788	PV841876
Moll74	PV765421	PV793451		PV362495	PV400556
Moll75	PV765399	PV793450	PV499171	PV362494	PV404208
Moll78	PV361708	PV849369	PV849155	PV362493	PV426728
Moll79	PV718012	PV793445	PV563597	PV652947	PV839901
Moll80	PV362735	PV793442	PV563779	PV362361	PV400540
Moll81	PV833936	PV793439	PV570380	PV362360	PV400435
Moll82	PV362638	PV793433	PV436809	PV362358	PV426855
Moll83	PV392231	PV793426	PV499170	PV533974	PV834026

7.11. Species-delineating ability of the primers used

7.11.1. Order Littorinimorpha - Family Naticidae

The family *Naticidae* is highly diverse and widely distributed globally, yet remains one of the least studied marine gastropod groups. In this study, *Paratectonatica tigrina*, *Neverita* sp., *Cryptonatica* sp., and *Natica* sp. were tested. *Paratectonatica tigrina* is the only confirmed representative of its genus. The other species require further taxonomical studies to resolve their species identification. The sample IDs Moll18 and Moll25 were identified upto the Family level and Moll 72 was identified upto species level, however mini-barcode sequences generated for each specimen showed high similarity to their respective reference sequences (Fig 15-32).

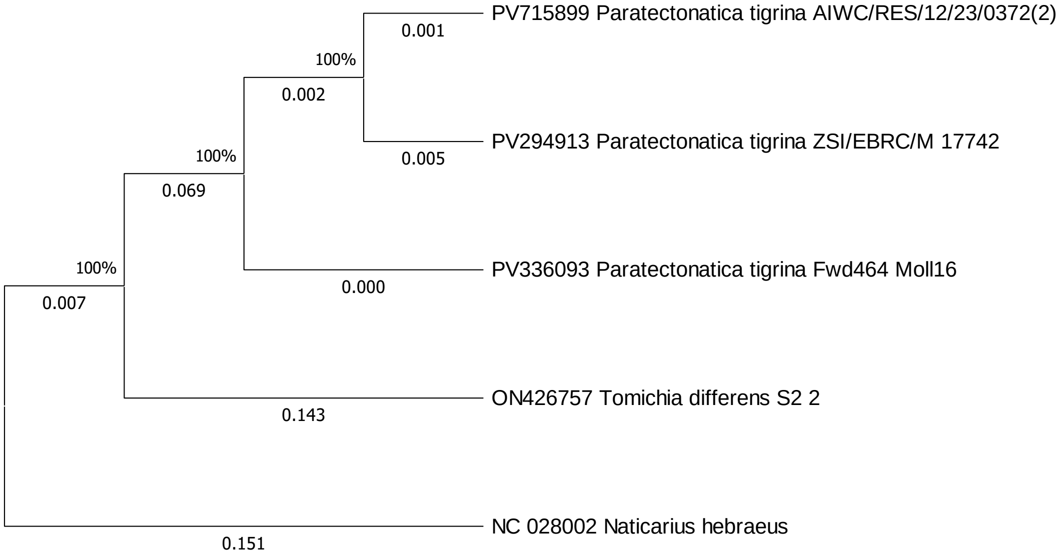
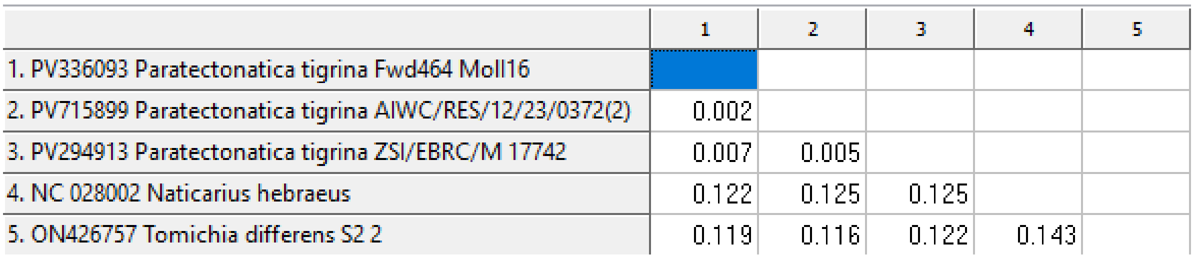


Figure 17: Dendrogram tree for Paratectonatica tigrina using Fwd 464 primers using HKY+G model. (All caps used for the species name, is it ok?) also in the Figures that follow this



	1	2	3	4	5
1. PV336093 Paratectonatica tigrina Fwd464 Moll16					
2. PV715899 Paratectonatica tigrina AIWC/RES/12/23/0372(2)	0.002				
3. PV294913 Paratectonatica tigrina ZSI/EBRC/M 17742	0.007	0.005			
4. NC 028002 Naticarius hebraeus	0.122	0.125	0.125		
5. ON426757 Tomichia differens S2 2	0.119	0.116	0.122	0.143	

Figure 18: K2P distance matrix for *Paratectonatica tigrina* using Fwd 464 primers

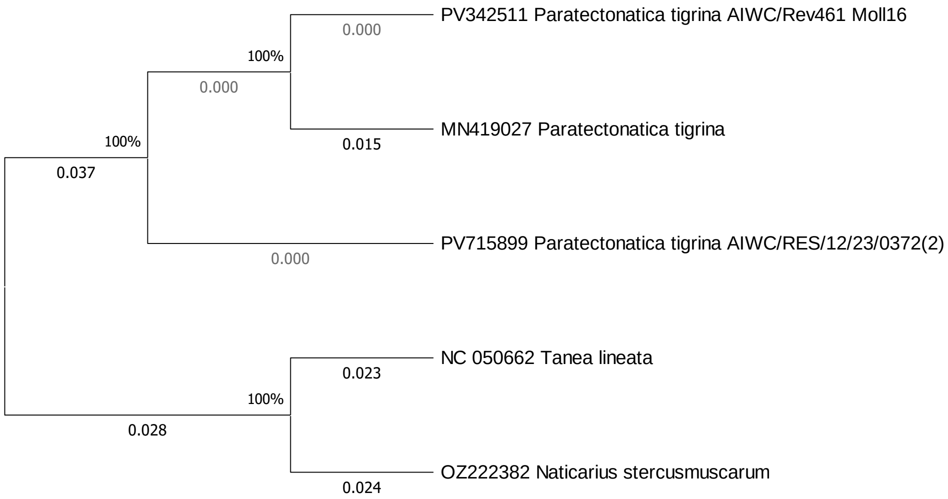
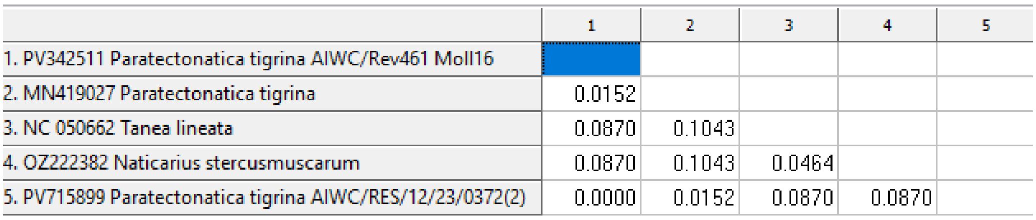


Figure 19: Dendrogram tree for *Paratectonatica tigrina* using Rev461 primers using T92 model



	1	2	3	4	5
1. PV342511 Paratectonatica tigrina AIWC/Rev461 Moll16					
2. MN419027 Paratectonatica tigrina	0.0152				
3. NC 050662 Tanea lineata	0.0870	0.1043			
4. OZ222382 Naticarius stercusmuscarum	0.0870	0.1043	0.0464		
5. PV715899 Paratectonatica tigrina AIWC/RES/12/23/0372(2)	0.0000	0.0152	0.0870	0.0870	

Figure 20 K2P distance matrix for *Paratectonatica tigrina* using Rev461 primers

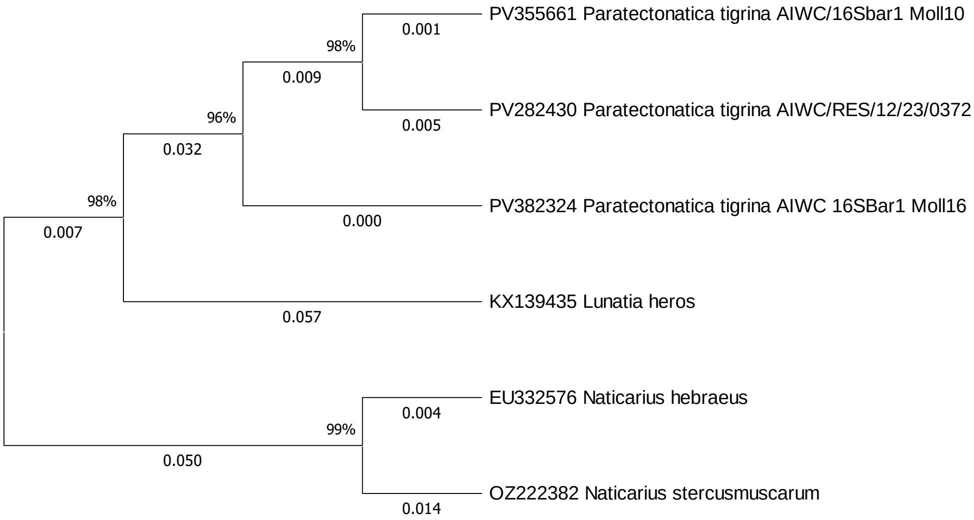
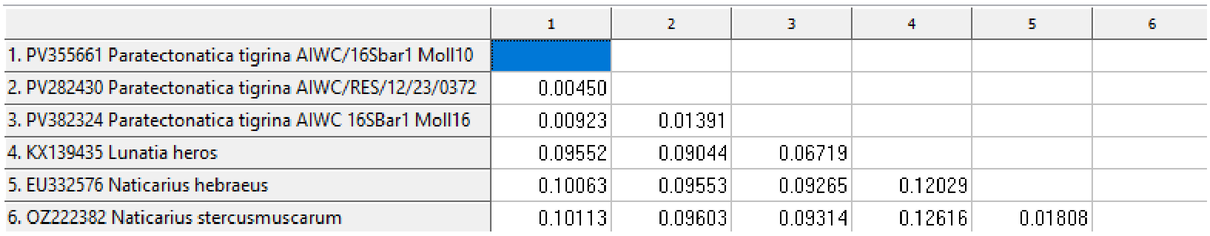


Figure 21 Dendrogram tree for *Paratectonatica tigrina* using 16Sbar primers using T92 model



	1	2	3	4	5	6
1. PV355661 Paratectonatica tigrina AIWC/16Sbar1 Moll10						
2. PV282430 Paratectonatica tigrina AIWC/RES/12/23/0372	0.00450					
3. PV382324 Paratectonatica tigrina AIWC 16SBar1 Moll16	0.00923	0.01391				
4. KX139435 Lunatia heros	0.09552	0.09044	0.06719			
5. EU332576 Naticarius hebraeus	0.10063	0.09553	0.09265	0.12029		
6. OZ222382 Naticarius stercusmuscarum	0.10113	0.09603	0.09314	0.12616	0.01808	

Figure 22: K2P distance matrix for *Paratectonatica tigrina* using 16Sbar primers

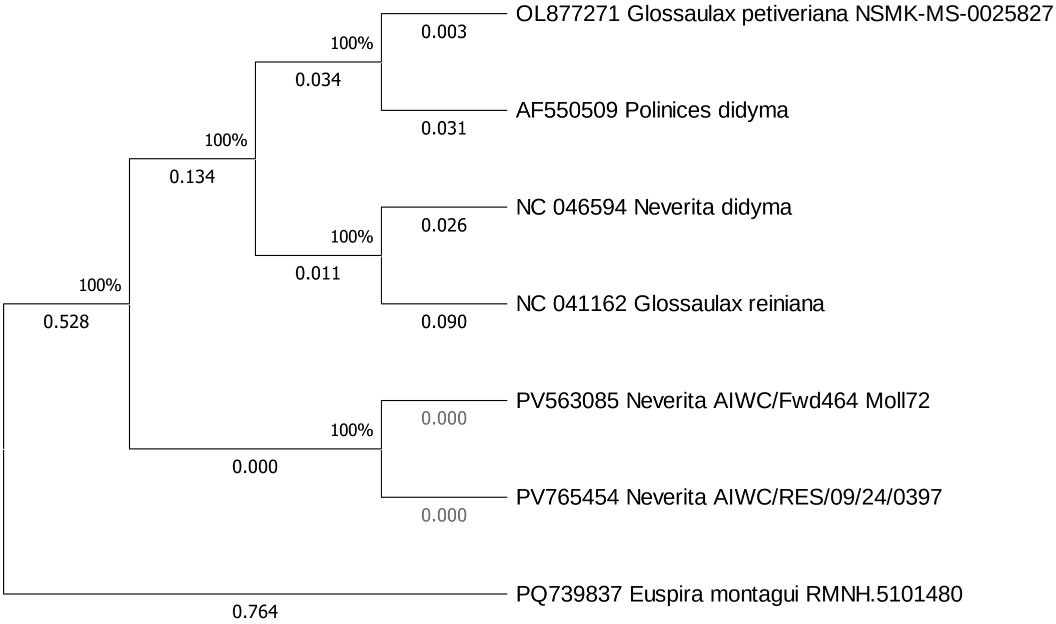
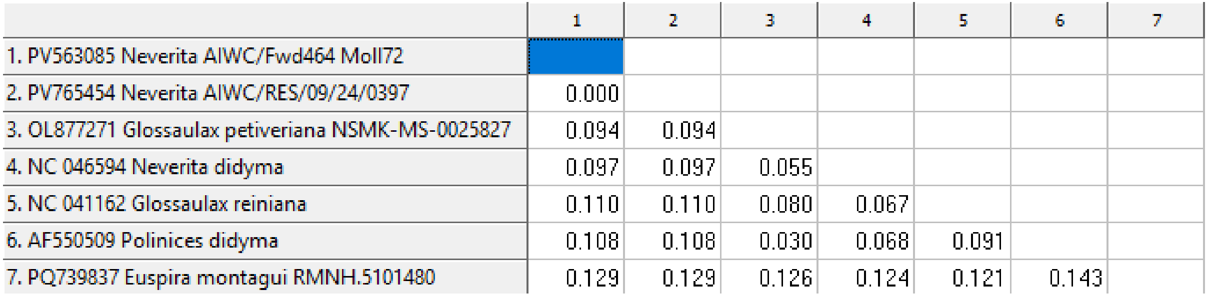


Figure 23: Dendrogram tree for *Neverita.sp* using Fwd464 primers using T92+G model



	1	2	3	4	5	6	7
1. PV563085 Neverita AIWC/Fwd464 Moll72							
2. PV765454 Neverita AIWC/RES/09/24/0397	0.000						
3. OL877271 Glossaulax petiveriana NSMK-MS-0025827	0.094	0.094					
4. NC 046594 Neverita didyma	0.097	0.097	0.055				
5. NC 041162 Glossaulax reiniana	0.110	0.110	0.080	0.067			
6. AF550509 Polinices didyma	0.108	0.108	0.030	0.068	0.091		
7. PQ739837 Euspira montagui RMNH.5101480	0.129	0.129	0.126	0.124	0.121	0.143	

Figure 24: K2P distance matrix for *Neverita.sp* using Fwd464 primers

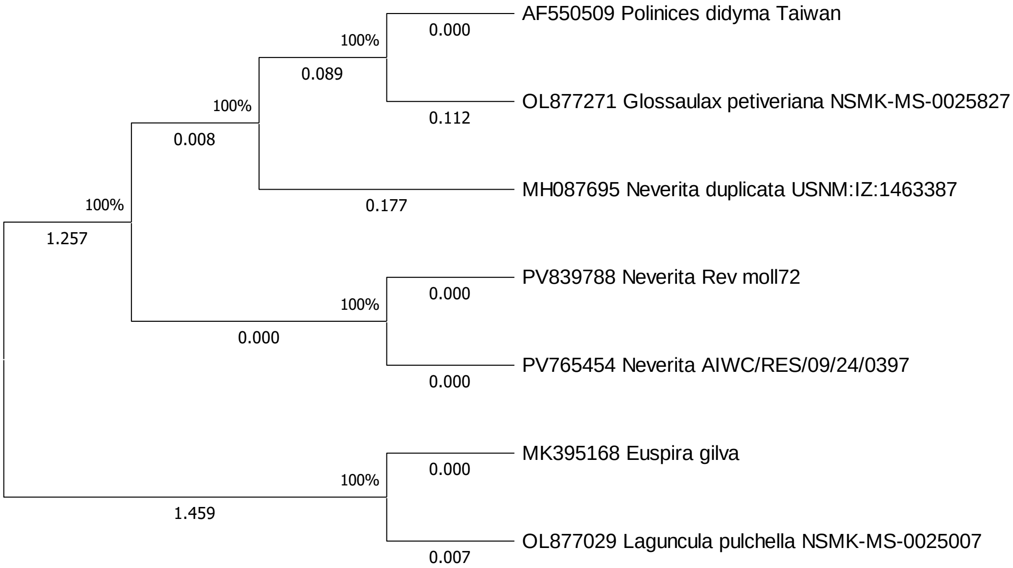
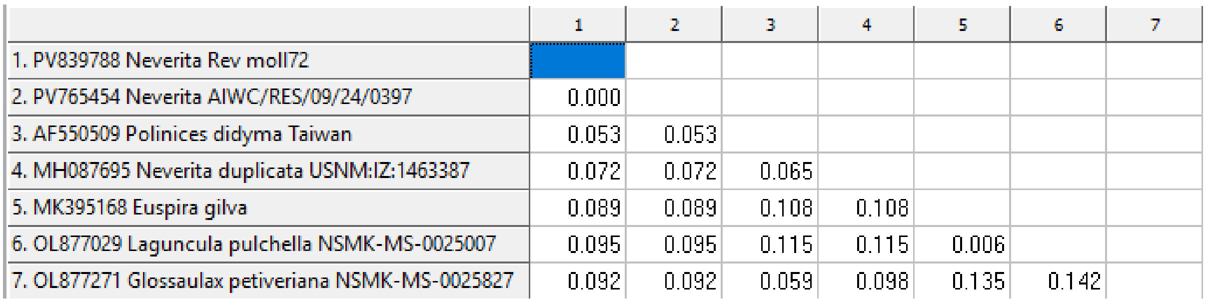


Figure 25: Dendrogram tree for *Neverita.sp* using Rev461 primers using T92+G model



	1	2	3	4	5	6	7
1. PV839788 Neverita Rev moll72							
2. PV765454 Neverita AIWC/RES/09/24/0397	0.000						
3. AF550509 Polinices didyma Taiwan	0.053	0.053					
4. MH087695 Neverita duplicata USNM:IZ:1463387	0.072	0.072	0.065				
5. MK395168 Euspira gilva	0.089	0.089	0.108	0.108			
6. OL877029 Laguncula pulchella NSMK-MS-0025007	0.095	0.095	0.115	0.115	0.006		
7. OL877271 Glossaulax petiveriana NSMK-MS-0025827	0.092	0.092	0.059	0.098	0.135	0.142	

Figure 26: K2P distance matrix for *Neverita.sp* using Rev461 primers

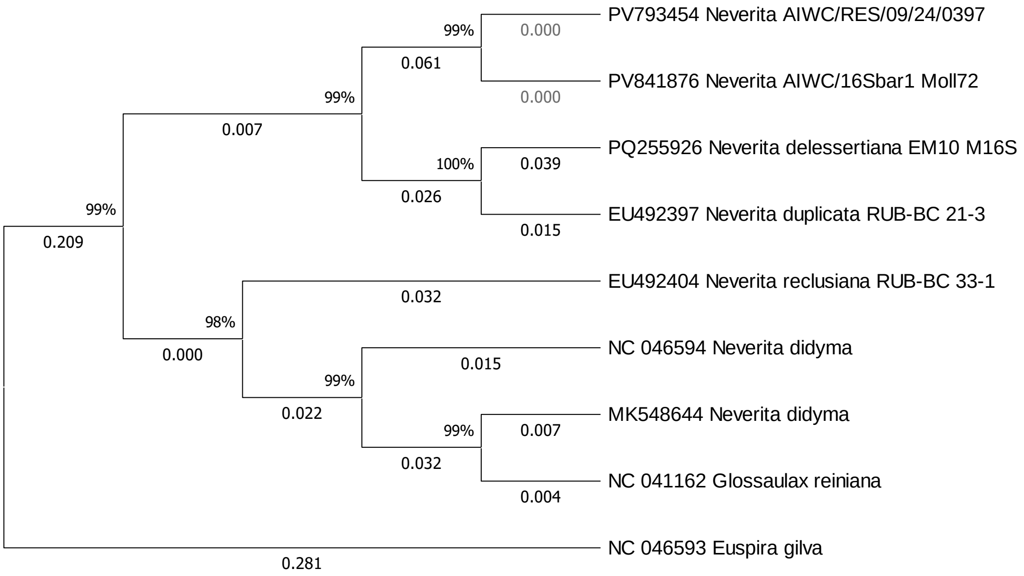
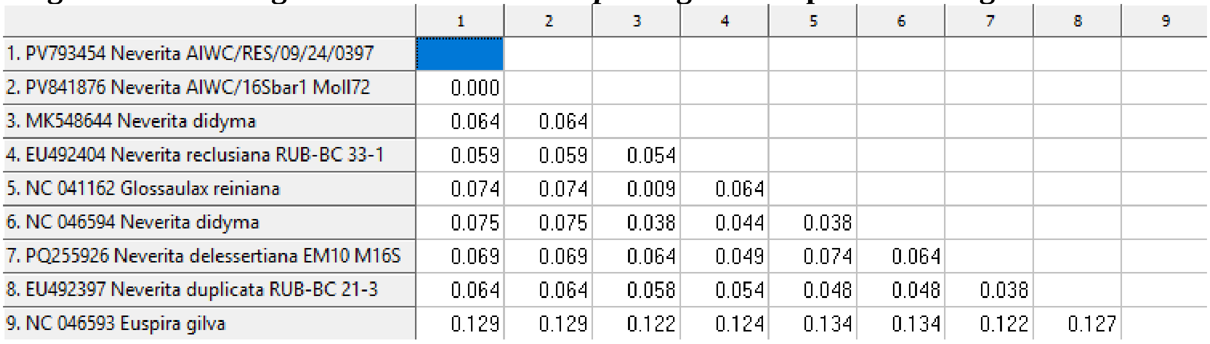


Figure 27: Dendrogram tree for *Neverita.sp* using 16Sbar primers using T92+G model



	1	2	3	4	5	6	7	8	9
1. PV793454 Neverita AIWC/RES/09/24/0397									
2. PV841876 Neverita AIWC/16Sbar1 Moll72	0.000								
3. MK548644 Neverita didyma	0.064	0.064							
4. EU492404 Neverita reclusiana RUB-BC 33-1	0.059	0.059	0.054						
5. NC 041162 Glossaulax reiniana	0.074	0.074	0.009	0.064					
6. NC 046594 Neverita didyma	0.075	0.075	0.038	0.044	0.038				
7. PQ255926 Neverita delessertiana EM10 M16S	0.069	0.069	0.064	0.049	0.074	0.064			
8. EU492397 Neverita duplicata RUB-BC 21-3	0.064	0.064	0.058	0.054	0.048	0.048	0.038		
9. NC 046593 Euspira gilva	0.129	0.129	0.122	0.124	0.134	0.134	0.122	0.127	

Figure 28: K2P distance matrix for *Neverita.sp* using 16Sbar primers

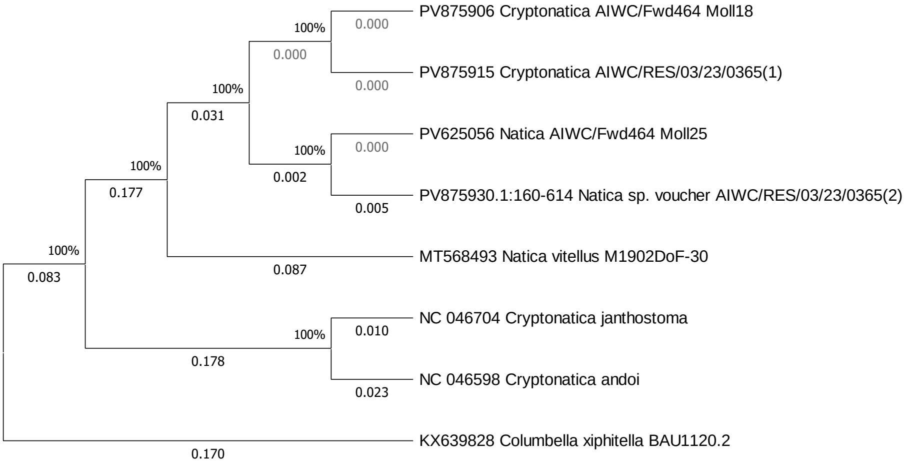
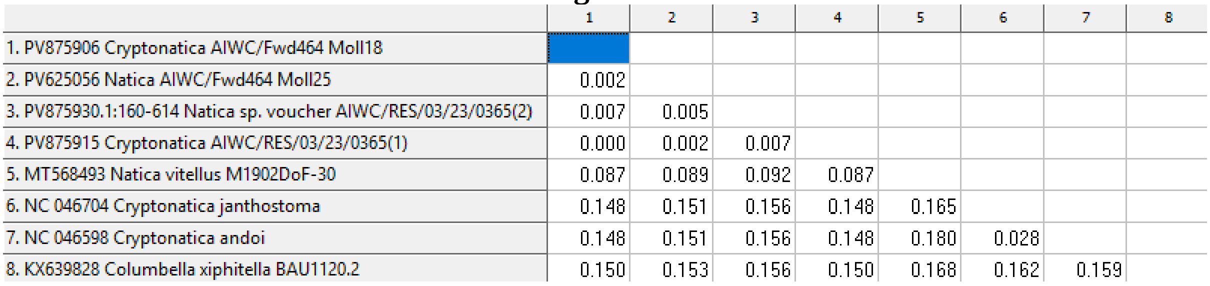


Figure 29: Dendrogram tree for *Cryptonatica.sp* and *Natica.sp* using Fwd464 primers using T93+G



	1	2	3	4	5	6	7	8
1. PV875906 Cryptonatica AIWC/Fwd464 Moll18								
2. PV625056 Natica AIWC/Fwd464 Moll25	0.002							
3. PV875930.1:160-614 Natica sp. voucher AIWC/RES/03/23/0365(2)	0.007	0.005						
4. PV875915 Cryptonatica AIWC/RES/03/23/0365(1)	0.000	0.002	0.007					
5. MT568493 Natica vitellus M1902DoF-30	0.087	0.089	0.092	0.087				
6. NC 046704 Cryptonatica janthostoma	0.148	0.151	0.156	0.148	0.165			
7. NC 046598 Cryptonatica andoi	0.148	0.151	0.156	0.148	0.180	0.028		
8. KX639828 Columbella xiphitella BAU1120.2	0.150	0.153	0.156	0.150	0.168	0.162	0.159	

Figure 30: K2P distance matrix for *Cryptonatica.sp* and *Natica.sp* using Fwd464 primers

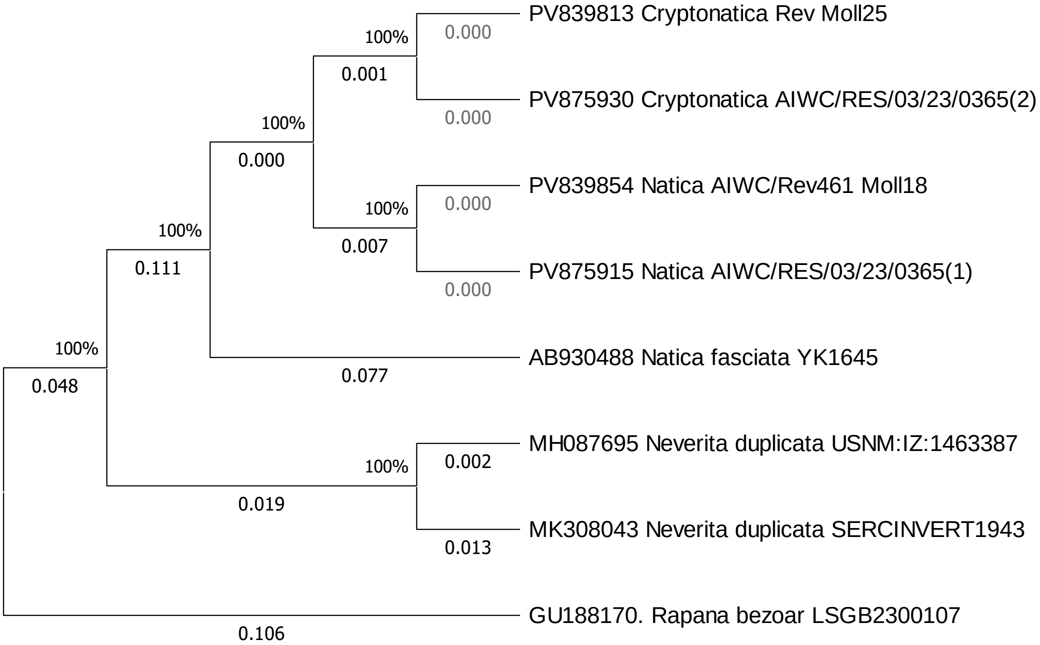
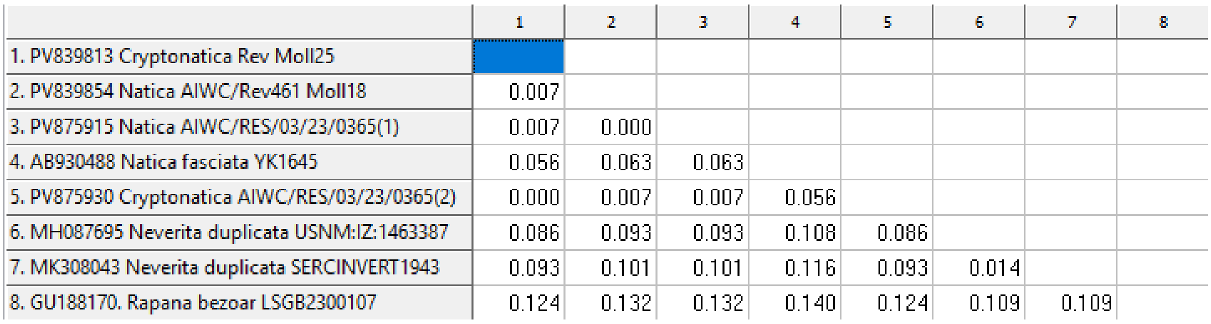


Figure 31: Dendrogram tree for *Cryptonatica.sp* and *Natica.sp* using Rev461 primers using T92+G



	1	2	3	4	5	6	7	8
1. PV839813 Cryptonatica Rev Moll25								
2. PV839854 Natica AIWC/Rev461 Moll18	0.007							
3. PV875915 Natica AIWC/RES/03/23/0365(1)	0.007	0.000						
4. AB930488 Natica fasciata YK1645	0.056	0.063	0.063					
5. PV875930 Cryptonatica AIWC/RES/03/23/0365(2)	0.000	0.007	0.007	0.056				
6. MH087695 Neverita duplicata USNM:IZ:1463387	0.086	0.093	0.093	0.108	0.086			
7. MK308043 Neverita duplicata SERCINVERT1943	0.093	0.101	0.101	0.116	0.093	0.014		
8. GU188170. Rapana bezoar LSGB2300107	0.124	0.132	0.132	0.140	0.124	0.109	0.109	

Figure 32: K2P distance matrix for *Cryptonatica.sp* and *Natica.sp* using Rev461 primers

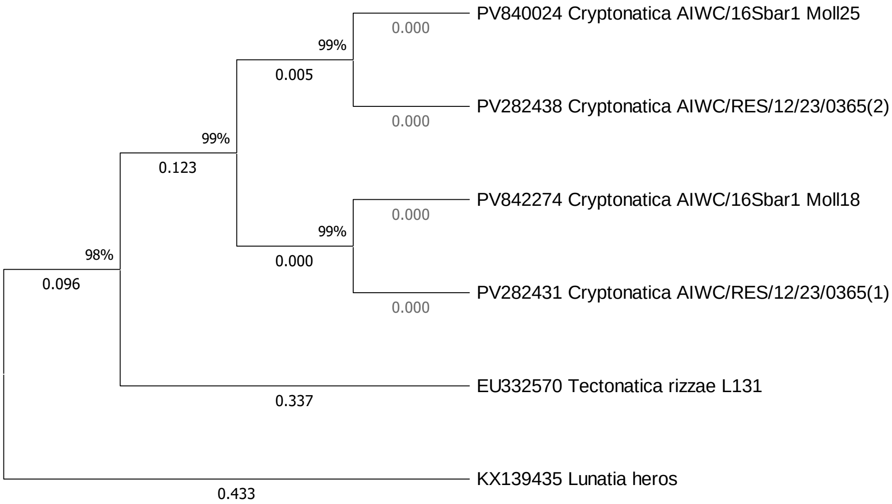
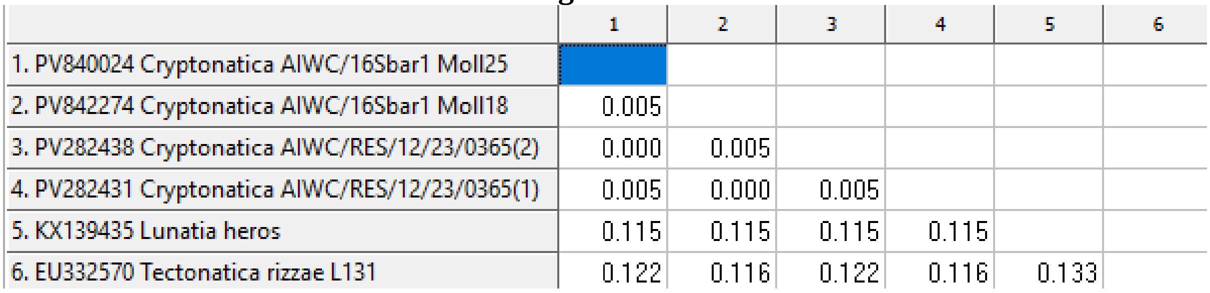


Figure 33: Dendrogram tree for *Cryptonatica.sp* and *Natica.sp* using 16Sbar primers using T92+G



	1	2	3	4	5	6
1. PV840024 Cryptonatica AIWC/16Sbar1 Moll25						
2. PV842274 Cryptonatica AIWC/16Sbar1 Moll18	0.005					
3. PV282438 Cryptonatica AIWC/RES/12/23/0365(2)	0.000	0.005				
4. PV282431 Cryptonatica AIWC/RES/12/23/0365(1)	0.005	0.000	0.005			
5. KX139435 Lunatia heros	0.115	0.115	0.115	0.115		
6. EU332570 Tectonatica rizzae L131	0.122	0.116	0.122	0.116	0.133	

Figure 34: K2P distance matrix for *Cryptonatica.sp* and *Natica.sp* using 16Sbar primers

7.11.2. Order Littorinimorpha - Family Tonnidae

The family Tonnidae comprises three genera, among which the genus Tonna includes approximately 30 recognized species (WoRMS, 2025). Despite its global distribution and commercial use for meat and shell, the phylogeny of this group remains understudied. As a result, very few reference sequences are currently available for interspecific comparisons. In the present study, *Tonna tessellata* was barcoded for the first time. Among the three primer sets used, the Rev461 primer failed to amplify the target region. The sequences generated using mini-barcodes showed high similarity to their respective reference sequences. However, the Fwd461 primers provided better resolution than the 16Sbar primers. Although the phylogenetic tree generated using the 16Sbar mini-barcode revealed distinct separation, the Maximum Likelihood (ML) tree showed low mean distance. Nevertheless, the distance matrix analysis clearly distinguished the species. (Fig 33-36)

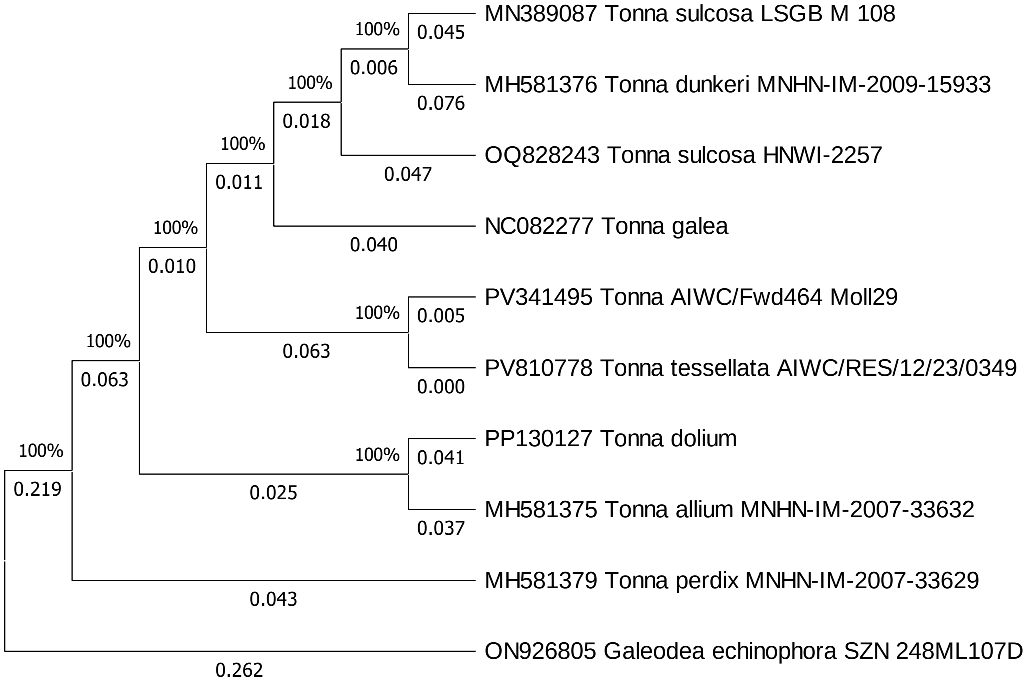
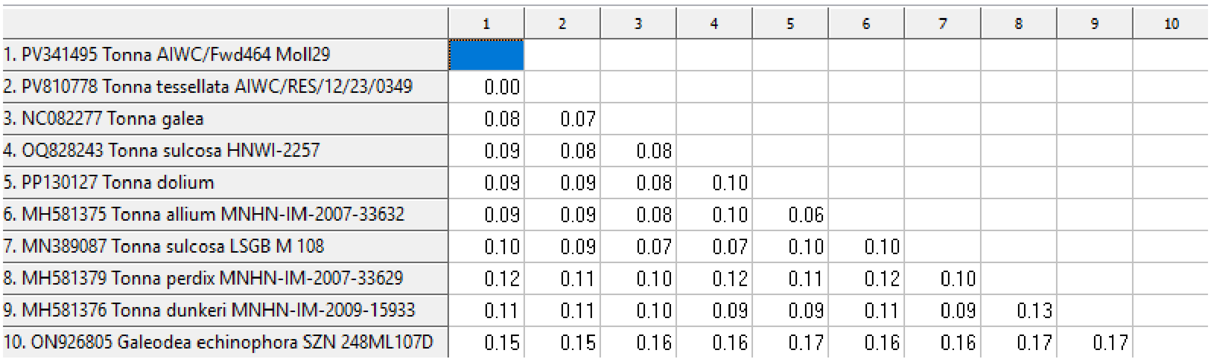


Figure 35: Dendrogram tree for *Tonna tessellata* using Fwd464 primers using HKY+G model



	1	2	3	4	5	6	7	8	9	10
1. PV341495 Tonna AIWC/Fwd464 Moll29										
2. PV810778 Tonna tessellata AIWC/RES/12/23/0349	0.00									
3. NC082277 Tonna galea	0.08	0.07								
4. OQ828243 Tonna sulcosa HNWI-2257	0.09	0.08	0.08							
5. PP130127 Tonna dolium	0.09	0.09	0.08	0.10						
6. MH581375 Tonna allium MNHN-IM-2007-33632	0.09	0.09	0.08	0.10	0.06					
7. MN389087 Tonna sulcosa LSGB M 108	0.10	0.09	0.07	0.07	0.10	0.10				
8. MH581379 Tonna perdix MNHN-IM-2007-33629	0.12	0.11	0.10	0.12	0.11	0.12	0.10			
9. MH581376 Tonna dunkeri MNHN-IM-2009-15933	0.11	0.11	0.10	0.09	0.09	0.11	0.09	0.13		
10. ON926805 Galeodea echinophora SZN 248ML107D	0.15	0.15	0.16	0.16	0.17	0.16	0.16	0.17	0.17	

Figure 36: K2P distance matrix for *Tonna tessellata* using Fwd464 primers

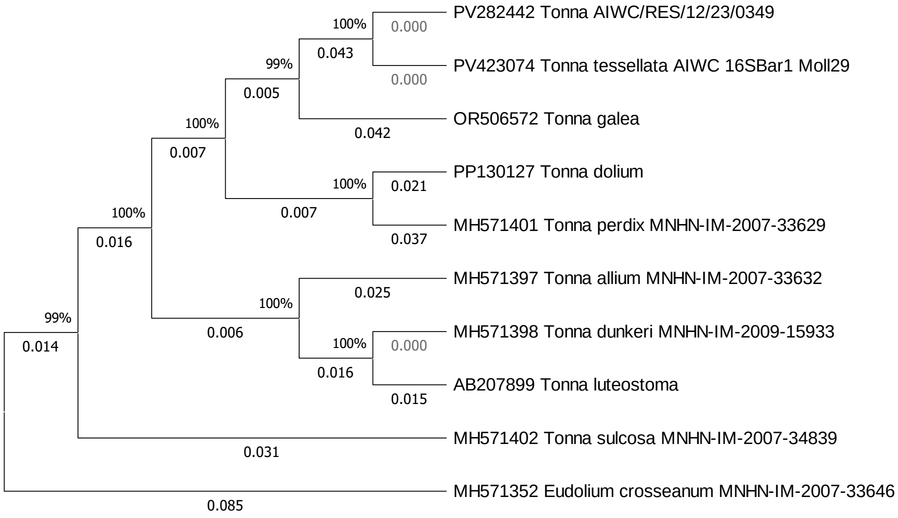
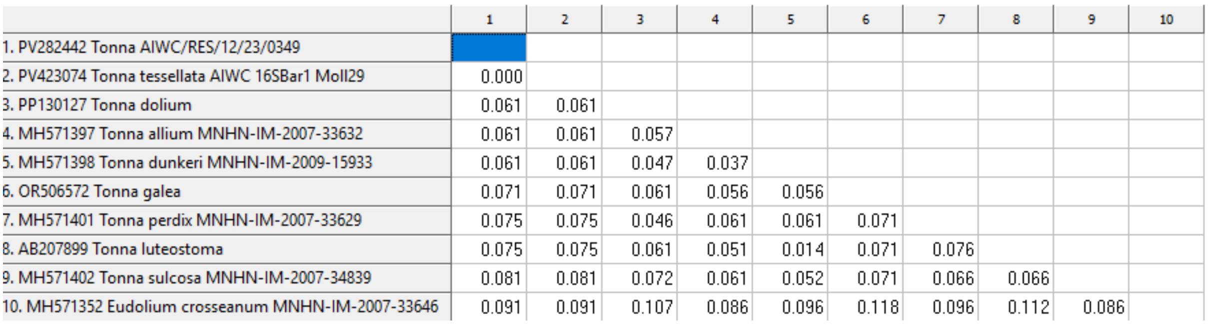


Figure 37: Dendrogram tree for *Tonna tessellata* using 16Sbar primers using *T92+G* model



	1	2	3	4	5	6	7	8	9	10
1. PV282442 Tonna AIWC/RES/12/23/0349										
2. PV423074 Tonna tessellata AIWC 16SBar1 Moll29	0.000									
3. PP130127 Tonna dolium	0.061	0.061								
4. MH571397 Tonna allium MNHN-IM-2007-33632	0.061	0.061	0.057							
5. MH571398 Tonna dunkeri MNHN-IM-2009-15933	0.061	0.061	0.047	0.037						
6. OR506572 Tonna galea	0.071	0.071	0.061	0.056	0.056					
7. MH571401 Tonna perdix MNHN-IM-2007-33629	0.075	0.075	0.046	0.061	0.061	0.071				
8. AB207899 Tonna luteostoma	0.075	0.075	0.061	0.051	0.014	0.071	0.076			
9. MH571402 Tonna sulcosa MNHN-IM-2007-34839	0.081	0.081	0.072	0.061	0.052	0.071	0.066	0.066		
10. MH571352 Eudolium crosseanum MNHN-IM-2007-33646	0.091	0.091	0.107	0.086	0.096	0.118	0.096	0.112	0.086	

Figure 38: K2P distance matrix for *Tonna tessellata* using 16Sbar primers

7.11.3. Order Littorinimorpha - Family Bursidae

The family Bursidae is a relatively well-studied group. In the present study, the 16S gene of *Bufonaria crumena* was barcoded and submitted to NCBI for the first time. Among the three primer sets used, the Rev461 primer failed to amplify the target region. The sequences generated using mini-barcodes showed high similarity to their respective reference sequences. Notably, the Fwd461 primers provided better resolution than the 16Sbar primers. Although the phylogenetic tree constructed using the 16Sbar mini-barcode revealed distinct species separation, the Maximum Likelihood (ML) tree indicated low mean genetic distances. However, genetic distance within the genus *Bufonaria* clearly distinguished *B. crumena* from other species (Fig 37 – 40).

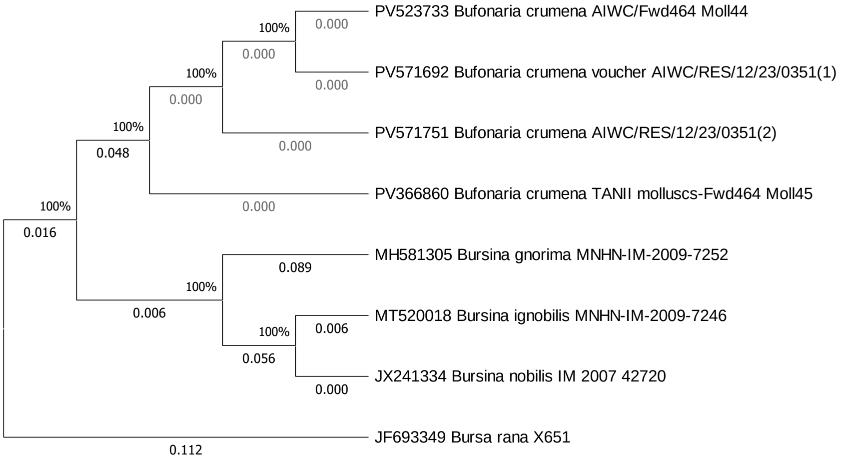
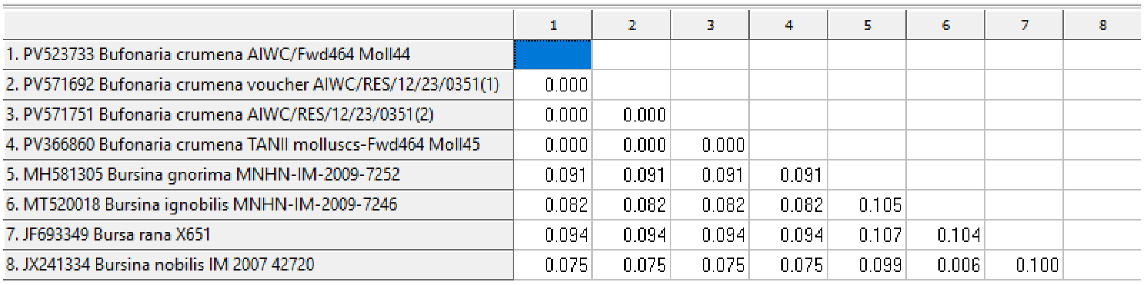


Figure 39: Dendrogram tree for *Bufonaria crumena* using Fwd464 primers using HKY+G model



	1	2	3	4	5	6	7	8
1. PV523733 Bufonaria crumena AIWC/Fwd464 Moll44								
2. PV571692 Bufonaria crumena voucher AIWC/RES/12/23/0351(1)	0.000							
3. PV571751 Bufonaria crumena AIWC/RES/12/23/0351(2)	0.000	0.000						
4. PV366860 Bufonaria crumena TANII molluscs-Fwd464 Moll45	0.000	0.000	0.000					
5. MH581305 Bursina gnorima MNHN-IM-2009-7252	0.091	0.091	0.091	0.091				
6. MT520018 Bursina ignobilis MNHN-IM-2009-7246	0.082	0.082	0.082	0.082	0.105			
7. JF693349 Bursa rana X651	0.094	0.094	0.094	0.094	0.107	0.104		
8. JX241334 Bursina nobilis IM 2007 42720	0.075	0.075	0.075	0.075	0.099	0.006	0.100	

Figure 40: K2P distance matrix for *Bufonaria crumena* using Fwd464 primers

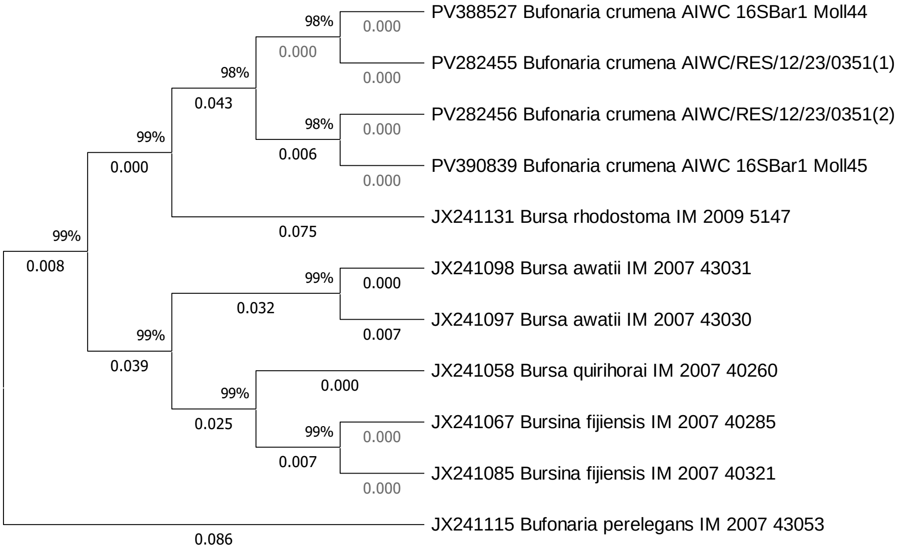
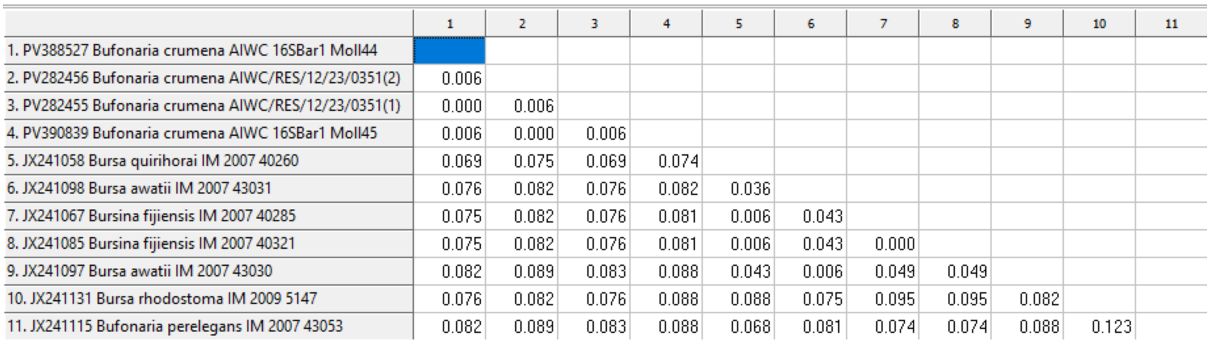


Figure 41: Dendrogram tree for *Bufonaria crumena* using 16Sbar primers using T92+G model



	1	2	3	4	5	6	7	8	9	10	11
1. PV388527 Bufonaria crumena AIWC 16SBar1 Moll44											
2. PV282456 Bufonaria crumena AIWC/RES/12/23/0351(2)	0.006										
3. PV282455 Bufonaria crumena AIWC/RES/12/23/0351(1)	0.000	0.006									
4. PV390839 Bufonaria crumena AIWC 16SBar1 Moll45	0.006	0.000	0.006								
5. JX241058 Bursa quirihorai IM 2007 40260	0.069	0.075	0.069	0.074							
6. JX241098 Bursa awatii IM 2007 43031	0.076	0.082	0.076	0.082	0.036						
7. JX241067 Bursina fijiensis IM 2007 40285	0.075	0.082	0.076	0.081	0.006	0.043					
8. JX241085 Bursina fijiensis IM 2007 40321	0.075	0.082	0.076	0.081	0.006	0.043	0.000				
9. JX241097 Bursa awatii IM 2007 43030	0.082	0.089	0.083	0.088	0.043	0.006	0.049	0.049			
10. JX241131 Bursa rhodostoma IM 2009 5147	0.076	0.082	0.076	0.088	0.088	0.075	0.095	0.095	0.082		
11. JX241115 Bufonaria perelegans IM 2007 43053	0.082	0.089	0.083	0.088	0.068	0.081	0.074	0.074	0.088	0.123	

Figure 42 K2P distance matrix for *Bufonaria crumena* using 16Sbar primers

7.11.4. Order Littorinimorpha – Family Strombidae

Laevistrombus canarium, *Lambis croata*, *Lambis lambis*, and *Harpago chiragra* from the family *Strombidae* were tested for primer efficiency. All three designed primers successfully amplified the target regions in all four specimens, resulting in clear species-level identification. Initially, there were no reference sequences available for the genus *Lambis*, except for *Lambis lambis*, which was used in primer design. However, during the course of this study, Irwin et al. (2024) published a comprehensive analysis of the family Stromboidea, including sequences from other protected *Lambis* species (Fig 41-58). In future studies, sequences from Irwin et al. (2024) can serve as standard references.

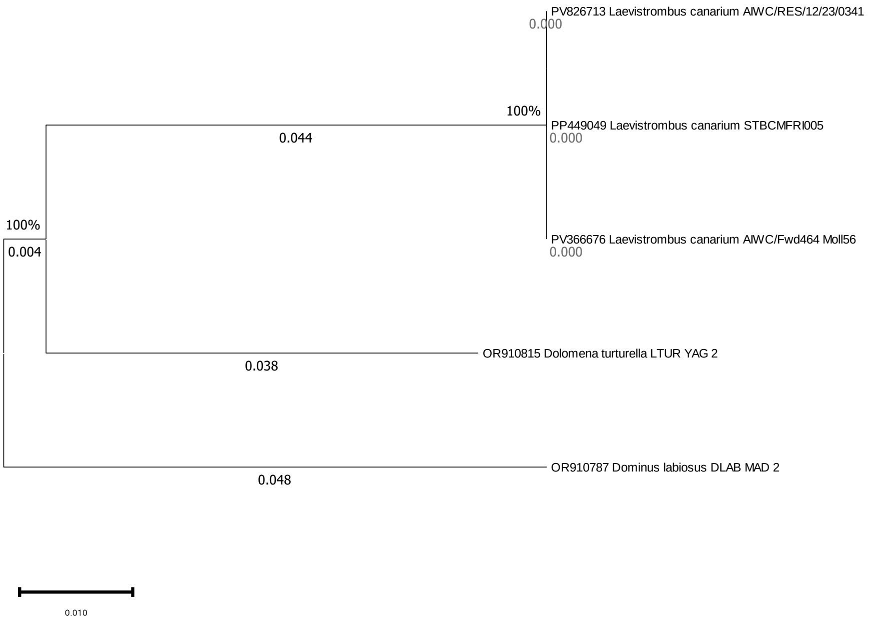
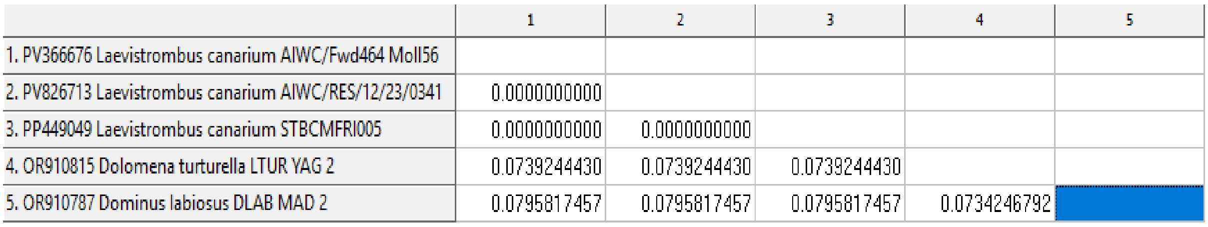


Figure 43: Dendrogram tree for *Laevistrombus canarium* using Fwd464 primers using HKY+G model



	1	2	3	4	5
1. PV366676 Laevistrombus canarium AIWC/Fwd464 Moll56					
2. PV826713 Laevistrombus canarium AIWC/RES/12/23/0341	0.0000000000				
3. PP449049 Laevistrombus canarium STBCMFRI005	0.0000000000	0.0000000000			
4. OR910815 Dolomena turturella LTUR YAG 2	0.0739244430	0.0739244430	0.0739244430		
5. OR910787 Dominus labiosus DLAB MAD 2	0.0795817457	0.0795817457	0.0795817457	0.0734246792	

Figure 44: K2P distance matrix for *Laevistrombus canarium* using Fwd 464 primers

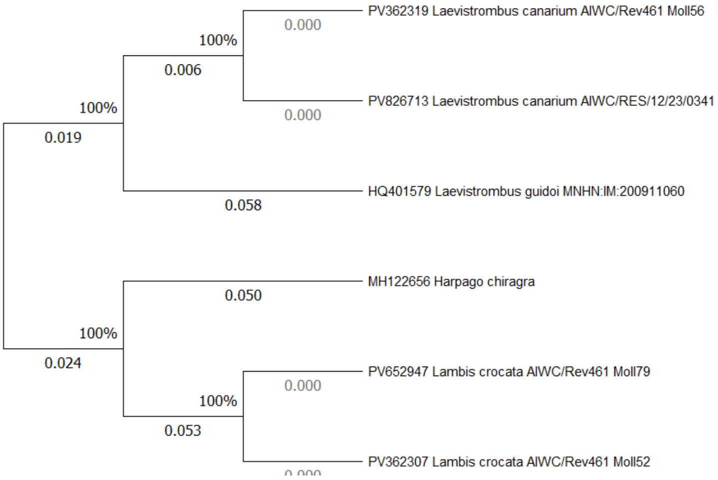
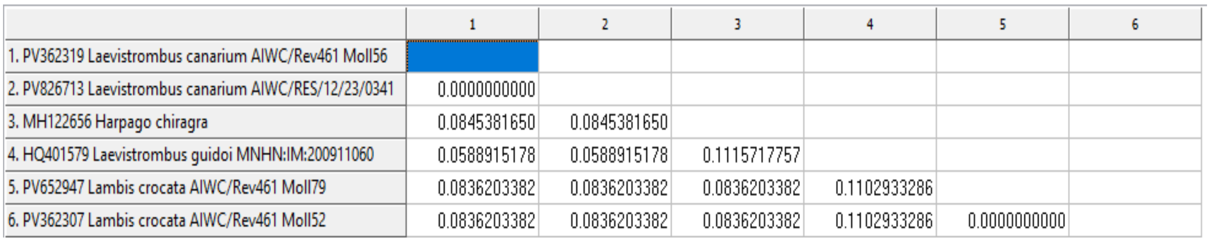


Figure 45: Dendrogram tree for *Laevistrombus canarium* using Rev461 primers using T92+G model



	1	2	3	4	5	6
1. PV362319 Laevistrombus canarium AIWC/Rev461 Moll56						
2. PV826713 Laevistrombus canarium AIWC/RES/12/23/0341	0.0000000000					
3. MH122656 Harpago chiragra	0.0845381650	0.0845381650				
4. HQ401579 Laevistrombus guidoi MNHN:IM:200911060	0.0588915178	0.0588915178	0.1115717757			
5. PV652947 Lambis crocata AIWC/Rev461 Moll79	0.0836203382	0.0836203382	0.0836203382	0.1102933286		
6. PV362307 Lambis crocata AIWC/Rev461 Moll52	0.0836203382	0.0836203382	0.0836203382	0.1102933286	0.0000000000	

Figure 46: K2P distance matrix for *Laevistrombus canarium* using Rev461 primers

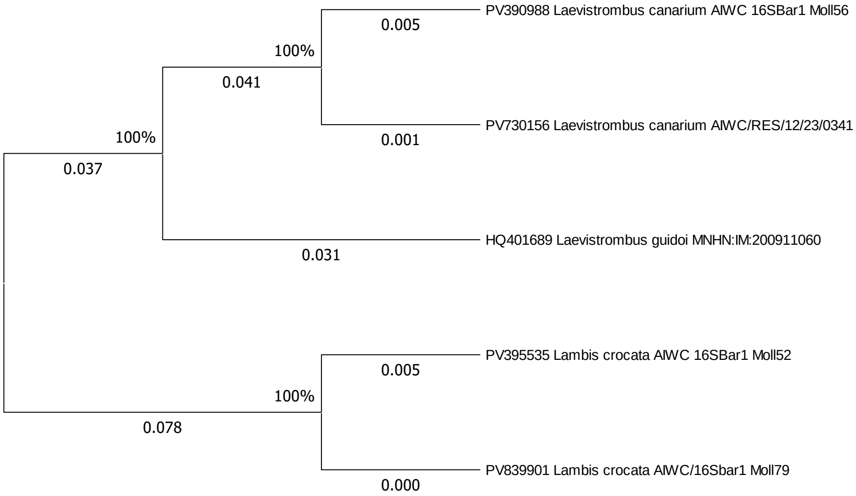
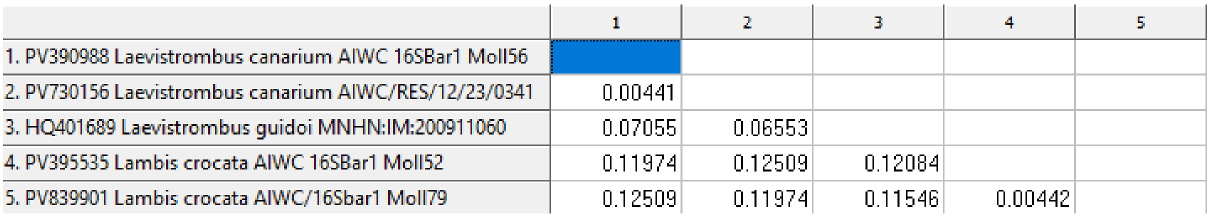


Figure 47: Dendrogram tree for *Laevistrombus canarium* using 16Sbar primers using T92+G model



	1	2	3	4	5
1. PV390988 Laevistrombus canarium AIWC 16SBar1 Moll56					
2. PV730156 Laevistrombus canarium AIWC/RES/12/23/0341	0.00441				
3. HQ401689 Laevistrombus guidoi MNHN:IM:200911060	0.07055	0.06553			
4. PV395535 Lambis crocata AIWC 16SBar1 Moll52	0.11974	0.12509	0.12084		
5. PV839901 Lambis crocata AIWC/16Sbar1 Moll79	0.12509	0.11974	0.11546	0.00442	

Figure 48: K2P distance matrix for *Laevistrombus canarium* using 16Sbar primers

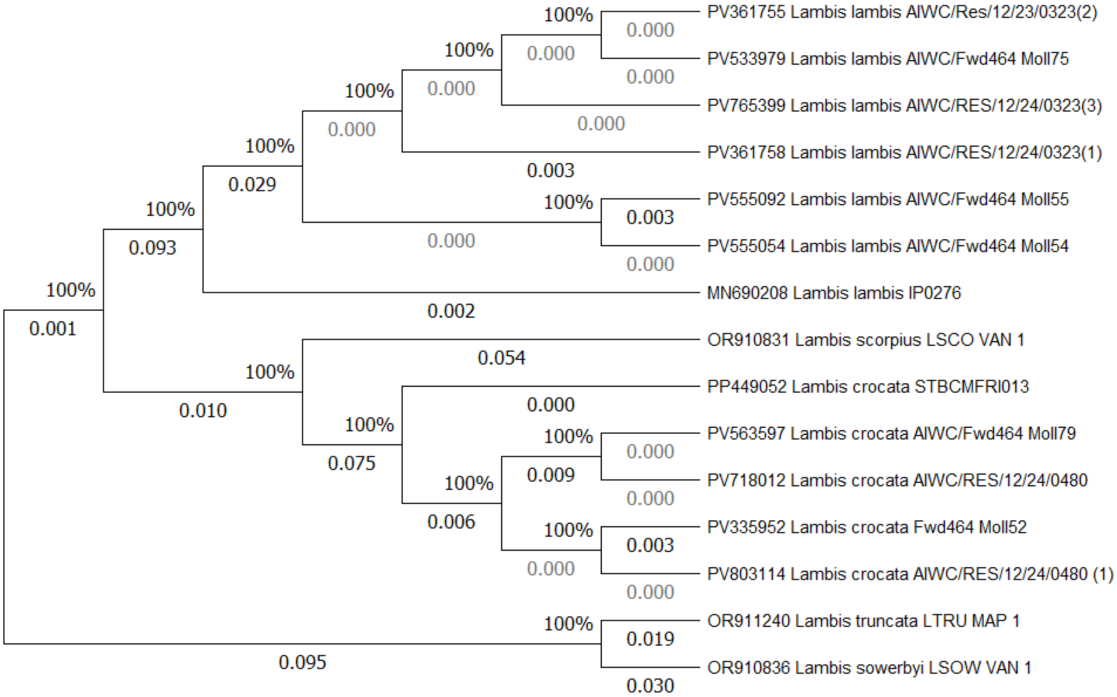
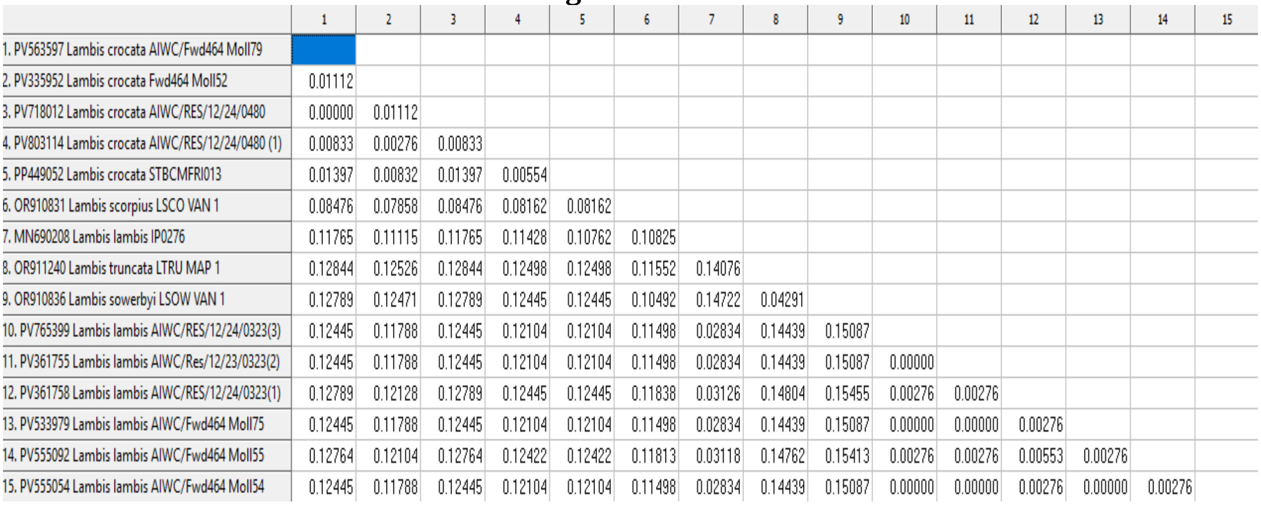


Figure 49: Dendrogram tree for *Lambis crocata* and *Lambis lambis* using Fwd464 primers using T92+G model



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. PV563597 Lambis crocata AIWC/Fwd464 Moll79															
2. PV335952 Lambis crocata Fwd464 Moll52	0.01112														
3. PV718012 Lambis crocata AIWC/RES/12/24/0480	0.00000	0.01112													
4. PV803114 Lambis crocata AIWC/RES/12/24/0480 (1)	0.00833	0.00276	0.00833												
5. PP449052 Lambis crocata STBCMFRI013	0.01397	0.00832	0.01397	0.00554											
6. OR910831 Lambis scorpius LSCO VAN 1	0.08476	0.07858	0.08476	0.08162	0.08162										
7. MN690208 Lambis lambis IP0276	0.11765	0.11115	0.11765	0.11428	0.10762	0.10825									
8. OR911240 Lambis truncata LTRU MAP 1	0.12844	0.12526	0.12844	0.12498	0.12498	0.11552	0.14076								
9. OR910836 Lambis sowerbyi LSOW VAN 1	0.12789	0.12471	0.12789	0.12445	0.12445	0.10492	0.14722	0.04291							
10. PV765399 Lambis lambis AIWC/RES/12/24/0323(3)	0.12445	0.11788	0.12445	0.12104	0.12104	0.11498	0.02834	0.14439	0.15087						
11. PV361755 Lambis lambis AIWC/Res/12/23/0323(2)	0.12445	0.11788	0.12445	0.12104	0.12104	0.11498	0.02834	0.14439	0.15087	0.00000					
12. PV361758 Lambis lambis AIWC/RES/12/24/0323(1)	0.12789	0.12128	0.12789	0.12445	0.12445	0.11838	0.03126	0.14804	0.15455	0.00276	0.00276				
13. PV533979 Lambis lambis AIWC/Fwd464 Moll75	0.12445	0.11788	0.12445	0.12104	0.12104	0.11498	0.02834	0.14439	0.15087	0.00000	0.00000	0.00276			
14. PV555092 Lambis lambis AIWC/Fwd464 Moll55	0.12764	0.12104	0.12764	0.12422	0.12422	0.11813	0.03118	0.14762	0.15413	0.00276	0.00276	0.00553	0.00276		
15. PV555054 Lambis lambis AIWC/Fwd464 Moll54	0.12445	0.11788	0.12445	0.12104	0.12104	0.11498	0.02834	0.14439	0.15087	0.00000	0.00000	0.00276	0.00000	0.00276	

Figure 50: K2P distance matrix for *Lambis crocata* and *Lambis lambis* using Fwd 464 primers

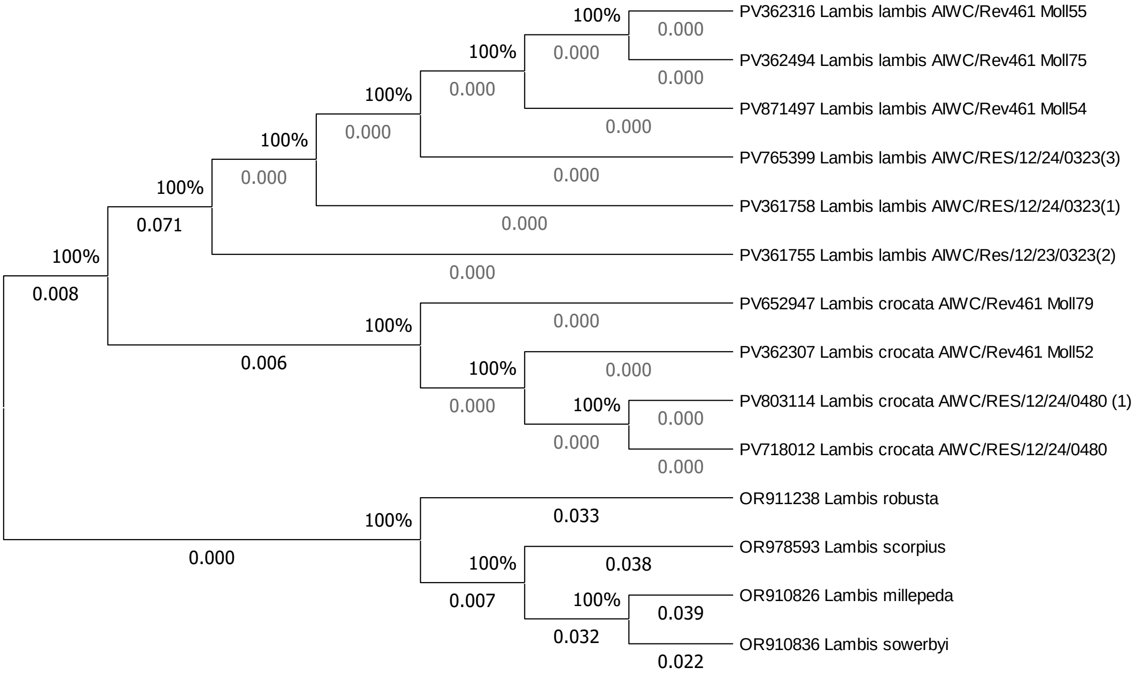
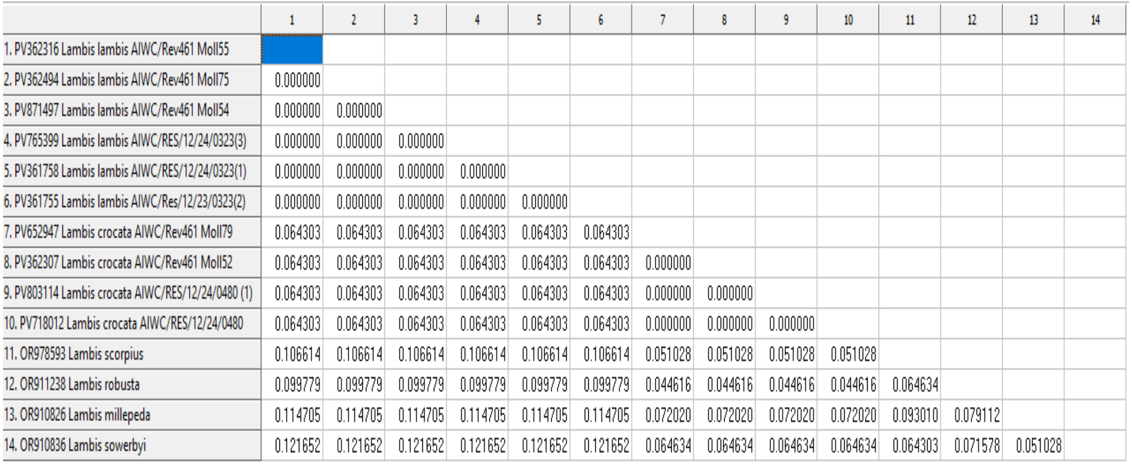


Figure 51: Dendrogram tree for *Lambis crocata* and *Lambis lambis* using Rev 461 primers using T92 model



	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. PV362316 Lambis lambis AIWC/Rev461 Moll55														
2. PV362494 Lambis lambis AIWC/Rev461 Moll75	0.000000													
3. PV871497 Lambis lambis AIWC/Rev461 Moll54	0.000000	0.000000												
4. PV765399 Lambis lambis AIWC/RES/12/24/0323(3)	0.000000	0.000000	0.000000											
5. PV361758 Lambis lambis AIWC/RES/12/24/0323(1)	0.000000	0.000000	0.000000	0.000000										
6. PV361755 Lambis lambis AIWC/Res/12/23/0323(2)	0.000000	0.000000	0.000000	0.000000	0.000000									
7. PV652947 Lambis crocata AIWC/Rev461 Moll79	0.064303	0.064303	0.064303	0.064303	0.064303	0.064303								
8. PV362307 Lambis crocata AIWC/Rev461 Moll52	0.064303	0.064303	0.064303	0.064303	0.064303	0.064303	0.000000							
9. PV803114 Lambis crocata AIWC/RES/12/24/0480 (1)	0.064303	0.064303	0.064303	0.064303	0.064303	0.064303	0.000000	0.000000						
10. PV718012 Lambis crocata AIWC/RES/12/24/0480	0.064303	0.064303	0.064303	0.064303	0.064303	0.064303	0.000000	0.000000	0.000000					
11. OR978593 Lambis scorpius	0.106614	0.106614	0.106614	0.106614	0.106614	0.106614	0.051028	0.051028	0.051028	0.051028				
12. OR911238 Lambis robusta	0.099779	0.099779	0.099779	0.099779	0.099779	0.099779	0.044616	0.044616	0.044616	0.044616	0.064634			
13. OR910826 Lambis millepeda	0.114705	0.114705	0.114705	0.114705	0.114705	0.114705	0.072020	0.072020	0.072020	0.072020	0.093010	0.079112		
14. OR910836 Lambis sowerbyi	0.121652	0.121652	0.121652	0.121652	0.121652	0.121652	0.064634	0.064634	0.064634	0.064634	0.064303	0.071578	0.051028	

Figure 52: K2P distance matrix for *Lambis crocata* and *Lambis lambis* using Rev 461 primers

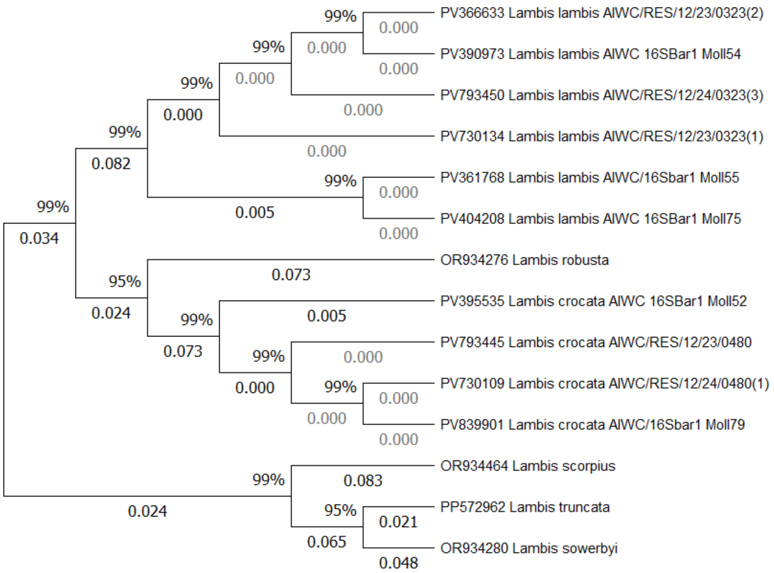
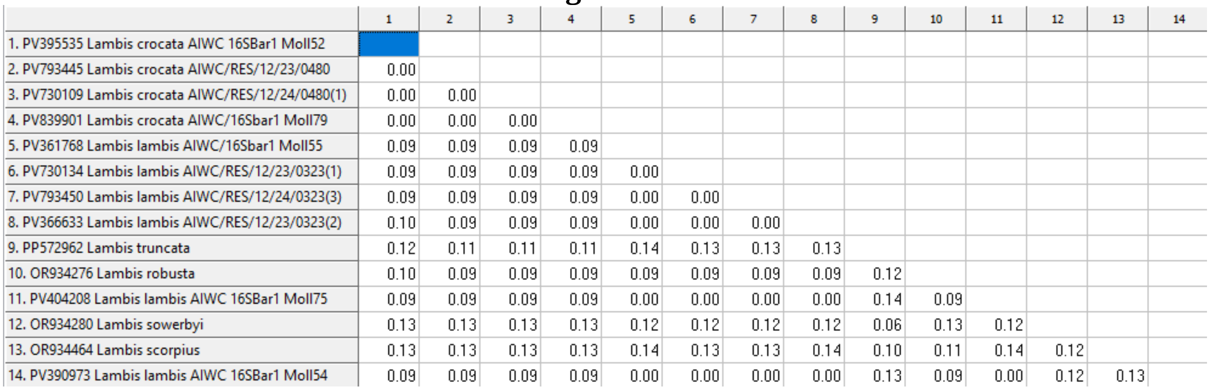


Figure 53: Dendrogram tree for *Lambis crocata* and *Lambis lambis* using 16Sbar primers using T92+G model



	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. PV395535 Lambis crocata AIWC 16SBar1 Moll52														
2. PV793445 Lambis crocata AIWC/RES/12/23/0480	0.00													
3. PV730109 Lambis crocata AIWC/RES/12/24/0480(1)	0.00	0.00												
4. PV839901 Lambis crocata AIWC/16Sbar1 Moll79	0.00	0.00	0.00											
5. PV361768 Lambis lambis AIWC/16Sbar1 Moll55	0.09	0.09	0.09	0.09										
6. PV730134 Lambis lambis AIWC/RES/12/23/0323(1)	0.09	0.09	0.09	0.09	0.00									
7. PV793450 Lambis lambis AIWC/RES/12/24/0323(3)	0.09	0.09	0.09	0.09	0.00	0.00								
8. PV366633 Lambis lambis AIWC/RES/12/23/0323(2)	0.10	0.09	0.09	0.09	0.00	0.00	0.00							
9. PP572962 Lambis truncata	0.12	0.11	0.11	0.11	0.14	0.13	0.13	0.13						
10. OR934276 Lambis robusta	0.10	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.12					
11. PV404208 Lambis lambis AIWC 16SBar1 Moll75	0.09	0.09	0.09	0.09	0.00	0.00	0.00	0.00	0.14	0.09				
12. OR934280 Lambis sowerbyi	0.13	0.13	0.13	0.13	0.12	0.12	0.12	0.12	0.06	0.13	0.12			
13. OR934464 Lambis scorpius	0.13	0.13	0.13	0.13	0.14	0.13	0.13	0.14	0.10	0.11	0.14	0.12		
14. PV390973 Lambis lambis AIWC 16SBar1 Moll54	0.09	0.09	0.09	0.09	0.00	0.00	0.00	0.00	0.13	0.09	0.00	0.12	0.13	

Figure 54: K2P distance matrix for *Lambis crocata* and *Lambis lambis* using 16Sbar primers

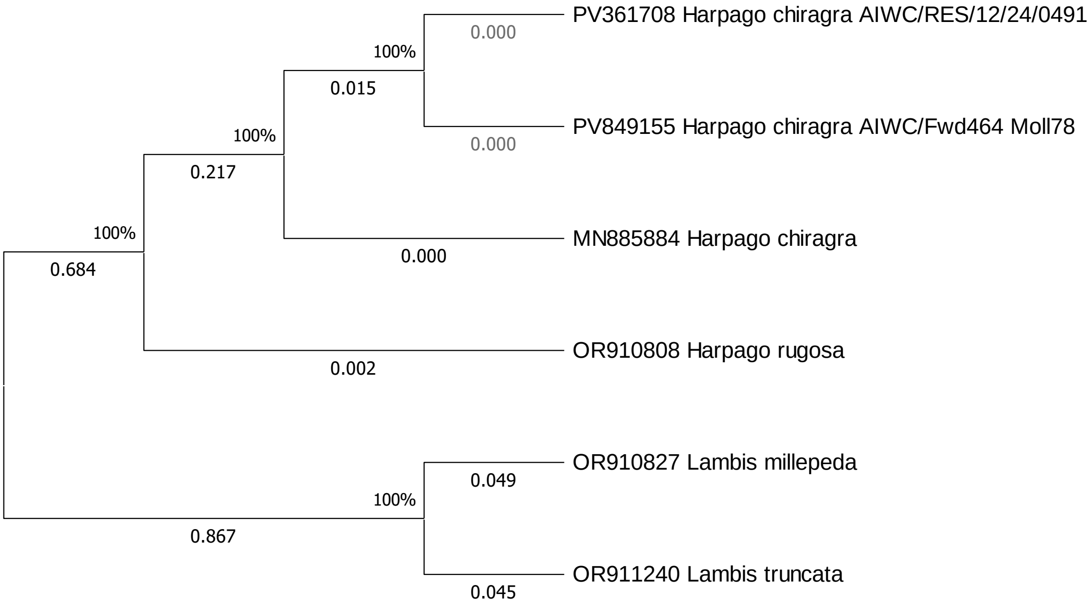
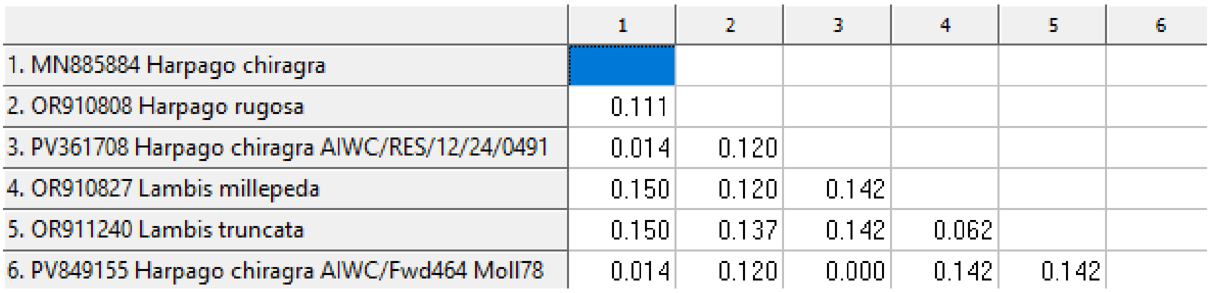


Figure 55: Dendrogram tree for *Harpago chiragra* using Fwd464 primers using T92+G model



	1	2	3	4	5	6
1. MN885884 Harpago chiragra						
2. OR910808 Harpago rugosa	0.111					
3. PV361708 Harpago chiragra AIWC/RES/12/24/0491	0.014	0.120				
4. OR910827 Lambis millepeda	0.150	0.120	0.142			
5. OR911240 Lambis truncata	0.150	0.137	0.142	0.062		
6. PV849155 Harpago chiragra AIWC/Fwd464 Moll78	0.014	0.120	0.000	0.142	0.142	

Figure 56: K2P distance matrix for *Harpago chiraga* using Fwd464 primers

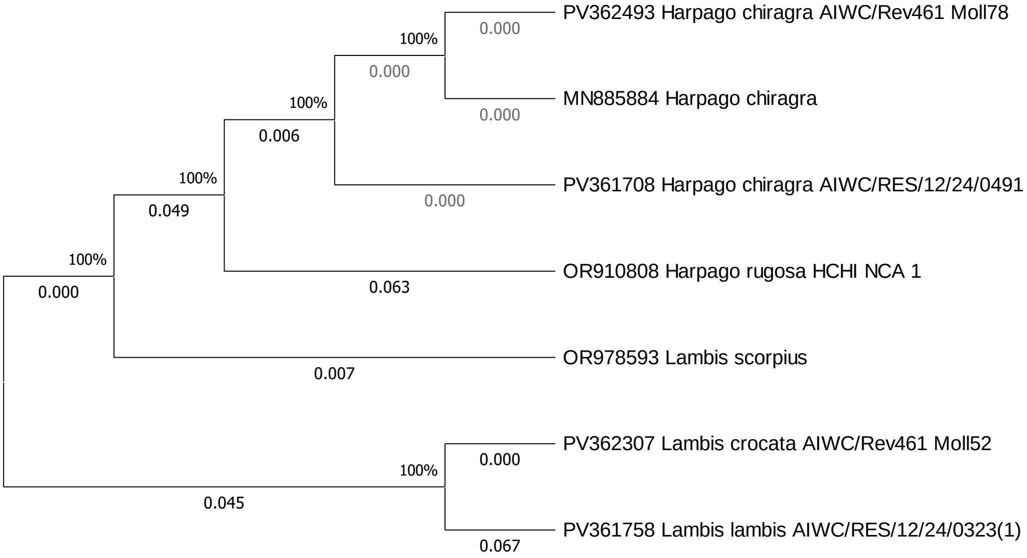
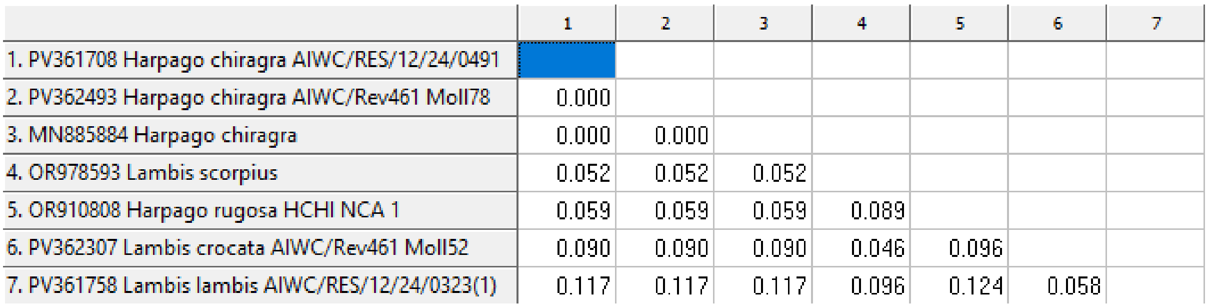


Figure 57: Dendrogram tree for *Harpago chiragra* using Rev461 primers using T92+G model



	1	2	3	4	5	6	7
1. PV361708 Harpago chiragra AIWC/RES/12/24/0491							
2. PV362493 Harpago chiragra AIWC/Rev461 Moll78	0.000						
3. MN885884 Harpago chiragra	0.000	0.000					
4. OR978593 Lambis scorpius	0.052	0.052	0.052				
5. OR910808 Harpago rugosa HCHI NCA 1	0.059	0.059	0.059	0.089			
6. PV362307 Lambis crocata AIWC/Rev461 Moll52	0.090	0.090	0.090	0.046	0.096		
7. PV361758 Lambis lambis AIWC/RES/12/24/0323(1)	0.117	0.117	0.117	0.096	0.124	0.058	

Figure 58: K2P distance matrix for *Harpago chiraga* using rev461 primers

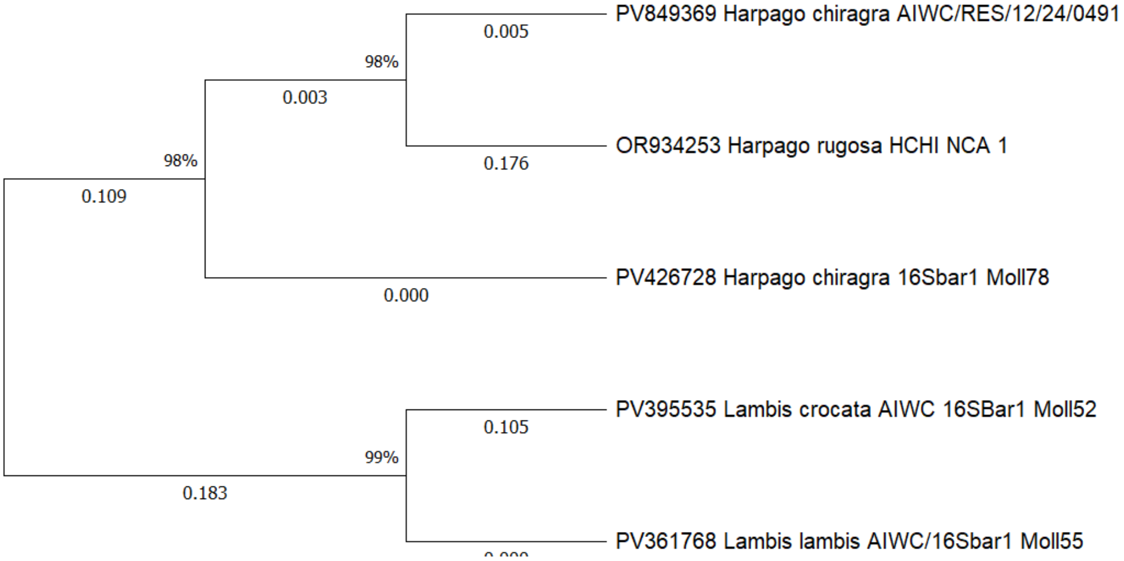
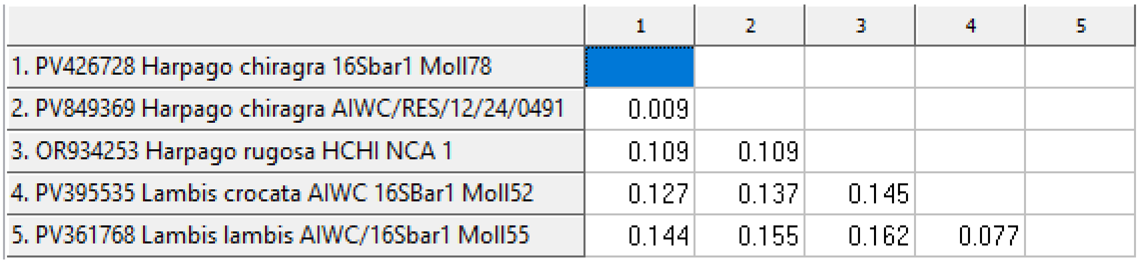


Figure 59: Dendrogram tree for *Harpago chiragra* using 16Sbar primers using T92+G model



	1	2	3	4	5
1. PV426728 Harpago chiragra 16Sbar1 Moll78					
2. PV849369 Harpago chiragra AIWC/RES/12/24/0491	0.009				
3. OR934253 Harpago rugosa HCHI NCA 1	0.109	0.109			
4. PV395535 Lambis crocata AIWC 16SBar1 Moll52	0.127	0.137	0.145		
5. PV361768 Lambis lambis AIWC/16Sbar1 Moll55	0.144	0.155	0.162	0.077	

Figure 60: K2P distance matrix for *Harpago chiraga* using 16Sbar primers

7.11.5. Order Littorinimorpha - Family Cassidae

Both the protected species *Cassis cornuta* and *Cypraecassis rufa* belong to the family *Cassidae*. During the study period, only a single specimen of Cassis cornuta was found as bycatch from the Thoothukudi Sea Harbour. In the present study, the 16S gene of Cassis cornuta was barcoded and submitted to NCBI for the first time. Of the three primers tested, both Fwd464 and 16Sbar successfully amplified the target region and clearly distinguished *Cassis cornuta* from other species within the genus Cassis *(Fig 59-62)*. However, publicly available sequences for the genus *Cassis* remain scarce.

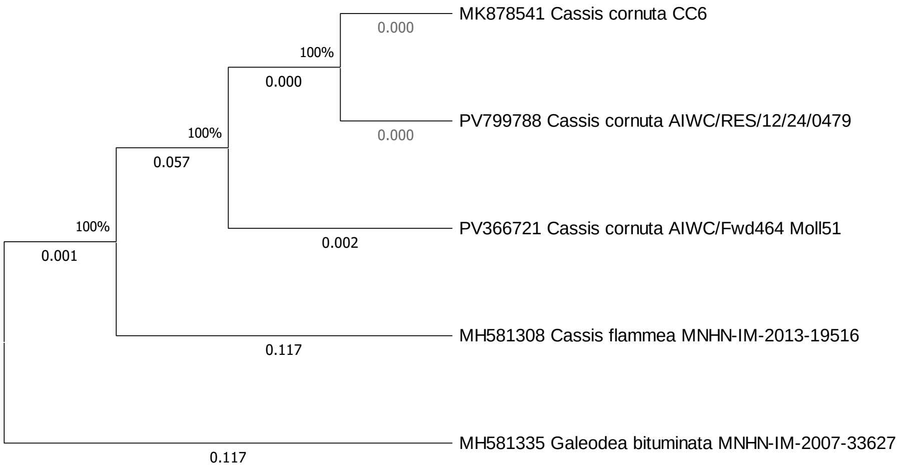
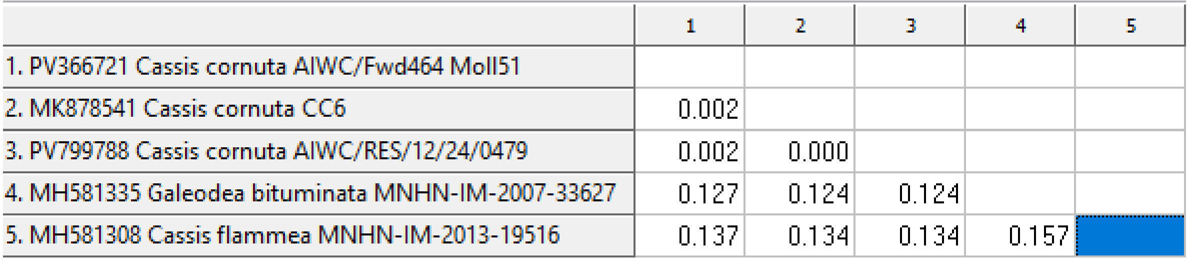


Figure 61: Dendrogram tree for *Cassis cornuta* using Fwd464 primers using HKY+G model



	1	2	3	4	5
1. PV366721 Cassis cornuta AIWC/Fwd464 Moll51					
2. MK878541 Cassis cornuta CC6	0.002				
3. PV799788 Cassis cornuta AIWC/RES/12/24/0479	0.002	0.000			
4. MH581335 Galeodea bituminata MNHN-IM-2007-33627	0.127	0.124	0.124		
5. MH581308 Cassis flammea MNHN-IM-2013-19516	0.137	0.134	0.134	0.157	

Figure 62: K2P distance matrix for *Cassis cornuta* using Fwd464 primers

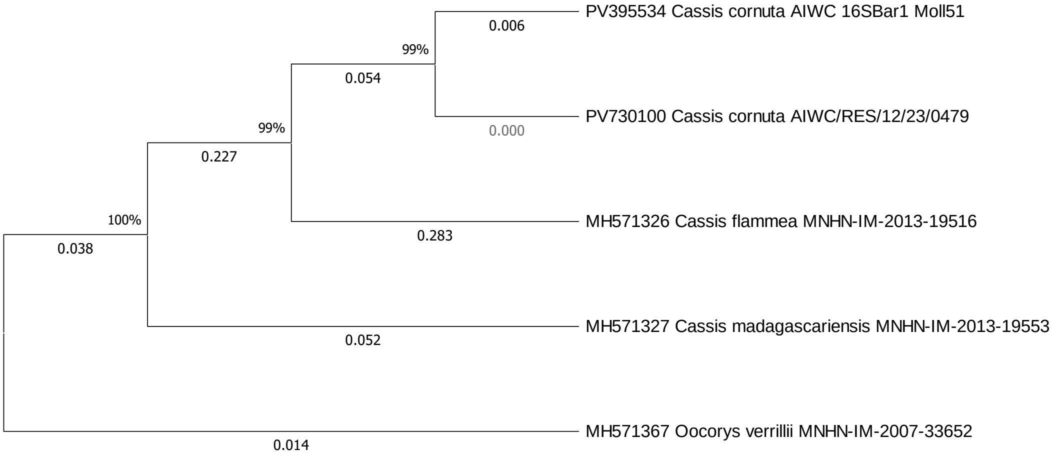
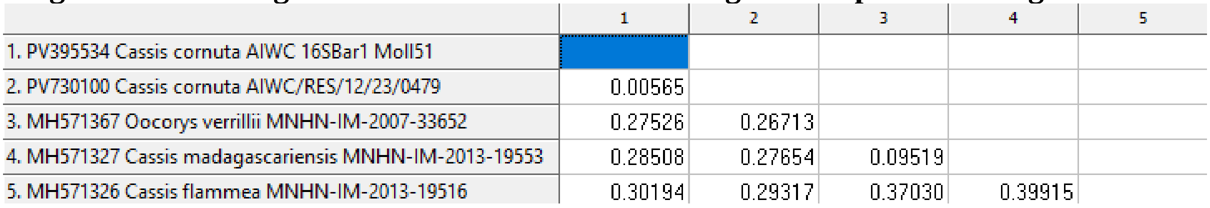


Figure 63: Dendrogram tree for *Cassis cornuta* using 16Sbar primers using T92model



	1	2	3	4	5
1. PV395534 Cassis cornuta AIWC 16SBar1 Moll51					
2. PV730100 Cassis cornuta AIWC/RES/12/23/0479	0.00565				
3. MH571367 Oocorys verrillii MNHN-IM-2007-33652	0.27526	0.26713			
4. MH571327 Cassis madagascariensis MNHN-IM-2013-19553	0.28508	0.27654	0.09519		
5. MH571326 Cassis flammea MNHN-IM-2013-19516	0.30194	0.29317	0.37030	0.39915	

Figure 64: K2P distance matrix for *Cassis cornuta* using 16Sbar primers

7.11.6. Order Neogastropoda - Family Babyloniidae

Babylonia spirata is a commercially important species, widely exploited for both its meat and shell. All three designed primers successfully amplified the target regions, and the resulting sequences matched the corresponding reference sequence. However, the mean genetic distance between *B. spirata* and other species was similar for both Rev461 and 16Sbar primers (Fig 63 -68), indicating limited discriminatory power between closely related *Babylonia* species.

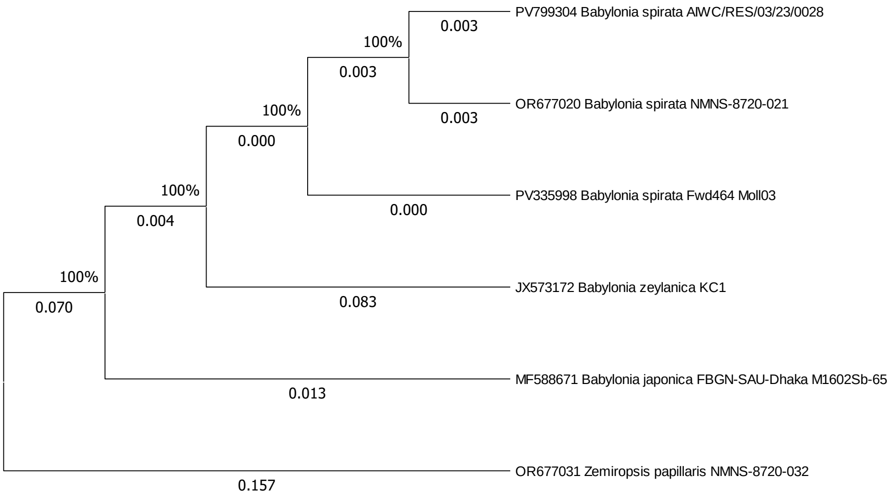
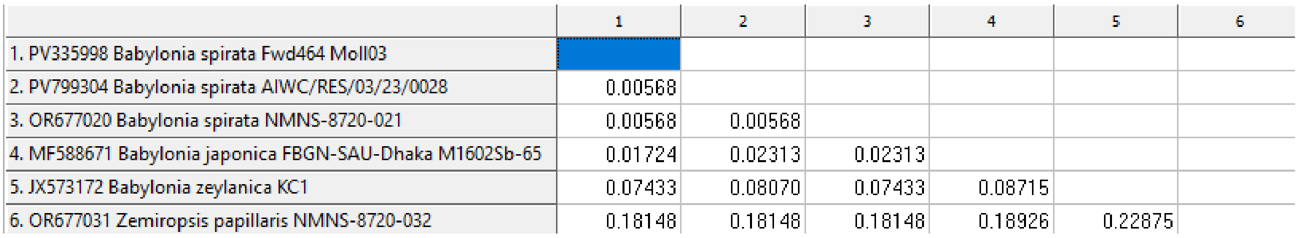


Figure 65: Dendrogram tree for *Babylonia spirata* using Fwd 464 primers using K2 model.



	1	2	3	4	5	6
1. PV335998 Babylonia spirata Fwd464 Moll03						
2. PV799304 Babylonia spirata AIWC/RES/03/23/0028	0.00568					
3. OR677020 Babylonia spirata NMNS-8720-021	0.00568	0.00568				
4. MF588671 Babylonia japonica FBGN-SAU-Dhaka M1602Sb-65	0.01724	0.02313	0.02313			
5. JX573172 Babylonia zeylanica KC1	0.07433	0.08070	0.07433	0.08715		
6. OR677031 Zemiropsis papillaris NMNS-8720-032	0.18148	0.18148	0.18148	0.18926	0.22875	

Figure 66: K2P distance matrix for *Babylonia spirata* using Fwd 464 primers

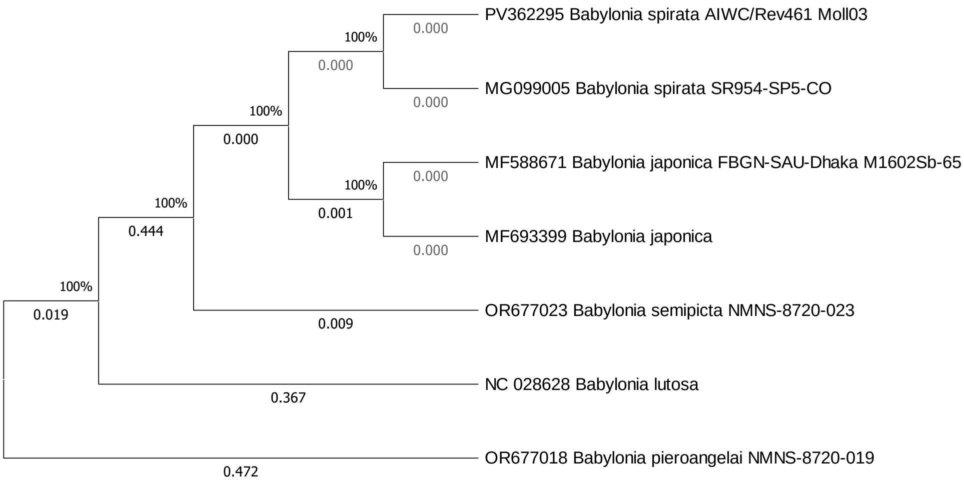
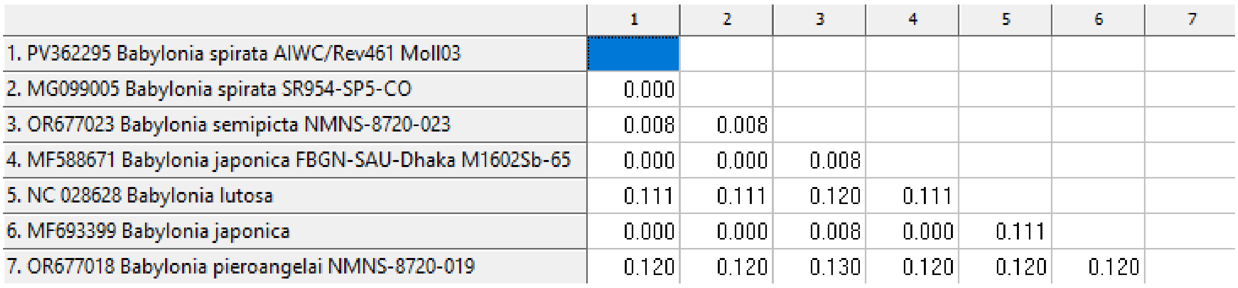


Figure 67: Dendrogram tree for *Babylonia spirata* using Rev 461 primers using T92 model.



	1	2	3	4	5	6	7
1. PV362295 Babylonia spirata AIWC/Rev461 Moll03							
2. MG099005 Babylonia spirata SR954-SP5-CO	0.000						
3. OR677023 Babylonia semipicta NMNS-8720-023	0.008	0.008					
4. MF588671 Babylonia japonica FBGN-SAU-Dhaka M1602Sb-65	0.000	0.000	0.008				
5. NC 028628 Babylonia lutosa	0.111	0.111	0.120	0.111			
6. MF693399 Babylonia japonica	0.000	0.000	0.008	0.000	0.111		
7. OR677018 Babylonia pieroangelai NMNS-8720-019	0.120	0.120	0.130	0.120	0.120	0.120	

Figure 68: K2P distance matrix for *Babylonia spirata* using Rev 461 primers

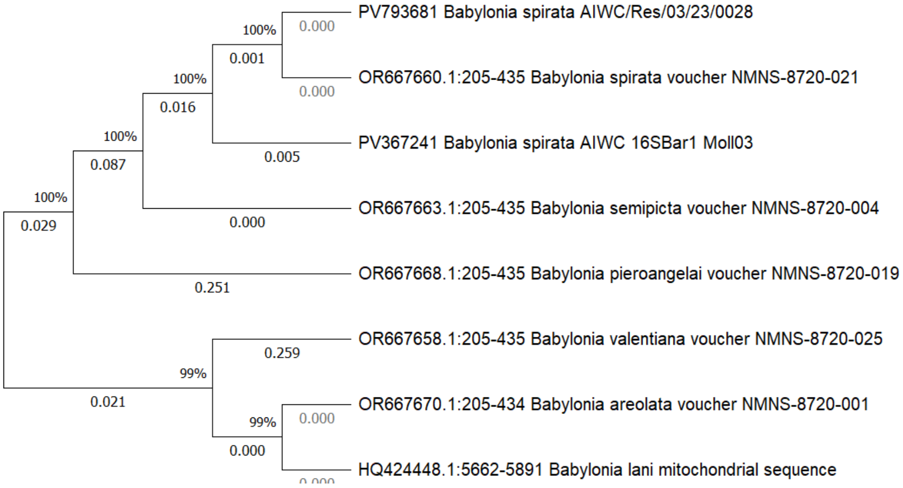
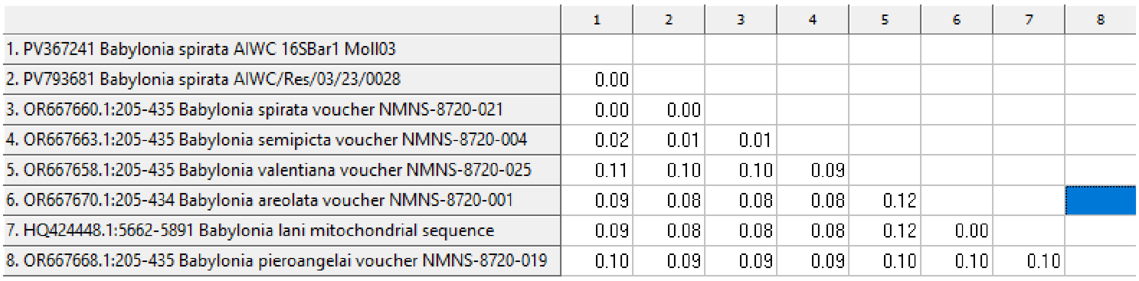


Figure 69: Dendrogram tree for *Babylonia spirata* using 16Sbar primers using T92 model



	1	2	3	4	5	6	7	8
1. PV367241 Babylonia spirata AIWC 16SBar1 Moll03								
2. PV793681 Babylonia spirata AIWC/Res/03/23/0028	0.00							
3. OR667660.1:205-435 Babylonia spirata voucher NMNS-8720-021	0.00	0.00						
4. OR667663.1:205-435 Babylonia semipicta voucher NMNS-8720-004	0.02	0.01	0.01					
5. OR667658.1:205-435 Babylonia valentiana voucher NMNS-8720-025	0.11	0.10	0.10	0.09				
6. OR667670.1:205-434 Babylonia areolata voucher NMNS-8720-001	0.09	0.08	0.08	0.08	0.12			
7. HQ424448.1:5662-5891 Babylonia lani mitochondrial sequence	0.09	0.08	0.08	0.08	0.12	0.00		
8. OR667668.1:205-435 Babylonia pieroangelai voucher NMNS-8720-019	0.10	0.09	0.09	0.09	0.10	0.10	0.10	

Figure 70: K2P distance matrix for *Babylonia spirata* using 16Sbar primers

7.11.7. Order Neogastropoda - Family Fasciolariidae

With 541 extant species across 51 genera worldwide (WoRMS, 2025), the family *Fasciolariidae* represents a diverse group of predatory marine molluscs inhabiting shallow to deep coastal waters, particularly favouring soft-bottom habitats (Leal, 1991). In the present study, *Fusinus* sp. was barcoded for the first time; however, further taxonomic identification is required. The interspecific genetic distances within this group were too less, and as a result, none of the tested primers was able to effectively differentiate between closely related species. Marmorofusus nicobaricus was sequenced for the first time. Among the three primer sets tested, the Fwd464 set showed better resolution compared to the other two. (Fig 69 -80)

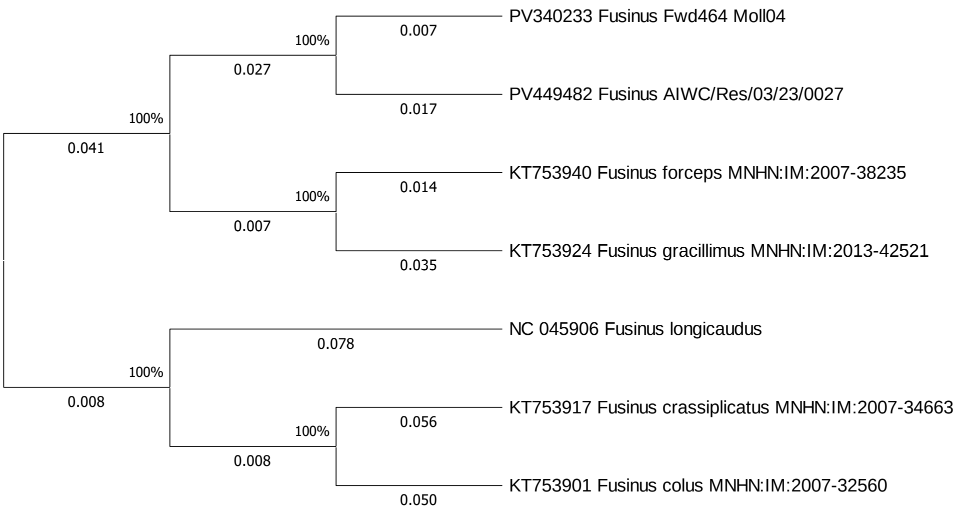
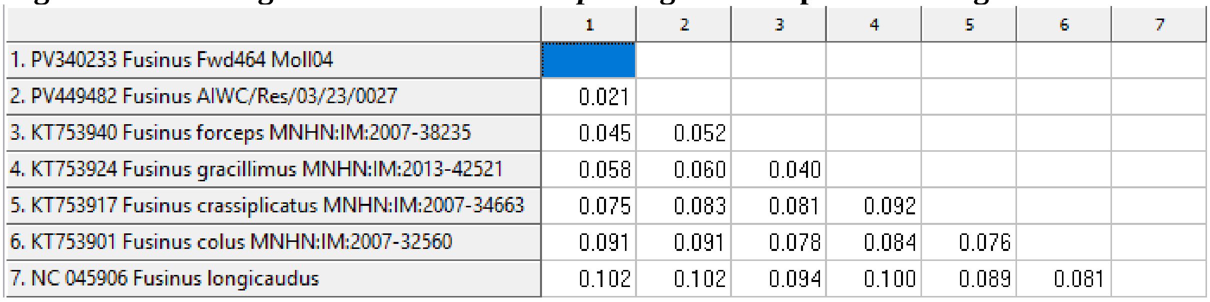


Figure 71: Dendrogram tree for *Fusinus.sp* using Fwd464 primers using HKY+G model



	1	2	3	4	5	6	7
1. PV340233 Fusinus Fwd464 Moll04							
2. PV449482 Fusinus AIWC/Res/03/23/0027	0.021						
3. KT753940 Fusinus forceps MNHN:IM:2007-38235	0.045	0.052					
4. KT753924 Fusinus gracillimus MNHN:IM:2013-42521	0.058	0.060	0.040				
5. KT753917 Fusinus crassiplicatus MNHN:IM:2007-34663	0.075	0.083	0.081	0.092			
6. KT753901 Fusinus colus MNHN:IM:2007-32560	0.091	0.091	0.078	0.084	0.076		
7. NC 045906 Fusinus longicaudus	0.102	0.102	0.094	0.100	0.089	0.081	

Figure 72: K2P distance matrix for *Fusinus.sp* using Fwd464 primers

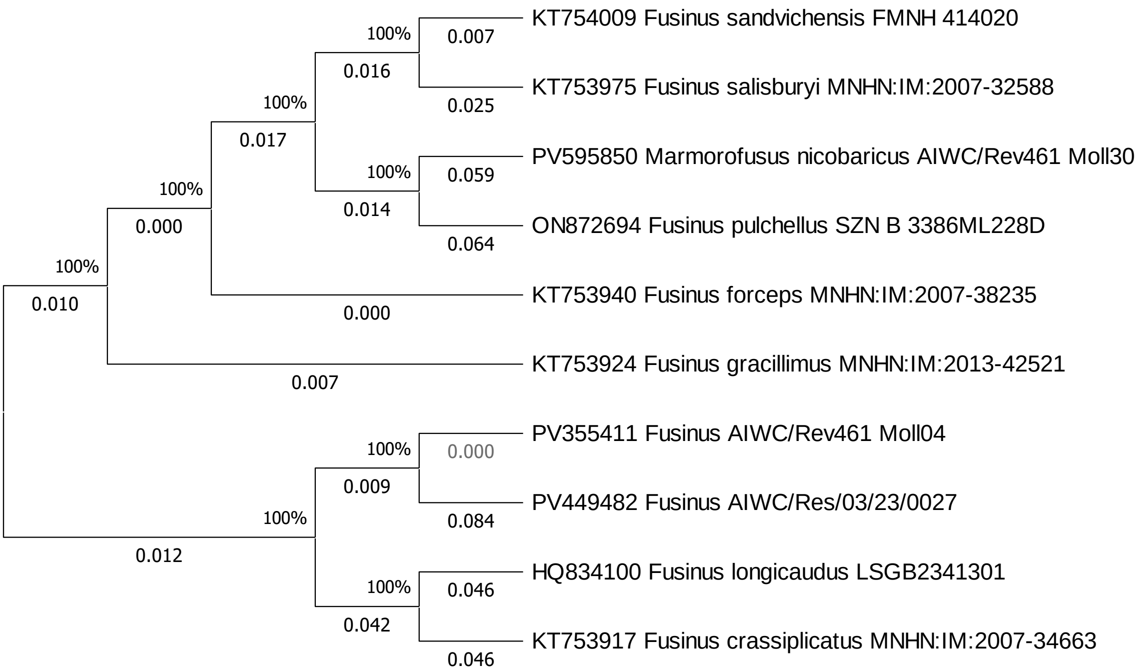
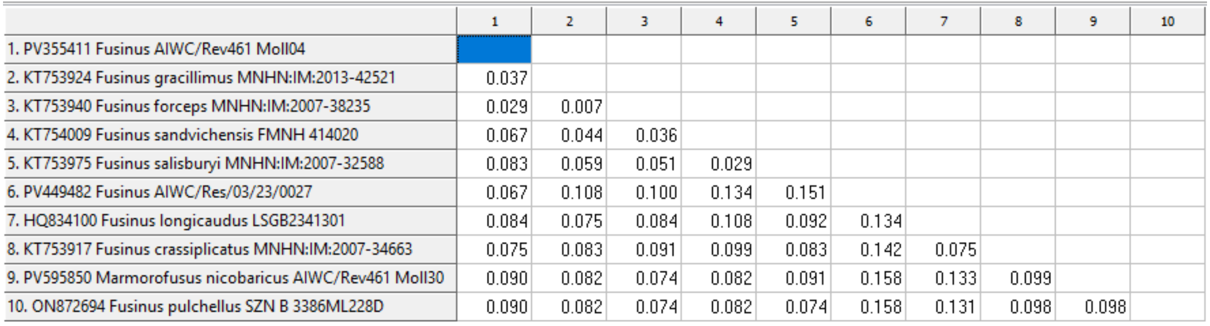


Figure 73: Dendrogram tree for *Fusinus.sp* using Rev461 primers using T92+G model



	1	2	3	4	5	6	7	8	9	10
1. PV355411 Fusinus AIWC/Rev461 Moll04										
2. KT753924 Fusinus gracillimus MNHN:IM:2013-42521	0.037									
3. KT753940 Fusinus forceps MNHN:IM:2007-38235	0.029	0.007								
4. KT754009 Fusinus sandvichensis FMNH 414020	0.067	0.044	0.036							
5. KT753975 Fusinus salisburyi MNHN:IM:2007-32588	0.083	0.059	0.051	0.029						
6. PV449482 Fusinus AIWC/Res/03/23/0027	0.067	0.108	0.100	0.134	0.151					
7. HQ834100 Fusinus longicaudus LSGB2341301	0.084	0.075	0.084	0.108	0.092	0.134				
8. KT753917 Fusinus crassiplicatus MNHN:IM:2007-34663	0.075	0.083	0.091	0.099	0.083	0.142	0.075			
9. PV595850 Marmorofusus nicobaricus AIWC/Rev461 Moll30	0.090	0.082	0.074	0.082	0.091	0.158	0.133	0.099		
10. ON872694 Fusinus pulchellus SZN B 3386ML228D	0.090	0.082	0.074	0.082	0.074	0.158	0.131	0.098	0.098	

Figure 74: K2P distance matrix for *Fusinus.sp* using Rev461 primers

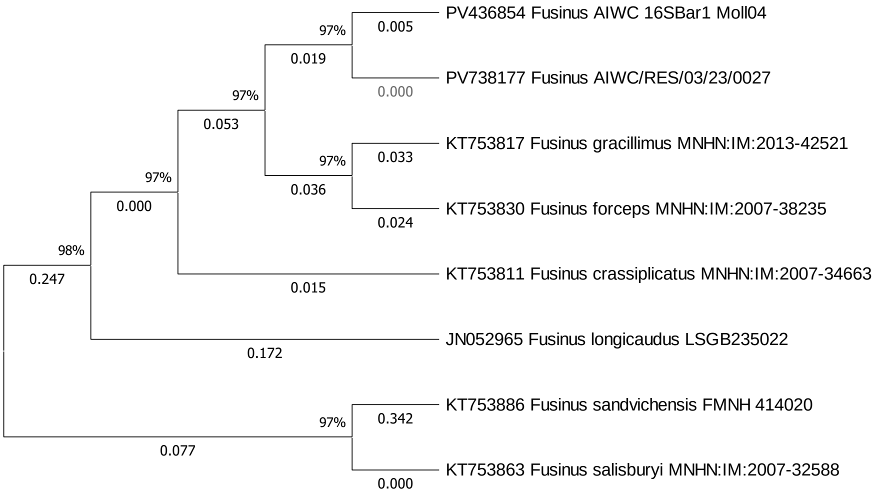
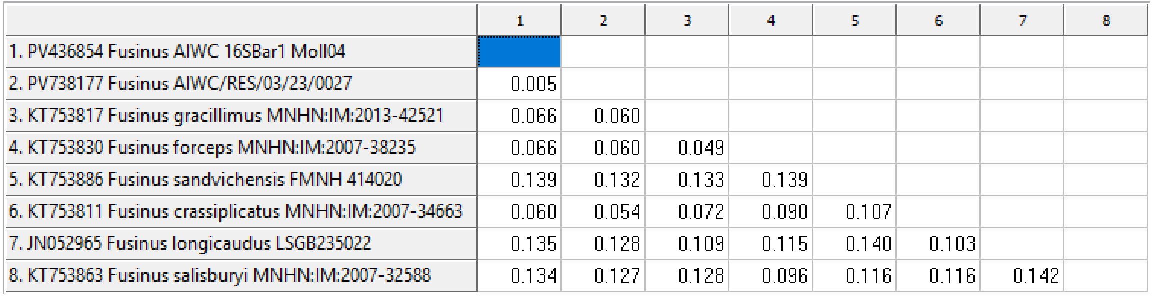


Figure 75: Dendrogram tree for *Fusinus.sp* using 16Sbar primers using T92+G model



	1	2	3	4	5	6	7	8
1. PV436854 Fusinus AIWC 16SBar1 Moll04								
2. PV738177 Fusinus AIWC/RES/03/23/0027	0.005							
3. KT753817 Fusinus gracillimus MNHN:IM:2013-42521	0.066	0.060						
4. KT753830 Fusinus forceps MNHN:IM:2007-38235	0.066	0.060	0.049					
5. KT753886 Fusinus sandvichensis FMNH 414020	0.139	0.132	0.133	0.139				
6. KT753811 Fusinus crassiplicatus MNHN:IM:2007-34663	0.060	0.054	0.072	0.090	0.107			
7. JN052965 Fusinus longicaudus LSGB235022	0.135	0.128	0.109	0.115	0.140	0.103		
8. KT753863 Fusinus salisburyi MNHN:IM:2007-32588	0.134	0.127	0.128	0.096	0.116	0.116	0.142	

Figure 76: K2P distance matrix for *Fusinus.sp* using 16Sbar primers

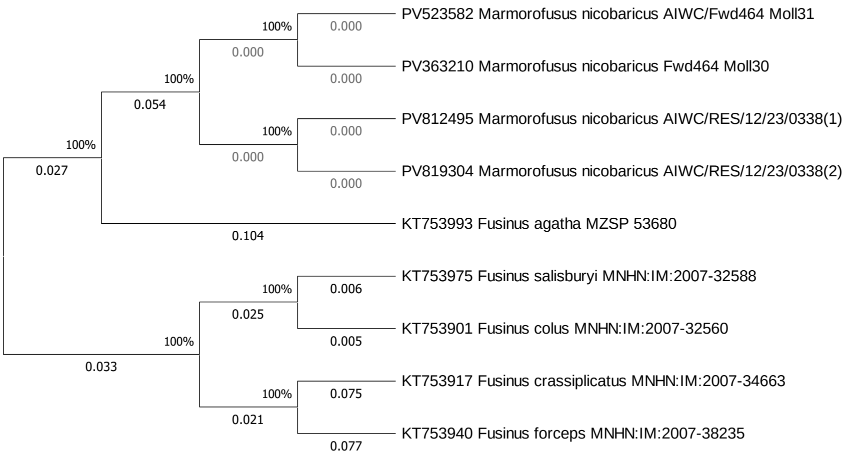
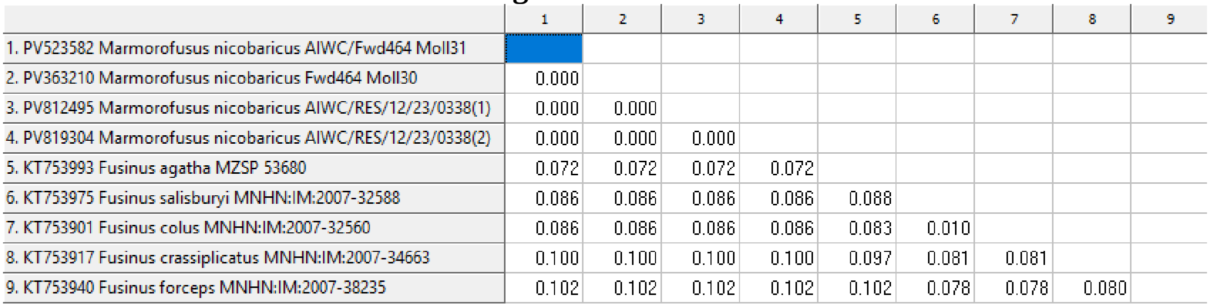


Figure 77: Dendrogram tree for *Marmorofuscus nicobaricus* using Fwd464 primers using T92+G model



	1	2	3	4	5	6	7	8	9
1. PV523582 Marmorofusus nicobaricus AIWC/Fwd464 Moll31									
2. PV363210 Marmorofusus nicobaricus Fwd464 Moll30	0.000								
3. PV812495 Marmorofusus nicobaricus AIWC/RES/12/23/0338(1)	0.000	0.000							
4. PV819304 Marmorofusus nicobaricus AIWC/RES/12/23/0338(2)	0.000	0.000	0.000						
5. KT753993 Fusinus agatha MZSP 53680	0.072	0.072	0.072	0.072					
6. KT753975 Fusinus salisburyi MNHN:IM:2007-32588	0.086	0.086	0.086	0.086	0.088				
7. KT753901 Fusinus colus MNHN:IM:2007-32560	0.086	0.086	0.086	0.086	0.083	0.010			
8. KT753917 Fusinus crassiplicatus MNHN:IM:2007-34663	0.100	0.100	0.100	0.100	0.097	0.081	0.081		
9. KT753940 Fusinus forceps MNHN:IM:2007-38235	0.102	0.102	0.102	0.102	0.102	0.078	0.078	0.080	

Figure 78: K2P distance matrix for *Marmorofuscus nicobarius* using Fwd464 primers

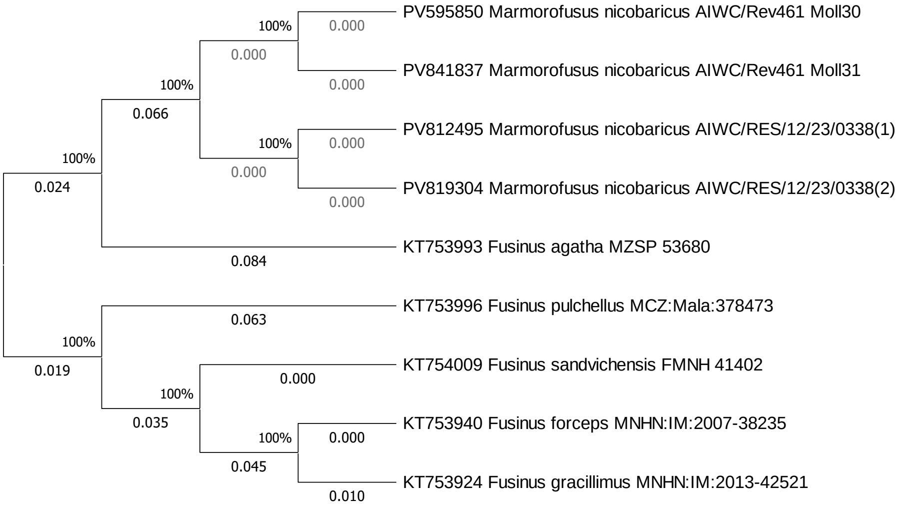
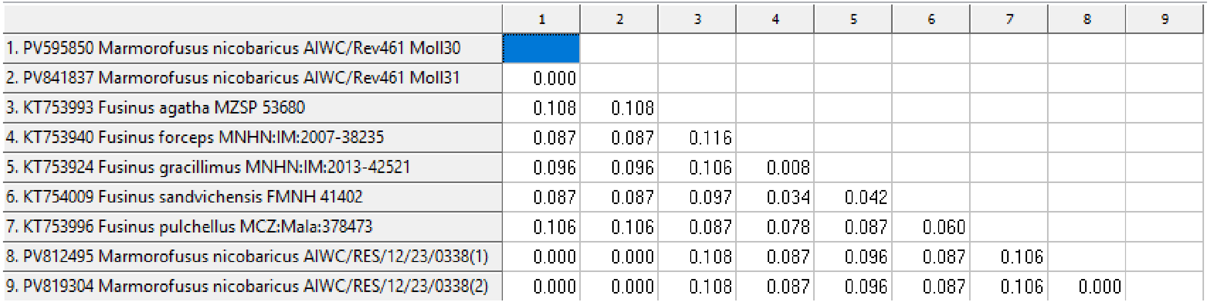


Figure 79: Dendrogram tree for *Marmorofuscus nicobaricus* using Rev461 primers using T92+G model



	1	2	3	4	5	6	7	8	9
1. PV595850 Marmorofusus nicobaricus AIWC/Rev461 Moll30									
2. PV841837 Marmorofusus nicobaricus AIWC/Rev461 Moll31	0.000								
3. KT753993 Fusinus agatha MZSP 53680	0.108	0.108							
4. KT753940 Fusinus forceps MNHN:IM:2007-38235	0.087	0.087	0.116						
5. KT753924 Fusinus gracillimus MNHN:IM:2013-42521	0.096	0.096	0.106	0.008					
6. KT754009 Fusinus sandvichensis FMNH 41402	0.087	0.087	0.097	0.034	0.042				
7. KT753996 Fusinus pulchellus MCZ:Mala:378473	0.106	0.106	0.087	0.078	0.087	0.060			
8. PV812495 Marmorofusus nicobaricus AIWC/RES/12/23/0338(1)	0.000	0.000	0.108	0.087	0.096	0.087	0.106		
9. PV819304 Marmorofusus nicobaricus AIWC/RES/12/23/0338(2)	0.000	0.000	0.108	0.087	0.096	0.087	0.106	0.000	

Figure 80: K2P distance matrix for *Marmorofuscus nicobarius* using Rev461 primers

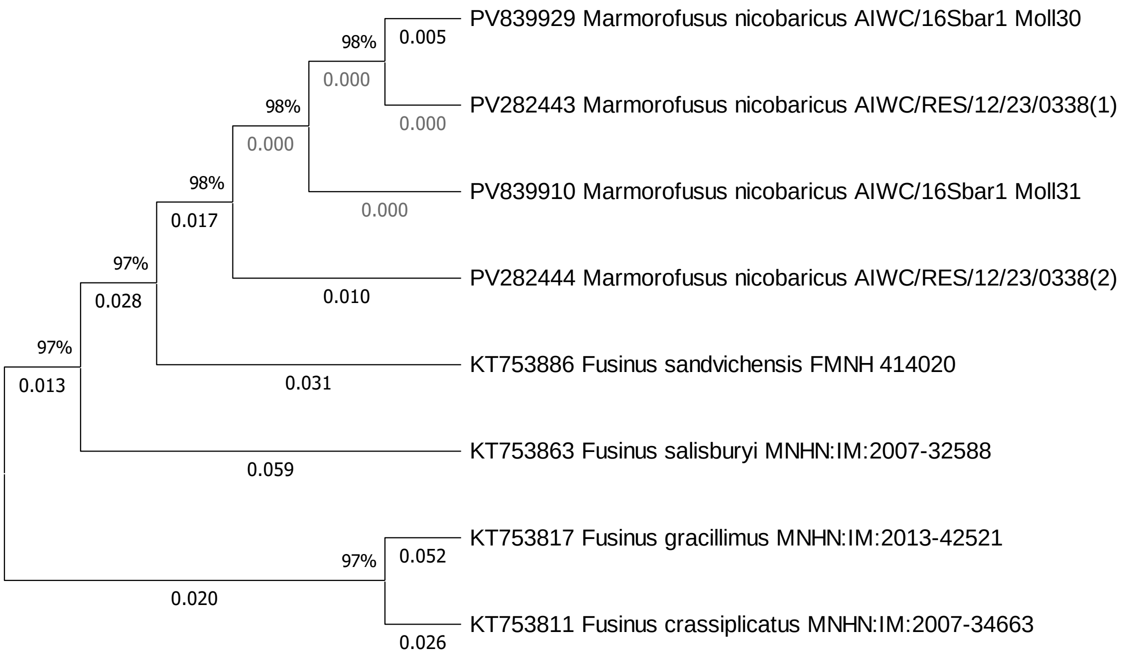
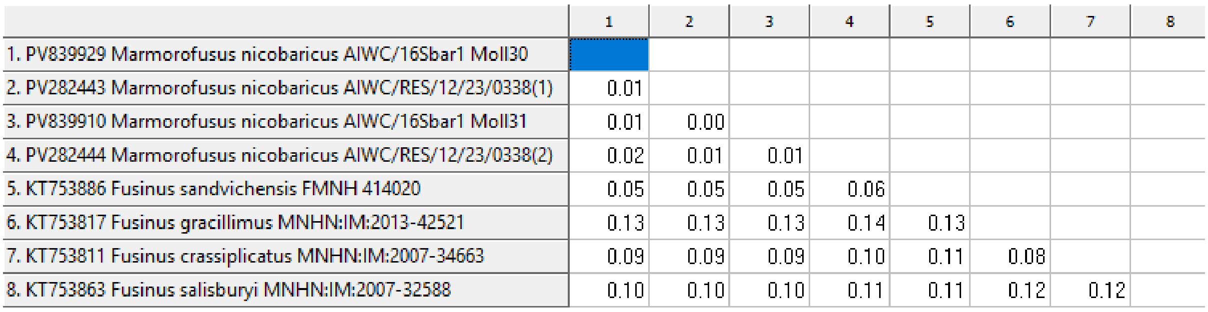


Figure 81: Dendrogram tree for *Marmorofuscus nicobaricus* using 16Sbar primers using T92 model



	1	2	3	4	5	6	7	8
1. PV839929 Marmorofusus nicobaricus AIWC/16Sbar1 Moll30								
2. PV282443 Marmorofusus nicobaricus AIWC/RES/12/23/0338(1)	0.01							
3. PV839910 Marmorofusus nicobaricus AIWC/16Sbar1 Moll31	0.01	0.00						
4. PV282444 Marmorofusus nicobaricus AIWC/RES/12/23/0338(2)	0.02	0.01	0.01					
5. KT753886 Fusinus sandvichensis FMNH 414020	0.05	0.05	0.05	0.06				
6. KT753817 Fusinus gracillimus MNHN:IM:2013-42521	0.13	0.13	0.13	0.14	0.13			
7. KT753811 Fusinus crassiplicatus MNHN:IM:2007-34663	0.09	0.09	0.09	0.10	0.11	0.08		
8. KT753863 Fusinus salisburyi MNHN:IM:2007-32588	0.10	0.10	0.10	0.11	0.11	0.12	0.12	

Figure 82: K2P distance matrix for Marmorofuscus nicobarius using 16Sbar primers

7.11.8. Order Neogastropoda – Genus Pleuroploca trapezium

The genus *Pleuroploca* comprises nine extant species in the marine ecosystem. However, limited information is available on their current distribution and molecular taxonomy. Couto et al. (2016) revised the family Fasciolariidae through molecular phylogenetic analyses. As part of that study, two specimens of *Pleuroploca trapezium* – MNHN:IM:2007-32591 from Vanuatu and MNHN:IM:2009-15358 from Madagascar – were sequenced for the genes **COX1**, **16S rRNA**, **28S rRNA**, and **Histone H1**. Specimens collected from Tamil Nadu closely matched MNHN:IM:2007-32591, as well as *Pleuroploca ponderosa*, which was independently sequenced from Tamil Nadu. Based on the currently available data, the genetic divergence between MNHN:IM:2007-32591 and MNHN:IM:2009-15358 is approximately 8%, suggesting that they are likely distinct species. However, formal species confirmation requires a comprehensive taxonomic revision, including the collection and sequencing of specimens from their respective type localities. Therefore, species-level identification based solely on phylogenetic tree placement is not feasible at this stage. Nonetheless, the distance matrix indicates a high similarity between the reference sequences and the sequences generated using mini-barcodes (Fig 81- 86).

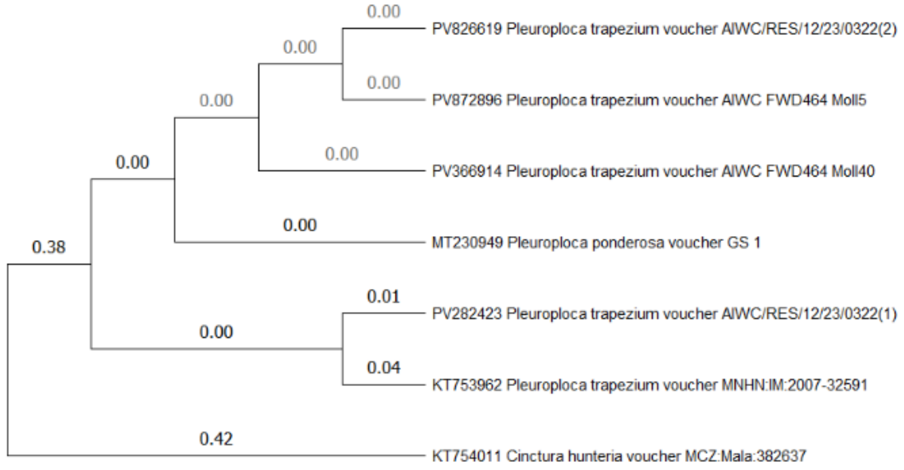
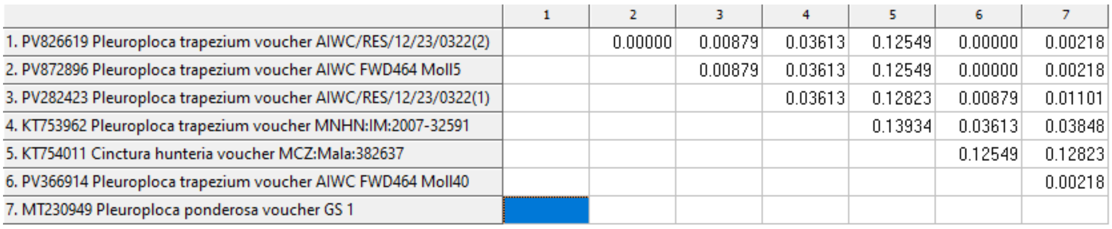


Figure 83 Dendrogram tree for *Pleuroploca trapezium* using Fwd 464 primers using T92+G model.



	1	2	3	4	5	6	7
1. PV826619 Pleuroploca trapezium voucher AIWC/RES/12/23/0322(2)		0.00000	0.00879	0.03613	0.12549	0.00000	0.00218
2. PV872896 Pleuroploca trapezium voucher AIWC FWD464 Moll5			0.00879	0.03613	0.12549	0.00000	0.00218
3. PV282423 Pleuroploca trapezium voucher AIWC/RES/12/23/0322(1)				0.03613	0.12823	0.00879	0.01101
4. KT753962 Pleuroploca trapezium voucher MNHN:IM:2007-32591					0.13934	0.03613	0.03848
5. KT754011 Cinctura hunteria voucher MCZ:Mala:382637						0.12549	0.12823
6. PV366914 Pleuroploca trapezium voucher AIWC FWD464 Moll40							0.00218
7. MT230949 Pleuroploca ponderosa voucher GS 1							

Figure 84 K2P distance matrix for *Pleuroploca trapezium* using Fwd 464 primers

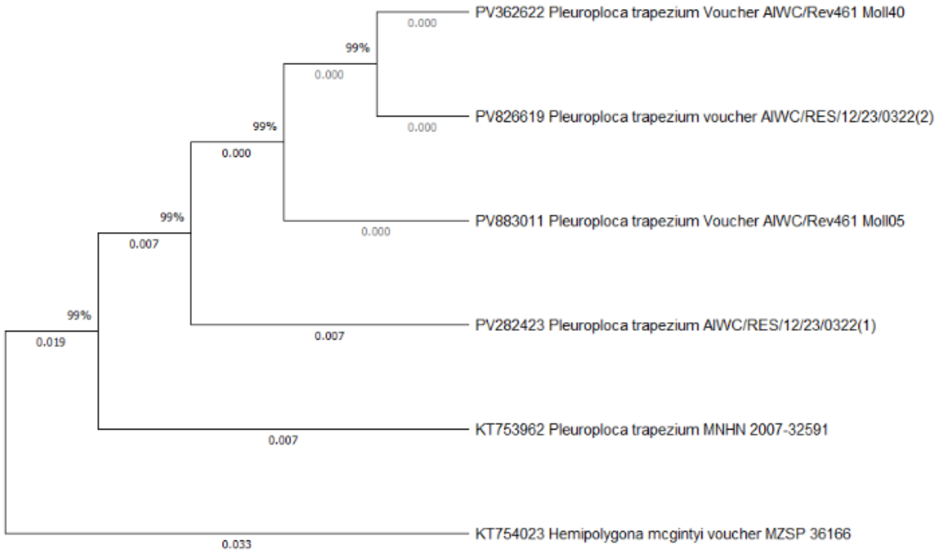
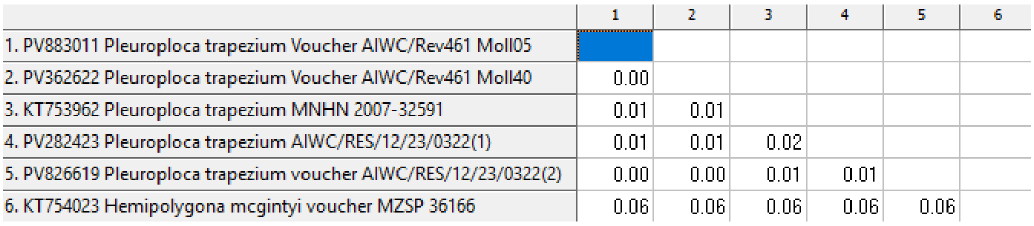


Figure 85: Dendrogram tree for *Pleuroploca trapezium* using Rev 461 primers using T92 model.



	1	2	3	4	5	6
1. PV883011 Pleuroploca trapezium Voucher AIWC/Rev461 Moll05						
2. PV362622 Pleuroploca trapezium Voucher AIWC/Rev461 Moll40	0.00					
3. KT753962 Pleuroploca trapezium MNHN 2007-32591	0.01	0.01				
4. PV282423 Pleuroploca trapezium AIWC/RES/12/23/0322(1)	0.01	0.01	0.02			
5. PV826619 Pleuroploca trapezium voucher AIWC/RES/12/23/0322(2)	0.00	0.00	0.01	0.01		
6. KT754023 Hemipolygona mcgintyi voucher MZSP 36166	0.06	0.06	0.06	0.06	0.06	

Figure 86: K2P distance matrix for *Pleuroploca trapezium* using Rev 461 primers

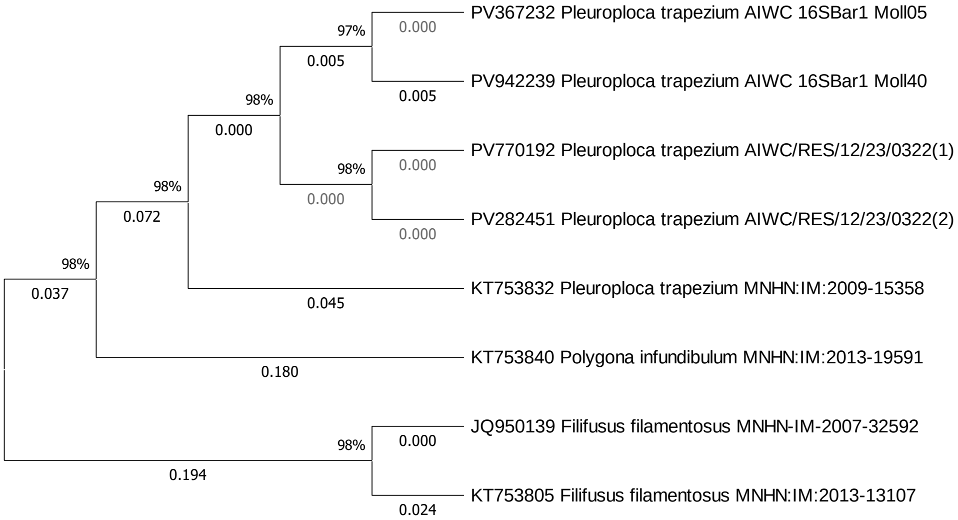
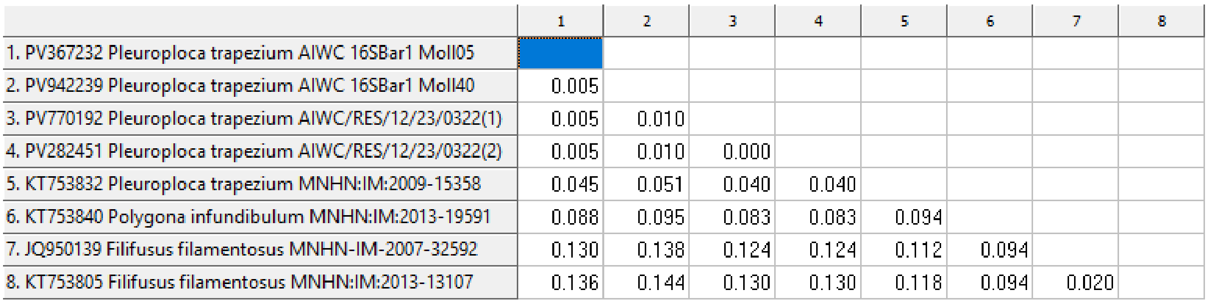


Figure 87: Dendrogram tree for *Pleuroploca trapezium* using 16Sbar primers using T92+G model



	1	2	3	4	5	6	7	8
1. PV367232 Pleuroploca trapezium AIWC 16SBar1 Moll05								
2. PV942239 Pleuroploca trapezium AIWC 16SBar1 Moll40	0.005							
3. PV770192 Pleuroploca trapezium AIWC/RES/12/23/0322(1)	0.005	0.010						
4. PV282451 Pleuroploca trapezium AIWC/RES/12/23/0322(2)	0.005	0.010	0.000					
5. KT753832 Pleuroploca trapezium MNHN:IM:2009-15358	0.045	0.051	0.040	0.040				
6. KT753840 Polygona infundibulum MNHN:IM:2013-19591	0.088	0.095	0.083	0.083	0.094			
7. JQ950139 Filifusus filamentosus MNHN-IM-2007-32592	0.130	0.138	0.124	0.124	0.112	0.094		
8. KT753805 Filifusus filamentosus MNHN:IM:2013-13107	0.136	0.144	0.130	0.130	0.118	0.094	0.020	

Figure 88: K2P distance matrix for *Pleuroploca trapezium* using 16Sbar primers

7.11.9. Order Neogastropoda – Family Muricidae

The family *Muricidae* is one of the most morphologically diverse groups of molluscs, comprising over 1,600 extant described species. For validation in the present study, five different genera were tested, including *Chicoreus ramosus*, *Purpura bufo*, *Indothais lacera*, *Rapana rapiformis*, and *Murex* sp.1. Given the commercial significance of *C. ramosus* and its considerable variation in shell morphology across growth stages, four individuals were included. All the mini-barcodes sequenced showed high similarity to their respective reference sequences, confirming accurate species identification. However, *Murex* sp. included in this study requires proper taxonomic identification. Except for *Murex* sp., which failed to amplify with the Fwd464 primer set, all other species were successfully amplified with all three primer sets showed clear species-level discrimination (Fig 87-114)

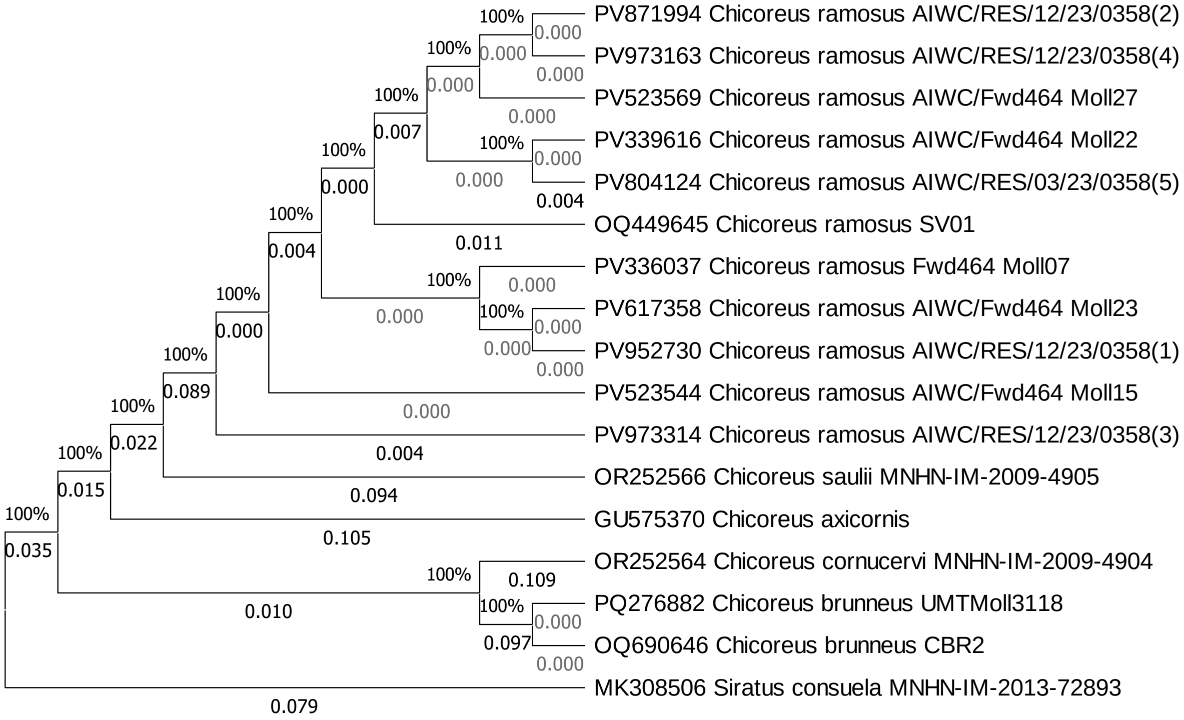
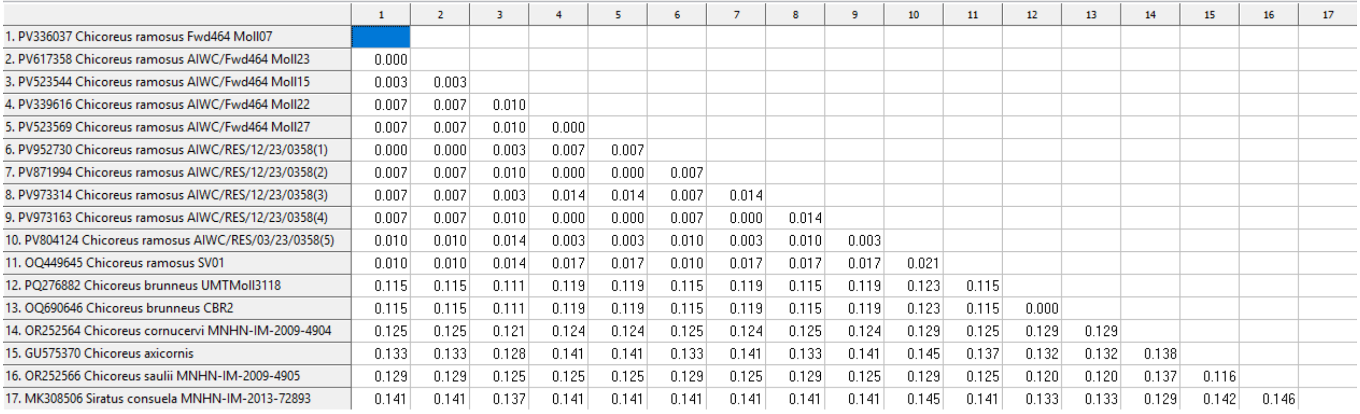


Figure 89: Dendrogram tree for *Chicoreus ramosus* using Fwd464 primers using T92+G



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. PV336037 Chicoreus ramosus Fwd464 Moll07																	
2. PV617358 Chicoreus ramosus AIWC/Fwd464 Moll23	0.000																
3. PV523544 Chicoreus ramosus AIWC/Fwd464 Moll15	0.003	0.003															
4. PV339616 Chicoreus ramosus AIWC/Fwd464 Moll22	0.007	0.007	0.010														
5. PV523569 Chicoreus ramosus AIWC/Fwd464 Moll27	0.007	0.007	0.010	0.000													
6. PV952730 Chicoreus ramosus AIWC/RES/12/23/0358(1)	0.000	0.000	0.003	0.007	0.007												
7. PV871994 Chicoreus ramosus AIWC/RES/12/23/0358(2)	0.007	0.007	0.010	0.000	0.000	0.007											
8. PV973314 Chicoreus ramosus AIWC/RES/12/23/0358(3)	0.007	0.007	0.003	0.014	0.014	0.007	0.014										
9. PV973163 Chicoreus ramosus AIWC/RES/12/23/0358(4)	0.007	0.007	0.010	0.000	0.000	0.007	0.000	0.014									
10. PV804124 Chicoreus ramosus AIWC/RES/03/23/0358(5)	0.010	0.010	0.014	0.003	0.003	0.010	0.003	0.010	0.003								
11. OQ449645 Chicoreus ramosus SV01	0.010	0.010	0.014	0.017	0.017	0.010	0.017	0.017	0.017	0.021							
12. PQ276882 Chicoreus brunneus UMTMoll3118	0.115	0.115	0.111	0.119	0.119	0.115	0.119	0.115	0.119	0.123	0.115						
13. OQ690646 Chicoreus brunneus CBR2	0.115	0.115	0.111	0.119	0.119	0.115	0.119	0.115	0.119	0.123	0.115	0.000					
14. OR252564 Chicoreus cornucervi MNHN-IM-2009-4904	0.125	0.125	0.121	0.124	0.124	0.125	0.124	0.125	0.124	0.129	0.125	0.129	0.129				
15. GU575370 Chicoreus axicornis	0.133	0.133	0.128	0.141	0.141	0.133	0.141	0.133	0.141	0.145	0.137	0.132	0.132	0.138			
16. OR252566 Chicoreus saulii MNHN-IM-2009-4905	0.129	0.129	0.125	0.125	0.125	0.129	0.125	0.129	0.125	0.129	0.125	0.120	0.120	0.137	0.116		
17. MK308506 Siratus consuela MNHN-IM-2013-72893	0.141	0.141	0.137	0.141	0.141	0.141	0.141	0.141	0.141	0.145	0.141	0.133	0.133	0.129	0.142	0.146	

Figure 90: K2P distance matrix for *Chicoreus ramosus* using Fwd464 primers

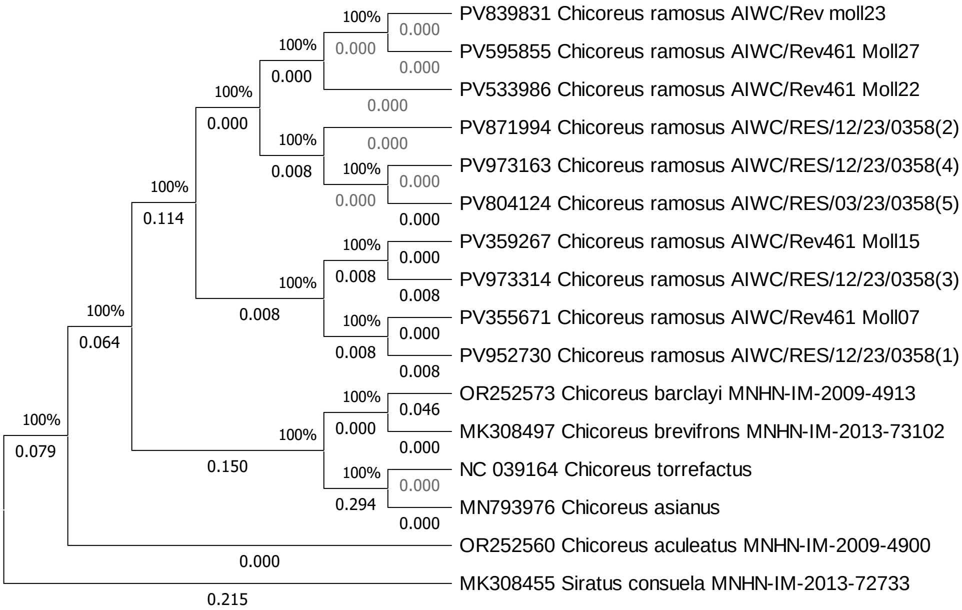
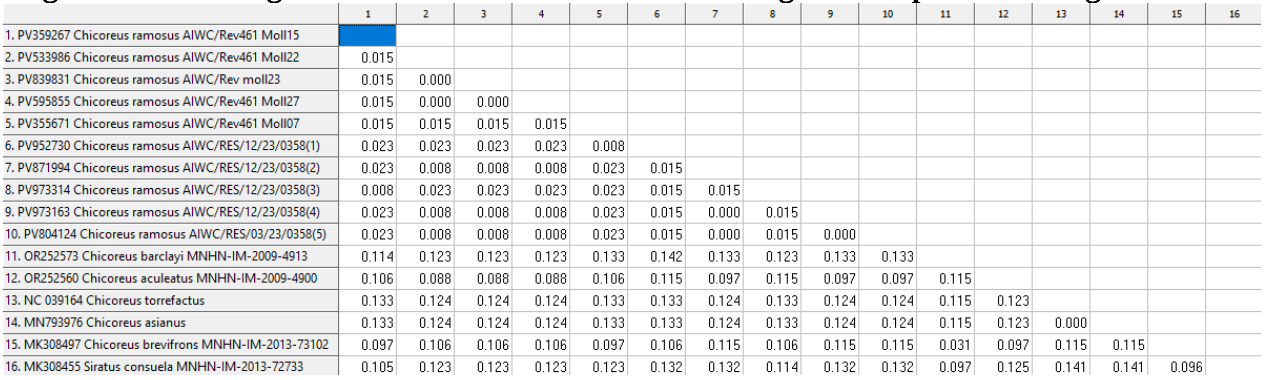


Figure 91: Dendrogram tree for *Chicoreus ramosus* using Rev461 primers using T92+I



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. PV359267 Chicoreus ramosus AIWC/Rev461 Moll15																
2. PV533986 Chicoreus ramosus AIWC/Rev461 Moll22	0.015															
3. PV839831 Chicoreus ramosus AIWC/Rev moll23	0.015	0.000														
4. PV595855 Chicoreus ramosus AIWC/Rev461 Moll27	0.015	0.000	0.000													
5. PV355671 Chicoreus ramosus AIWC/Rev461 Moll07	0.015	0.015	0.015	0.015												
6. PV952730 Chicoreus ramosus AIWC/RES/12/23/0358(1)	0.023	0.023	0.023	0.023	0.008											
7. PV871994 Chicoreus ramosus AIWC/RES/12/23/0358(2)	0.023	0.008	0.008	0.008	0.023	0.015										
8. PV973314 Chicoreus ramosus AIWC/RES/12/23/0358(3)	0.008	0.023	0.023	0.023	0.023	0.015	0.015									
9. PV973163 Chicoreus ramosus AIWC/RES/12/23/0358(4)	0.023	0.008	0.008	0.008	0.023	0.015	0.000	0.015								
10. PV804124 Chicoreus ramosus AIWC/RES/03/23/0358(5)	0.023	0.008	0.008	0.008	0.023	0.015	0.000	0.015	0.000							
11. OR252573 Chicoreus barclayi MNHN-IM-2009-4913	0.114	0.123	0.123	0.123	0.133	0.142	0.133	0.123	0.133	0.133						
12. OR252560 Chicoreus aculeatus MNHN-IM-2009-4900	0.106	0.088	0.088	0.088	0.106	0.115	0.097	0.115	0.097	0.097	0.115					
13. NC 039164 Chicoreus torrefactus	0.133	0.124	0.124	0.124	0.133	0.133	0.124	0.133	0.124	0.124	0.115	0.123				
14. MN793976 Chicoreus asianus	0.133	0.124	0.124	0.124	0.133	0.133	0.124	0.133	0.124	0.124	0.115	0.123	0.000			
15. MK308497 Chicoreus brevifrons MNHN-IM-2013-73102	0.097	0.106	0.106	0.106	0.097	0.106	0.115	0.106	0.115	0.115	0.031	0.097	0.115	0.115		
16. MK308455 Siratus consuela MNHN-IM-2013-72733	0.105	0.123	0.123	0.123	0.123	0.132	0.132	0.114	0.132	0.132	0.097	0.125	0.141	0.141	0.096	

Figure 92: K2P distance matrix for *Chicoreus ramosus* using Rev461 primers

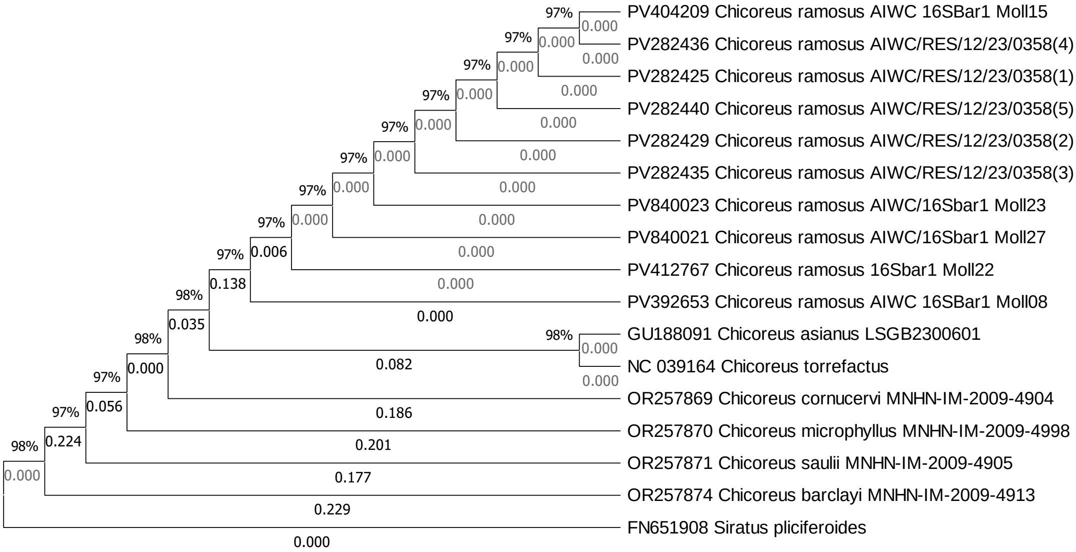
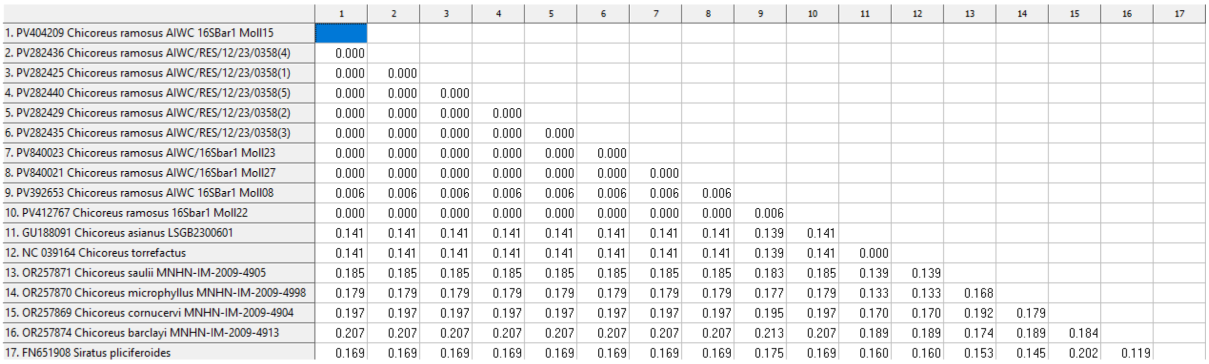


Figure 93: Dendrogram tree for *Chicoreus ramosus* using 16Sbar primers using T92+G



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. PV404209 Chicoreus ramosus AIWC 16SBar1 Moll15																	
2. PV282436 Chicoreus ramosus AIWC/RES/12/23/0358(4)	0.000																
3. PV282425 Chicoreus ramosus AIWC/RES/12/23/0358(1)	0.000	0.000															
4. PV282440 Chicoreus ramosus AIWC/RES/12/23/0358(5)	0.000	0.000	0.000														
5. PV282429 Chicoreus ramosus AIWC/RES/12/23/0358(2)	0.000	0.000	0.000	0.000													
6. PV282435 Chicoreus ramosus AIWC/RES/12/23/0358(3)	0.000	0.000	0.000	0.000	0.000												
7. PV840023 Chicoreus ramosus AIWC/16Sbar1 Moll23	0.000	0.000	0.000	0.000	0.000	0.000											
8. PV840021 Chicoreus ramosus AIWC/16Sbar1 Moll27	0.000	0.000	0.000	0.000	0.000	0.000	0.000										
9. PV392653 Chicoreus ramosus AIWC 16SBar1 Moll08	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006									
10. PV412767 Chicoreus ramosus 16Sbar1 Moll22	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006								
11. GU188091 Chicoreus asianus LSGB2300601	0.141	0.141	0.141	0.141	0.141	0.141	0.141	0.141	0.139	0.141							
12. NC 039164 Chicoreus torrefactus	0.141	0.141	0.141	0.141	0.141	0.141	0.141	0.141	0.139	0.141	0.000						
13. OR257871 Chicoreus saulii MNHN-IM-2009-4905	0.185	0.185	0.185	0.185	0.185	0.185	0.185	0.185	0.183	0.185	0.139	0.139					
14. OR257870 Chicoreus microphyllus MNHN-IM-2009-4998	0.179	0.179	0.179	0.179	0.179	0.179	0.179	0.179	0.177	0.179	0.133	0.133	0.168				
15. OR257869 Chicoreus cornucervi MNHN-IM-2009-4904	0.197	0.197	0.197	0.197	0.197	0.197	0.197	0.197	0.195	0.197	0.170	0.170	0.192	0.179			
16. OR257874 Chicoreus barclayi MNHN-IM-2009-4913	0.207	0.207	0.207	0.207	0.207	0.207	0.207	0.207	0.213	0.207	0.189	0.189	0.174	0.189	0.184		
17. FN651908 Siratus pliciferoides	0.169	0.169	0.169	0.169	0.169	0.169	0.169	0.169	0.175	0.169	0.160	0.160	0.153	0.145	0.202	0.119	

Figure 94: K2P distance matrix for *Chicoreus ramosus* using 16Sbar primers

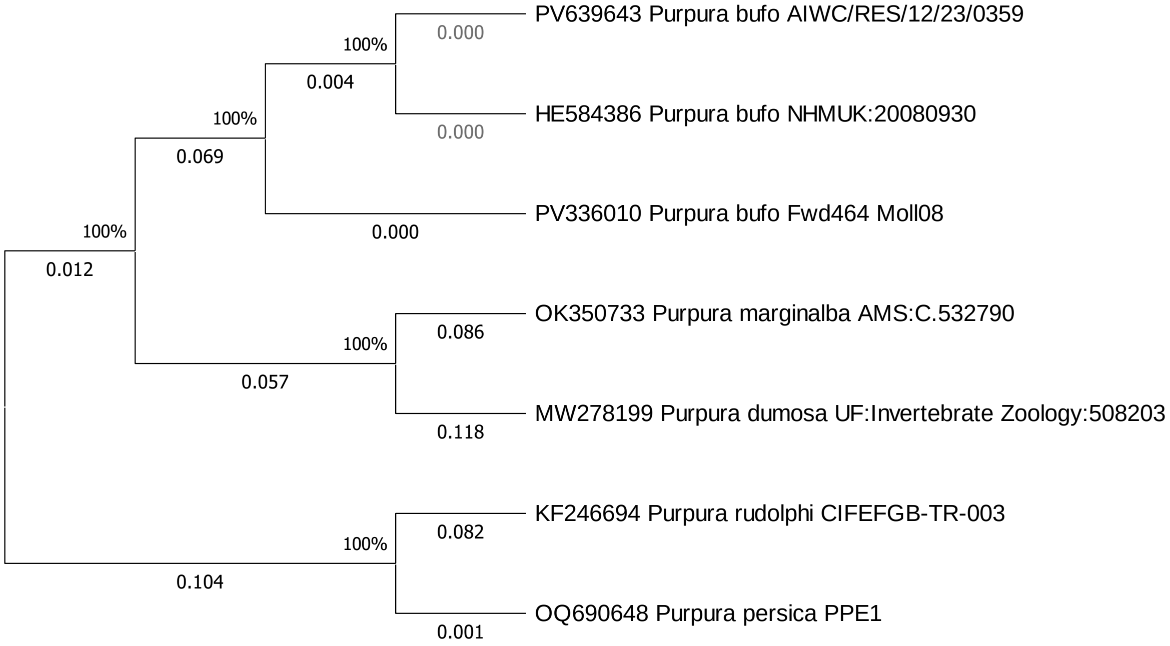
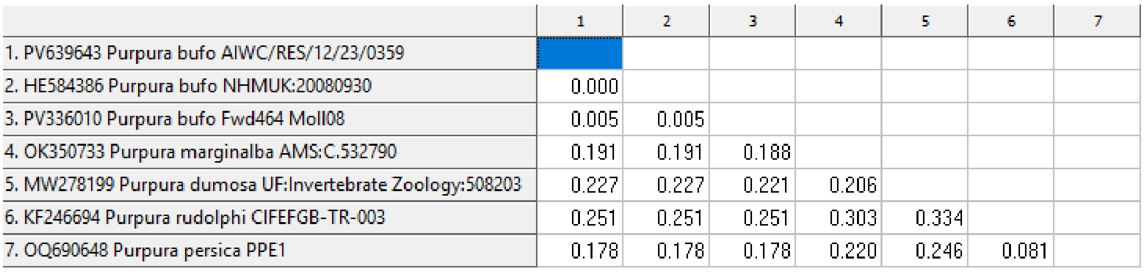


Figure 95: Dendrogram tree for *Purpura bufo* using Fwd 464 primers using HKY+G model



	1	2	3	4	5	6	7
1. PV639643 Purpura bufo AIWC/RES/12/23/0359							
2. HE584386 Purpura bufo NHMUK:20080930	0.000						
3. PV336010 Purpura bufo Fwd464 Moll08	0.005	0.005					
4. OK350733 Purpura marginalba AMS:C.532790	0.191	0.191	0.188				
5. MW278199 Purpura dumosa UF:Invertebrate Zoology:508203	0.227	0.227	0.221	0.206			
6. KF246694 Purpura rudolphi CIFEFGB-TR-003	0.251	0.251	0.251	0.303	0.334		
7. OQ690648 Purpura persica PPE1	0.178	0.178	0.178	0.220	0.246	0.081	

Figure 96: K2P distance matrix for *Purpura bufo* using Fwd 464 primers

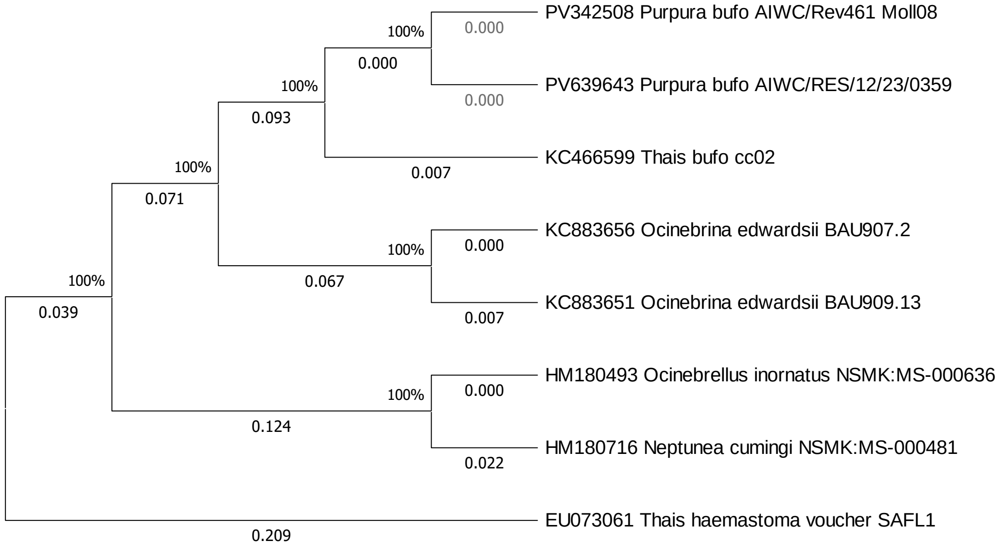
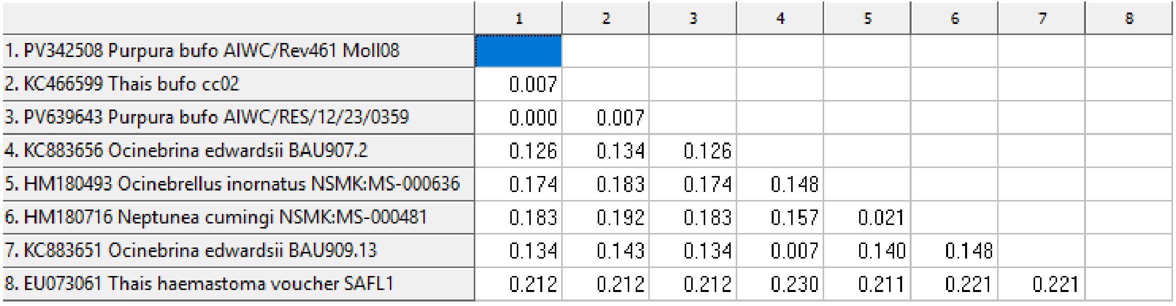


Figure 97: Dendrogram tree for *Purpura bufo* using Rev461 primers using T92+I model



	1	2	3	4	5	6	7	8
1. PV342508 Purpura bufo AIWC/Rev461 Moll08								
2. KC466599 Thais bufo cc02	0.007							
3. PV639643 Purpura bufo AIWC/RES/12/23/0359	0.000	0.007						
4. KC883656 Ocinebrina edwardsii BAU907.2	0.126	0.134	0.126					
5. HM180493 Ocinebrellus inornatus NSMK:MS-000636	0.174	0.183	0.174	0.148				
6. HM180716 Neptunea cumingi NSMK:MS-000481	0.183	0.192	0.183	0.157	0.021			
7. KC883651 Ocinebrina edwardsii BAU909.13	0.134	0.143	0.134	0.007	0.140	0.148		
8. EU073061 Thais haemastoma voucher SAFL1	0.212	0.212	0.212	0.230	0.211	0.221	0.221	

Figure 98: K2P distance matrix for *Purpura bufo* using Rev461 primers

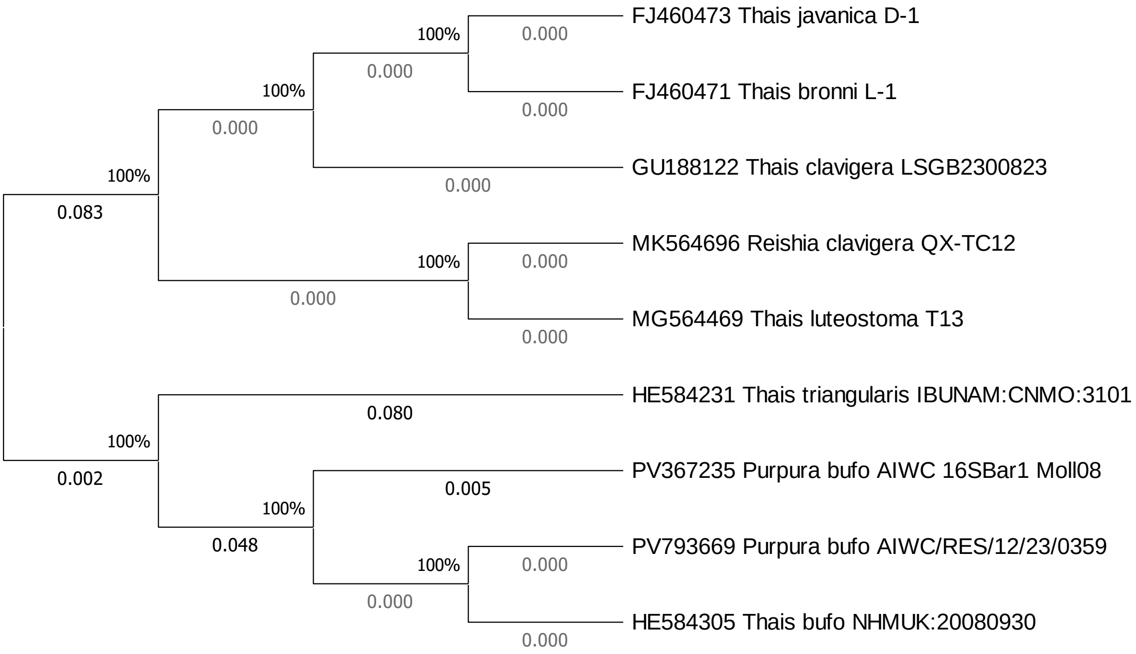
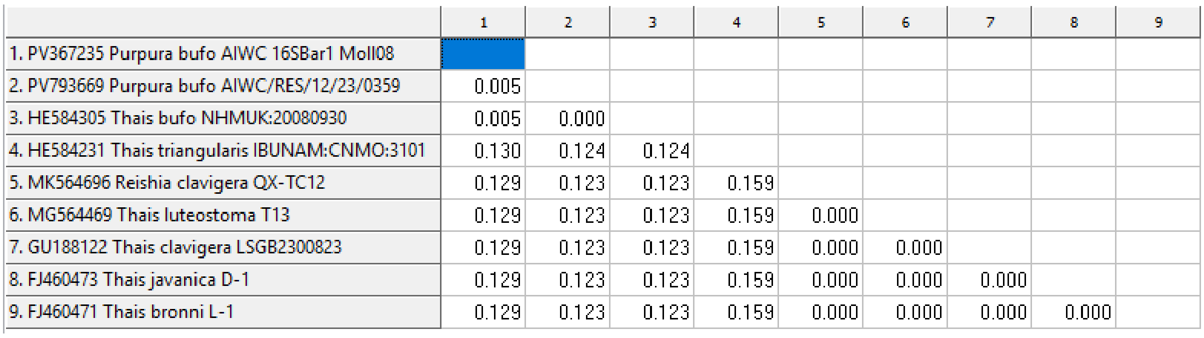


Figure 99: Dendrogram tree for *Purpura bufo* using 16Sbar primers using T92 model



	1	2	3	4	5	6	7	8	9
1. PV367235 Purpura bufo AIWC 16SBar1 Moll08									
2. PV793669 Purpura bufo AIWC/RES/12/23/0359	0.005								
3. HE584305 Thais bufo NHMUK:20080930	0.005	0.000							
4. HE584231 Thais triangularis IBUNAM:CNMO:3101	0.130	0.124	0.124						
5. MK564696 Reishia clavigera QX-TC12	0.129	0.123	0.123	0.159					
6. MG564469 Thais luteostoma T13	0.129	0.123	0.123	0.159	0.000				
7. GU188122 Thais clavigera LSGB2300823	0.129	0.123	0.123	0.159	0.000	0.000			
8. FJ460473 Thais javanica D-1	0.129	0.123	0.123	0.159	0.000	0.000	0.000		
9. FJ460471 Thais bronni L-1	0.129	0.123	0.123	0.159	0.000	0.000	0.000	0.000	

Figure 100: K2P distance matrix for *Purpura bufo* using 16Sbar primers

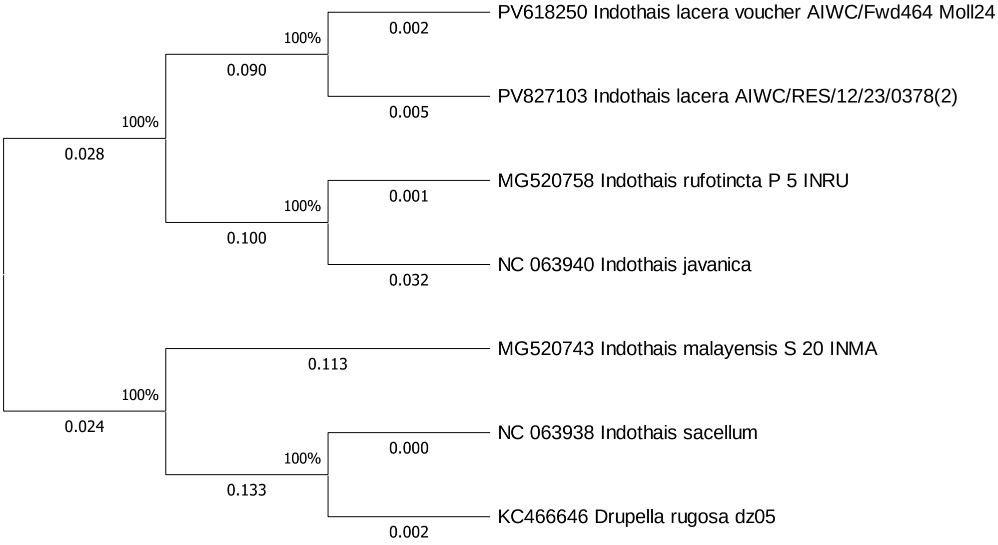
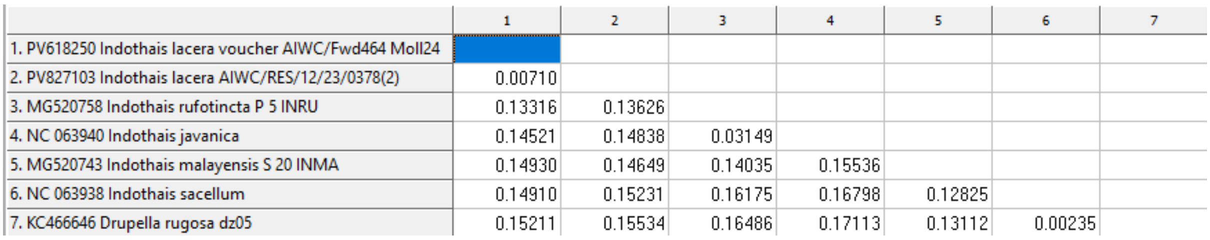


Figure 101: Dendrogram tree for *Indothais lacera* using Fwd464 primers using HKY+I model



	1	2	3	4	5	6	7
1. PV618250 Indothais lacera voucher AIWC/Fwd464 Moll24							
2. PV827103 Indothais lacera AIWC/RES/12/23/0378(2)	0.00710						
3. MG520758 Indothais rufotincta P 5 INRU	0.13316	0.13626					
4. NC 063940 Indothais javanica	0.14521	0.14838	0.03149				
5. MG520743 Indothais malayensis S 20 INMA	0.14930	0.14649	0.14035	0.15536			
6. NC 063938 Indothais sacellum	0.14910	0.15231	0.16175	0.16798	0.12825		
7. KC466646 Drupella rugosa dz05	0.15211	0.15534	0.16486	0.17113	0.13112	0.00235	

Figure 102: K2P distance matrix for *Indothais lacera* using Fwd464 primers

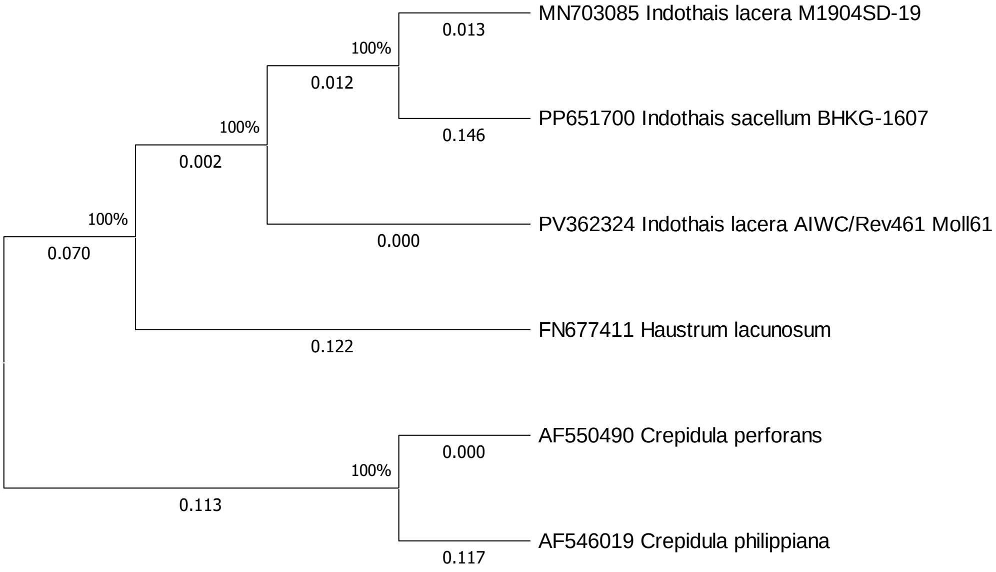
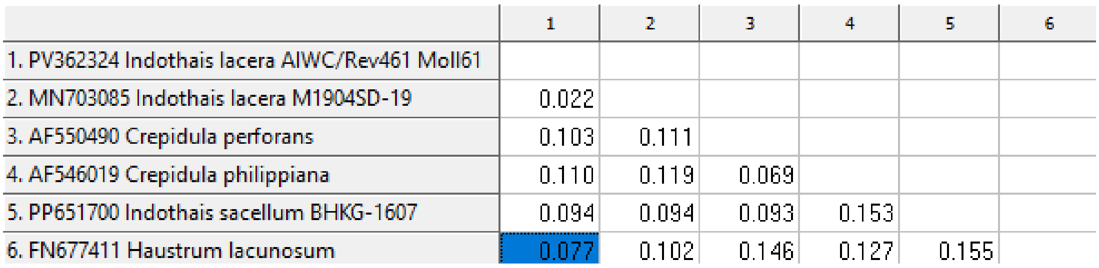


Figure 103: Dendrogram tree for *Indothais lacera* using Rev461 primers using T92+G model



	1	2	3	4	5	6
1. PV362324 Indothais lacera AIWC/Rev461 Moll61						
2. MN703085 Indothais lacera M1904SD-19	0.022					
3. AF550490 Crepidula perforans	0.103	0.111				
4. AF546019 Crepidula philippiana	0.110	0.119	0.069			
5. PP651700 Indothais sacellum BHKG-1607	0.094	0.094	0.093	0.153		
6. FN677411 Haustrum lacunosum	0.077	0.102	0.146	0.127	0.155	

Figure 104: K2P distance matrix for *Indothais lacera* using Rev461 primers

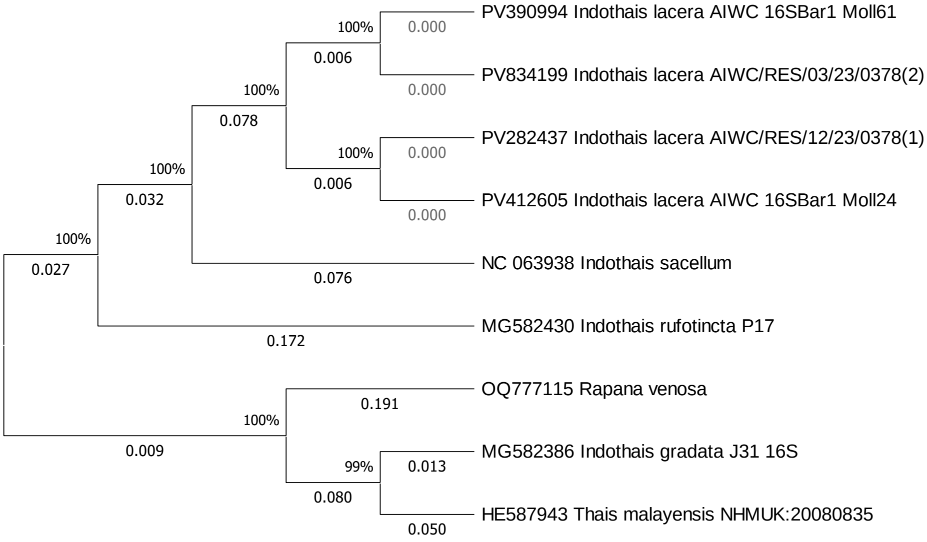
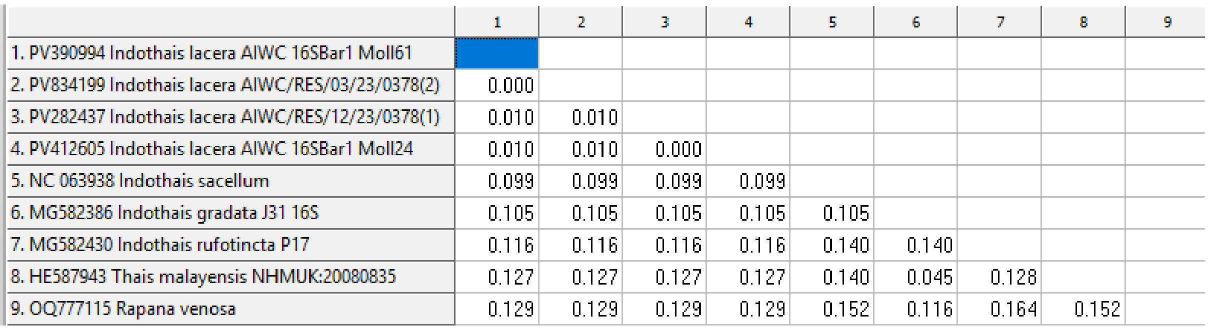


Figure 105: Dendrogram tree for *Indothais lacera* using 16Sbar primers using T92+G model



	1	2	3	4	5	6	7	8	9
1. PV390994 Indothais lacera AIWC 16SBar1 Moll61									
2. PV834199 Indothais lacera AIWC/RES/03/23/0378(2)	0.000								
3. PV282437 Indothais lacera AIWC/RES/12/23/0378(1)	0.010	0.010							
4. PV412605 Indothais lacera AIWC 16SBar1 Moll24	0.010	0.010	0.000						
5. NC 063938 Indothais sacellum	0.099	0.099	0.099	0.099					
6. MG582386 Indothais gradata J31 16S	0.105	0.105	0.105	0.105	0.105				
7. MG582430 Indothais rufotincta P17	0.116	0.116	0.116	0.116	0.140	0.140			
8. HE587943 Thais malayensis NHMUK:20080835	0.127	0.127	0.127	0.127	0.140	0.045	0.128		
9. OQ777115 Rapana venosa	0.129	0.129	0.129	0.129	0.152	0.116	0.164	0.152	

Figure 106: K2P distance matrix for *Indothais lacera* using 16Sbar primers

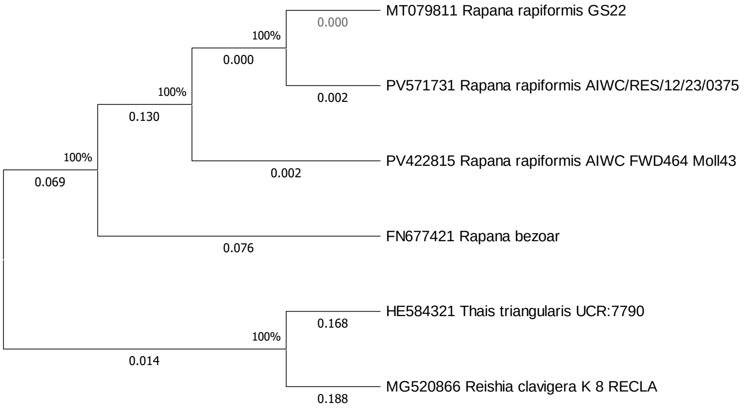
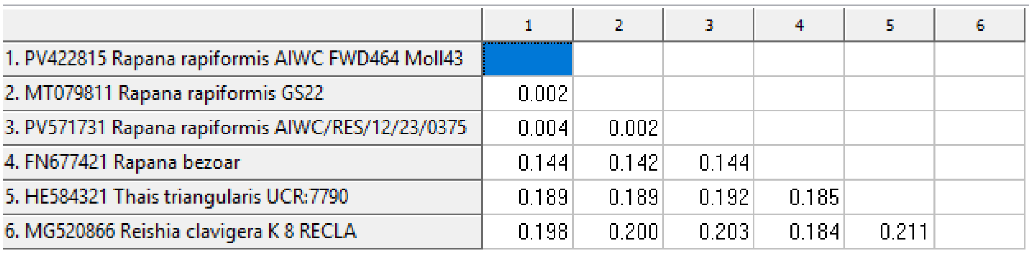


Figure 107: Dendrogram tree for *Rapana rapiformis* using Fwd464 primers using HKY+I model



	1	2	3	4	5	6
1. PV422815 Rapana rapiformis AIWC FWD464 Moll43						
2. MT079811 Rapana rapiformis GS22	0.002					
3. PV571731 Rapana rapiformis AIWC/RES/12/23/0375	0.004	0.002				
4. FN677421 Rapana bezoar	0.144	0.142	0.144			
5. HE584321 Thais triangularis UCR:7790	0.189	0.189	0.192	0.185		
6. MG520866 Reishia clavigera K 8 RECLA	0.198	0.200	0.203	0.184	0.211	

Figure 108: K2P distance matrix for *Rapana rapiformis* using Fwd464 primers

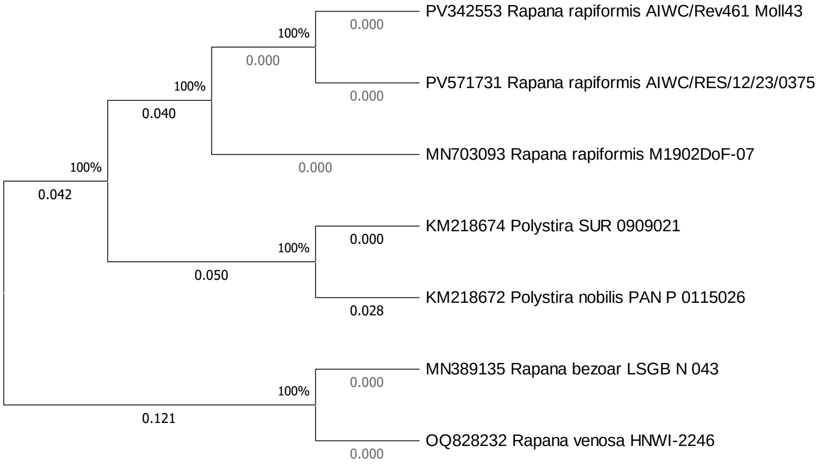
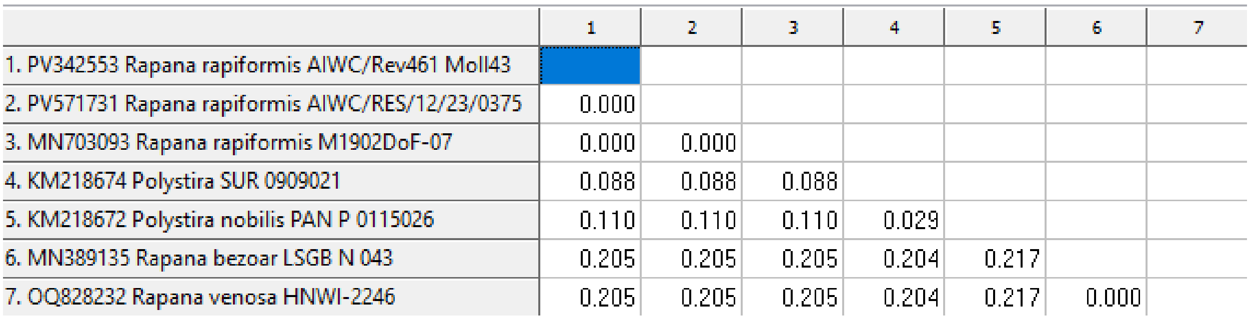


Figure 109: Dendrogram tree for *Rapana rapiformis* using Rev461 primers using T92 model



	1	2	3	4	5	6	7
1. PV342553 Rapana rapiformis AIWC/Rev461 Moll43							
2. PV571731 Rapana rapiformis AIWC/RES/12/23/0375	0.000						
3. MN703093 Rapana rapiformis M1902DoF-07	0.000	0.000					
4. KM218674 Polystira SUR 0909021	0.088	0.088	0.088				
5. KM218672 Polystira nobilis PAN P 0115026	0.110	0.110	0.110	0.029			
6. MN389135 Rapana bezoar LSGB N 043	0.205	0.205	0.205	0.204	0.217		
7. OQ828232 Rapana venosa HNWI-2246	0.205	0.205	0.205	0.204	0.217	0.000	

Figure 110: K2P distance matrix for *Rapana rapiformis* using Rev461 primers

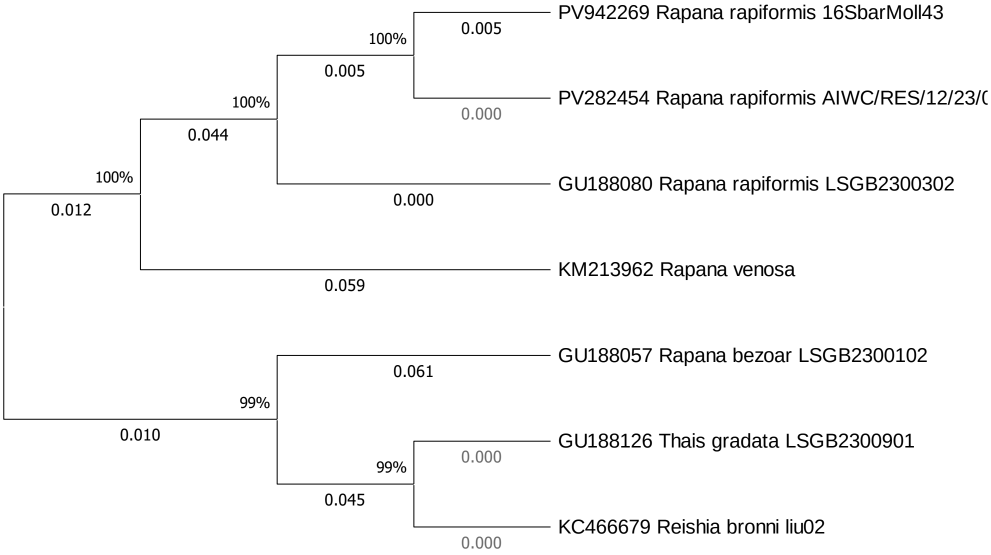
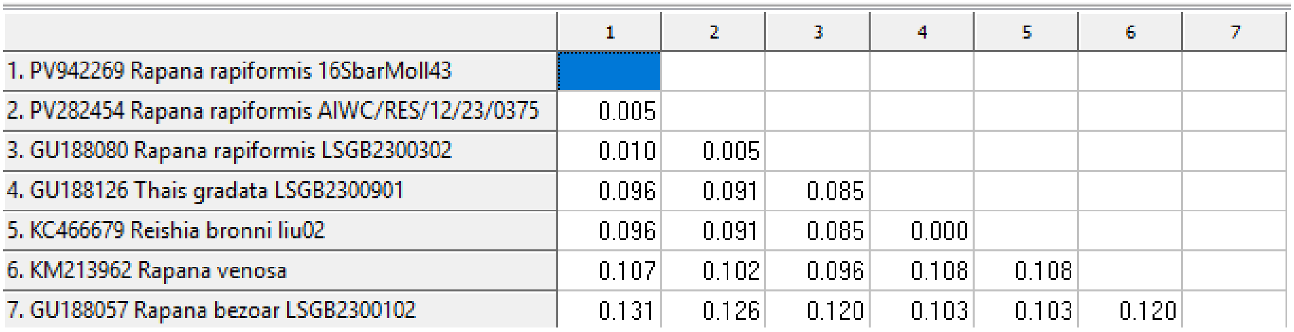


Figure 111: Dendrogram tree for *Rapana rapiformis* using 16Sbar primers using T92+G model



	1	2	3	4	5	6	7
1. PV942269 Rapana rapiformis 16SbarMoll43							
2. PV282454 Rapana rapiformis AIWC/RES/12/23/0375	0.005						
3. GU188080 Rapana rapiformis LSGB2300302	0.010	0.005					
4. GU188126 Thais gradata LSGB2300901	0.096	0.091	0.085				
5. KC466679 Reishia bronni liu02	0.096	0.091	0.085	0.000			
6. KM213962 Rapana venosa	0.107	0.102	0.096	0.108	0.108		
7. GU188057 Rapana bezoar LSGB2300102	0.131	0.126	0.120	0.103	0.103	0.120	

Figure 112: K2P distance matrix for *Rapana rapiformis* using 16Sbar primers

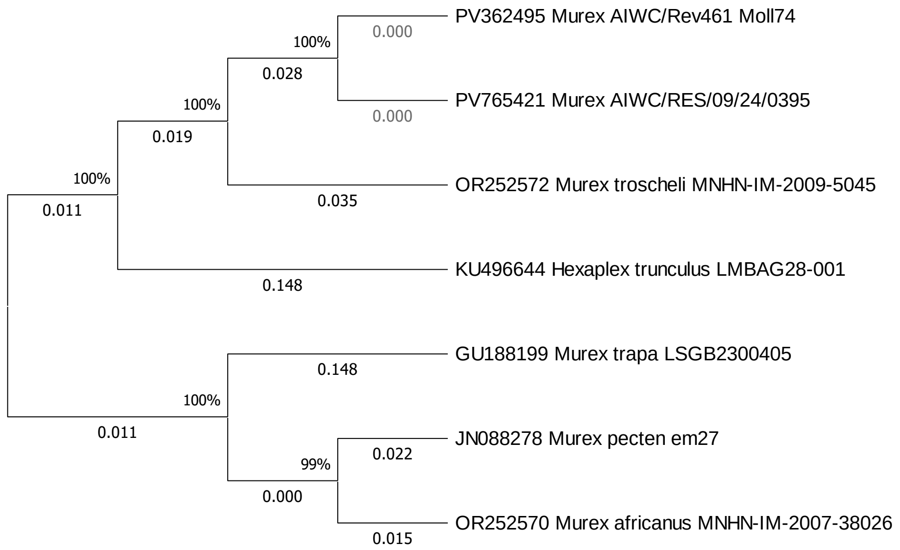
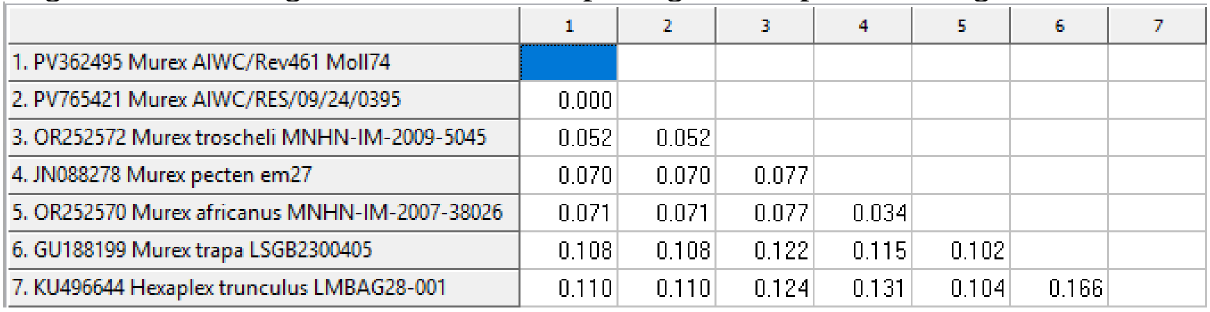


Figure 113: Dendrogram tree for *Murex.sp* using Rev461 primers using HKY+I model



	1	2	3	4	5	6	7
1. PV362495 Murex AIWC/Rev461 Moll74							
2. PV765421 Murex AIWC/RES/09/24/0395	0.000						
3. OR252572 Murex troscheli MNHN-IM-2009-5045	0.052	0.052					
4. JN088278 Murex pecten em27	0.070	0.070	0.077				
5. OR252570 Murex africanus MNHN-IM-2007-38026	0.071	0.071	0.077	0.034			
6. GU188199 Murex trapa LSGB2300405	0.108	0.108	0.122	0.115	0.102		
7. KU496644 Hexaplex trunculus LMBAG28-001	0.110	0.110	0.124	0.131	0.104	0.166	

Figure 114: K2P distance matrix for *Murex. sp* using Rev461 primers

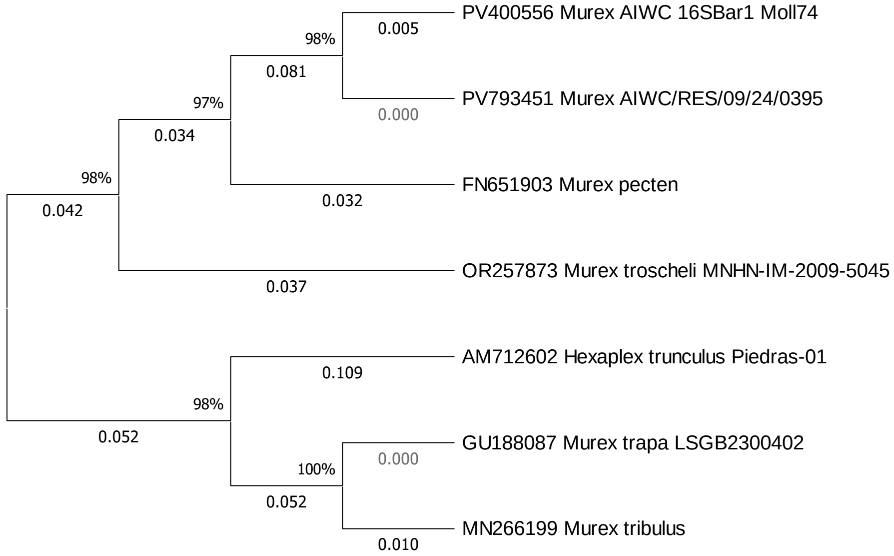
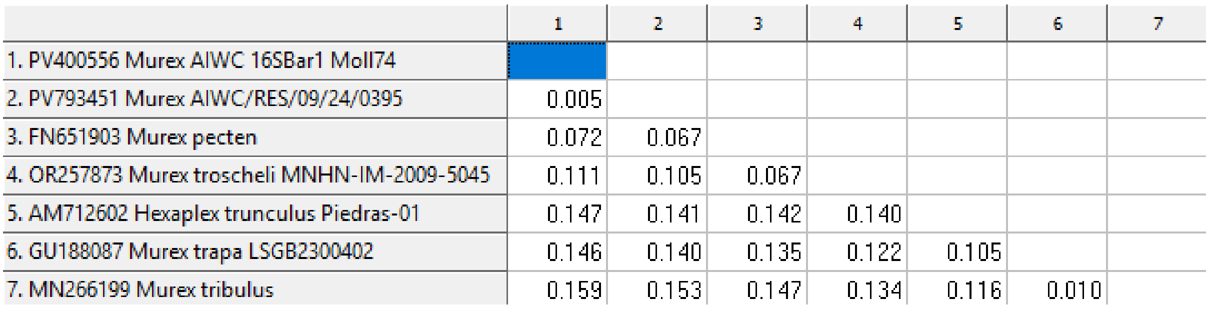


Figure 115: Dendrogram tree for *Murex.sp* using 16Sbar primers using T92+G model



	1	2	3	4	5	6	7
1. PV400556 Murex AIWC 16SBar1 Moll74							
2. PV793451 Murex AIWC/RES/09/24/0395	0.005						
3. FN651903 Murex pecten	0.072	0.067					
4. OR257873 Murex troscheli MNHN-IM-2009-5045	0.111	0.105	0.067				
5. AM712602 Hexaplex trunculus Piedras-01	0.147	0.141	0.142	0.140			
6. GU188087 Murex trapa LSGB2300402	0.146	0.140	0.135	0.122	0.105		
7. MN266199 Murex tribulus	0.159	0.153	0.147	0.134	0.116	0.010	

Figure 116: K2P distance matrix for *Murex.sp* using 16Sbar primers

7.11.10. Order Littorinimorpha – Family Cypraeidae

The genus *Erronea* belongs to the family *Cypraeidae* (cowries). Despite two rounds of DNA extraction, PCR amplification was successful only with the Rev461 primer set. Amplification attempts using multiplex mastermix, both with and without the addition of Bovine Serum Albumin (BSA), did not yield results with other primers. The amplified fragment did not clearly distinguish between *Erronea onyx* and *E.adusta* (Fig 115 & 116).

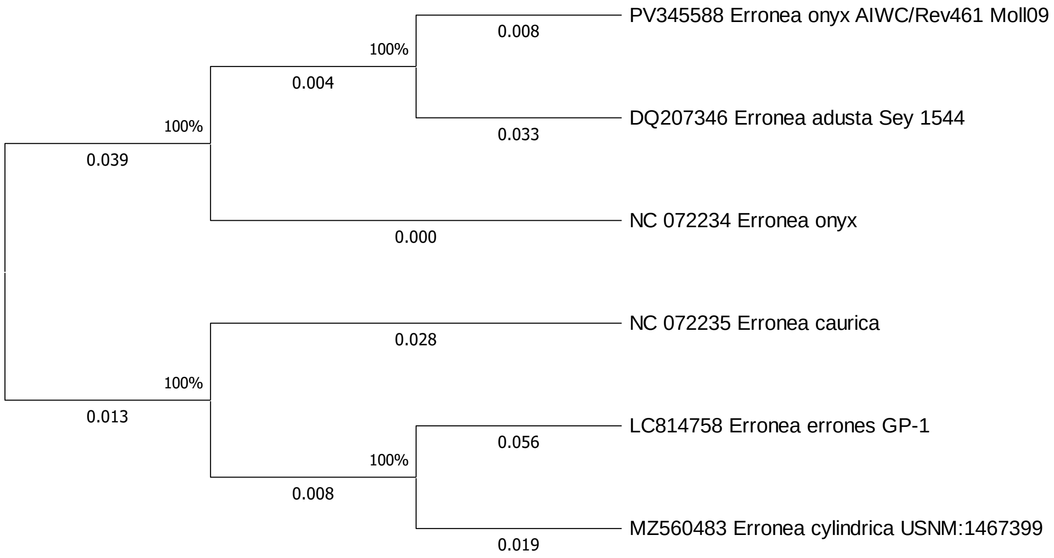
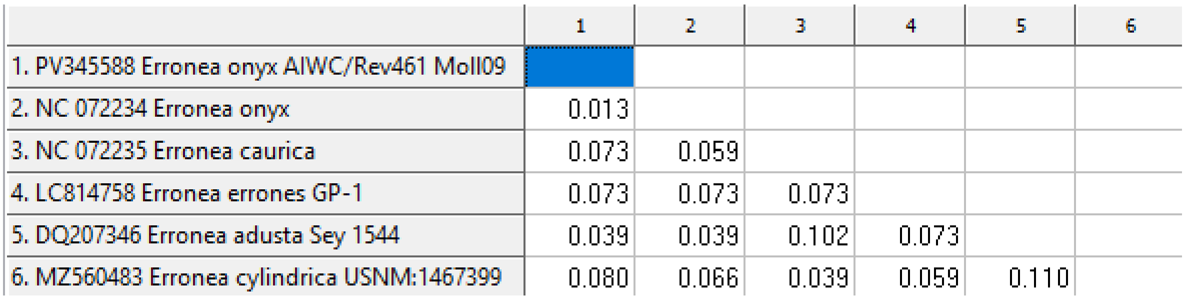


Figure 117: Dendrogram tree for *Erronea onyx* using Rev461 primers using T92+G model



	1	2	3	4	5	6
1. PV345588 Erronea onyx AIWC/Rev461 Moll09						
2. NC 072234 Erronea onyx	0.013					
3. NC 072235 Erronea caurica	0.073	0.059				
4. LC814758 Erronea errones GP-1	0.073	0.073	0.073			
5. DQ207346 Erronea adusta Sey 1544	0.039	0.039	0.102	0.073		
6. MZ560483 Erronea cylindrica USNM:1467399	0.080	0.066	0.039	0.059	0.110	

Figure 118 K2P distance matrix for *Erronea onyx* using Rev461 primers

7.11.11. Order Neogastropoda – Family Nassariidae

The *Nassariidae* is a diverse family of mud whelks predominantly found in tropical waters. Using three mitochondrial and two nuclear gene markers, Galindo et al. (2016) constructed a comprehensive molecular phylogeny that refined and expanded the previously recognized limits of the group. The family currently comprises about 603 valid extant species (Molluscabase, 2020a). Two different *Nassarius* species were barcoded through this project but the species are yet to be identified. All the three primer sets got successfully amplified, yet the inter-species distance is below 3%, hence cannot used for confirming species.

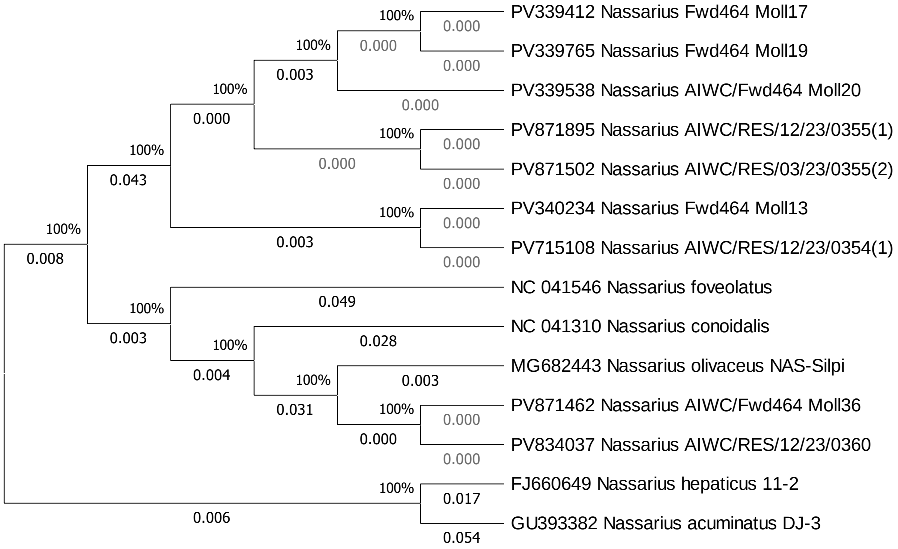
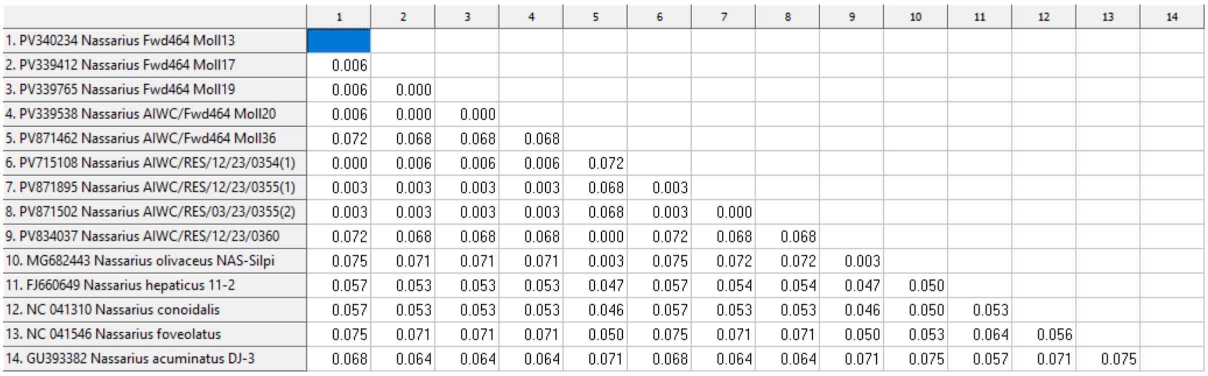


Figure 119: Dendrogram tree for *Nassarius sp* using Fwd464 primers using TN92+G model



	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. PV340234 Nassarius Fwd464 Moll13														
2. PV339412 Nassarius Fwd464 Moll17	0.006													
3. PV339765 Nassarius Fwd464 Moll19	0.006	0.000												
4. PV339538 Nassarius AIWC/Fwd464 Moll20	0.006	0.000	0.000											
5. PV871462 Nassarius AIWC/Fwd464 Moll36	0.072	0.068	0.068	0.068										
6. PV715108 Nassarius AIWC/RES/12/23/0354(1)	0.000	0.006	0.006	0.006	0.072									
7. PV871895 Nassarius AIWC/RES/12/23/0355(1)	0.003	0.003	0.003	0.003	0.068	0.003								
8. PV871502 Nassarius AIWC/RES/03/23/0355(2)	0.003	0.003	0.003	0.003	0.068	0.003	0.000							
9. PV834037 Nassarius AIWC/RES/12/23/0360	0.072	0.068	0.068	0.068	0.000	0.072	0.068	0.068						
10. MG682443 Nassarius olivaceus NAS-Silpi	0.075	0.071	0.071	0.071	0.003	0.075	0.072	0.072	0.003					
11. FJ660649 Nassarius hepaticus 11-2	0.057	0.053	0.053	0.053	0.047	0.057	0.054	0.054	0.047	0.050				
12. NC 041310 Nassarius conoidalis	0.057	0.053	0.053	0.053	0.046	0.057	0.053	0.053	0.046	0.050	0.053			
13. NC 041546 Nassarius foveolatus	0.075	0.071	0.071	0.071	0.050	0.075	0.071	0.071	0.050	0.053	0.064	0.056		
14. GU393382 Nassarius acuminatus DJ-3	0.068	0.064	0.064	0.064	0.071	0.068	0.064	0.064	0.071	0.075	0.057	0.071	0.075	

Figure 120: K2P distance matrix for *Nassarius.sp* using Fwd464 primers

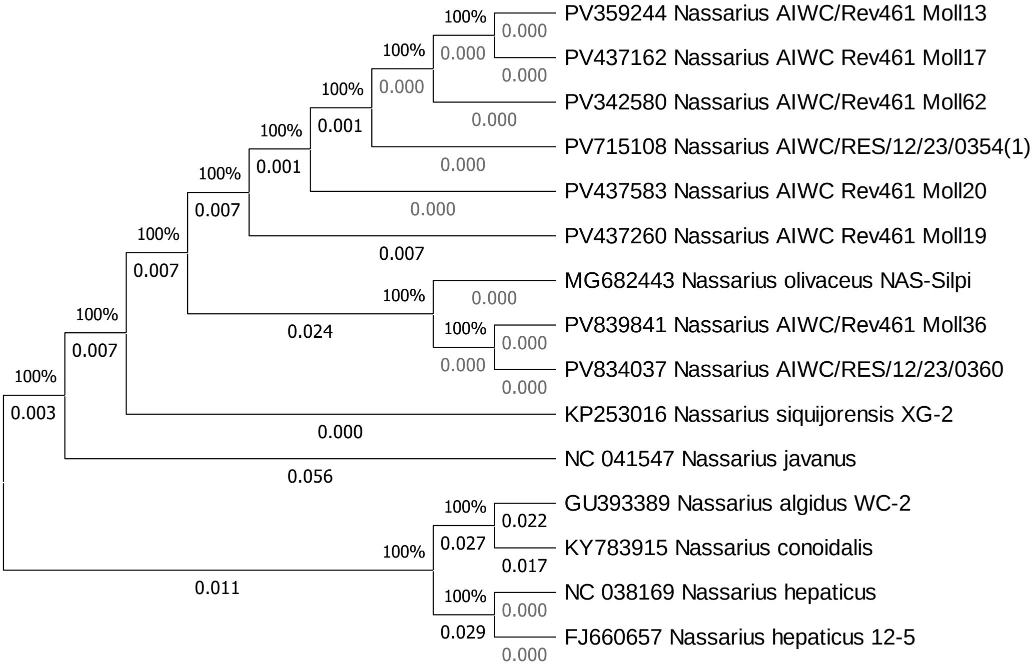
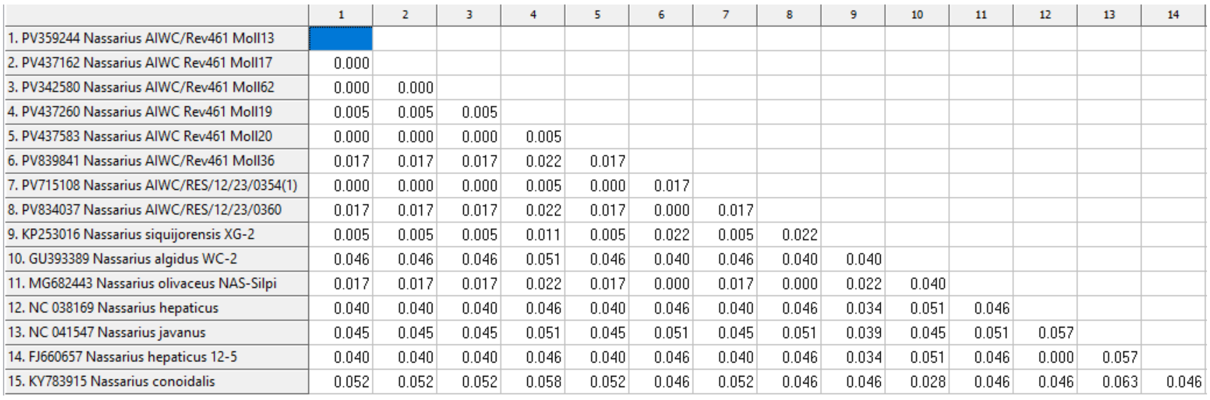


Figure 121: Dendrogram tree for *Nassarius sp* using Rev461 primers using TN92+G model



	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. PV359244 Nassarius AIWC/Rev461 Moll13														
2. PV437162 Nassarius AIWC Rev461 Moll17	0.000													
3. PV342580 Nassarius AIWC/Rev461 Moll62	0.000	0.000												
4. PV437260 Nassarius AIWC Rev461 Moll19	0.005	0.005	0.005											
5. PV437583 Nassarius AIWC Rev461 Moll20	0.000	0.000	0.000	0.005										
6. PV839841 Nassarius AIWC/Rev461 Moll36	0.017	0.017	0.017	0.022	0.017									
7. PV715108 Nassarius AIWC/RES/12/23/0354(1)	0.000	0.000	0.000	0.005	0.000	0.017								
8. PV834037 Nassarius AIWC/RES/12/23/0360	0.017	0.017	0.017	0.022	0.017	0.000	0.017							
9. KP253016 Nassarius siquijorensis XG-2	0.005	0.005	0.005	0.011	0.005	0.022	0.005	0.022						
10. GU393389 Nassarius algidus WC-2	0.046	0.046	0.046	0.051	0.046	0.040	0.046	0.040	0.040					
11. MG682443 Nassarius olivaceus NAS-Silpi	0.017	0.017	0.017	0.022	0.017	0.000	0.017	0.000	0.022	0.040				
12. NC 038169 Nassarius hepaticus	0.040	0.040	0.040	0.046	0.040	0.046	0.040	0.046	0.034	0.051	0.046			
13. NC 041547 Nassarius javanus	0.045	0.045	0.045	0.051	0.045	0.051	0.045	0.051	0.039	0.045	0.051	0.057		
14. FJ660657 Nassarius hepaticus 12-5	0.040	0.040	0.040	0.046	0.040	0.046	0.040	0.046	0.034	0.051	0.046	0.000	0.057	
15. KY783915 Nassarius conoidalis	0.052	0.052	0.052	0.058	0.052	0.046	0.052	0.046	0.046	0.028	0.046	0.046	0.063	0.046

Figure 122: K2P distance matrix for *Nassarius.sp* using Rev461 primers

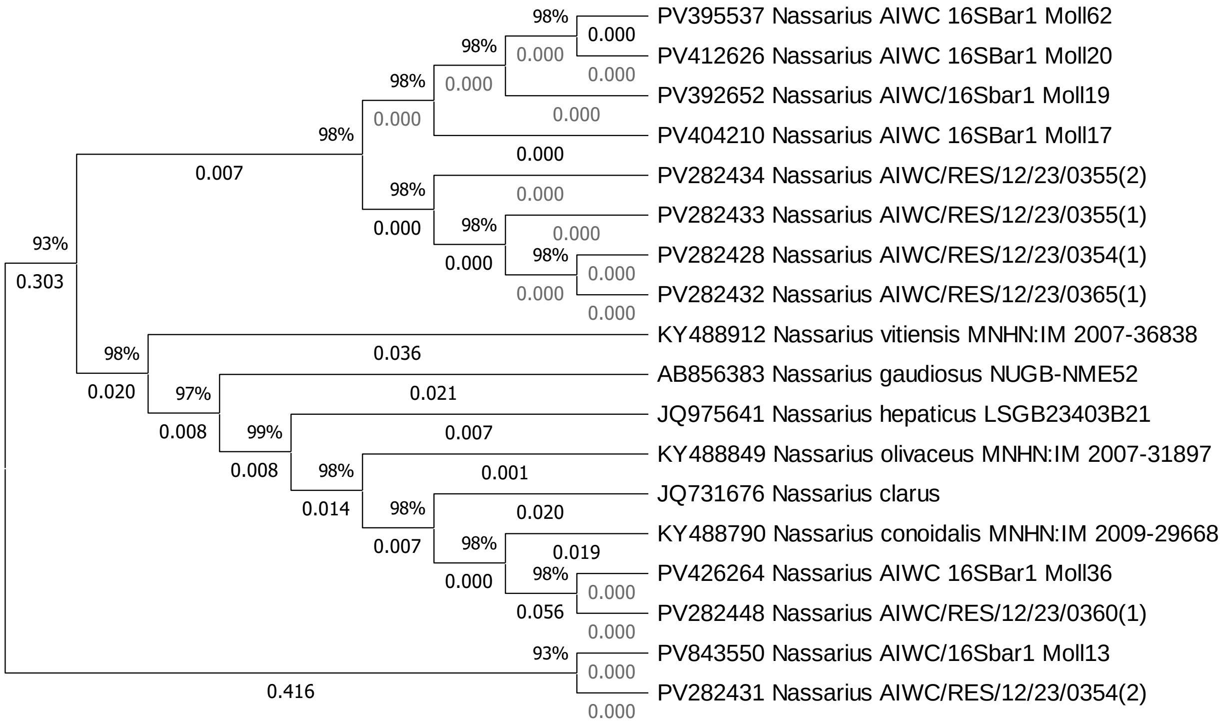
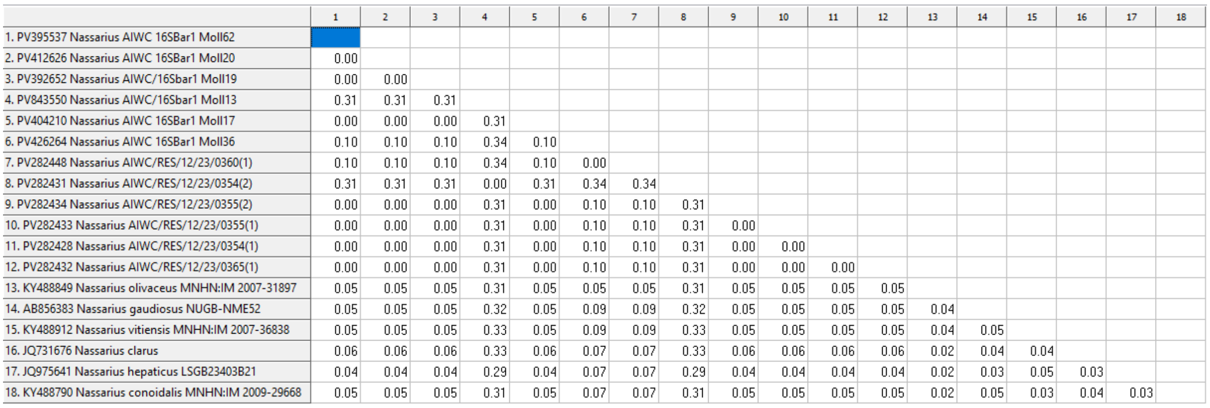


Figure 123: Dendrogram tree for *Nassarius sp* using 16Sbar primers using TN92+G model



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. PV395537 Nassarius AIWC 16SBar1 Moll62																		
2. PV412626 Nassarius AIWC 16SBar1 Moll20	0.00																	
3. PV392652 Nassarius AIWC/16Sbar1 Moll19	0.00	0.00																
4. PV843550 Nassarius AIWC/16Sbar1 Moll13	0.31	0.31	0.31															
5. PV404210 Nassarius AIWC 16SBar1 Moll17	0.00	0.00	0.00	0.31														
6. PV426264 Nassarius AIWC 16SBar1 Moll36	0.10	0.10	0.10	0.34	0.10													
7. PV282448 Nassarius AIWC/RES/12/23/0360(1)	0.10	0.10	0.10	0.34	0.10	0.00												
8. PV282431 Nassarius AIWC/RES/12/23/0354(2)	0.31	0.31	0.31	0.00	0.31	0.34	0.34											
9. PV282434 Nassarius AIWC/RES/12/23/0355(2)	0.00	0.00	0.00	0.31	0.00	0.10	0.10	0.31										
10. PV282433 Nassarius AIWC/RES/12/23/0355(1)	0.00	0.00	0.00	0.31	0.00	0.10	0.10	0.31	0.00									
11. PV282428 Nassarius AIWC/RES/12/23/0354(1)	0.00	0.00	0.00	0.31	0.00	0.10	0.10	0.31	0.00	0.00								
12. PV282432 Nassarius AIWC/RES/12/23/0365(1)	0.00	0.00	0.00	0.31	0.00	0.10	0.10	0.31	0.00	0.00	0.00							
13. KY488849 Nassarius olivaceus MNHN:IM 2007-31897	0.05	0.05	0.05	0.31	0.05	0.05	0.05	0.31	0.05	0.05	0.05	0.05						
14. AB856383 Nassarius gaudiosus NUGB-NME52	0.05	0.05	0.05	0.32	0.05	0.09	0.09	0.32	0.05	0.05	0.05	0.05	0.04					
15. KY488912 Nassarius vitiensis MNHN:IM 2007-36838	0.05	0.05	0.05	0.33	0.05	0.09	0.09	0.33	0.05	0.05	0.05	0.05	0.04	0.05				
16. JQ731676 Nassarius clarus	0.06	0.06	0.06	0.33	0.06	0.07	0.07	0.33	0.06	0.06	0.06	0.06	0.02	0.04	0.04			
17. JQ975641 Nassarius hepaticus LSGB23403B21	0.04	0.04	0.04	0.29	0.04	0.07	0.07	0.29	0.04	0.04	0.04	0.04	0.02	0.03	0.05	0.03		
18. KY488790 Nassarius conoidalis MNHN:IM 2009-29668	0.05	0.05	0.05	0.31	0.05	0.07	0.07	0.31	0.05	0.05	0.05	0.05	0.02	0.05	0.03	0.04	0.03	

Figure 124: K2P distance matrix for *Nassarius.sp* using 16Sbar primers

7.11.12. *Order Neogastropoda - Family Melongenidae*

Due to the limited number of genetic studies on the family Melongenidae, the efficiency of the primers could not be thoroughly evaluated, even though all three primer sets successfully amplified sequences closely matching the available reference sequence (Fig 123-128).

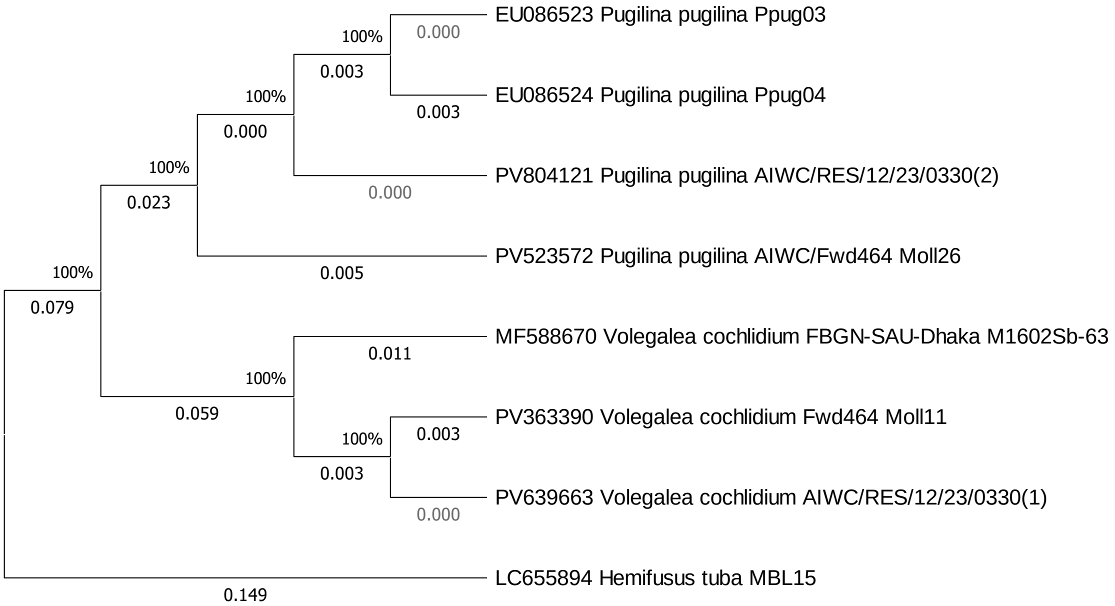
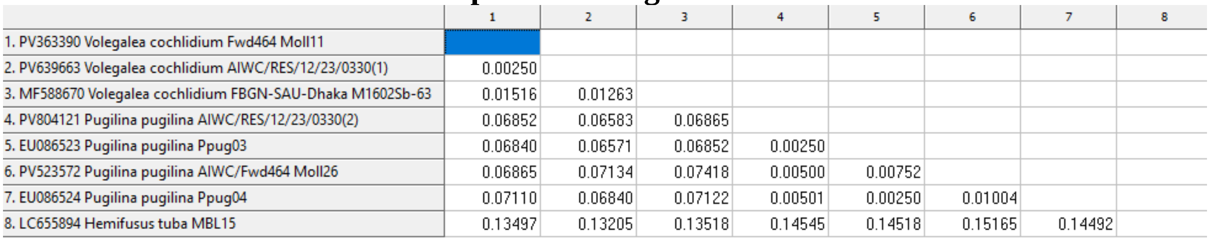


Figure 125: Dendrogram tree for *Pugilina pugilina* and *Volegalea cochlidium* using Fwd464 primers using T92+G model



	1	2	3	4	5	6	7	8
1. PV363390 Volegalea cochlidium Fwd464 Moll11								
2. PV639663 Volegalea cochlidium AIWC/RES/12/23/0330(1)	0.00250							
3. MF588670 Volegalea cochlidium FBGN-SAU-Dhaka M1602Sb-63	0.01516	0.01263						
4. PV804121 Pugilina pugilina AIWC/RES/12/23/0330(2)	0.06852	0.06583	0.06865					
5. EU086523 Pugilina pugilina Ppug03	0.06840	0.06571	0.06852	0.00250				
6. PV523572 Pugilina pugilina AIWC/Fwd464 Moll26	0.06865	0.07134	0.07418	0.00500	0.00752			
7. EU086524 Pugilina pugilina Ppug04	0.07110	0.06840	0.07122	0.00501	0.00250	0.01004		
8. LC655894 Hemifusus tuba MBL15	0.13497	0.13205	0.13518	0.14545	0.14518	0.15165	0.14492	

Figure 126: K2P distance matrix for *Pugilina pugilina* and *Volegalea cochlidium* using Fwd464 primers

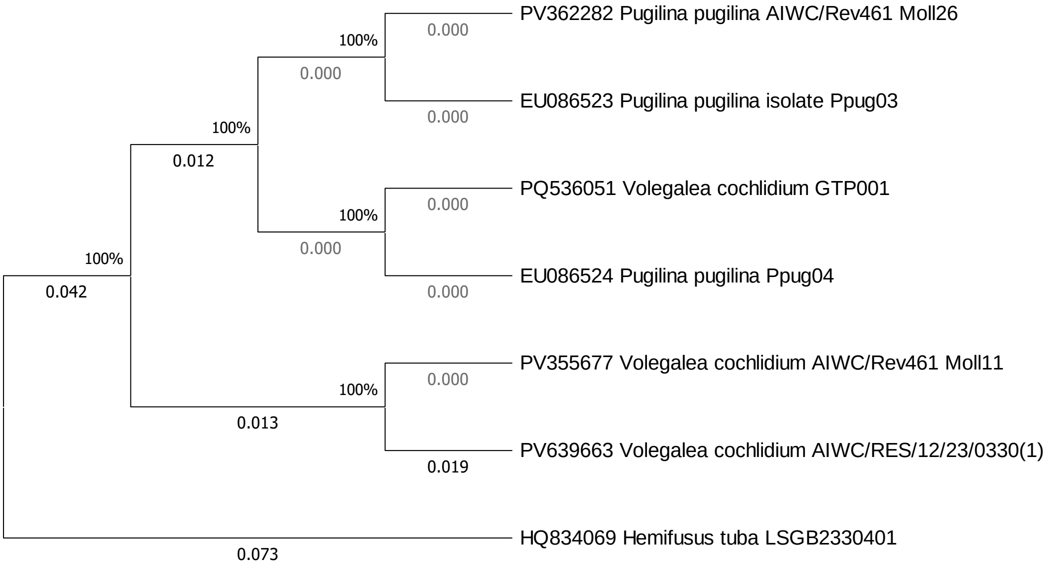
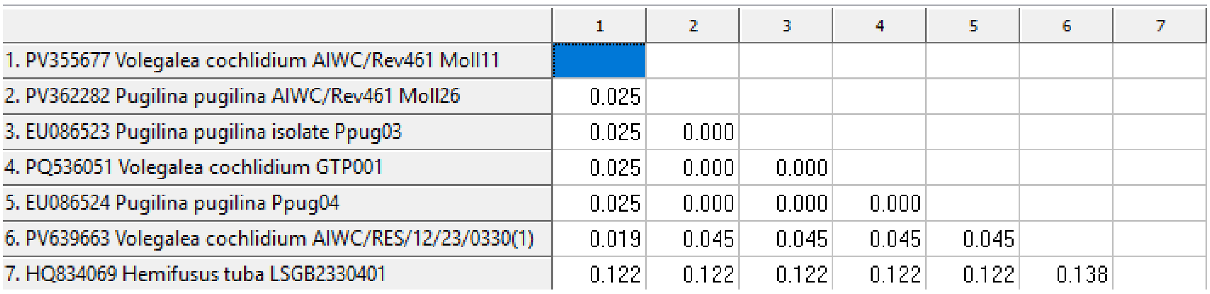


Figure 127: Dendrogram tree for *Pugilina pugilina* and *Volegalea cochlidium* using Rev461 primers using T92 model



	1	2	3	4	5	6	7
1. PV355677 Volegalea cochlidium AIWC/Rev461 Moll11							
2. PV362282 Pugilina pugilina AIWC/Rev461 Moll26	0.025						
3. EU086523 Pugilina pugilina isolate Ppug03	0.025	0.000					
4. PQ536051 Volegalea cochlidium GTP001	0.025	0.000	0.000				
5. EU086524 Pugilina pugilina Ppug04	0.025	0.000	0.000	0.000			
6. PV639663 Volegalea cochlidium AIWC/RES/12/23/0330(1)	0.019	0.045	0.045	0.045	0.045		
7. HQ834069 Hemifusus tuba LSGB2330401	0.122	0.122	0.122	0.122	0.122	0.138	

Figure 128: K2P distance matrix for *Pugilina pugilina* and *Volegalea cochlidium* using Rev461 primers

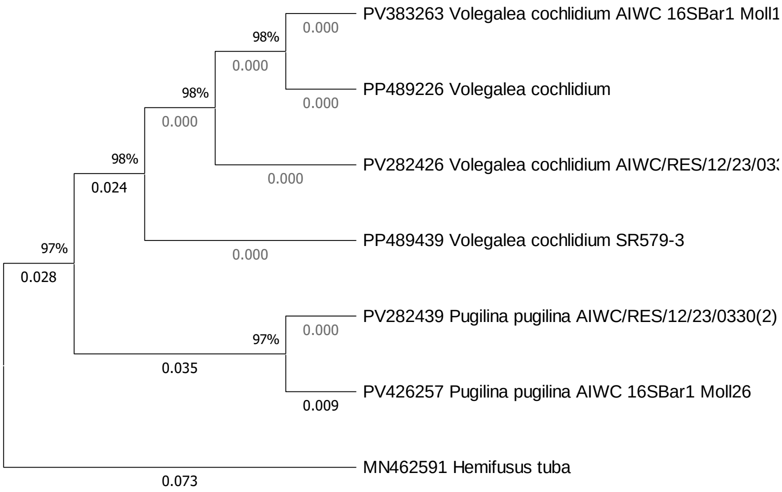
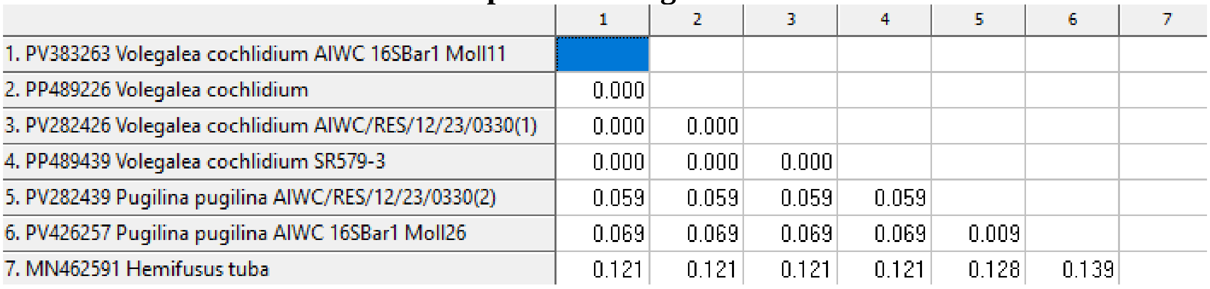


Figure 129: Dendrogram tree for *Pugilina pugilina* and *Volegalea cochlidium* using 16Sbar primers using T92 model



	1	2	3	4	5	6	7
1. PV383263 Volegalea cochlidium AIWC 16SBar1 Moll11							
2. PP489226 Volegalea cochlidium	0.000						
3. PV282426 Volegalea cochlidium AIWC/RES/12/23/0330(1)	0.000	0.000					
4. PP489439 Volegalea cochlidium SR579-3	0.000	0.000	0.000				
5. PV282439 Pugilina pugilina AIWC/RES/12/23/0330(2)	0.059	0.059	0.059	0.059			
6. PV426257 Pugilina pugilina AIWC 16SBar1 Moll26	0.069	0.069	0.069	0.069	0.009		
7. MN462591 Hemifusus tuba	0.121	0.121	0.121	0.121	0.128	0.139	

Figure 130: K2P distance matrix for *Pugilina pugilina* and *Volegalea cochlidium* using 16Sbar primers

7.11.13. Order Littorinimorpha - Family Cymatiidae

Lotoria triangularis and *Ranularia oboesa* were sequenced and submitted to NCBI for the first time through this study. The recent revision of the superfamily Tonnoidea by Strong et al. (2019) provided a modern framework for re-evaluating traditional taxonomy. Both *Lotoria* and *Ranularia* were confirmed as monophyletic clades in their analysis, aligning with our phylogenetic findings. Due to the limited availability of sequence data within these genera, closely related taxa from the same family were used for comparative analysis.

All three primer sets successfully amplified *Ranularia oboesa*, whereas the Fwd464 primer set failed to amplify for *Lotoria triangularis*. Despite this, both Maximum Likelihood (ML) phylogenetic reconstruction and pairwise distance matrix analyses clearly resolved the species included in the comparison (Fig 129 – 138). The sequences generated using mini-barcodes showed high similarity to their respective references, supporting their taxonomic identity and utility for species-level discrimination within the family Cymatiidae.

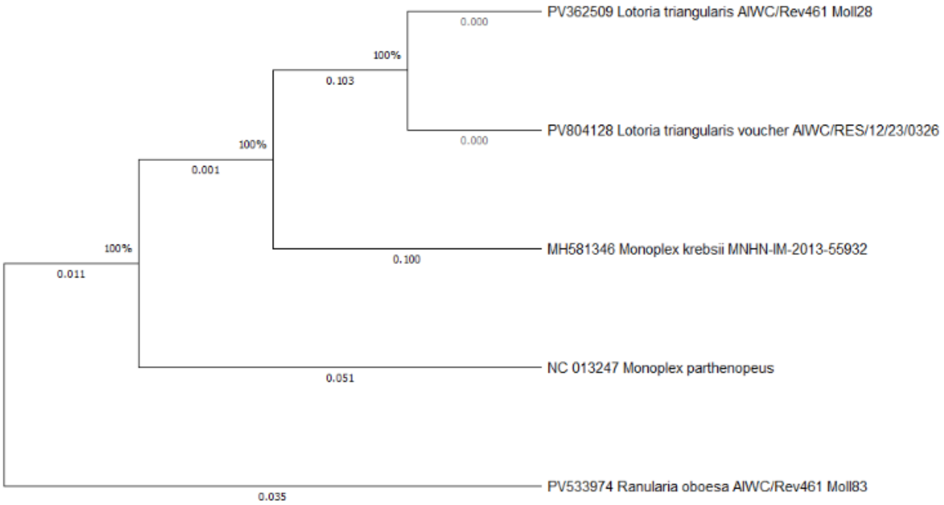
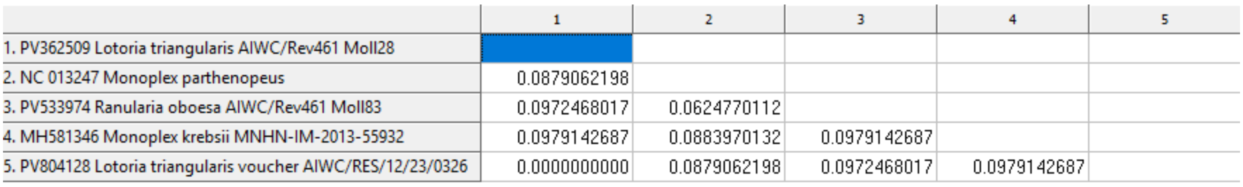


Figure 131: Dendrogram tree for *Lotoria triangularis* using Rev 461 primers using T92+ G model



	1	2	3	4	5
1. PV362509 Lotoria triangularis AIWC/Rev461 Moll28					
2. NC 013247 Monoplex parthenopeus	0.0879062198				
3. PV533974 Ranularia oboesa AIWC/Rev461 Moll83	0.0972468017	0.0624770112			
4. MH581346 Monoplex krebsii MNHN-IM-2013-55932	0.0979142687	0.0883970132	0.0979142687		
5. PV804128 Lotoria triangularis voucher AIWC/RES/12/23/0326	0.0000000000	0.0879062198	0.0972468017	0.0979142687	

Figure 132: K2P distance matrix for *Lotoria triangularis* using Rev 461 primers

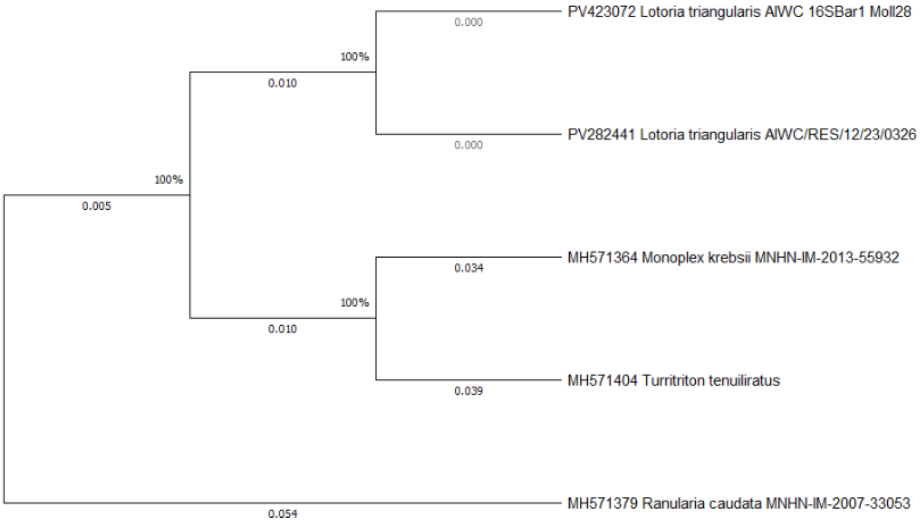
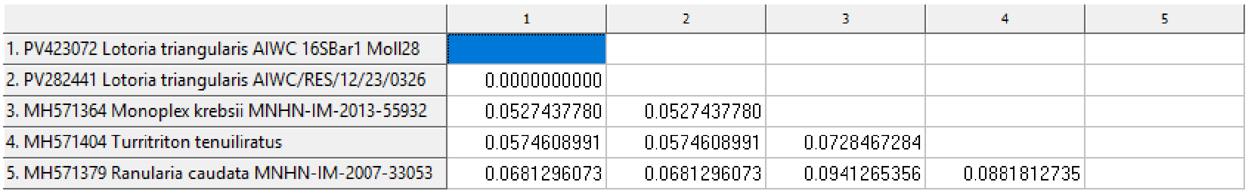


Figure 133: Dendrogram tree for *Lotoria triangularis* using 16Sbar primers using T92 model



	1	2	3	4	5
1. PV423072 Lotoria triangularis AIWC 16SBar1 Moll28					
2. PV282441 Lotoria triangularis AIWC/RES/12/23/0326	0.0000000000				
3. MH571364 Monoplex krebsii MNHN-IM-2013-55932	0.0527437780	0.0527437780			
4. MH571404 Turritriton tenuiliratus	0.0574608991	0.0574608991	0.0728467284		
5. MH571379 Ranularia caudata MNHN-IM-2007-33053	0.0681296073	0.0681296073	0.0941265356	0.0881812735	

Figure 134: K2P distance matrix for *Lotoria triangularis* using 16Sbar

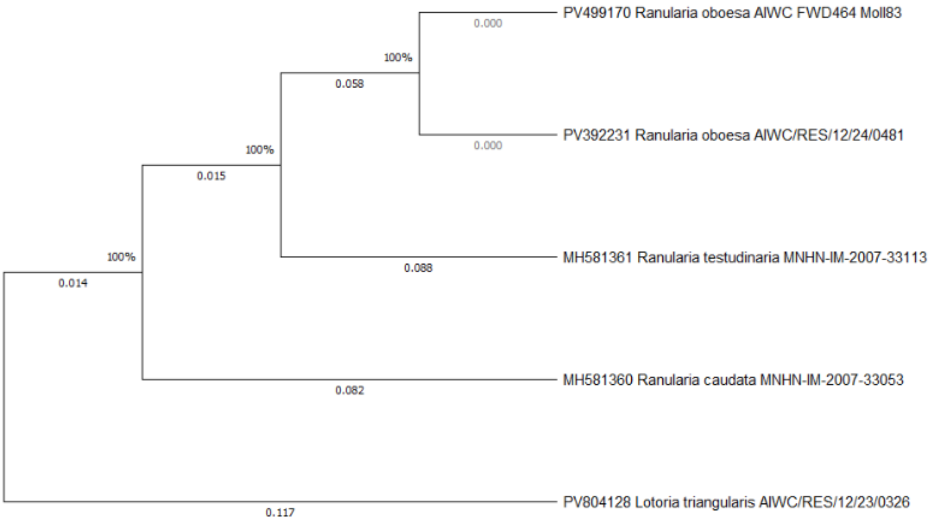
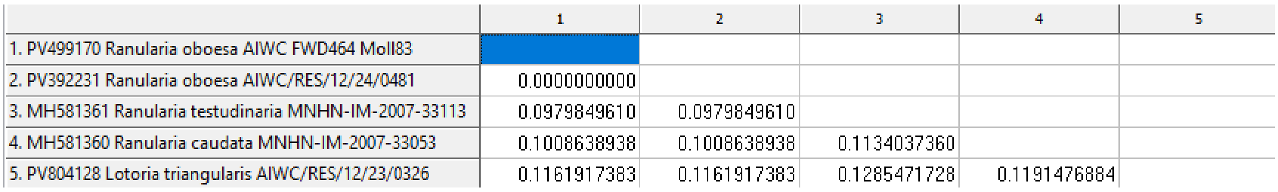


Figure 135: Dendrogram tree for *Ranularia oboesa* using Fwd464 primers using T92 model



	1	2	3	4	5
1. PV499170 Ranularia oboesa AIWC FWD464 Moll83					
2. PV392231 Ranularia oboesa AIWC/RES/12/24/0481	0.0000000000				
3. MH581361 Ranularia testudinaria MNHN-IM-2007-33113	0.0979849610	0.0979849610			
4. MH581360 Ranularia caudata MNHN-IM-2007-33053	0.1008638938	0.1008638938	0.1134037360		
5. PV804128 Lotoria triangularis AIWC/RES/12/23/0326	0.1161917383	0.1161917383	0.1285471728	0.1191476884	

Figure 136: K2P distance matrix for *Ranularia oboesa* using Fwd 464 primers

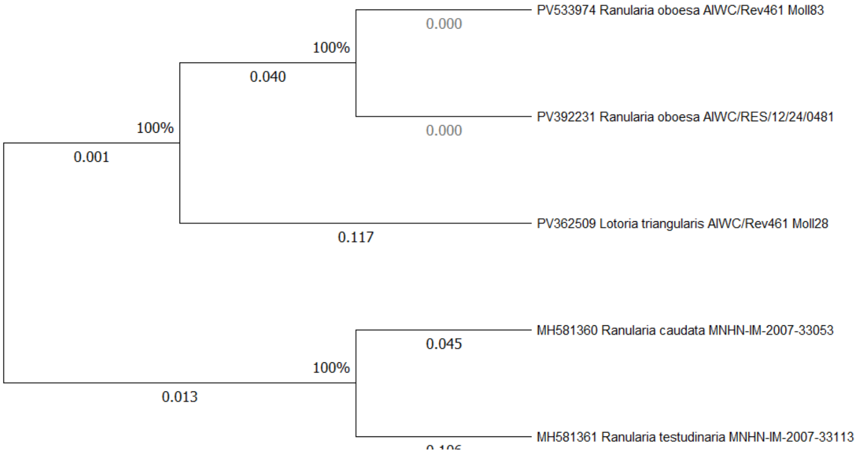
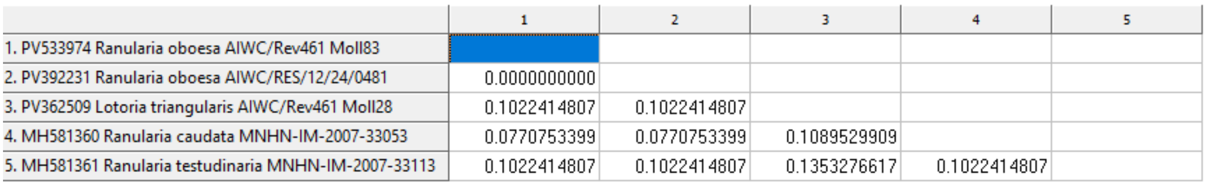


Figure 137: Dendrogram tree for *Ranularia oboesa* using Rev461 primers using T92+G model



	1	2	3	4	5
1. PV533974 Ranularia oboesa AIWC/Rev461 Moll83					
2. PV392231 Ranularia oboesa AIWC/RES/12/24/0481	0.0000000000				
3. PV362509 Lotoria triangularis AIWC/Rev461 Moll28	0.1022414807	0.1022414807			
4. MH581360 Ranularia caudata MNHN-IM-2007-33053	0.0770753399	0.0770753399	0.1089529909		
5. MH581361 Ranularia testudinaria MNHN-IM-2007-33113	0.1022414807	0.1022414807	0.1353276617	0.1022414807	

Figure 138: K2P distance matrix for *Ranularia oboesa* using Rev 461 primers

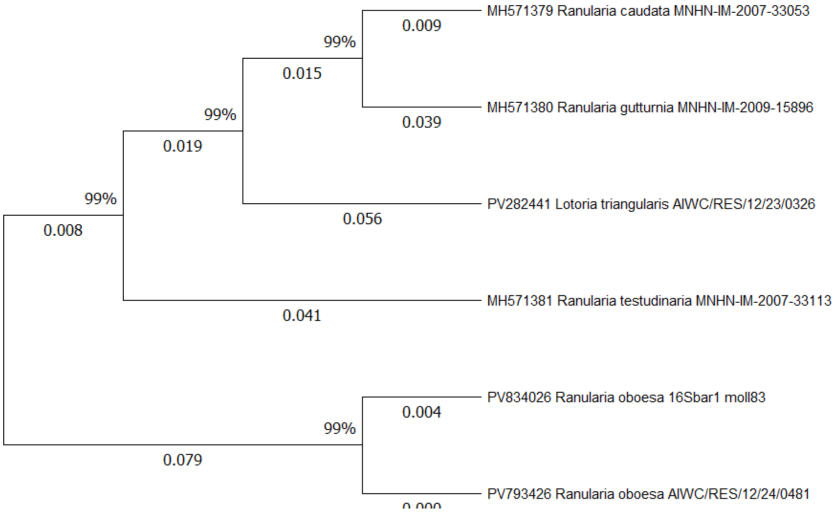
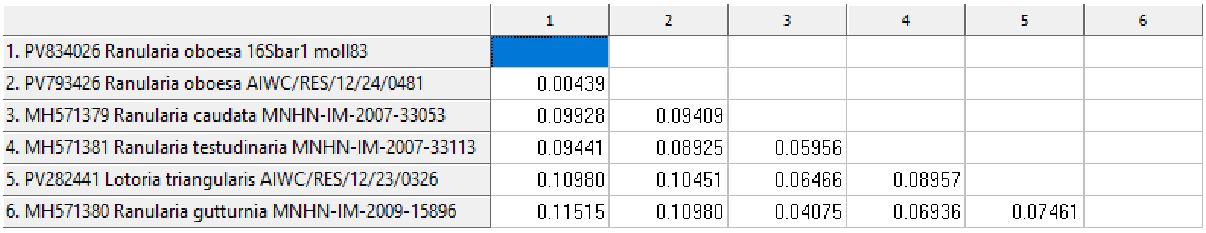


Figure 139: Dendrogram tree for *Ranularia oboesa* using 16Sbar primers using T92+G model



	1	2	3	4	5	6
1. PV834026 Ranularia oboesa 16Sbar1 moll83						
2. PV793426 Ranularia oboesa AIWC/RES/12/24/0481	0.00439					
3. MH571379 Ranularia caudata MNHN-IM-2007-33053	0.09928	0.09409				
4. MH571381 Ranularia testudinaria MNHN-IM-2007-33113	0.09441	0.08925	0.05956			
5. PV282441 Lotoria triangularis AIWC/RES/12/23/0326	0.10980	0.10451	0.06466	0.08957		
6. MH571380 Ranularia gutturnia MNHN-IM-2009-15896	0.11515	0.10980	0.04075	0.06936	0.07461	

Figure 140: K2P distance matrix for *Ranularia oboesa* using 16Sbar primers

7.11.14. Order Neogastropoda - Family Conidae

The family Conidae has more than 360 valid extant species of which 60 species were recorded from the coast of Tamil Nadu (Franklin et.al., 2009). *Conus milneedwardsi* is the protected species in this family. Althought it was recorded previously by Franklin et.al (2009), it was not recorded in the current study. For testing the primers, *Conus amadis*, which is commonly found in the coast of Tamil Nadu and *Conus araneousus* is only restricted to Gulf of Mannar,India. Even though sequences from all the three primers amplified successfully and gap distance to identify the species was significantly high, the distance to discrimate within other species was not sufficient. Among the three, Fwd464 primer set showed better resolution (Fig 139 – 150).

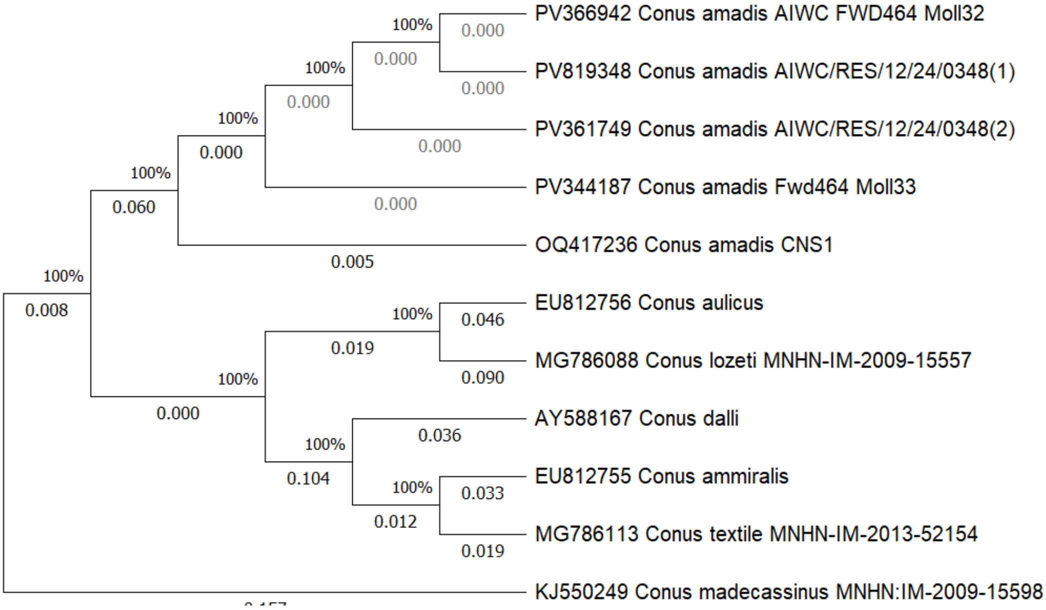
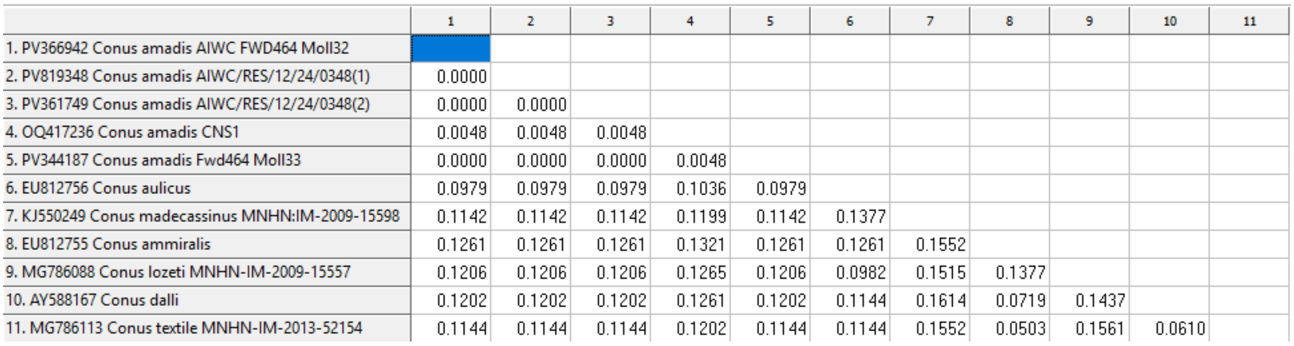


Figure 141: Dendrogram tree for *Conus amadis* using Fwd464 primers using T92+G model.



	1	2	3	4	5	6	7	8	9	10	11
1. PV366942 Conus amadis AIWC FWD464 Moll32											
2. PV819348 Conus amadis AIWC/RES/12/24/0348(1)	0.0000										
3. PV361749 Conus amadis AIWC/RES/12/24/0348(2)	0.0000	0.0000									
4. OQ417236 Conus amadis CNS1	0.0048	0.0048	0.0048								
5. PV344187 Conus amadis Fwd464 Moll33	0.0000	0.0000	0.0000	0.0048							
6. EU812756 Conus aulicus	0.0979	0.0979	0.0979	0.1036	0.0979						
7. KJ550249 Conus madecassinus MNHN:IM-2009-15598	0.1142	0.1142	0.1142	0.1199	0.1142	0.1377					
8. EU812755 Conus ammiralis	0.1261	0.1261	0.1261	0.1321	0.1261	0.1261	0.1552				
9. MG786088 Conus lozeti MNHN-IM-2009-15557	0.1206	0.1206	0.1206	0.1265	0.1206	0.0982	0.1515	0.1377			
10. AY588167 Conus dalli	0.1202	0.1202	0.1202	0.1261	0.1202	0.1144	0.1614	0.0719	0.1437		
11. MG786113 Conus textile MNHN-IM-2013-52154	0.1144	0.1144	0.1144	0.1202	0.1144	0.1144	0.1552	0.0503	0.1561	0.0610	

Figure 142: K2P distance matrix for *Conus amadis* using Fwd464 primers

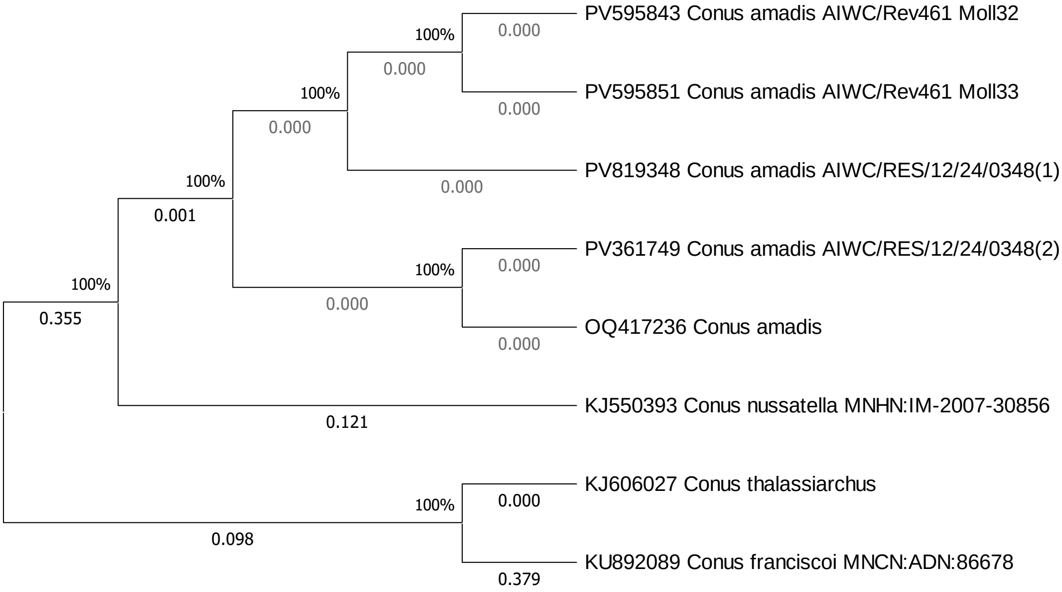
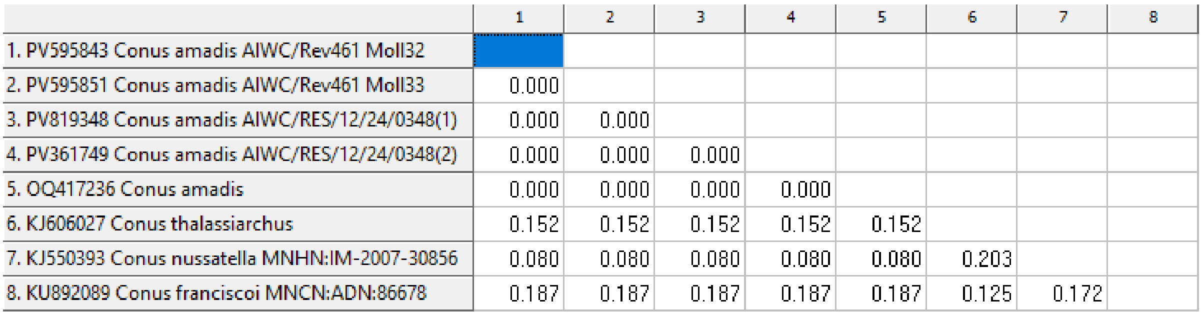


Figure 143: Dendrogram tree for *Conus amadis* using Rev461 primers using T92+G model



	1	2	3	4	5	6	7	8
1. PV595843 Conus amadis AIWC/Rev461 Moll32								
2. PV595851 Conus amadis AIWC/Rev461 Moll33	0.000							
3. PV819348 Conus amadis AIWC/RES/12/24/0348(1)	0.000	0.000						
4. PV361749 Conus amadis AIWC/RES/12/24/0348(2)	0.000	0.000	0.000					
5. OQ417236 Conus amadis	0.000	0.000	0.000	0.000				
6. KJ606027 Conus thalassiarchus	0.152	0.152	0.152	0.152	0.152			
7. KJ550393 Conus nussatella MNHN:IM-2007-30856	0.080	0.080	0.080	0.080	0.080	0.203		
8. KU892089 Conus franciscoi MNCN:ADN:86678	0.187	0.187	0.187	0.187	0.187	0.125	0.172	

Figure 144: K2P distance matrix for *Conus amadis* using Rev461 primers

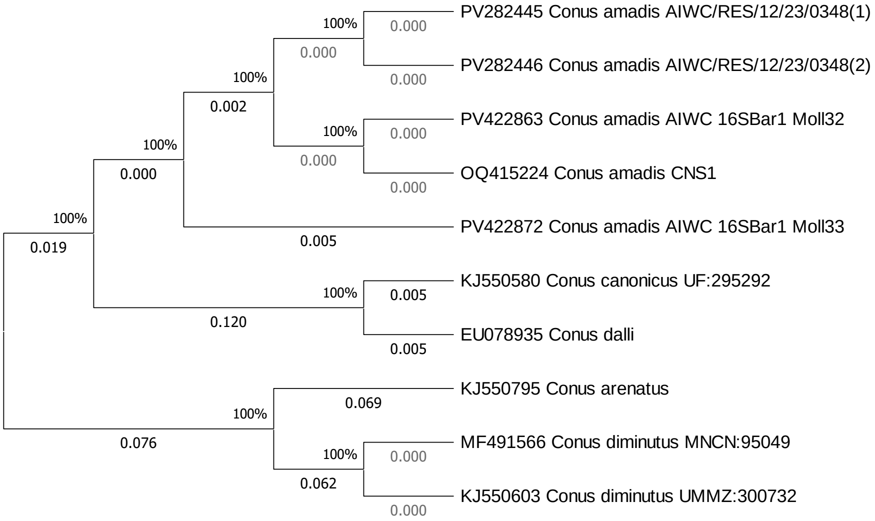
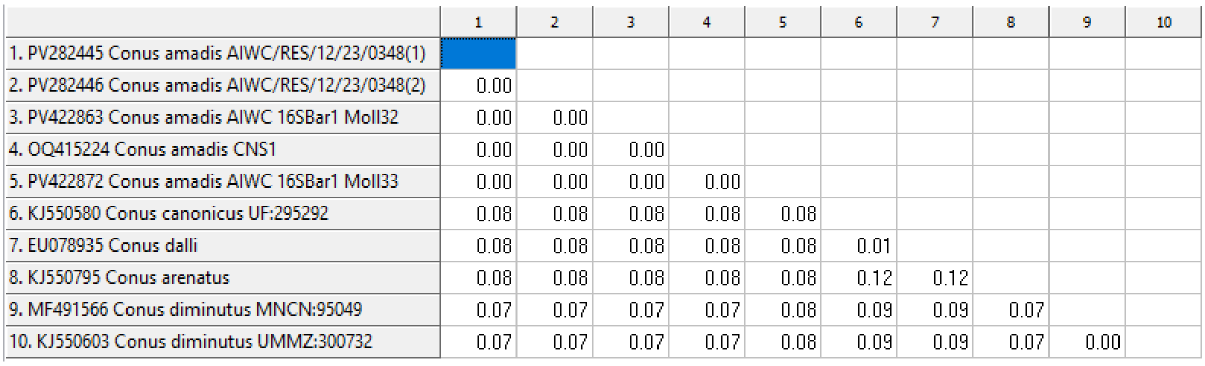


Figure 145: Dendrogram tree for *Conus amadis* using 16Sbar primers using T92+G model



	1	2	3	4	5	6	7	8	9	10
1. PV282445 Conus amadis AIWC/RES/12/23/0348(1)										
2. PV282446 Conus amadis AIWC/RES/12/23/0348(2)	0.00									
3. PV422863 Conus amadis AIWC 16SBar1 Moll32	0.00	0.00								
4. OQ415224 Conus amadis CNS1	0.00	0.00	0.00							
5. PV422872 Conus amadis AIWC 16SBar1 Moll33	0.00	0.00	0.00	0.00						
6. KJ550580 Conus canonicus UF:295292	0.08	0.08	0.08	0.08	0.08					
7. EU078935 Conus dalli	0.08	0.08	0.08	0.08	0.08	0.01				
8. KJ550795 Conus arenatus	0.08	0.08	0.08	0.08	0.08	0.12	0.12			
9. MF491566 Conus diminutus MNCN:95049	0.07	0.07	0.07	0.07	0.08	0.09	0.09	0.07		
10. KJ550603 Conus diminutus UMMZ:300732	0.07	0.07	0.07	0.07	0.08	0.09	0.09	0.07	0.00	

Figure 146: K2P distance matrix for *Conus amadis* using 16Sbar primers

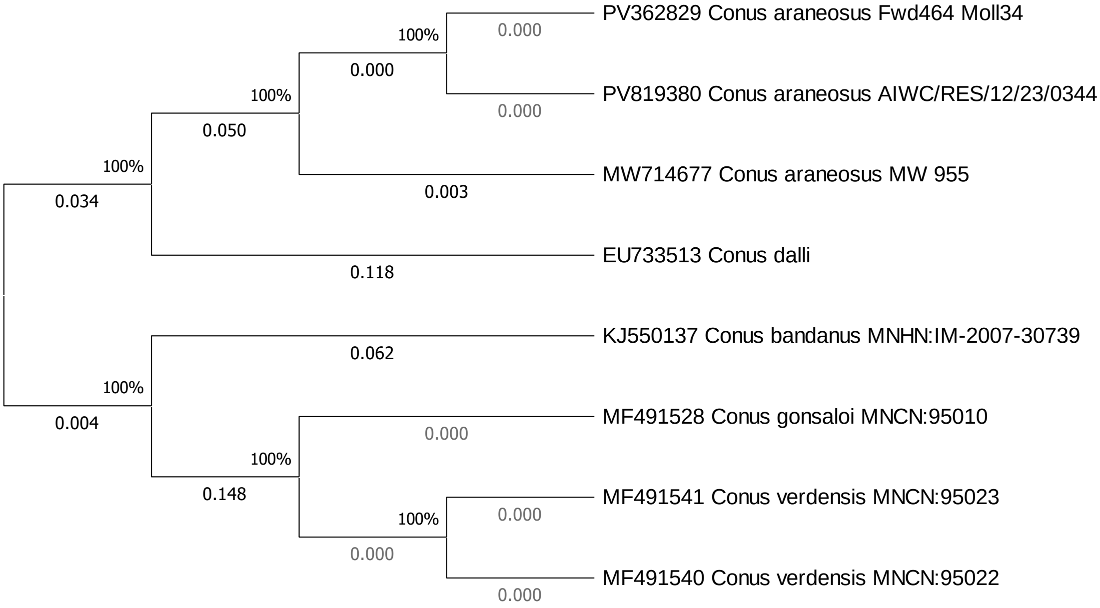
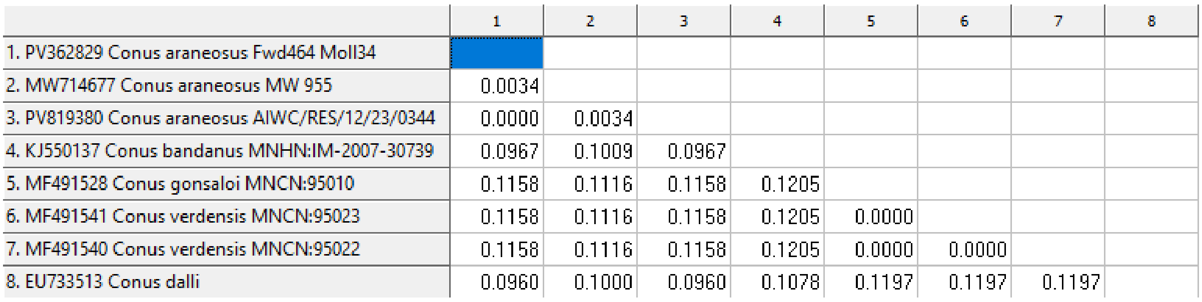


Figure 147: Dendrogram tree for *Conus araneousus* using Fwd464 primers using T92+G model



	1	2	3	4	5	6	7	8
1. PV362829 Conus araneosus Fwd464 Moll34								
2. MW714677 Conus araneosus MW 955	0.0034							
3. PV819380 Conus araneosus AIWC/RES/12/23/0344	0.0000	0.0034						
4. KJ550137 Conus bandanus MNHN:IM-2007-30739	0.0967	0.1009	0.0967					
5. MF491528 Conus gonsaloi MNCN:95010	0.1158	0.1116	0.1158	0.1205				
6. MF491541 Conus verdensis MNCN:95023	0.1158	0.1116	0.1158	0.1205	0.0000			
7. MF491540 Conus verdensis MNCN:95022	0.1158	0.1116	0.1158	0.1205	0.0000	0.0000		
8. EU733513 Conus dalli	0.0960	0.1000	0.0960	0.1078	0.1197	0.1197	0.1197	

Figure 148: K2P distance matrix for *Conus araneousus* using Fwd464 primers

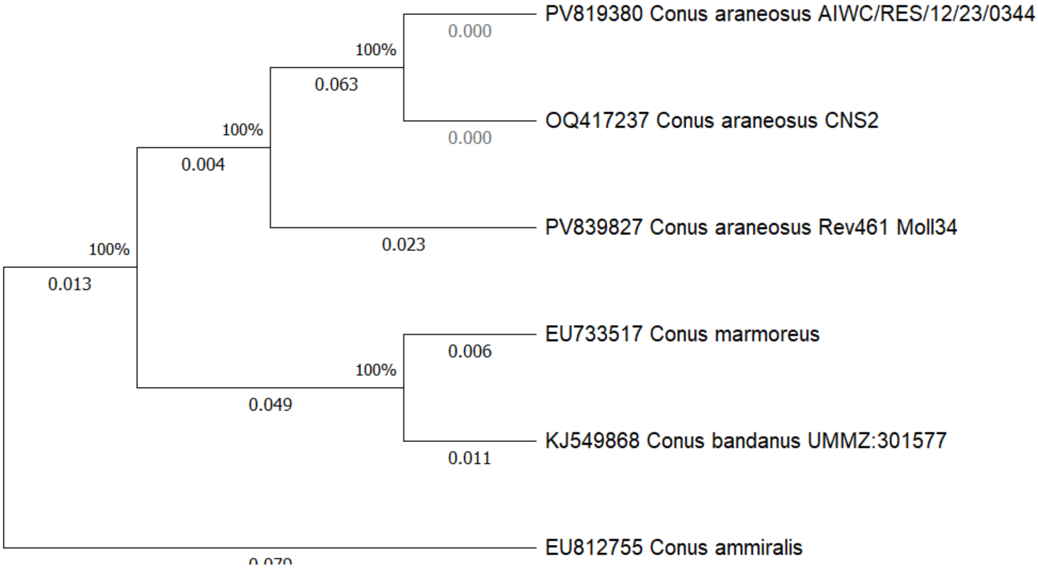
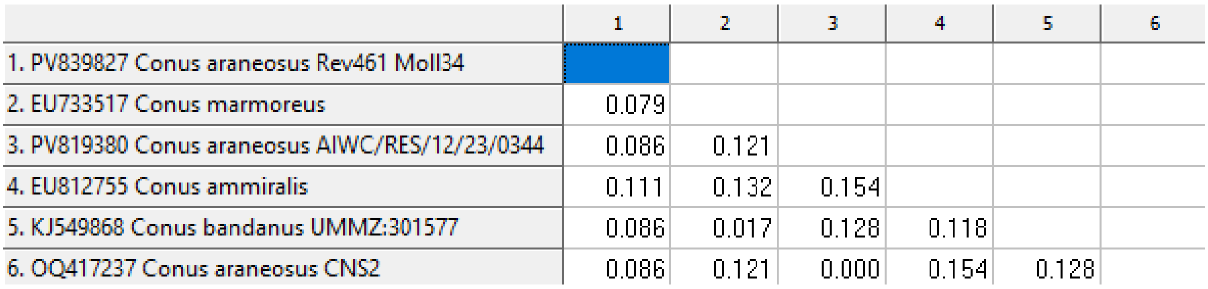


Figure 149: Dendrogram tree for *Conus araneousus* using Rev461 primers using HKY model



	1	2	3	4	5	6
1. PV839827 Conus araneosus Rev461 Moll34						
2. EU733517 Conus marmoreus	0.079					
3. PV819380 Conus araneosus AIWC/RES/12/23/0344	0.086	0.121				
4. EU812755 Conus ammiralis	0.111	0.132	0.154			
5. KJ549868 Conus bandanus UMMZ:301577	0.086	0.017	0.128	0.118		
6. OQ417237 Conus araneosus CNS2	0.086	0.121	0.000	0.154	0.128	

Figure 150: K2P distance matrix for *Conus araneousus* using Rev461 primers

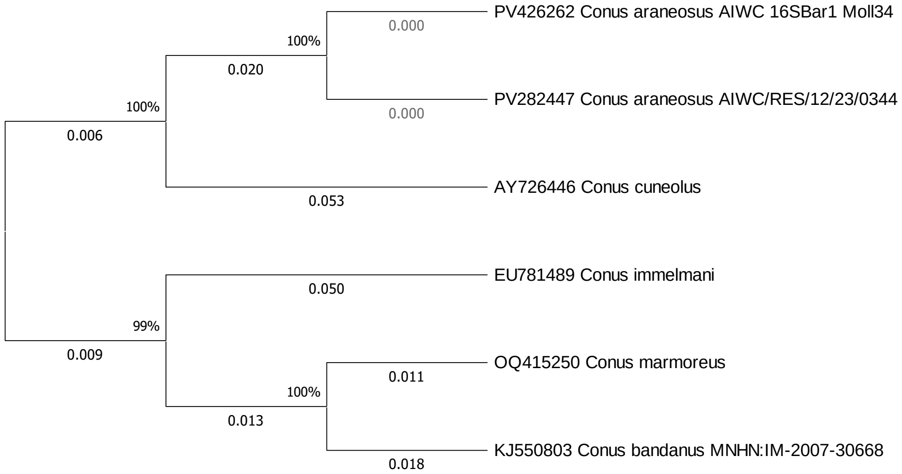
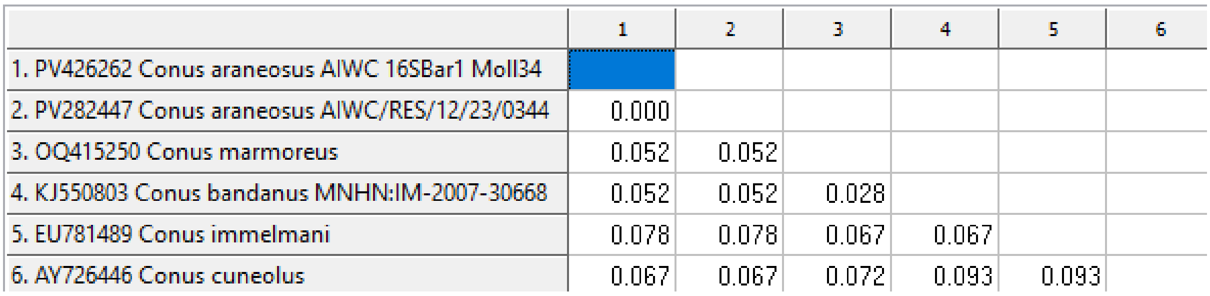


Figure 151: Dendrogram tree for *Conus araneousus* using 16Sbar primers using T92+G model



	1	2	3	4	5	6
1. PV426262 Conus araneosus AIWC 16SBar1 Moll34						
2. PV282447 Conus araneosus AIWC/RES/12/23/0344	0.000					
3. OQ415250 Conus marmoreus	0.052	0.052				
4. KJ550803 Conus bandanus MNHN:IM-2007-30668	0.052	0.052	0.028			
5. EU781489 Conus immelmani	0.078	0.078	0.067	0.067		
6. AY726446 Conus cuneolus	0.067	0.067	0.072	0.093	0.093	

Figure 152: K2P distance matrix for *Conus araneousus* using 16Sbar primers

7.11.15. Order Neogastropoda - Family Volutidae

The genus Harpulina, belonging to the family Volutidae, comprises four extant species. In this study, sequences from this genus were generated and submitted for the first time. As no reference sequences are currently available in public databases, comparative analyses could not be performed (Fig 151 -154). Therefore, the efficacy of the primers (both Rev641 and 16Sbar) cannot be determined.

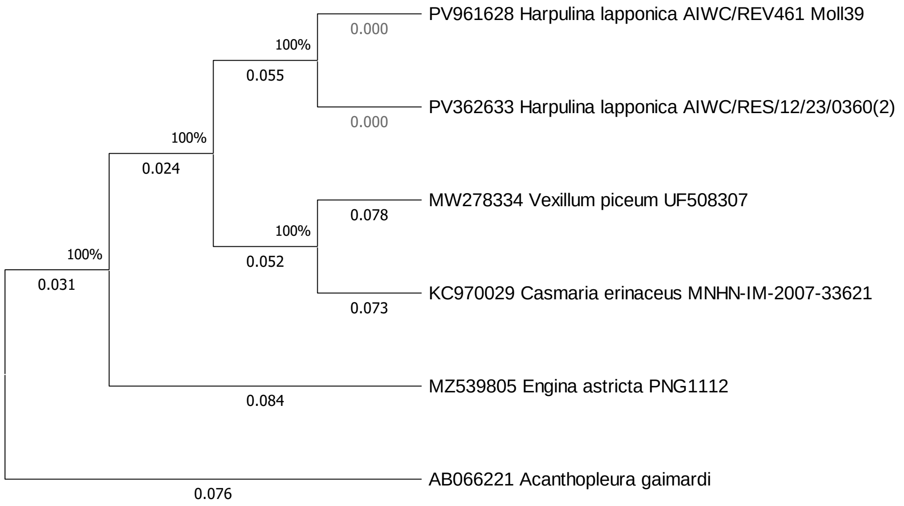
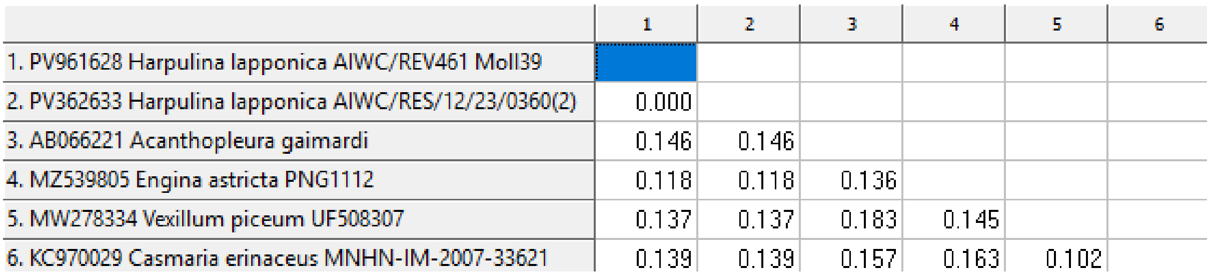


Figure 153: Dendrogram tree for *Harpulina lapponica* using Rev461 primers using T92+G



	1	2	3	4	5	6
1. PV961628 Harpulina lapponica AIWC/REV461 Moll39						
2. PV362633 Harpulina lapponica AIWC/RES/12/23/0360(2)	0.000					
3. AB066221 Acanthopleura gaimardi	0.146	0.146				
4. MZ539805 Engina astricta PNG1112	0.118	0.118	0.136			
5. MW278334 Vexillum piceum UF508307	0.137	0.137	0.183	0.145		
6. KC970029 Casmaria erinaceus MNHN-IM-2007-33621	0.139	0.139	0.157	0.163	0.102	

Figure 154: K2P distance matrix for *Harpulina lapponica* using Rev461 primers

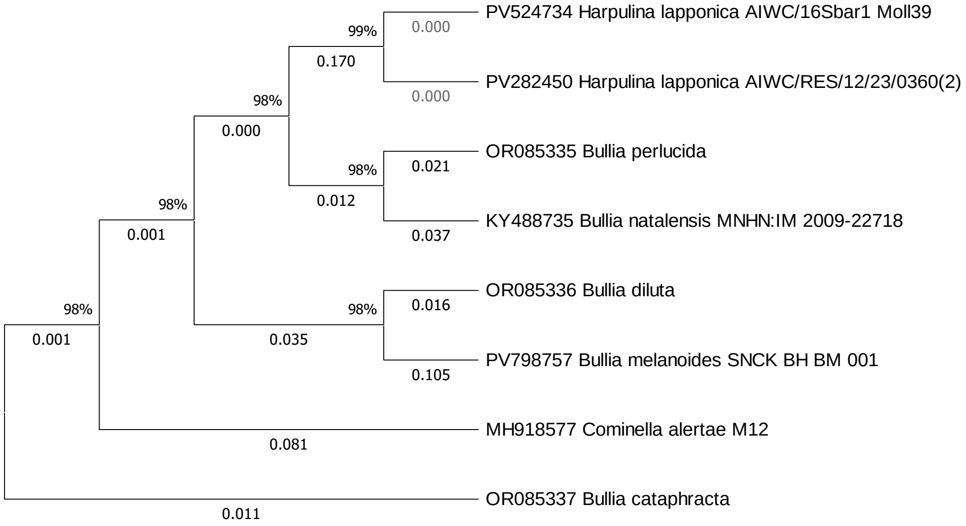
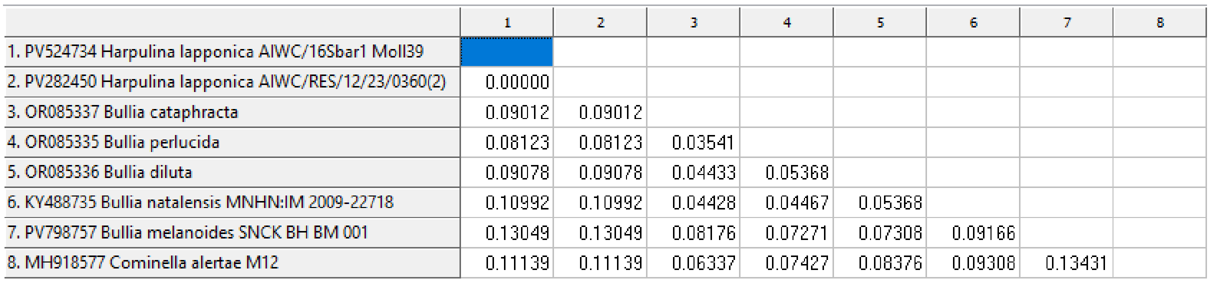


Figure 155: Dendrogram tree for *Harpulina lapponica* using 16Sbar primers using T92+G



	1	2	3	4	5	6	7	8
1. PV524734 Harpulina lapponica AIWC/16Sbar1 Moll39								
2. PV282450 Harpulina lapponica AIWC/RES/12/23/0360(2)	0.00000							
3. OR085337 Bullia cataphracta	0.09012	0.09012						
4. OR085335 Bullia perlucida	0.08123	0.08123	0.03541					
5. OR085336 Bullia diluta	0.09078	0.09078	0.04433	0.05368				
6. KY488735 Bullia natalensis MNHN:IM 2009-22718	0.10992	0.10992	0.04428	0.04467	0.05368			
7. PV798757 Bullia melanoides SNCK BH BM 001	0.13049	0.13049	0.08176	0.07271	0.07308	0.09166		
8. MH918577 Cominella alertae M12	0.11139	0.11139	0.06337	0.07427	0.08376	0.09308	0.13431	

Figure 156: K2P distance matrix for *Harpulina lapponica* using 16Sbar primers

7.11.16. Order Neogastropoda - Family Olividae

The genus *Agaronia*, belonging to the family Olividae, comprises 24 extant species. In this study, sequences from Agaronia gibbosa were generated using Rev461 primers and submitted for the first time. As no reference sequences are currently available in public databases, comparative analyses could not be performed. Therefore, the efficacy of Rev641 primers cannot be determined (Fig 155 & 156).

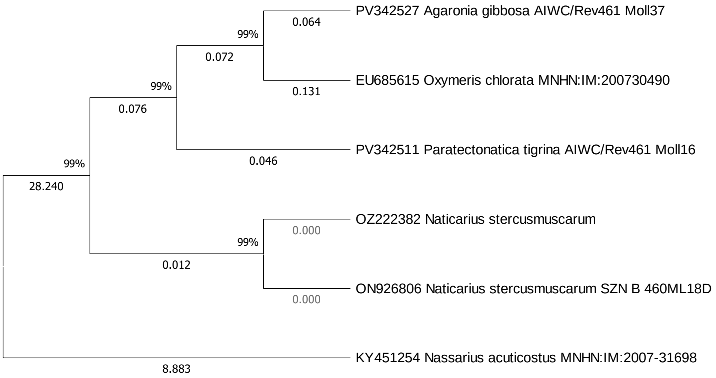
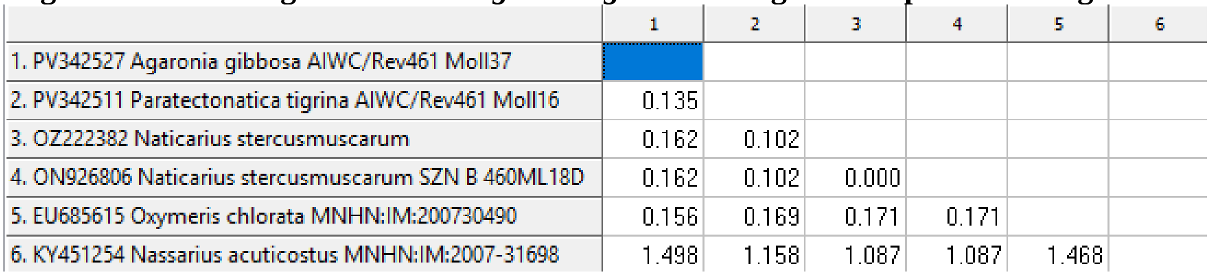


Figure 157: Dendrogram tree for *Agaronia gibbosa* using Rev461 primers using T92+G



	1	2	3	4	5	6
1. PV342527 Agaronia gibbosa AIWC/Rev461 Moll37						
2. PV342511 Paratectonatica tigrina AIWC/Rev461 Moll16	0.135					
3. OZ222382 Naticarius stercusmuscarum	0.162	0.102				
4. ON926806 Naticarius stercusmuscarum SZN B 460ML18D	0.162	0.102	0.000			
5. EU685615 Oxymeris chlorata MNHN:IM:200730490	0.156	0.169	0.171	0.171		
6. KY451254 Nassarius acuticostus MNHN:IM:2007-31698	1.498	1.158	1.087	1.087	1.468	

Figure 158 K2P distance matrix for *Agaronia gibbosa* using Rev461 primers

7.11.17. Order Neogastropoda - Family Harpidae

Integrated taxonomic studies on the family *Harpidae* remain poorly explored. Although the genus *Harpa* comprises 14 recognized species, reference sequences are currently available only for *Harpa harpa*. The species collected in the present study requires further identification. Both the Fwd464 and 16Sbar primer sets successfully amplified the target region, and the resulting sequences were identical to the available reference sequence of *Harpa*.sp (Fig 157 – 160). However, given the limited studies available on *Harpa* species, additional morphological and molecular analyses are necessary to confirm the species identity.

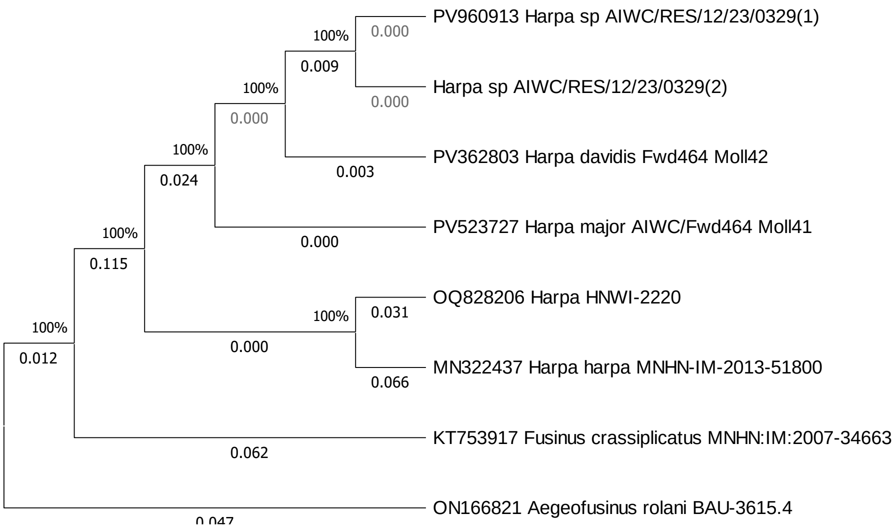
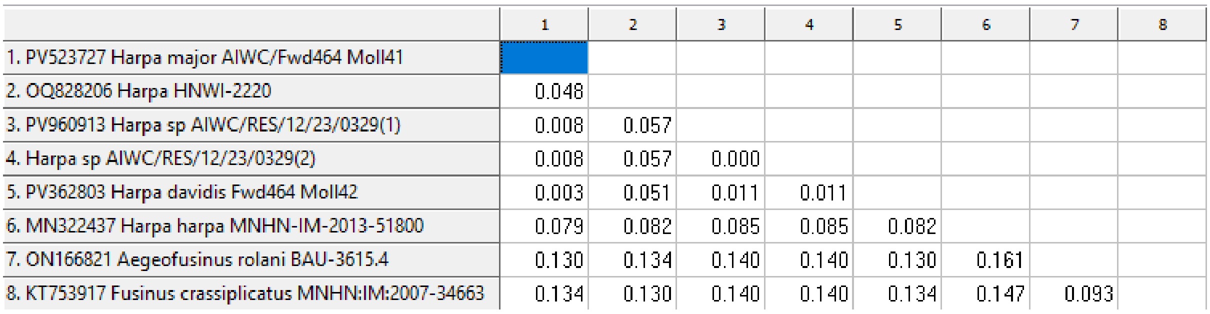


Figure 159: Dendrogram tree for *Harpa.sp1 &2* using Fwd464 primers using T92+G model



	1	2	3	4	5	6	7	8
1. PV523727 Harpa major AIWC/Fwd464 Moll41								
2. OQ828206 Harpa HNWI-2220	0.048							
3. PV960913 Harpa sp AIWC/RES/12/23/0329(1)	0.008	0.057						
4. Harpa sp AIWC/RES/12/23/0329(2)	0.008	0.057	0.000					
5. PV362803 Harpa davidis Fwd464 Moll42	0.003	0.051	0.011	0.011				
6. MN322437 Harpa harpa MNHN-IM-2013-51800	0.079	0.082	0.085	0.085	0.082			
7. ON166821 Aegeofusinus rolani BAU-3615.4	0.130	0.134	0.140	0.140	0.130	0.161		
8. KT753917 Fusinus crassiplicatus MNHN:IM:2007-34663	0.134	0.130	0.140	0.140	0.134	0.147	0.093	

Figure 160: K2P distance matrix for *Harpa.sp1 & 2* using Fwd464 primers

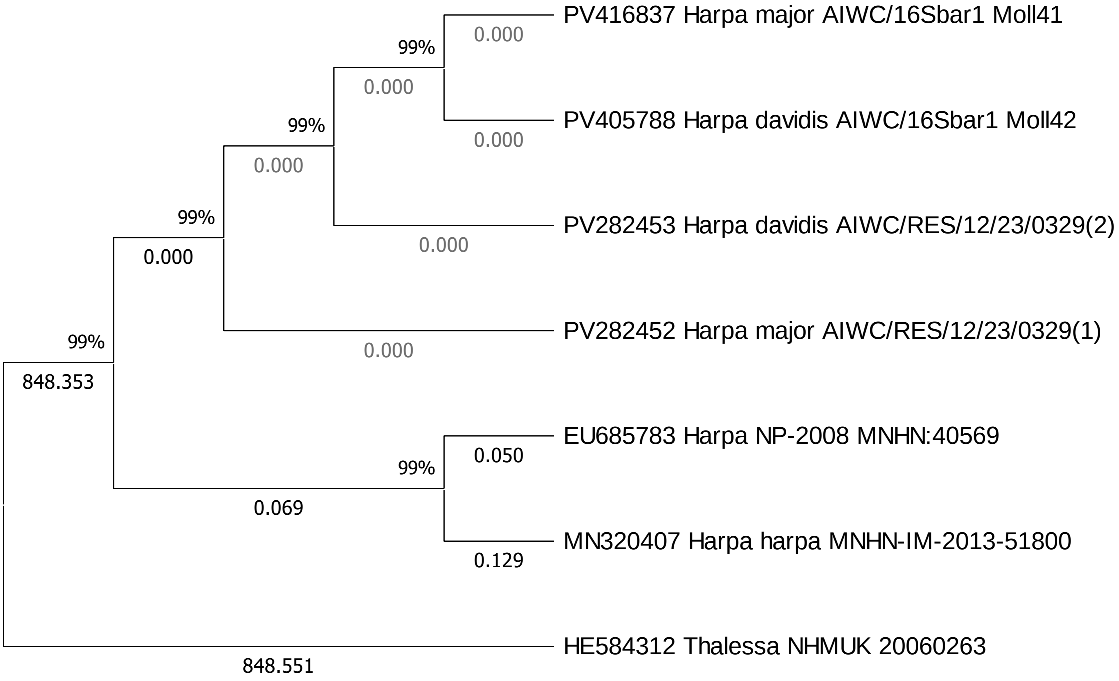
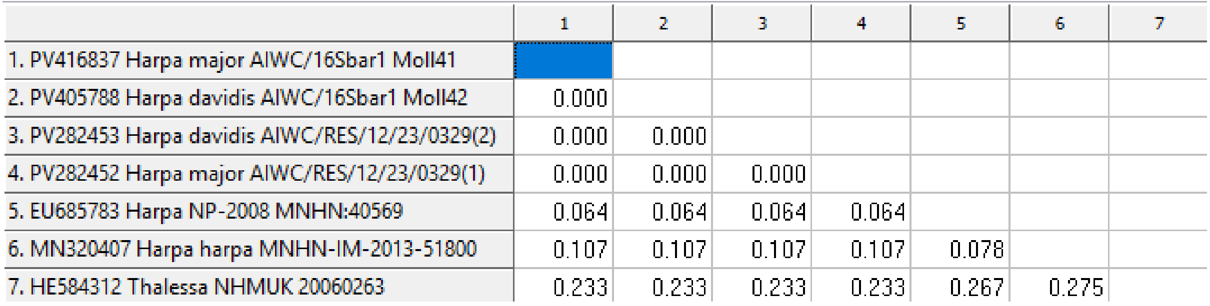


Figure 161: Dendrogram tree for *Harpa.sp1 &2* using 16Sbar primers using T92+G model



	1	2	3	4	5	6	7
1. PV416837 Harpa major AIWC/16Sbar1 Moll41							
2. PV405788 Harpa davidis AIWC/16Sbar1 Moll42	0.000						
3. PV282453 Harpa davidis AIWC/RES/12/23/0329(2)	0.000	0.000					
4. PV282452 Harpa major AIWC/RES/12/23/0329(1)	0.000	0.000	0.000				
5. EU685783 Harpa NP-2008 MNHN:40569	0.064	0.064	0.064	0.064			
6. MN320407 Harpa harpa MNHN-IM-2013-51800	0.107	0.107	0.107	0.107	0.078		
7. HE584312 Thalessa NHMUK 20060263	0.233	0.233	0.233	0.233	0.267	0.275	

Figure 162: K2P distance matrix for *Harpa.sp1 & 2* using 16Sbar primers

7.11.18. Order Nudibranchia - Family Glaucidae

The family *Glaucidae* includes pelagic nudibranchs known for their vivid coloration and they remain underrepresented in molecular and taxonomic studies (Churchill et al., 2013). *Glaucus atlanticus* is the only representative of the genus *Glaucus*, which exhibited a sufficient barcode gap to clearly distinct it from closely related species. In this study, both Fwd464 and Rev461 primer sets successfully amplified the target region, enabling reliable species-level identification (Fig 161-164).

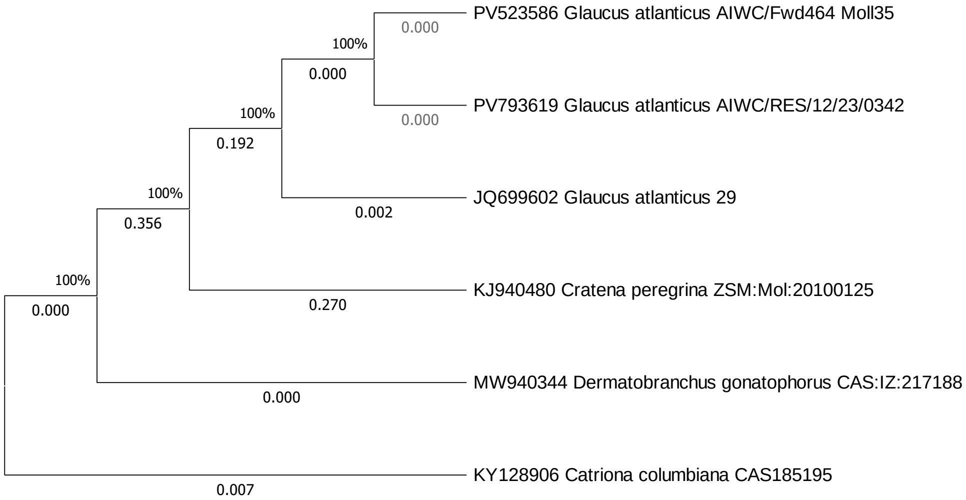
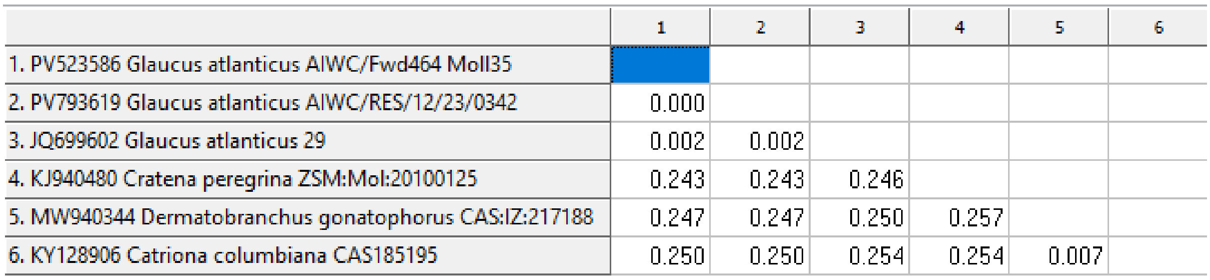


Figure 163: Dendrogram tree for *Glaucus atlanticus* using Fwd464 primers using HKY+G



	1	2	3	4	5	6
1. PV523586 Glaucus atlanticus AIWC/Fwd464 Moll35						
2. PV793619 Glaucus atlanticus AIWC/RES/12/23/0342	0.000					
3. JQ699602 Glaucus atlanticus 29	0.002	0.002				
4. KJ940480 Cratena peregrina ZSM:Mol:20100125	0.243	0.243	0.246			
5. MW940344 Dermatobranchus gonatophorus CAS:IZ:217188	0.247	0.247	0.250	0.257		
6. KY128906 Catriona columbiana CAS185195	0.250	0.250	0.254	0.254	0.007	

Figure 164: K2P distance matrix for *Glaucus atlanticus* using Fwd464 primers

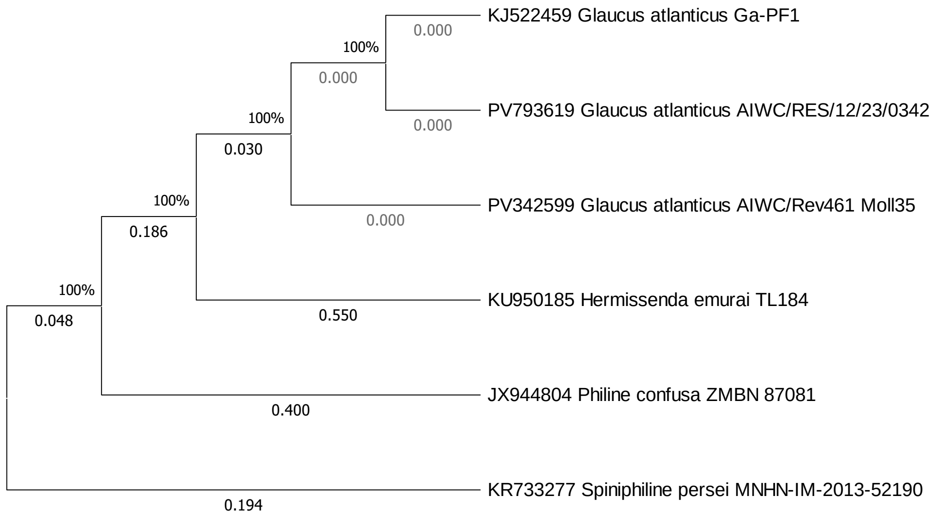
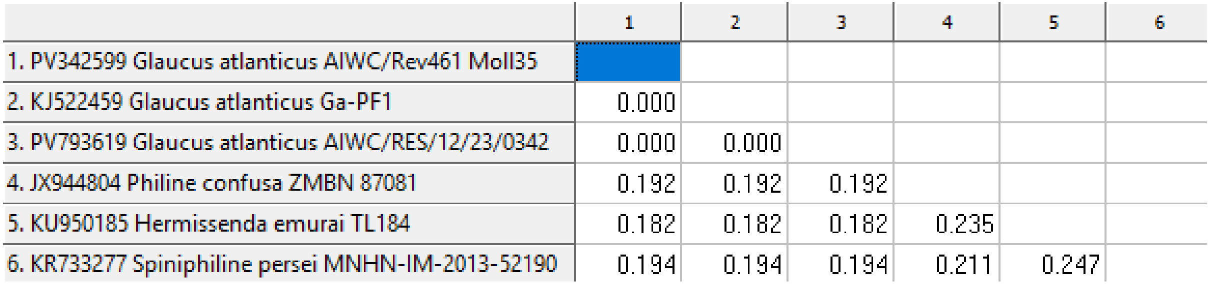


Figure 165: Dendrogram tree for *Glaucus atlanticus* using Rev461 primers using T92+G



	1	2	3	4	5	6
1. PV342599 Glaucus atlanticus AIWC/Rev461 Moll35						
2. KJ522459 Glaucus atlanticus Ga-PF1	0.000					
3. PV793619 Glaucus atlanticus AIWC/RES/12/23/0342	0.000	0.000				
4. JX944804 Philine confusa ZMBN 87081	0.192	0.192	0.192			
5. KU950185 Hermissenda emurai TL184	0.182	0.182	0.182	0.235		
6. KR733277 Spiniphiline persei MNHN-IM-2013-52190	0.194	0.194	0.194	0.211	0.247	

Figure 166: K2P distance matrix for *Glaucus atlanticus* using Rev461 primers

7.11.19. Order Patellogastropoda - Family Nacellidae

Limpets are very primitive taxonomical group and comprises three genera – Cellana H Adams, 1869, Naccula Iredale, 1924 and Nacella Schumacher, 1817 (WoRMS Editorial Board, 2025). The genus Cellana, comprising over 37 species, exhibits a remarkable distribution across the Indo-Pacific Ocean (Lindberg and Hickman 1986). To date, the presence of five species of Cellana - *C.rota*, *C.radiata*, *C.karachiensis*, *C.consilata and C.cylindrica* have been reported throughout the mainland India (Vakani et al 2020). All the three primers successfully amplified and showed clear distance between the species (Fig 165 -170).

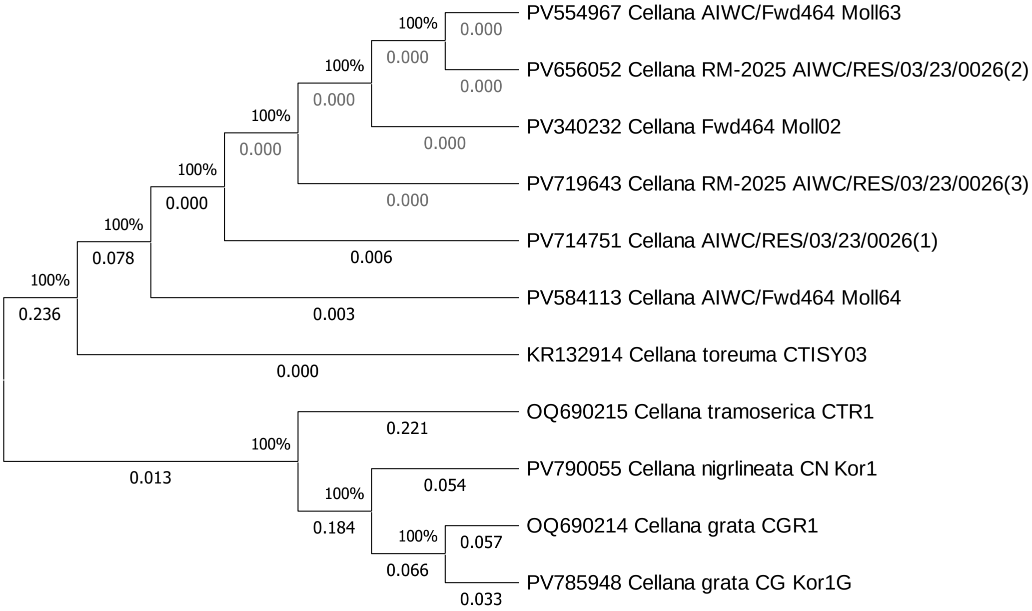
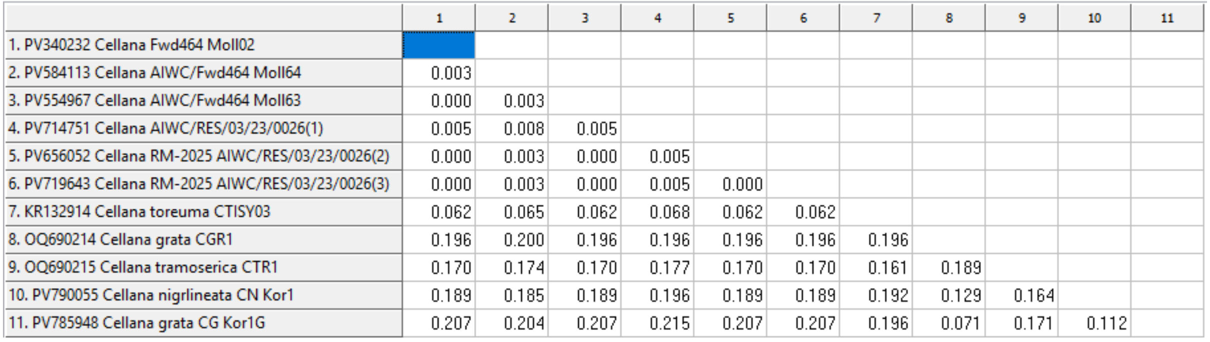


Figure 167: Dendrogram tree for *Cellana.sp* using Fwd464 primers using TN92+I model



	1	2	3	4	5	6	7	8	9	10	11
1. PV340232 Cellana Fwd464 Moll02											
2. PV584113 Cellana AIWC/Fwd464 Moll64	0.003										
3. PV554967 Cellana AIWC/Fwd464 Moll63	0.000	0.003									
4. PV714751 Cellana AIWC/RES/03/23/0026(1)	0.005	0.008	0.005								
5. PV656052 Cellana RM-2025 AIWC/RES/03/23/0026(2)	0.000	0.003	0.000	0.005							
6. PV719643 Cellana RM-2025 AIWC/RES/03/23/0026(3)	0.000	0.003	0.000	0.005	0.000						
7. KR132914 Cellana toreuma CTISY03	0.062	0.065	0.062	0.068	0.062	0.062					
8. OQ690214 Cellana grata CGR1	0.196	0.200	0.196	0.196	0.196	0.196	0.196				
9. OQ690215 Cellana tramoserica CTR1	0.170	0.174	0.170	0.177	0.170	0.170	0.161	0.189			
10. PV790055 Cellana nigrlineata CN Kor1	0.189	0.185	0.189	0.196	0.189	0.189	0.192	0.129	0.164		
11. PV785948 Cellana grata CG Kor1G	0.207	0.204	0.207	0.215	0.207	0.207	0.196	0.071	0.171	0.112	

Figure 168: K2P distance matrix for *Cellana.sp* using Fwd464 primers

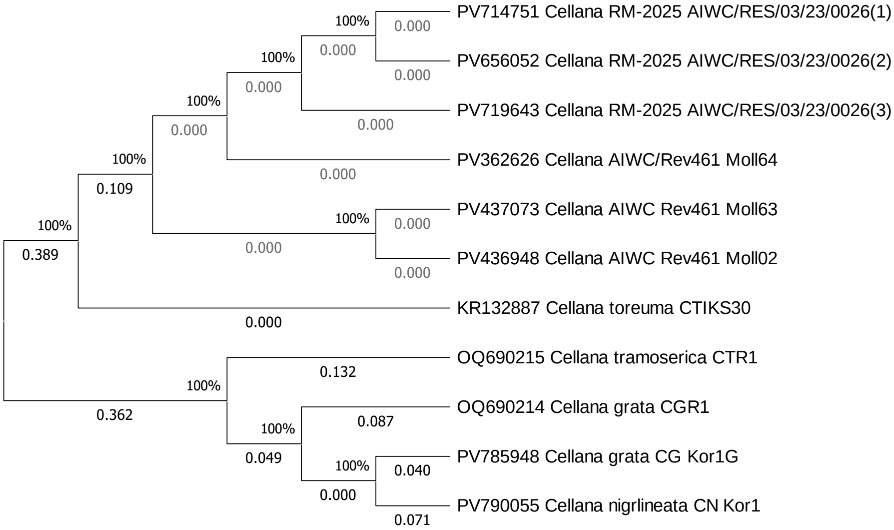
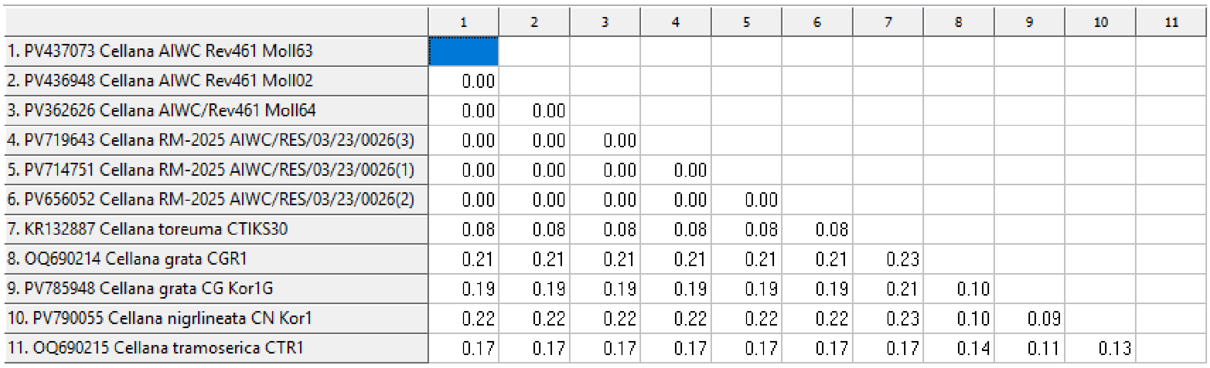


Figure 169: Dendrogram tree for *Cellana.sp* using Rev461 primers using TN92+G model



	1	2	3	4	5	6	7	8	9	10	11
1. PV437073 Cellana AIWC Rev461 Moll63											
2. PV436948 Cellana AIWC Rev461 Moll02	0.00										
3. PV362626 Cellana AIWC/Rev461 Moll64	0.00	0.00									
4. PV719643 Cellana RM-2025 AIWC/RES/03/23/0026(3)	0.00	0.00	0.00								
5. PV714751 Cellana RM-2025 AIWC/RES/03/23/0026(1)	0.00	0.00	0.00	0.00							
6. PV656052 Cellana RM-2025 AIWC/RES/03/23/0026(2)	0.00	0.00	0.00	0.00	0.00						
7. KR132887 Cellana toreuma CTIKS30	0.08	0.08	0.08	0.08	0.08	0.08					
8. OQ690214 Cellana grata CGR1	0.21	0.21	0.21	0.21	0.21	0.21	0.23				
9. PV785948 Cellana grata CG Kor1G	0.19	0.19	0.19	0.19	0.19	0.19	0.21	0.10			
10. PV790055 Cellana nigrlineata CN Kor1	0.22	0.22	0.22	0.22	0.22	0.22	0.23	0.10	0.09		
11. OQ690215 Cellana tramoserica CTR1	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.14	0.11	0.13	

Figure 170: K2P distance matrix for *Cellana.sp* using Rev461 primers

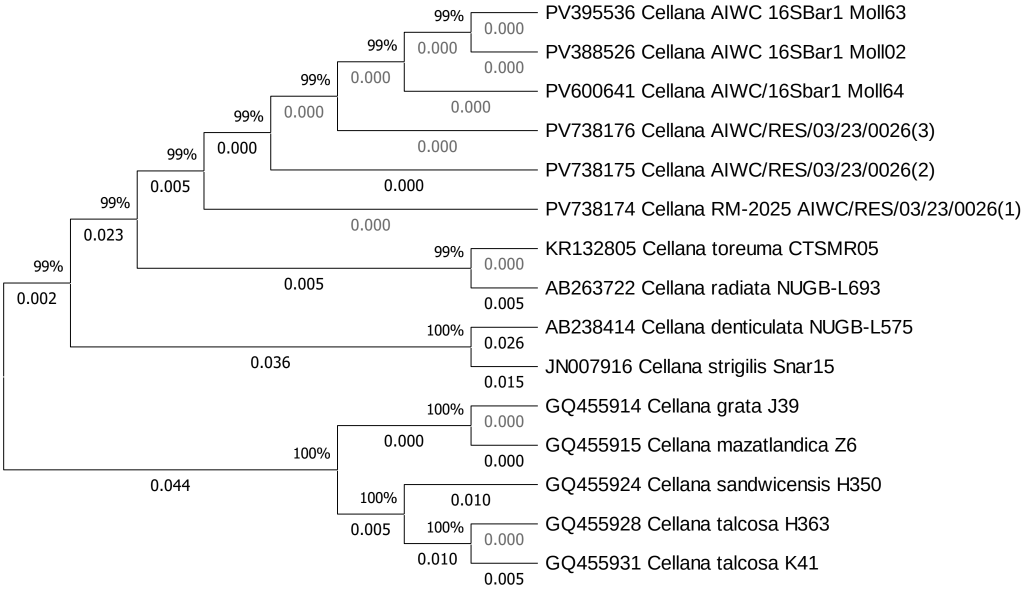
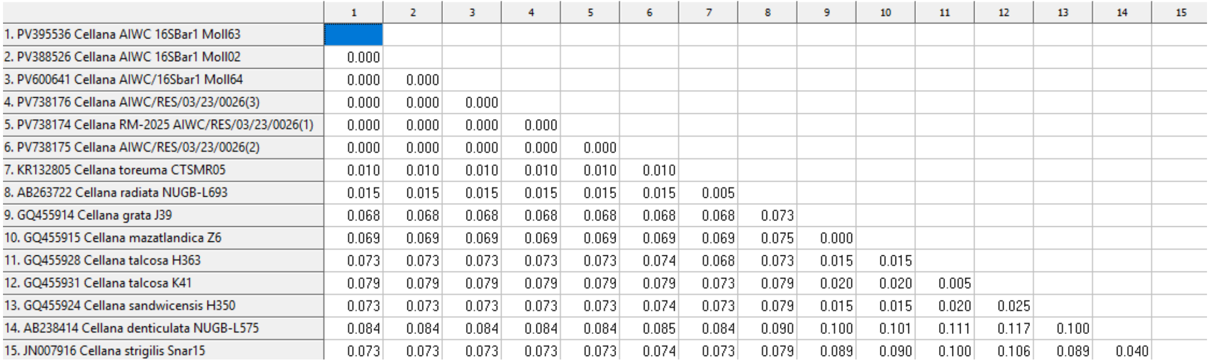


Figure 171: Dendrogram tree for *Cellana.sp* using 16Sbar primers using TN92+G model



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. PV395536 Cellana AIWC 16SBar1 Moll63															
2. PV388526 Cellana AIWC 16SBar1 Moll02	0.000														
3. PV600641 Cellana AIWC/16Sbar1 Moll64	0.000	0.000													
4. PV738176 Cellana AIWC/RES/03/23/0026(3)	0.000	0.000	0.000												
5. PV738174 Cellana RM-2025 AIWC/RES/03/23/0026(1)	0.000	0.000	0.000	0.000											
6. PV738175 Cellana AIWC/RES/03/23/0026(2)	0.000	0.000	0.000	0.000	0.000										
7. KR132805 Cellana toreuma CTSMR05	0.010	0.010	0.010	0.010	0.010	0.010									
8. AB263722 Cellana radiata NUGB-L693	0.015	0.015	0.015	0.015	0.015	0.015	0.005								
9. GQ455914 Cellana grata J39	0.068	0.068	0.068	0.068	0.068	0.068	0.068	0.073							
10. GQ455915 Cellana mazatlandica Z6	0.069	0.069	0.069	0.069	0.069	0.069	0.069	0.075	0.000						
11. GQ455928 Cellana talcosa H363	0.073	0.073	0.073	0.073	0.073	0.074	0.068	0.073	0.015	0.015					
12. GQ455931 Cellana talcosa K41	0.079	0.079	0.079	0.079	0.079	0.079	0.073	0.079	0.020	0.020	0.005				
13. GQ455924 Cellana sandwicensis H350	0.073	0.073	0.073	0.073	0.073	0.074	0.073	0.079	0.015	0.015	0.020	0.025			
14. AB238414 Cellana denticulata NUGB-L575	0.084	0.084	0.084	0.084	0.084	0.085	0.084	0.090	0.100	0.101	0.111	0.117	0.100		
15. JN007916 Cellana strigilis Snar15	0.073	0.073	0.073	0.073	0.073	0.074	0.073	0.079	0.089	0.090	0.100	0.106	0.089	0.040	

Figure 172: K2P distance matrix for *Cellana.sp* using 16Sbar primers

7.11.20. Order Octopoda – Family Octopodidae

Integrated taxonomic studies on *Amphioctopus* are limited from India. Norman (2000) have acknowledged the presence of species complex in the Genus *Amphioctopus*. For the study, *Amphioctopus aegina* was used for validation. All the three primer sets successfully validated the species, however Fwd464 primers showed better resolution than other two. *A.kagoshimensis* and *A.fangsiao* got clearly separated in two different clades in the previous study by Vargheese.et. al (2022) but in the current study they were resolved as single clade. Therefore, the source of sequence needs to be re-checked for further confirmation (Fig 171-176).

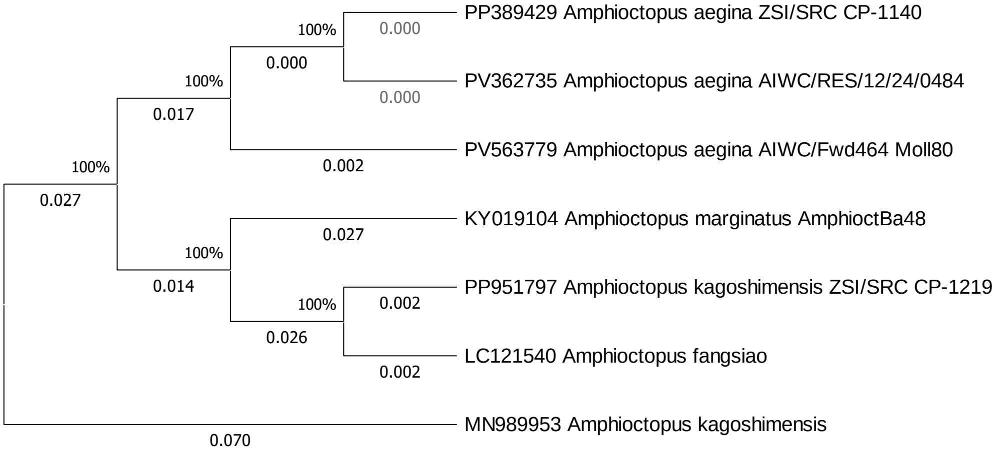
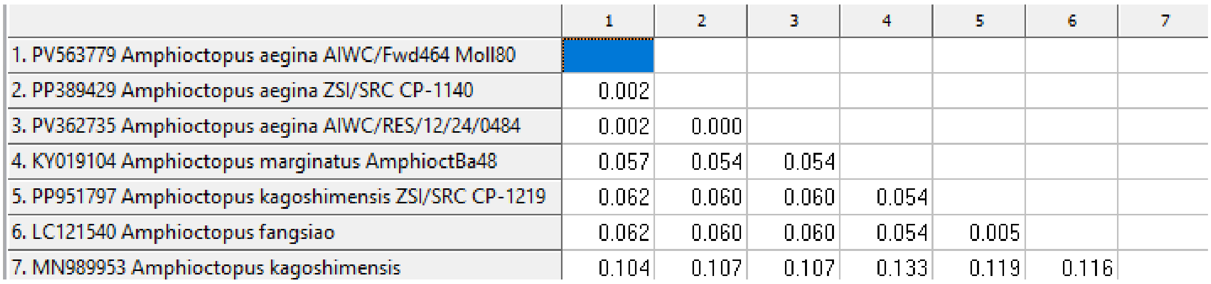


Figure 173: Dendrogram tree for *Amphioctopus aegina* using Fwd464 primers using T92 modelT92



	1	2	3	4	5	6	7
1. PV563779 Amphioctopus aegina AIWC/Fwd464 Moll80							
2. PP389429 Amphioctopus aegina ZSI/SRC CP-1140	0.002						
3. PV362735 Amphioctopus aegina AIWC/RES/12/24/0484	0.002	0.000					
4. KY019104 Amphioctopus marginatus AmphioctBa48	0.057	0.054	0.054				
5. PP951797 Amphioctopus kagoshimensis ZSI/SRC CP-1219	0.062	0.060	0.060	0.054			
6. LC121540 Amphioctopus fangsiao	0.062	0.060	0.060	0.054	0.005		
7. MN989953 Amphioctopus kagoshimensis	0.104	0.107	0.107	0.133	0.119	0.116	

Figure 174: K2P distance matrix for *Amphioctopus aegina* using Fwd464 primers

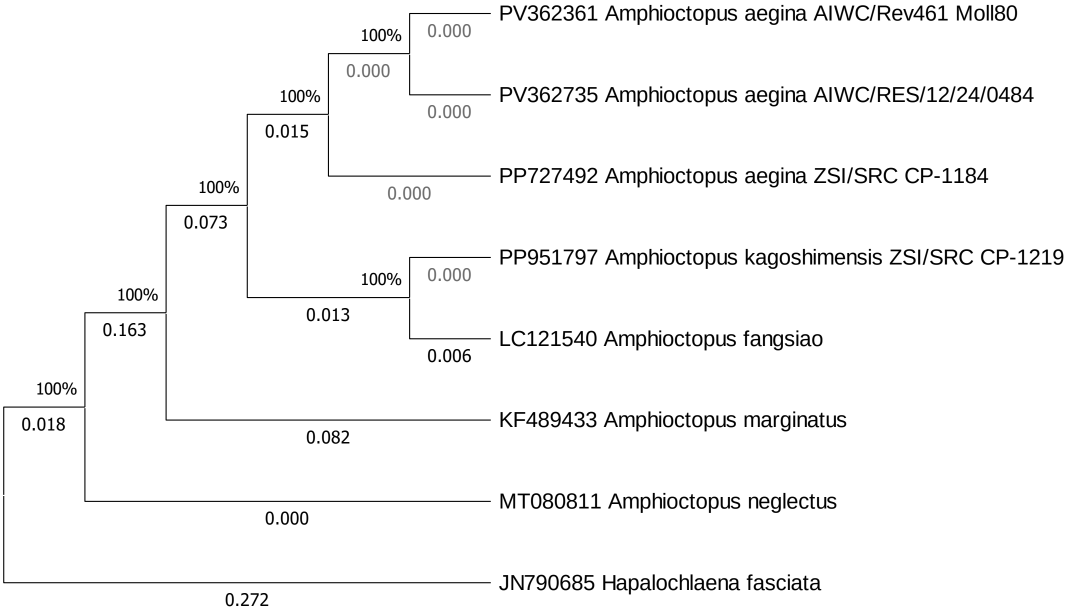
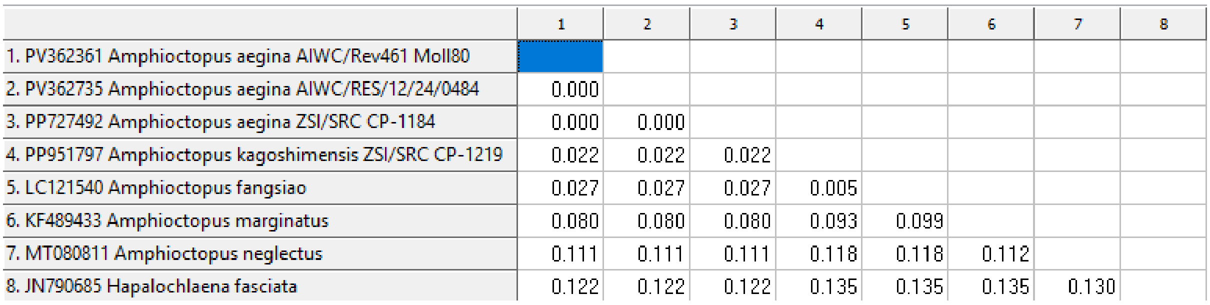


Figure 175: Dendrogram tree for *Amphioctopus aegina* using Rev461primers using T92+G model



	1	2	3	4	5	6	7	8
1. PV362361 Amphioctopus aegina AIWC/Rev461 Moll80								
2. PV362735 Amphioctopus aegina AIWC/RES/12/24/0484	0.000							
3. PP727492 Amphioctopus aegina ZSI/SRC CP-1184	0.000	0.000						
4. PP951797 Amphioctopus kagoshimensis ZSI/SRC CP-1219	0.022	0.022	0.022					
5. LC121540 Amphioctopus fangsiao	0.027	0.027	0.027	0.005				
6. KF489433 Amphioctopus marginatus	0.080	0.080	0.080	0.093	0.099			
7. MT080811 Amphioctopus neglectus	0.111	0.111	0.111	0.118	0.118	0.112		
8. JN790685 Hapalochlaena fasciata	0.122	0.122	0.122	0.135	0.135	0.135	0.130	

Figure 176: K2P distance matrix for *Amphioctopus aegina* using Rev461 primers

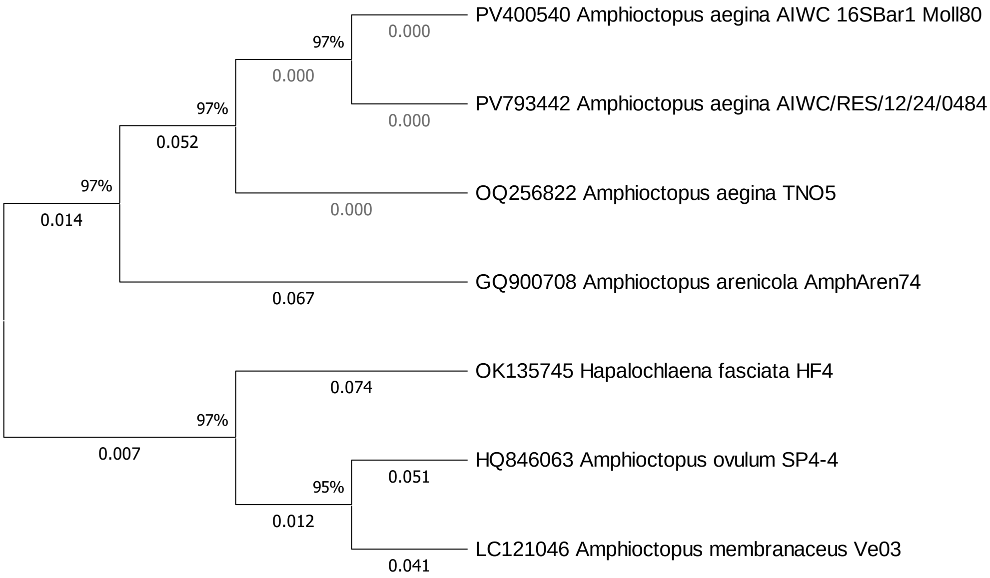
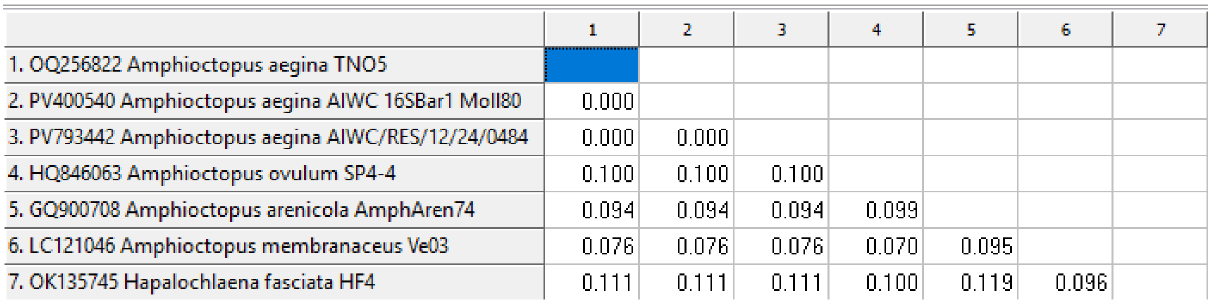


Figure 177: Dendrogram tree for *Amphioctopus aegina* using 16Sbar primers using T92+G model



	1	2	3	4	5	6	7
1. OQ256822 Amphioctopus aegina TNO5							
2. PV400540 Amphioctopus aegina AIWC 16SBar1 Moll80	0.000						
3. PV793442 Amphioctopus aegina AIWC/RES/12/24/0484	0.000	0.000					
4. HQ846063 Amphioctopus ovulum SP4-4	0.100	0.100	0.100				
5. GQ900708 Amphioctopus arenicola AmphAren74	0.094	0.094	0.094	0.099			
6. LC121046 Amphioctopus membranaceus Ve03	0.076	0.076	0.076	0.070	0.095		
7. OK135745 Hapalochlaena fasciata HF4	0.111	0.111	0.111	0.100	0.119	0.096	

Figure 178: K2P distance matrix for *Amphioctopus aegina* using 16Sbar primers

7.11.21. Order Sepiida - Family Sepiidae

The genus *Sepiella* comprises seven species of cuttlefishes globally and are commercially important marine bio-resource. In Indian waters, *Sepia pharaonis*, *Sepia aculeata*, *Sepia elliptica*, *Sepia prashadi*, *Sepia brevimana*, and *Sepiella inermis* are among the commonly landed cuttlefish species (Meiyappan and Mohamed, 2003; Silas et al., 1982). *Sepiella inermis* is widely distributed along both the east and west coasts of India and is predominantly harvested using trawl nets (Mahadwala et al., 2023). At present, *S. inermis* is listed as Data Deficient on the IUCN Red List (Barratt and Allcock, 2012). In the current study, all three primer sets successfully amplified and identified *S. inermis* (Fig.177 -182). However, the genetic distance between other two *Sepiella* - *S. japonica* and *S. maindroni* were found to be low, limiting the ability to distinguish between these two species based on the current primers.

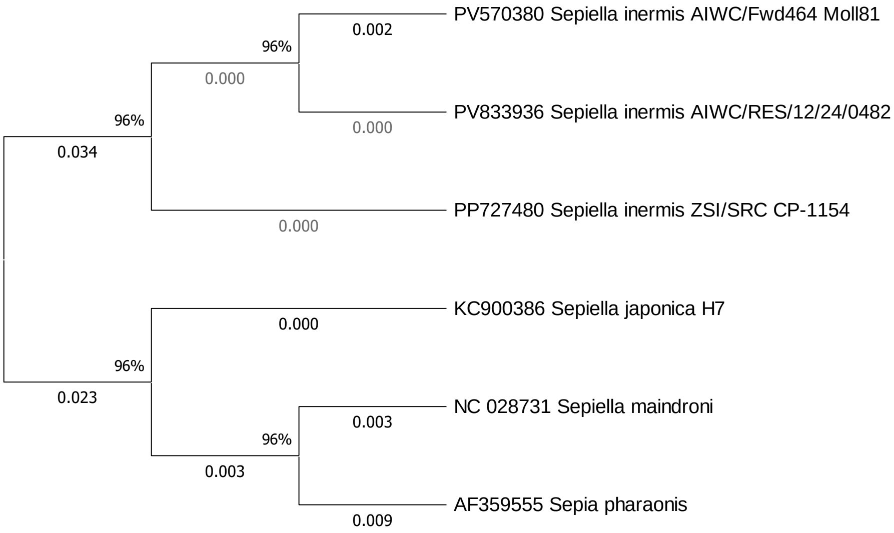
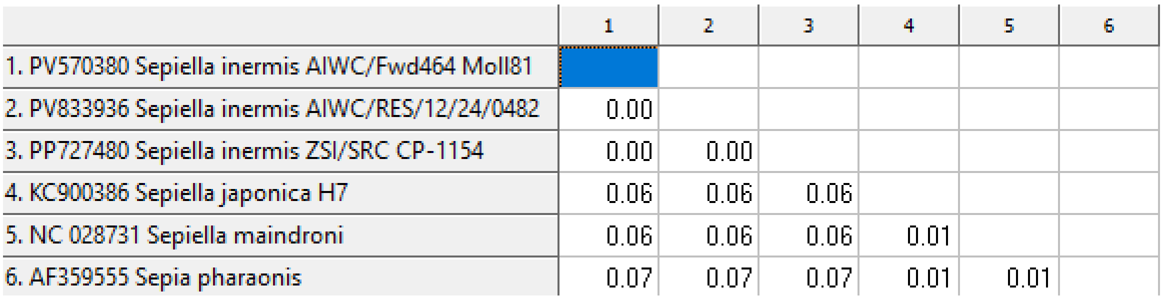


Figure 179 Dendrogram tree for *Sepiella inermis* using Fwd464 primers using HKY model



	1	2	3	4	5	6
1. PV570380 Sepiella inermis AIWC/Fwd464 Moll81						
2. PV833936 Sepiella inermis AIWC/RES/12/24/0482	0.00					
3. PP727480 Sepiella inermis ZSI/SRC CP-1154	0.00	0.00				
4. KC900386 Sepiella japonica H7	0.06	0.06	0.06			
5. NC 028731 Sepiella maindroni	0.06	0.06	0.06	0.01		
6. AF359555 Sepia pharaonis	0.07	0.07	0.07	0.01	0.01	

Figure 180: K2P distance matrix for *Sepiella inermis* using Fwd464 primers

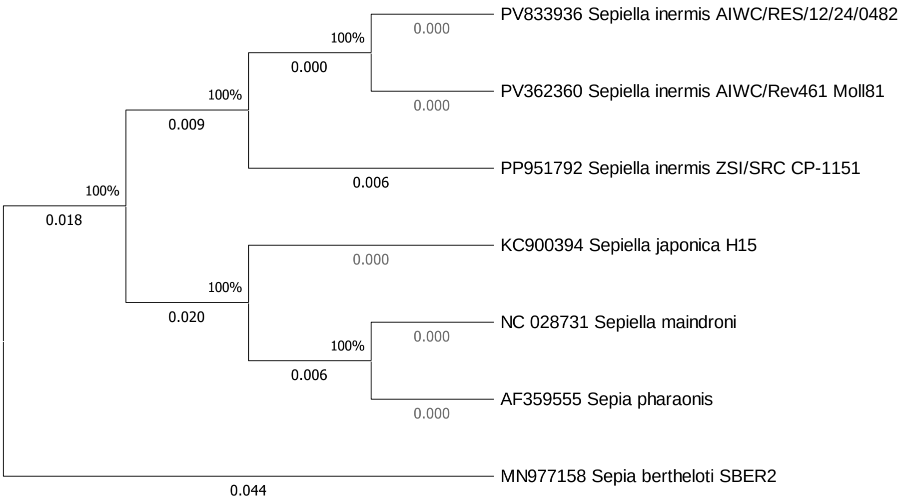
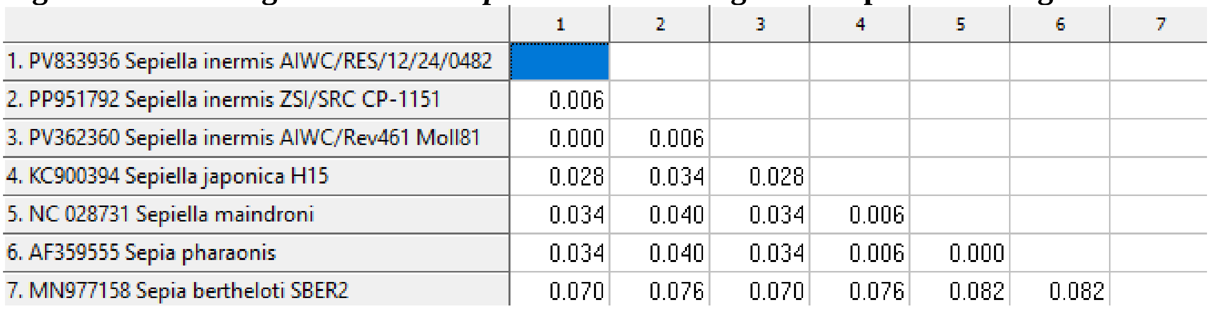


Figure 181 Dendrogram tree for *Sepiella inermis* using Rev461 primers using T92 model



	1	2	3	4	5	6	7
1. PV833936 Sepiella inermis AIWC/RES/12/24/0482							
2. PP951792 Sepiella inermis ZSI/SRC CP-1151	0.006						
3. PV362360 Sepiella inermis AIWC/Rev461 Moll81	0.000	0.006					
4. KC900394 Sepiella japonica H15	0.028	0.034	0.028				
5. NC 028731 Sepiella maindroni	0.034	0.040	0.034	0.006			
6. AF359555 Sepia pharaonis	0.034	0.040	0.034	0.006	0.000		
7. MN977158 Sepia bertheloti SBER2	0.070	0.076	0.070	0.076	0.082	0.082	

Figure 182: K2P distance matrix for *Sepiella inermis* using Rev461 primers

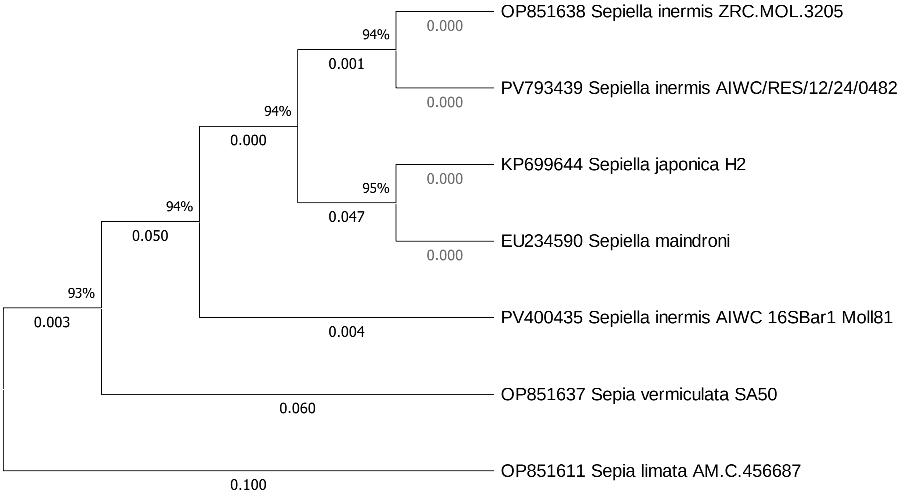
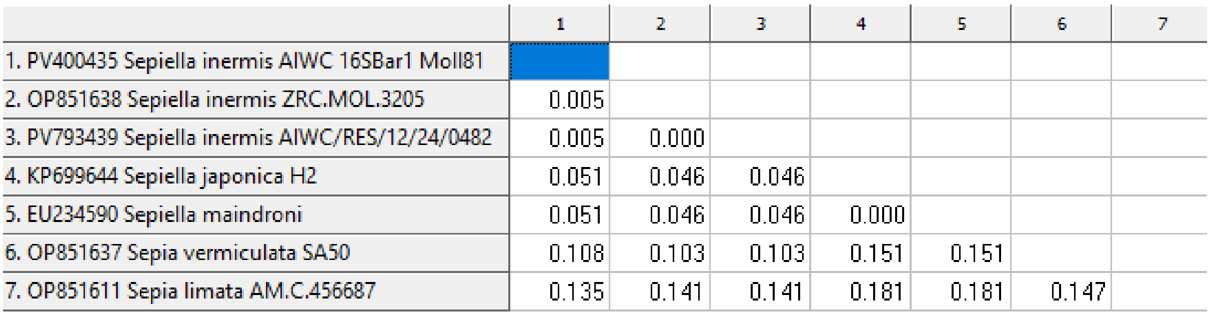


Figure 183: Dendrogram tree for *Sepiella inermis* using 16Sbar primers using T92 model



	1	2	3	4	5	6	7
1. PV400435 Sepiella inermis AIWC 16SBar1 Moll81							
2. OP851638 Sepiella inermis ZRC.MOL.3205	0.005						
3. PV793439 Sepiella inermis AIWC/RES/12/24/0482	0.005	0.000					
4. KP699644 Sepiella japonica H2	0.051	0.046	0.046				
5. EU234590 Sepiella maindroni	0.051	0.046	0.046	0.000			
6. OP851637 Sepia vermiculata SA50	0.108	0.103	0.103	0.151	0.151		
7. OP851611 Sepia limata AM.C.456687	0.135	0.141	0.141	0.181	0.181	0.147	

Figure 184: K2P distance matrix for *Sepiella inermis* using 16Sbar primers

7.11.22. Order Myopsida - Family Loliginidae

The genus *Uroteuthis* includes several species of neritic squids and is widely distributed in tropical and subtropical waters. This genus constitutes of many economically important species for cephalopod fishery. In Indian waters, *Uroteuthis duvaucelii*, *U. bengalensis*, *U.singhalensis* , and *Uroteuthis sibogae* are commonly reported species (Ramakrishna & Dey, 2010; Dey & Ramakrishna, 2007; Jena & Gopalakrishnan, 2012). Among these, *U. duvaucelii* is the most abundant and are extensively harvested along both the east and west coasts, primarily through trawl and hook-and-line fisheries. The species is a major contributor to cephalopod landings in India (Jena & Gopalakrishnan, 2012). In the present study, all three primer sets successfully amplified and identified *U. duvaucelii* (Fig.183 - 188). However, the genetic distance between *U. edulis* and *U. singhalensis* was observed to be low, making it difficult to reliably distinguish between the two species using the current primer sets.

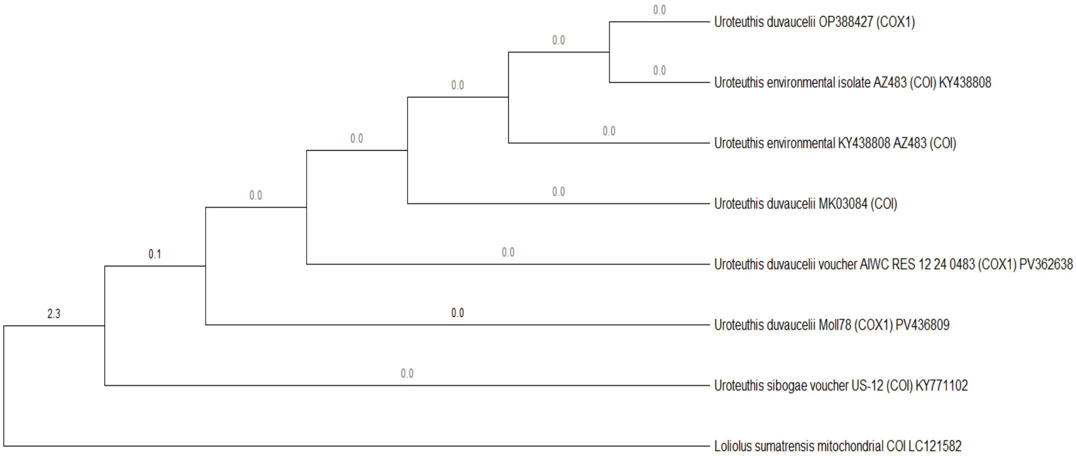
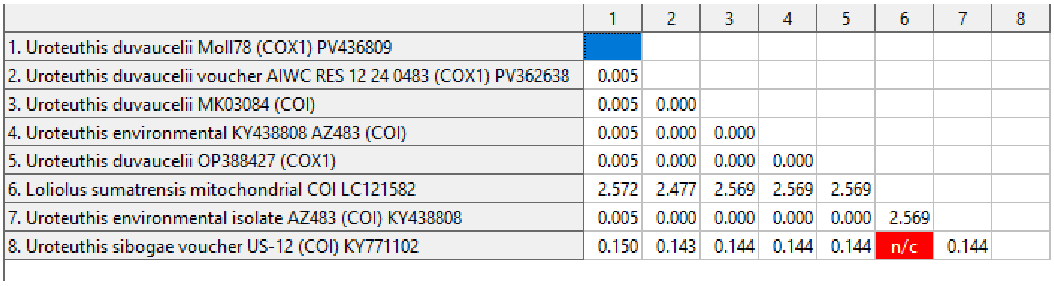


Figure 185: Dendrogram tree for *Uroteuthis duvaucelii* using Fwd464 primers using T92 model



	1	2	3	4	5	6	7	8
1. Uroteuthis duvaucelii Moll78 (COX1) PV436809								
2. Uroteuthis duvaucelii voucher AIWC RES 12 24 0483 (COX1) PV362638	0.005							
3. Uroteuthis duvaucelii MK03084 (COI)	0.005	0.000						
4. Uroteuthis environmental KY438808 AZ483 (COI)	0.005	0.000	0.000					
5. Uroteuthis duvaucelii OP388427 (COX1)	0.005	0.000	0.000	0.000				
6. Loliolus sumatrensis mitochondrial COI LC121582	2.572	2.477	2.569	2.569	2.569			
7. Uroteuthis environmental isolate AZ483 (COI) KY438808	0.005	0.000	0.000	0.000	0.000	2.569		
8. Uroteuthis sibogae voucher US-12 (COI) KY771102	0.150	0.143	0.144	0.144	0.144	n/c	0.144	

Figure 186: K2P distance matrix for *Uroteuthis duvaucelii* using Fwd464 primers

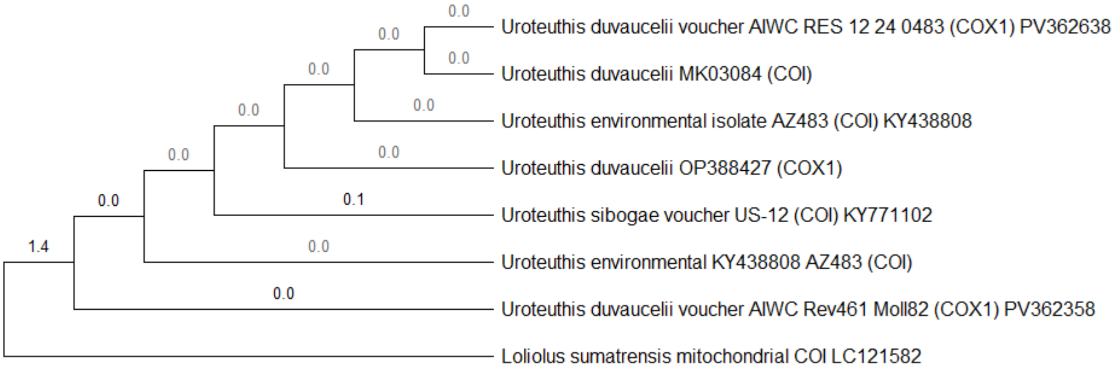
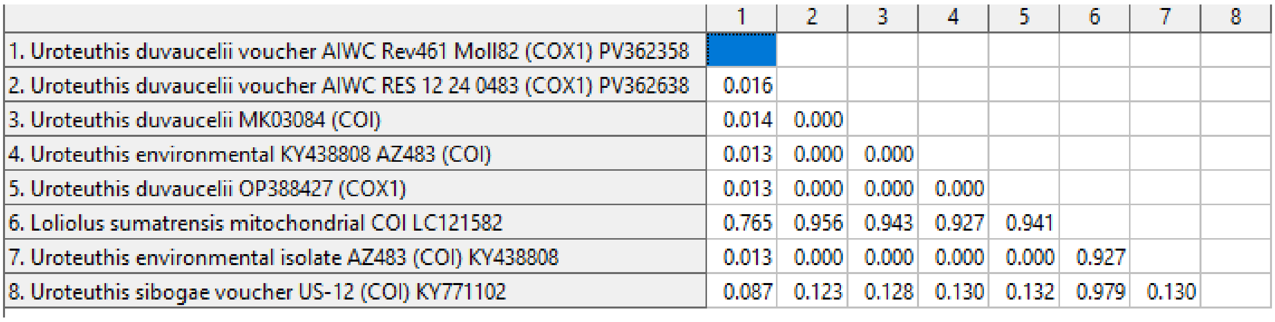


Figure 187: Dendrogram tree for *Uroteuthis duvaucelii* using Rev461 primers using T92+I model



	1	2	3	4	5	6	7	8
1. Uroteuthis duvaucelii voucher AIWC Rev461 Moll82 (COX1) PV362358								
2. Uroteuthis duvaucelii voucher AIWC RES 12 24 0483 (COX1) PV362638	0.016							
3. Uroteuthis duvaucelii MK03084 (COI)	0.014	0.000						
4. Uroteuthis environmental KY438808 AZ483 (COI)	0.013	0.000	0.000					
5. Uroteuthis duvaucelii OP388427 (COX1)	0.013	0.000	0.000	0.000				
6. Loliolus sumatrensis mitochondrial COI LC121582	0.765	0.956	0.943	0.927	0.941			
7. Uroteuthis environmental isolate AZ483 (COI) KY438808	0.013	0.000	0.000	0.000	0.000	0.927		
8. Uroteuthis sibogae voucher US-12 (COI) KY771102	0.087	0.123	0.128	0.130	0.132	0.979	0.130	

Figure 188: K2P distance matrix for *Uroteuthis duvaucelii* using Rev461 primers

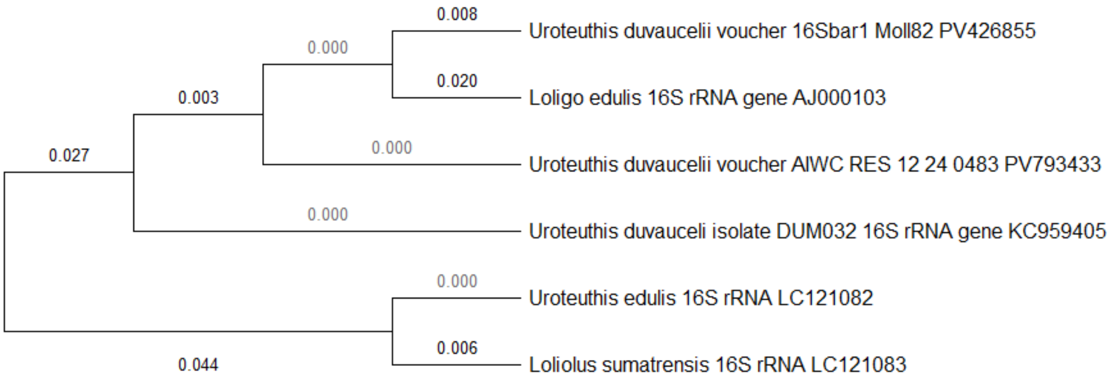
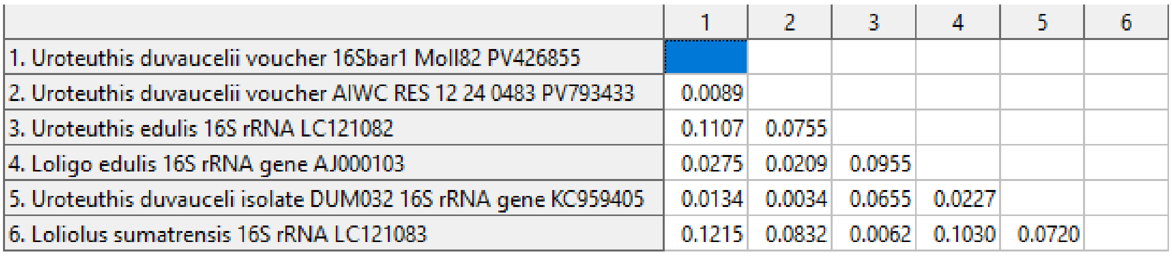


Figure 189: Dendrogram tree for *Uroteuthis duvaucelii* using 16Sbar primers using T92+I model



	1	2	3	4	5	6
1. Uroteuthis duvaucelii voucher 16Sbar1 Moll82 PV426855						
2. Uroteuthis duvaucelii voucher AIWC RES 12 24 0483 PV793433	0.0089					
3. Uroteuthis edulis 16S rRNA LC121082	0.1107	0.0755				
4. Loligo edulis 16S rRNA gene AJ000103	0.0275	0.0209	0.0955			
5. Uroteuthis duvauceli isolate DUM032 16S rRNA gene KC959405	0.0134	0.0034	0.0655	0.0227		
6. Loliolus sumatrensis 16S rRNA LC121083	0.1215	0.0832	0.0062	0.1030	0.0720	

Figure 190: K2P distance matrix for *Uroteuthis duvaucelii* using 16Sbar primers

7.11.23. Order Venerida - Family Veneridae

Protapes gallus is one of the common Bivalvia in the coast of Tamil Nadu. Even though all the three primer sets got amplified, the inter-species gap between was not sufficient enough to distinguish between the species (Fig 189 – 194).

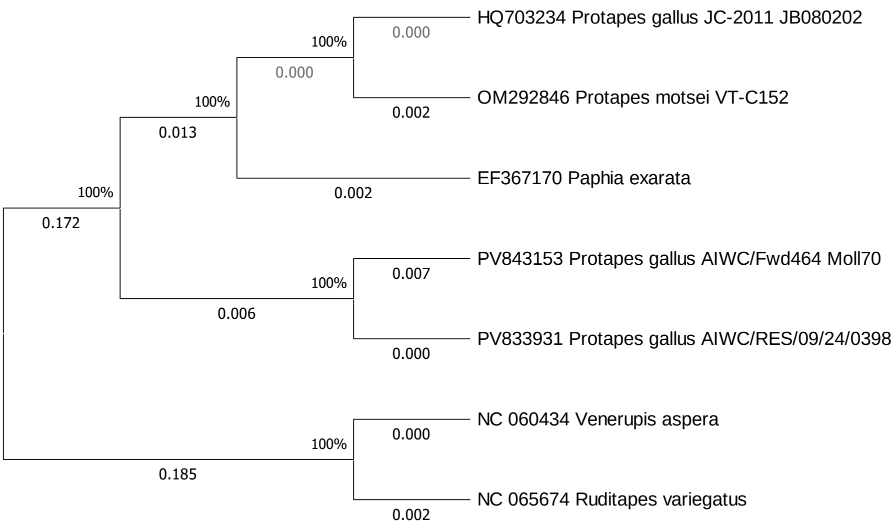
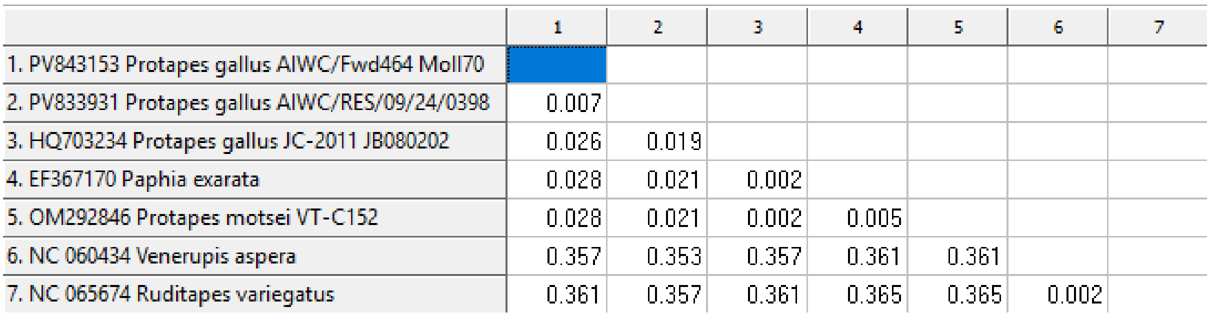


Figure 191 Dendrogram tree for *Protapes gallus* using Fwd464 primers using HKY model



	1	2	3	4	5	6	7
1. PV843153 Protapes gallus AIWC/Fwd464 Moll70							
2. PV833931 Protapes gallus AIWC/RES/09/24/0398	0.007						
3. HQ703234 Protapes gallus JC-2011 JB080202	0.026	0.019					
4. EF367170 Paphia exarata	0.028	0.021	0.002				
5. OM292846 Protapes motsei VT-C152	0.028	0.021	0.002	0.005			
6. NC 060434 Venerupis aspera	0.357	0.353	0.357	0.361	0.361		
7. NC 065674 Ruditapes variegatus	0.361	0.357	0.361	0.365	0.365	0.002	

Figure 192: K2P distance matrix for *Protapes gallus* using Fwd464 primers

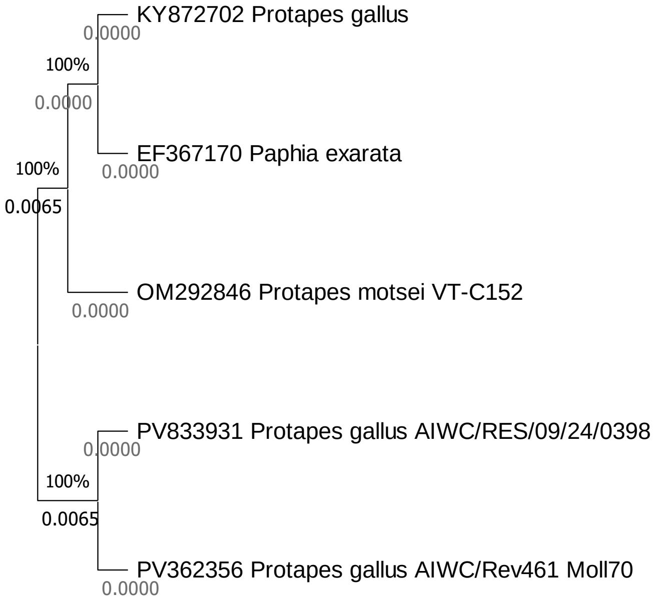
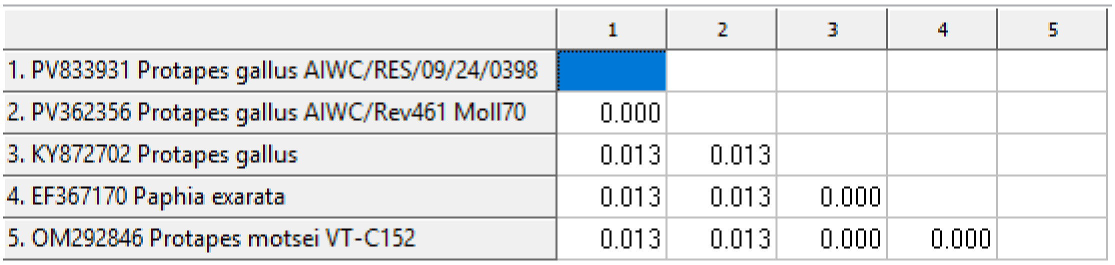


Figure 193: Dendrogram tree for *Protapes gallus* using Rev464 primers using HKY model



	1	2	3	4	5
1. PV833931 Protapes gallus AIWC/RES/09/24/0398					
2. PV362356 Protapes gallus AIWC/Rev461 Moll70	0.000				
3. KY872702 Protapes gallus	0.013	0.013			
4. EF367170 Paphia exarata	0.013	0.013	0.000		
5. OM292846 Protapes motsei VT-C152	0.013	0.013	0.000	0.000	

Figure 194: K2P distance matrix for *Protapes gallus* using Rev461primers

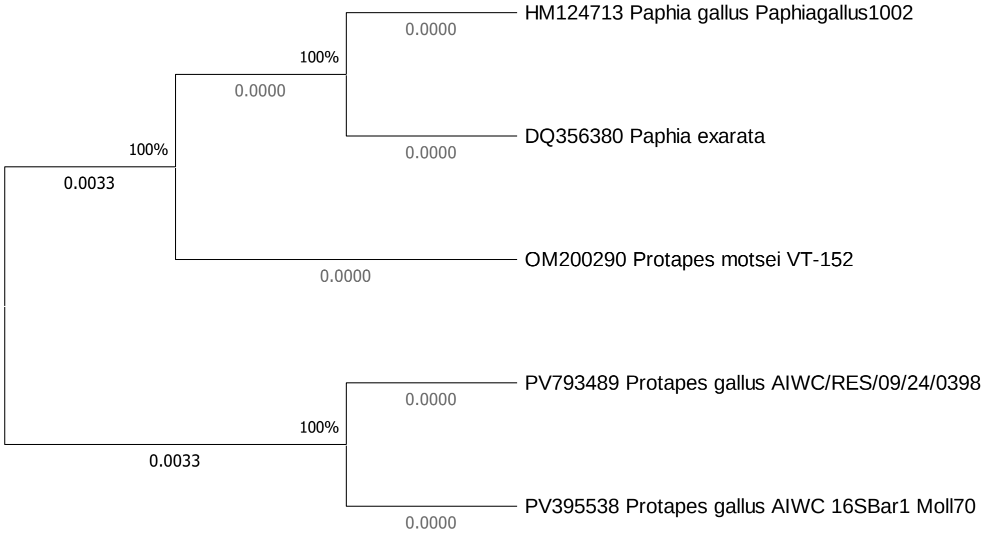
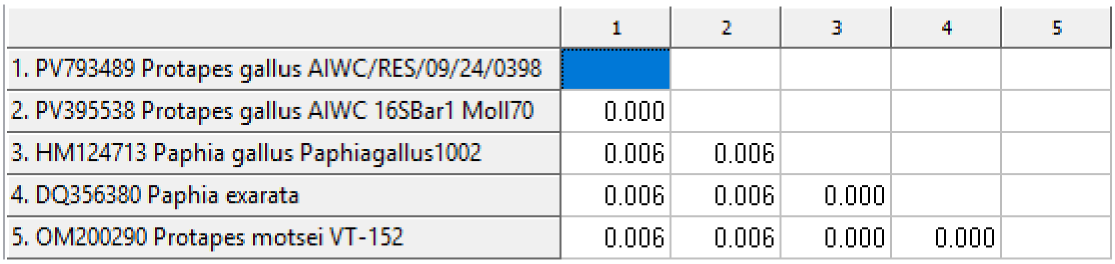


Figure 195: Dendrogram tree for *Protapes gallus* using 16Sbar primers using HKY model



	1	2	3	4	5
1. PV793489 Protapes gallus AIWC/RES/09/24/0398					
2. PV395538 Protapes gallus AIWC 16SBar1 Moll70	0.000				
3. HM124713 Paphia gallus Paphiagallus1002	0.006	0.006			
4. DQ356380 Paphia exarata	0.006	0.006	0.000		
5. OM200290 Protapes motsei VT-152	0.006	0.006	0.000	0.000	

Figure 196: K2P distance matrix for *Protapes gallus* using 16Sbar primers

7.11.24. Order Anomiida – Family Placunidae

The family Placunidae is comprised of the genera *Placunanomia* and *Placuna*, which is reported from Indo-West Pacific region. So far, three species from the Genus Placuna – *P.quadrangula, P. Ephippium and P. Placenta* have been reported from the coast of Tamil Nadu (Das et al, 2019). P. placenta is one the protected species and has been recognised as highly cryptic species. Lin.et al (2024) reported two new species from Indo-Pacific region – *P. Vitream* and *P. Aestuaria* from China, which was earlier known to be a single species as *P.placenta*. The species which is collected from Gulf of Mannar is closely related to *P. vitream*. Of the three primer sets, only Fwd464 primers amplified and showed a significant difference between the species (Fig 195 & 196).

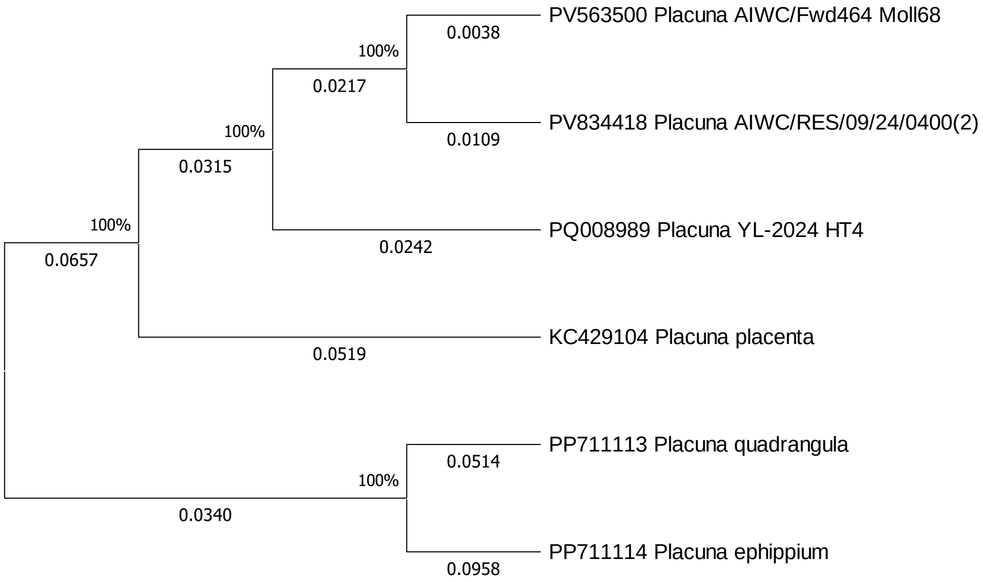
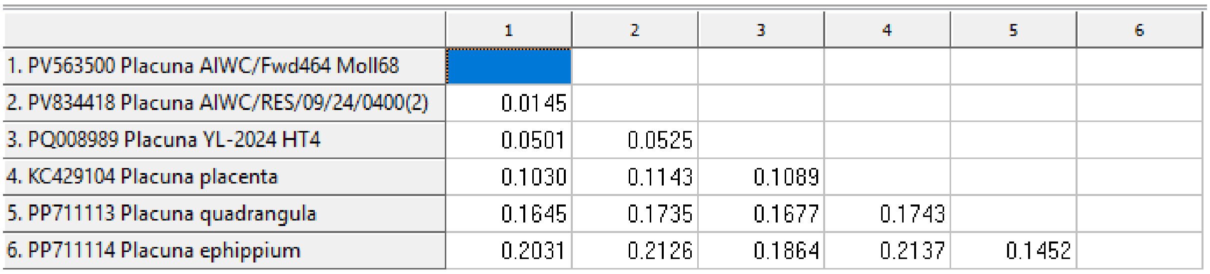


Figure 197: Dendrogram tree for *Placuna.sp* using Fwd464 primers using HKY+G model



	1	2	3	4	5	6
1. PV563500 Placuna AIWC/Fwd464 Moll68						
2. PV834418 Placuna AIWC/RES/09/24/0400(2)	0.0145					
3. PQ008989 Placuna YL-2024 HT4	0.0501	0.0525				
4. KC429104 Placuna placenta	0.1030	0.1143	0.1089			
5. PP711113 Placuna quadrangula	0.1645	0.1735	0.1677	0.1743		
6. PP711114 Placuna ephippium	0.2031	0.2126	0.1864	0.2137	0.1452	

Figure 198: K2P distance matrix for *Placuna.sp* using Fwd464 primers

7.11.25. Order Mytiloida – Family Mytilidae

Perna viridis is a predominant species in bivalve culture in India with a total production of 20,000 tonnes (Imelda J & B Ignatius, 2016). The amplified sequences clearly showed distinct difference between species of Genus *Perna.sp* for the sequences, which were amplified.

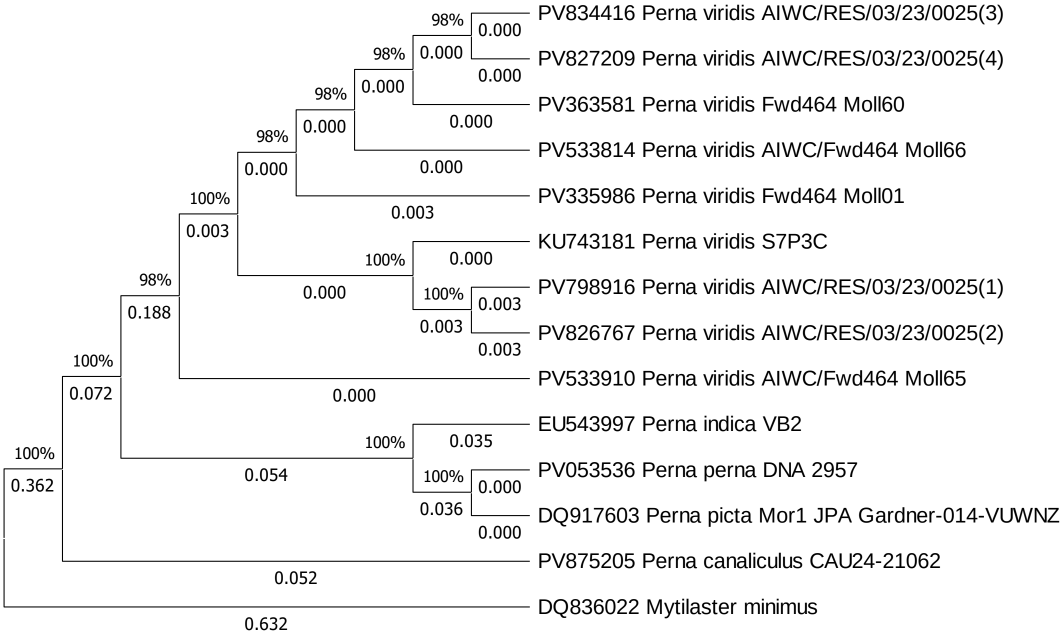
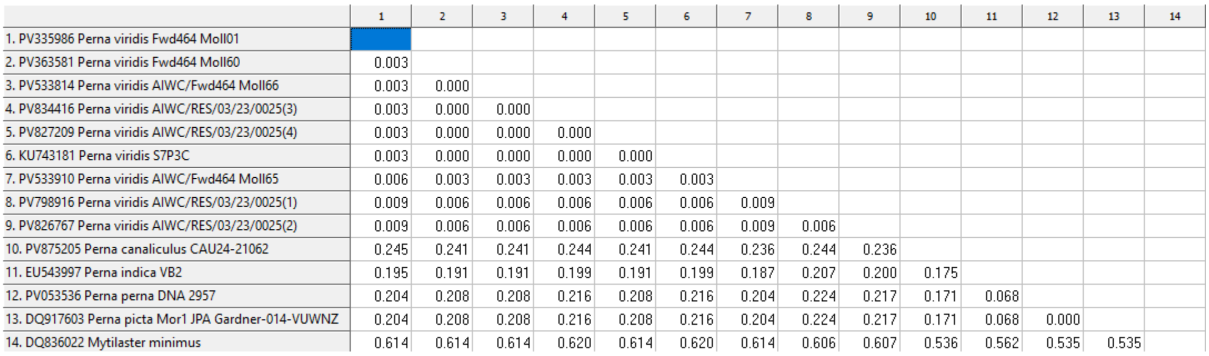


Figure 199: Dendrogram tree for *Perna viridis* using Fwd464 primers using TN92+I model



	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. PV335986 Perna viridis Fwd464 Moll01														
2. PV363581 Perna viridis Fwd464 Moll60	0.003													
3. PV533814 Perna viridis AIWC/Fwd464 Moll66	0.003	0.000												
4. PV834416 Perna viridis AIWC/RES/03/23/0025(3)	0.003	0.000	0.000											
5. PV827209 Perna viridis AIWC/RES/03/23/0025(4)	0.003	0.000	0.000	0.000										
6. KU743181 Perna viridis S7P3C	0.003	0.000	0.000	0.000	0.000									
7. PV533910 Perna viridis AIWC/Fwd464 Moll65	0.006	0.003	0.003	0.003	0.003	0.003								
8. PV798916 Perna viridis AIWC/RES/03/23/0025(1)	0.009	0.006	0.006	0.006	0.006	0.006	0.009							
9. PV826767 Perna viridis AIWC/RES/03/23/0025(2)	0.009	0.006	0.006	0.006	0.006	0.006	0.009	0.006						
10. PV875205 Perna canaliculus CAU24-21062	0.245	0.241	0.241	0.244	0.241	0.244	0.236	0.244	0.236					
11. EU543997 Perna indica VB2	0.195	0.191	0.191	0.199	0.191	0.199	0.187	0.207	0.200	0.175				
12. PV053536 Perna perna DNA 2957	0.204	0.208	0.208	0.216	0.208	0.216	0.204	0.224	0.217	0.171	0.068			
13. DQ917603 Perna picta Mor1 JPA Gardner-014-VUWNZ	0.204	0.208	0.208	0.216	0.208	0.216	0.204	0.224	0.217	0.171	0.068	0.000		
14. DQ836022 Mytilaster minimus	0.614	0.614	0.614	0.620	0.614	0.620	0.614	0.606	0.607	0.536	0.562	0.535	0.535	

Figure 200: K2P distance matrix for *Perna viridis* using Fwd464 primers

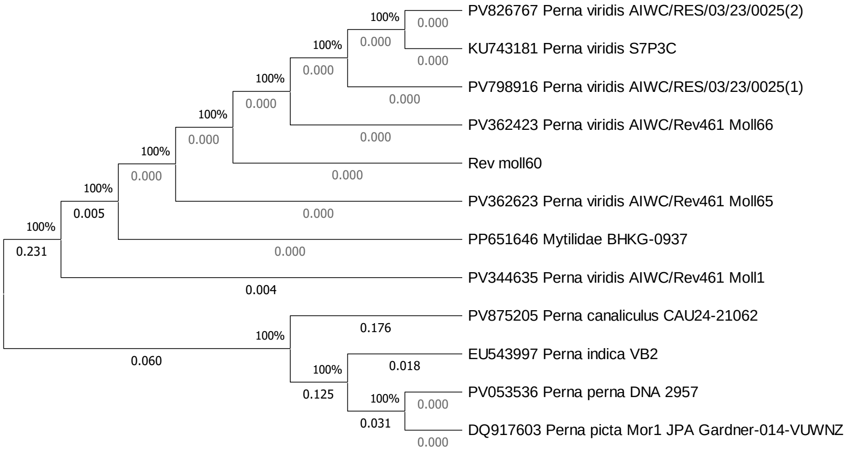
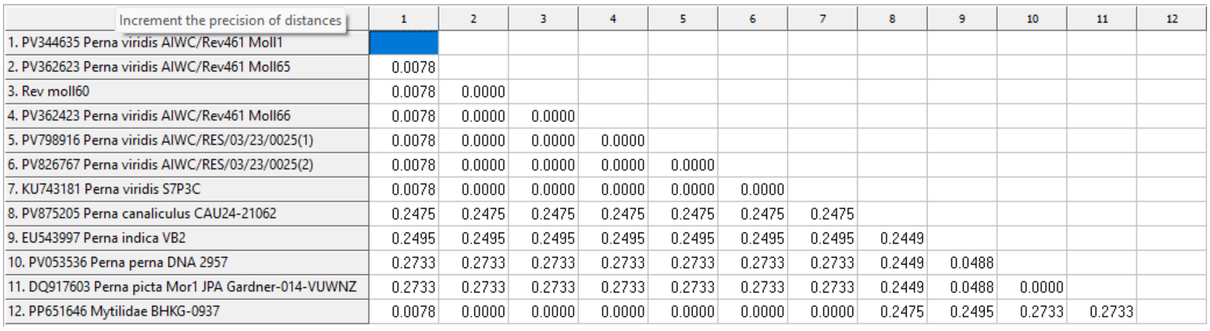


Figure 201: Dendrogram tree for *Perna viridis* using Rev461 primers using TN92+G model



	1	2	3	4	5	6	7	8	9	10	11	12
1. PV344635 Perna viridis AIWC/Rev461 Moll1												
2. PV362623 Perna viridis AIWC/Rev461 Moll65	0.0078											
3. Rev moll60	0.0078	0.0000										
4. PV362423 Perna viridis AIWC/Rev461 Moll66	0.0078	0.0000	0.0000									
5. PV798916 Perna viridis AIWC/RES/03/23/0025(1)	0.0078	0.0000	0.0000	0.0000								
6. PV826767 Perna viridis AIWC/RES/03/23/0025(2)	0.0078	0.0000	0.0000	0.0000	0.0000							
7. KU743181 Perna viridis S7P3C	0.0078	0.0000	0.0000	0.0000	0.0000	0.0000						
8. PV875205 Perna canaliculus CAU24-21062	0.2475	0.2475	0.2475	0.2475	0.2475	0.2475	0.2475					
9. EU543997 Perna indica VB2	0.2495	0.2495	0.2495	0.2495	0.2495	0.2495	0.2495	0.2449				
10. PV053536 Perna perna DNA 2957	0.2733	0.2733	0.2733	0.2733	0.2733	0.2733	0.2733	0.2449	0.0488			
11. DQ917603 Perna picta Mor1 JPA Gardner-014-VUWNZ	0.2733	0.2733	0.2733	0.2733	0.2733	0.2733	0.2733	0.2449	0.0488	0.0000		
12. PP651646 Mytilidae BHKG-0937	0.0078	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.2475	0.2495	0.2733	0.2733	

Figure 202: K2P distance matrix for *Perna viridis* using Rev461 primers

7.12. Blind sample validation

Ten unknown blind samples were given to five different participants for validation. Each participant was given one/two samples from which molecular species identification were not done so far. Tissue samples that were not of molluscs and were outgroup were also included. Outgroup samples were research samples for which species identification was already completed, and it was taken as internal control, and results are shown in table 9.

Sequencing success

Table 12 Sequencing success and species identification of Blind samples. Green indicate Successful and Red indicate failure.

S.No	Blind validator	Research ID	Species	Fwd464	Rev461	16Sbar1
1	1	AIWC/RES/12/24/0489	*Lambis crocata*	🟩	🟩	🟩
2	2	AIWC/RES/12/23/0322	*Pleuroploca trapezium*	🟩	🟩	🟩
3	1	AIWC/RES/12/24/0489	*Lambis crocata*	🟩	🟩	🟩
4	2	AIWC/RES/12/23/0322	*Pleuroploca trapezium*	🟩	🟩	🟩
5	3	AIWC/RES/10/22/0115	*Wild boar*	🟩	🟩	🟩
6	4	AIWC/RES/10/22/0121	*Elephant*	🟩	🟥	🟥
7	5	AIWC/RES/09/23/0212	*Sambar*	🟩	🟩	🟩
8	6	AIWC/RES/09/23/0010	*Spotted deer*	🟩	🟩	🟩
9	7	AIWC/RES/03/24/0070	*Domestic cattle*	🟩	🟩	🟩
10	8	AIWC/RES/10/22/0115	*Wild boar*	🟩	🟩	🟩
14	6	AIWC/RES/10/23/0265	*Sepia pharaonis*	🟩	🟩	🟩

7.13. DNA sequencing from Shell

Within the stipulated time, an attempt was made to amplify DNA using a standardized primer. DNA amplification was assessed only once using the Fwd464 primer across all three tested protocols. Of these, only Method 1 yielded successful amplification in 3 out of 18 samples. The resulting amplicons were sequenced using both Sanger and Nanopore platforms. The Nanopore sequencing reads demonstrated higher quality compared to the Sanger reads (Fig. 202). Among the three successfully amplified samples, only one produced sequence data with Sanger sequencing, whereas all three generated high-quality sequences with Nanopore sequencing. However, a larger sample size is required to conclude which platform is recommended for sequencing shell samples.

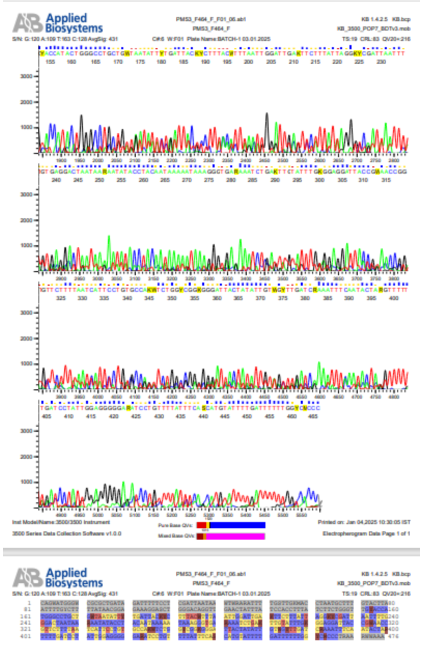


Figure 203: Electropherogram of the sequence through Sanger sequencer

7.14. Capacity building

During the tenure, various stakeholders including field officers, judicial magistrates and forensic research persons were given training on different classes of shells, protected molluscs and distinguishing them from similar non-protected molluscs, and Nanopore sequencer and its application in the field (plate 1).

8. Conclusion

DNA barcoding is an indispensable tool in molluscan taxonomy, particularly for cryptic species. For example, using an integrative taxonomic approach, *Placuna placenta* previously as reported as a single species was reclassified into two distinct new species from the Indo-Pacific by Lin et al. (2024). Zhang et al. (2016) underlined the need for a comprehensive DNA reference library, highlighting the limitations of morphology-based species identification, particularly due to phenotypic plasticity and the presence of cryptic species. (This sentence needs reframing, please do it). Through this study, a total of 289 sequences representing 41 species and 65 individuals were sequenced and uploaded in NCBI (Table 8). The genetic information of several species—*Lotoria triangularis, Marmorofuscus nicobaricus, Murex sp., Harpa davidis, Harpa major, Ranularia oboesa, Cellana sp., Provanna sp., Nassarius sp., and Neverida sp.* were submitted to GenBank for the first time. There is a need for furtherstudies to be carried out in Indian waters especially from Tamil Nadu. By doing so, it will help resolve ambiguous juvenile and morphologically challenging specimens in both gastropods and bivalves (Casu et al., 2021)

Currently, the DNA barcode gap required for delimitating species is 3-5% for COI gene. Studies from Ma. et.al (2022), Chen et al (2010) emphasised on integrative study by using morphology, genetics and geography rather than a single threshold cut-off value due to the presence of errors such as specimen misidentification, sample confusion, and contamination. Given the urgent need for accurate species inventories, integrating COI DNA barcoding with traditional taxonomic methods is essential for refining species delimitation. In the current study, for Genera such as *Protapes, Uroteuthis, Sepiella*, the barcode gap is below 3%. Therefore, we need more sequences from same Genus for determining accurate barcode gap.

Through this study, three set of primers were designed and validated both using in-silico analysis and wet lab. Although the mini-barcodes designed in the present study successfully amplified the terminal portion of the Folmer COI region, their utility for species-level discrimination was compromised during phylogenetic reconstruction. This limitation is due to the fact that publicly available reference sequences covering this specific segment are limited, and the acquired mini-barcode data were further shortened during sequence alignment. Consequently, the discriminatory potential of mini-barcodes depends largely on the completeness and taxonomic coverage of reference datasets in GenBank. Geller et al. (2013) similarly noted that when barcode sequences are truncated, the retained fragment often lacks sufficient overlap with public reference data, thereby reducing the reliability of species identification.

Sequencing through Oxford Nanopore Technologies' MinION Mk1B enables fast, real-time sequencing and immediate initiation of downstream analyses (Loose et al., 2016). While reference-free (de novo) alignment is already available for the Native Barcoding workflow, this study has for the first time standardized and validated a reference-free alignment pipeline for the Rapid Barcoding method. Demonstrating that the Rapid workflow can generate high-quality consensus sequences without relying on existing reference genomes. This workflow significantly enhances the use of Nanopore sequencer for non-human forensic applications (Stevanović A.L. et al. 2021).

9. Further Studies

Given that molluscs constitute the second-largest metazoan taxon, there is a critical need for comprehensive and integrative taxonomic studies to resolve species identities and uncover cryptic diversity. Further investigations into the ecology of protected molluscs, including detailed mapping of their distribution across Tamil Nadu, are essential for informed conservation planning. Moreover, public awareness initiatives targeting the illegal trade of molluscs are necessary to reduce anthropogenic pressures and promote sustainable conservation practices.

Being a long-read sequencer, Oxford Nanopore sequencing can be potentially used in future studies – in addition to DNA barcoding – for whole-genome analyses and metabarcoding, particularly for protected mollusc species whose genetic data are lacking. Approaches such as genome skimming like in sharks, could provide broader genomic insights beyond mini-barcodes, facilitating more robust phylogenetic and population-level studies.

10. Photos of various activities

Plate 1: Different capacity-building activities carried out during the project.

Plate 2: Photos from the field taken during shell collection

Plate 3: Photos from the field taken during shell collection

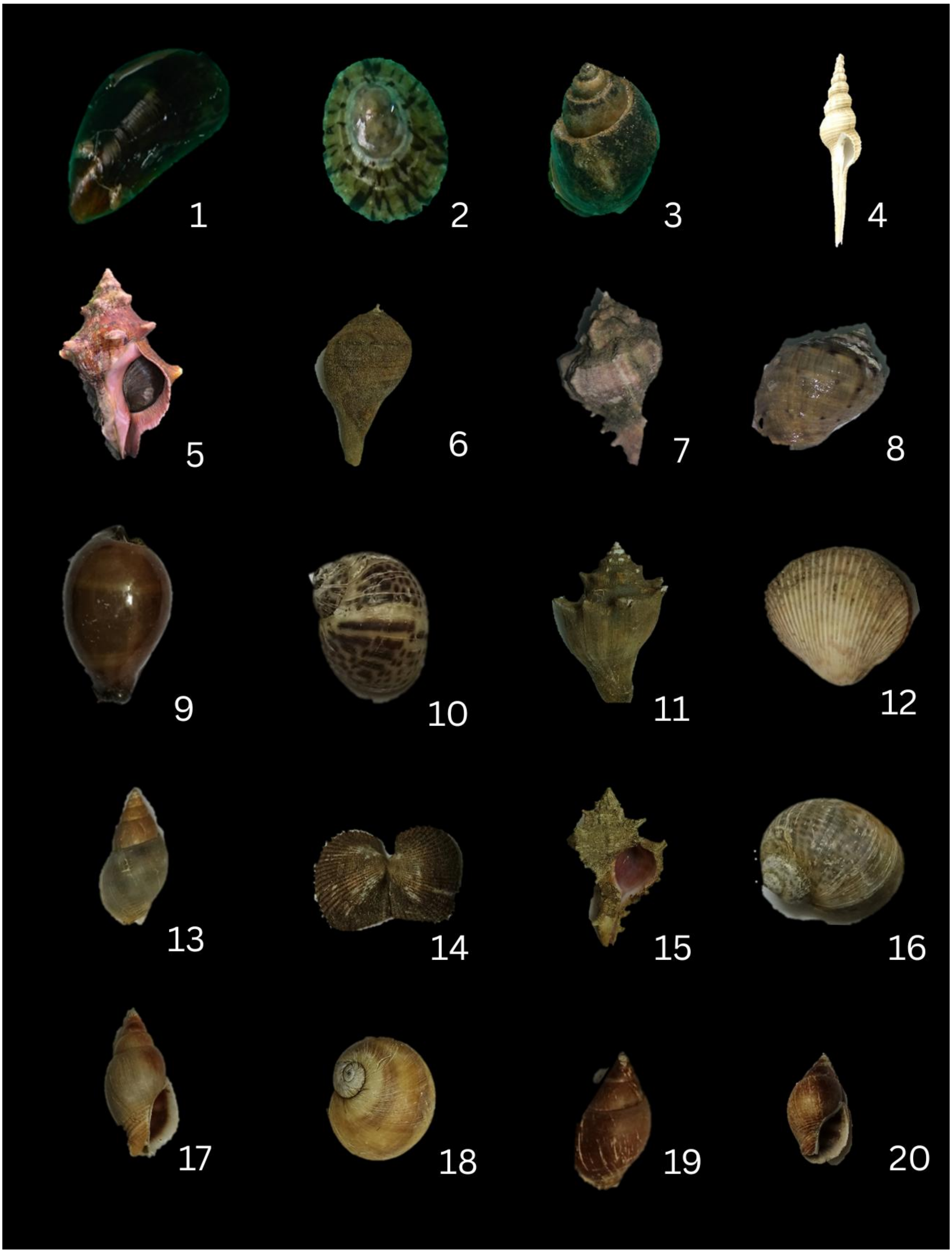


Plate 4: Photos of the shell voucher samples used for in-vivo primer validation.

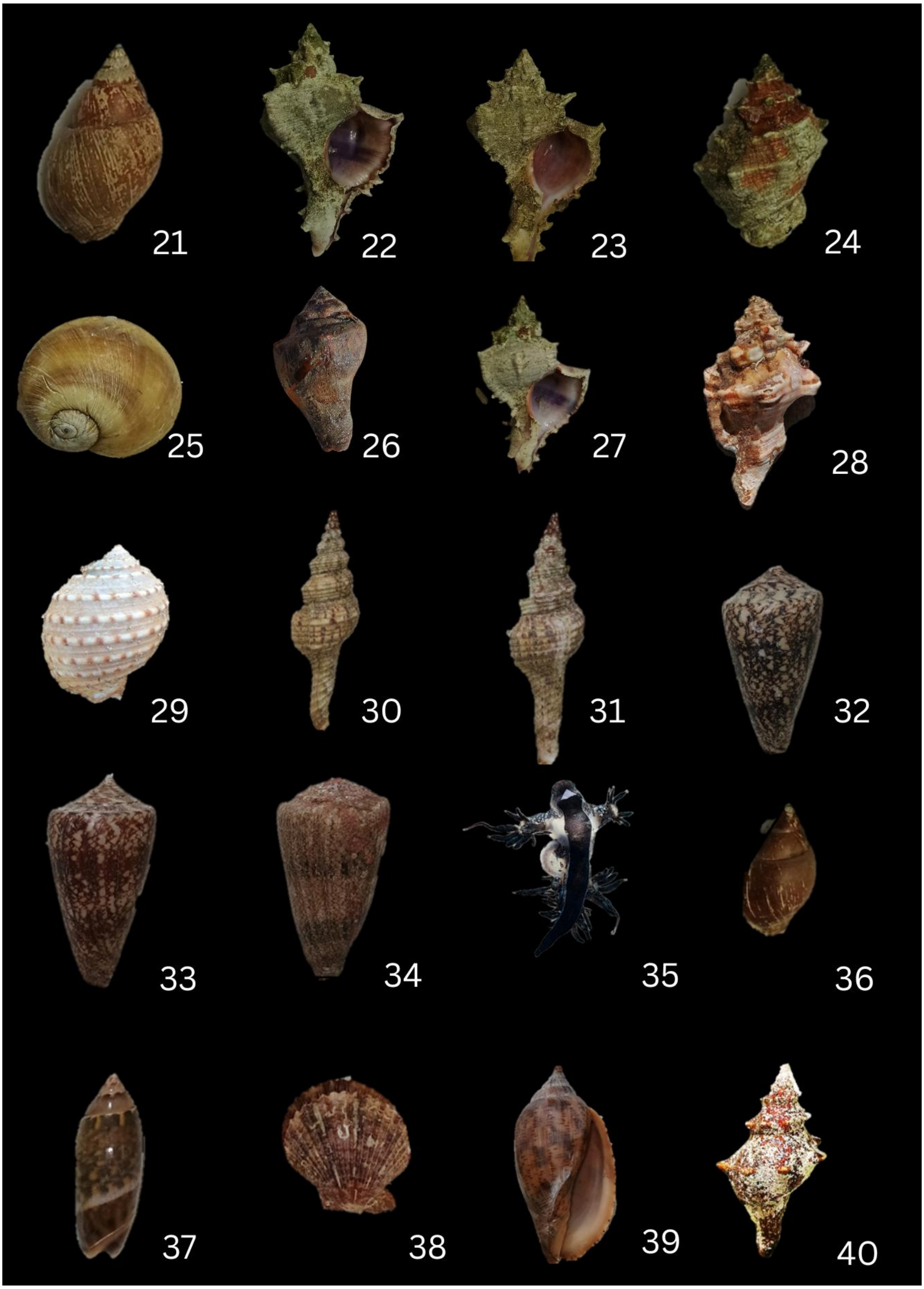


Plate 5: Photos of the shell voucher samples used for in-vivo primer validation.

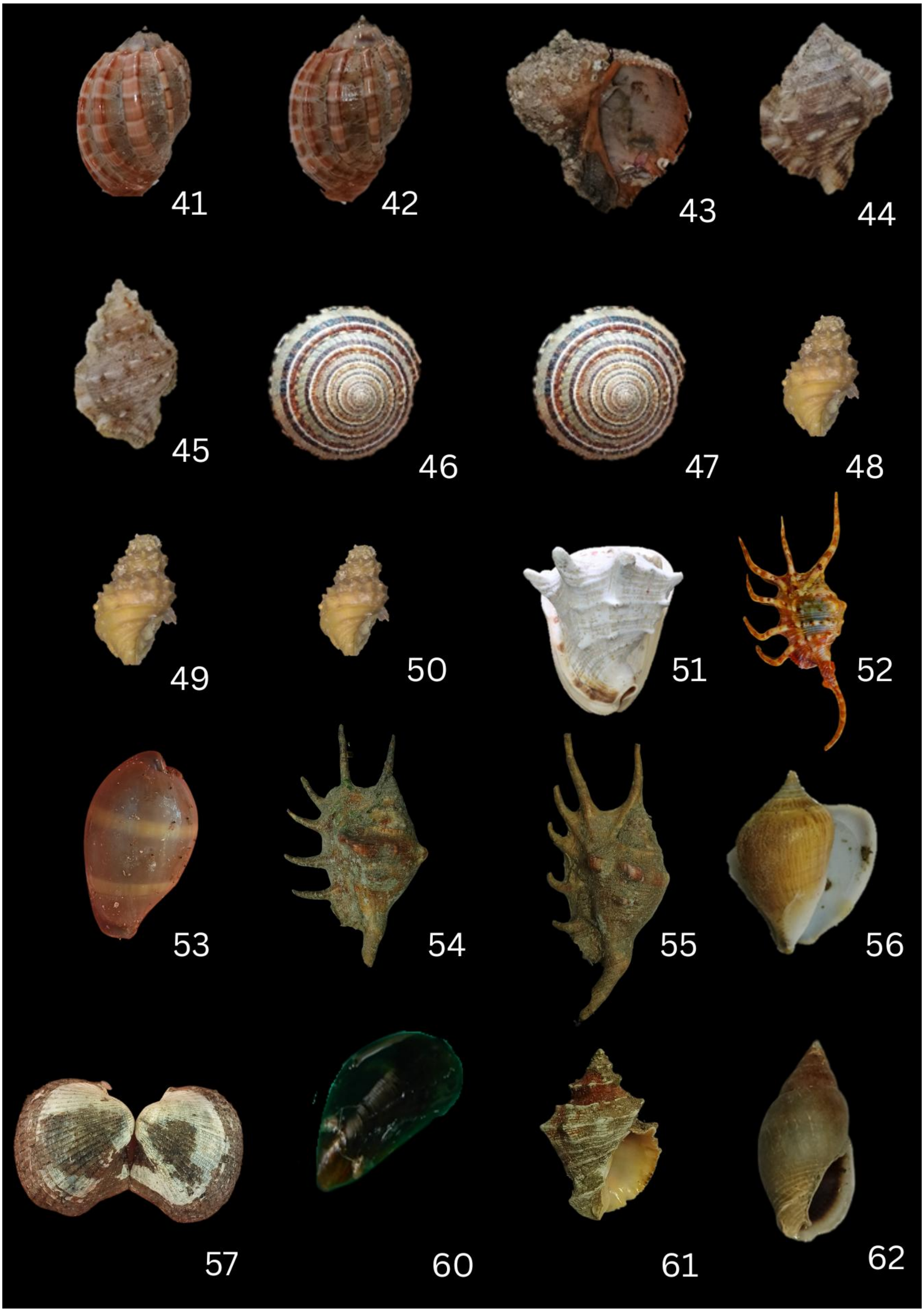


Plate 6: Photos of the shell voucher samples used for in-vivo primer validation.

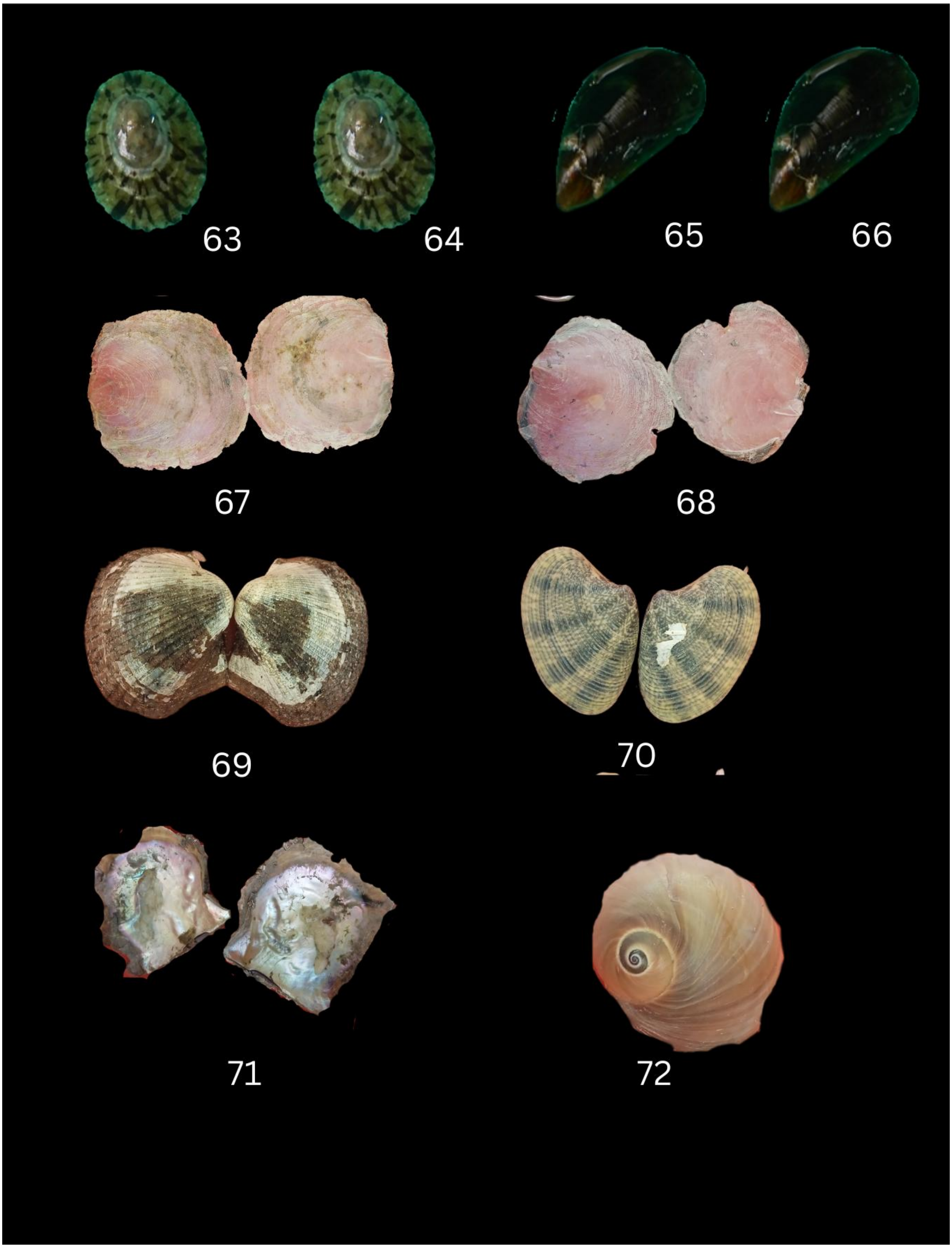


Plate 7: Photos of the shell voucher samples used for in vivo primer validation.

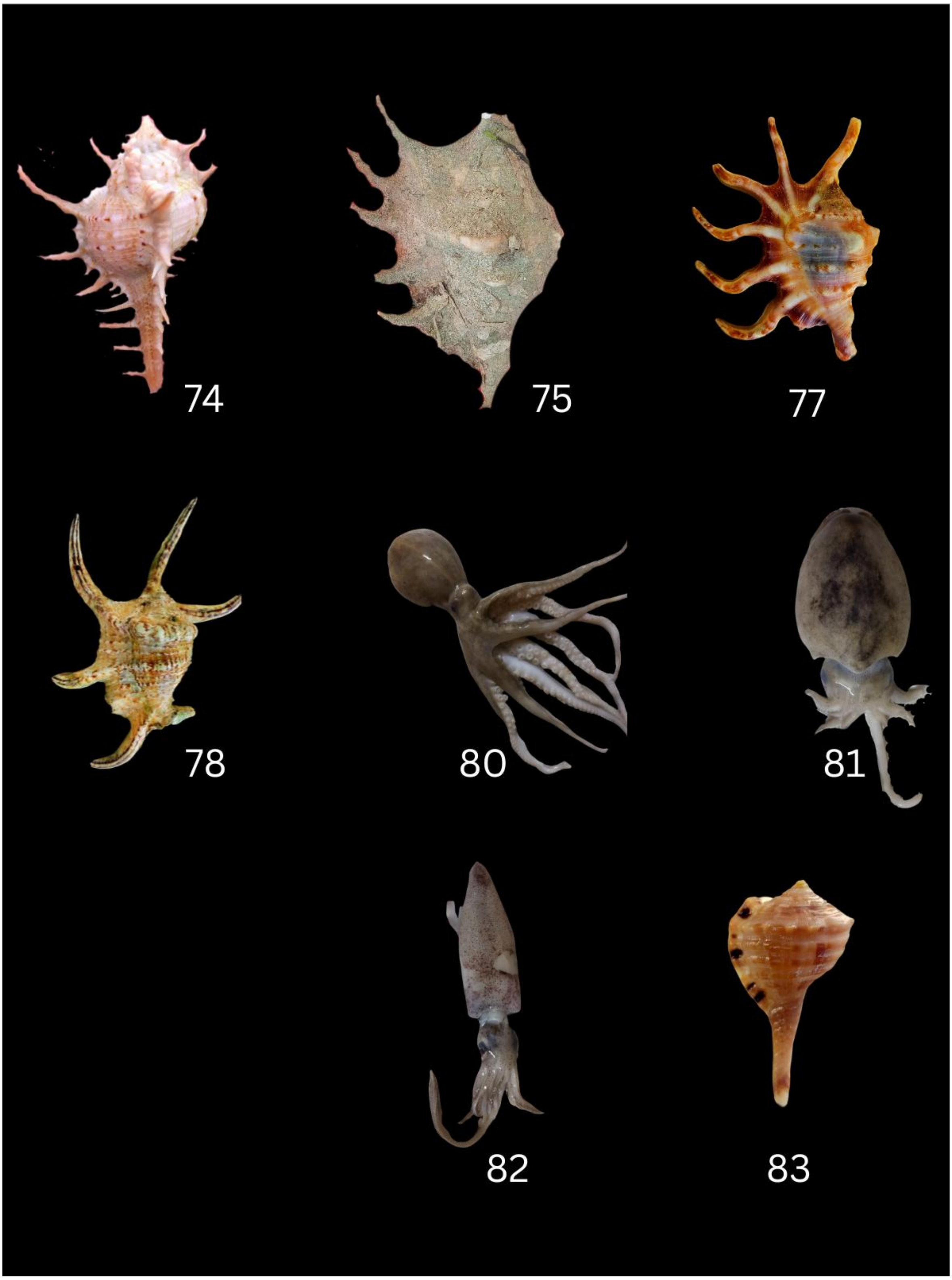
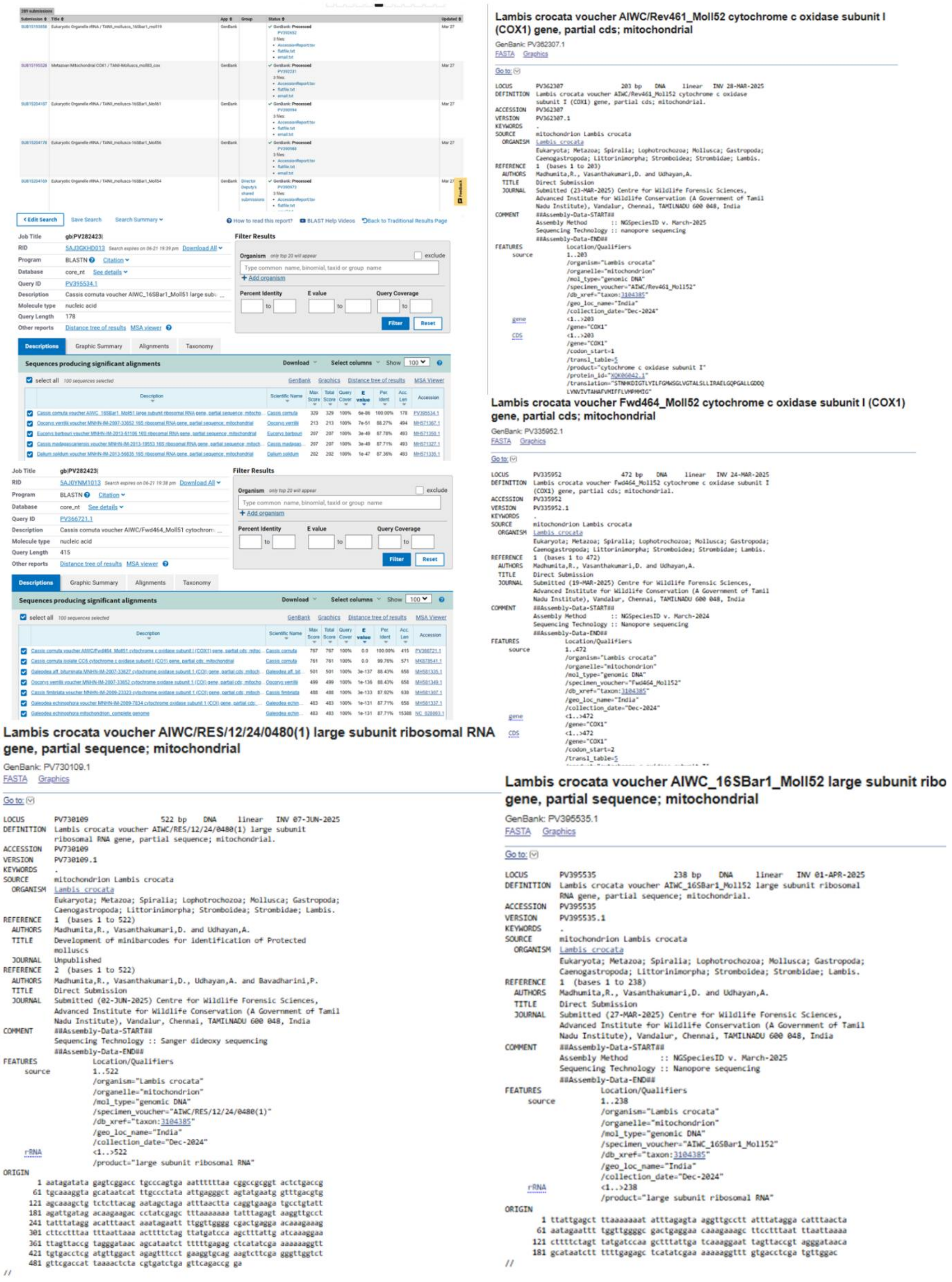


Plate 8: Photos of the shell voucher samples used for in-vivo primer validation.



Job Title: gb|PV282423|
RID: 5AJ3GKHD013 Search expires on 06-21 19:39 pm Download All
Program: BLASTN Citation
Database: core_nt See details
Query ID: PV395534.1
Description: Cassis cornuta voucher AIWC_16SBar1_Moll51 large sub: ...
Molecule type: nucleic acid
Query Length: 178
Other reports: Distance tree of results MSA viewer

Sequences producing significant alignments

☑ select all 100 sequences selected

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Cassis cornuta voucher AIWC_16SBar1_Moll51 large subunit ribosomal RNA gene, partial sequence; mitocho...	Cassis cornuta	329	329	100%	6e-86	100.00%	178	PV395534.1
☑	Oocorys verrilli voucher MNHN-IM-2007-33652 16S ribosomal RNA gene, partial sequence; mitochondrial	Oocorys verrilli	213	213	100%	7e-51	88.27%	494	MH571367.1
☑	Eucorys barbouri voucher MNHN-IM-2013-61106 16S ribosomal RNA gene, partial sequence; mitochondrial	Eucorys barbouri	207	207	100%	3e-49	87.78%	493	MH571359.1
☑	Cassis madagascariensis voucher MNHN-IM-2013-19553 16S ribosomal RNA gene, partial sequence; mitoch...	Cassis madagas...	207	207	100%	3e-49	87.71%	493	MH571327.1
☑	Dalium solidum voucher MNHN-IM-2013-56835 16S ribosomal RNA gene, partial sequence; mitochondrial	Dalium solidum	202	202	100%	1e-47	87.36%	493	MH571335.1

Job Title: gb|PV282423|
RID: 5AJ0YNM1013 Search expires on 06-21 19:38 pm Download All
Program: BLASTN Citation
Database: core_nt See details
Query ID: PV366721.1
Description: Cassis cornuta voucher AIWC/Fwd464_Moll51 cytochrom: ...
Molecule type: nucleic acid
Query Length: 415
Other reports: Distance tree of results MSA viewer

Sequences producing significant alignments

☑ select all 100 sequences selected

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Cassis cornuta voucher AIWC/Fwd464_Moll51 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitoc...	Cassis cornuta	767	767	100%	0.0	100.00%	415	PV366721.1
☑	Cassis cornuta isolate CC6 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	Cassis cornuta	761	761	100%	0.0	99.76%	571	MK878541.1
☑	Galeodea aff. bituminata MNHN-IM-2007-33627 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitoch...	Galeodea aff. bit...	501	501	100%	3e-137	88.43%	658	MH581335.1
☑	Oocorys verrilli voucher MNHN-IM-2007-33652 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitocho...	Oocorys verrilli	499	499	100%	1e-136	88.43%	658	MH581349.1
☑	Cassis fimbriata voucher MNHN-IM-2009-23323 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitoch...	Cassis fimbriata	488	488	100%	3e-133	87.92%	638	MH581307.1
☑	Galeodea echinophora voucher MNHN-IM-2009-7834 cytochrome oxidase subunit 1 (COI) gene, partial cds; ...	Galeodea echin...	483	483	100%	1e-131	87.71%	658	MH581337.1
☑	Galeodea echinophora mitochondrion, complete genome	Galeodea echin...	483	483	100%	1e-131	87.71%	15388	NC_028003.1

Lambis crocata voucher AIWC/RES/12/24/0480(1) large subunit ribosomal RNA gene, partial sequence; mitochondrial

GenBank: PV730109.1

```
LOCUS       PV730109                 522 bp    DNA     linear   INV 07-JUN-2025
DEFINITION  Lambis crocata voucher AIWC/RES/12/24/0480(1) large subunit
            ribosomal RNA gene, partial sequence; mitochondrial.
ACCESSION   PV730109
VERSION     PV730109.1
KEYWORDS    .
SOURCE      mitochondrion Lambis crocata
  ORGANISM  Lambis crocata
            Eukaryota; Metazoa; Spiralia; Lophotrochozoa; Mollusca; Gastropoda;
            Caenogastropoda; Littorinimorpha; Stromboidea; Strombidae; Lambis.
REFERENCE   1  (bases 1 to 522)
  AUTHORS   Madhumita,R., Vasanthakumari,D. and Udhayan,A.
  TITLE     Development of minibarcodes for identification of Protected
            molluscs
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 522)
  AUTHORS   Madhumita,R., Vasanthakumari,D., Udhayan,A. and Bavadharini,P.
  TITLE     Direct Submission
  JOURNAL   Submitted (02-JUN-2025) Centre for Wildlife Forensic Sciences,
            Advanced Institute for Wildlife Conservation (A Government of Tamil
            Nadu Institute), Vandalur, Chennai, TAMILNADU 600 048, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
     source          1..522
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                     /organelle="mitochondrion"
                     /mol_type="genomic DNA"
                     /specimen_voucher="AIWC/RES/12/24/0480(1)"
                     /db_xref="taxon:3104385"
                     /geo_loc_name="India"
                     /collection_date="Dec-2024"
     rRNA            <1..>522
                     /product="large subunit ribosomal RNA"
ORIGIN
        1 aatagatata gagtcggacc tgcccagtga aattttttaa cggccgcggt actctgaccg
       61 tgcaaaggta gcataatcat ttgccctata attgagggct agtatgaatg gtttgacgtg
      121 agcaaagctg tctcttacag aatagctaga atttaactta caggtgaaga tgcctgtatt
      181 agattgatag acaagaagac cctatcgagc tttaaaaaaa tatttagagt aaggttgcct
      241 tatttatagg acatttaact aaatagaatt ttggttgggg cgactgagga acaaagaaag
      301 cttcctttaa tttaattaaa acttttctag ttatgatcca agctttattg atcaaaggaa
      361 ttagttaccg tagggataac agcataatct tttttgagag ctcatatcga aaaaaaggtt
      421 tgtgacctcg atgttggact agagtttcct gaaggtgcag aagtcttcga gggttggtct
      481 gttcgaccat taaaactcta cgtgatctga gttcagaccg ga
//
```

Lambis crocata voucher AIWC/Rev461_Moll52 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial

GenBank: PV362307.1

```
LOCUS       PV362307                 203 bp    DNA     linear   INV 28-MAR-2025
DEFINITION  Lambis crocata voucher AIWC/Rev461_Moll52 cytochrome c oxidase
            subunit I (COX1) gene, partial cds; mitochondrial.
ACCESSION   PV362307
VERSION     PV362307.1
KEYWORDS    .
SOURCE      mitochondrion Lambis crocata
  ORGANISM  Lambis crocata
            Eukaryota; Metazoa; Spiralia; Lophotrochozoa; Mollusca; Gastropoda;
            Caenogastropoda; Littorinimorpha; Stromboidea; Strombidae; Lambis.
REFERENCE   1  (bases 1 to 203)
  AUTHORS   Madhumita,R., Vasanthakumari,D. and Udhayan,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-MAR-2025) Centre for Wildlife Forensic Sciences,
            Advanced Institute for Wildlife Conservation (A Government of Tamil
            Nadu Institute), Vandalur, Chennai, TAMILNADU 600 048, India
COMMENT     ##Assembly-Data-START##
            Assembly Method       :: NGSpeciesID v. March-2025
            Sequencing Technology :: nanopore sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /organism="Lambis crocata"
                     /organelle="mitochondrion"
                     /mol_type="genomic DNA"
                     /specimen_voucher="AIWC/Rev461_Moll52"
                     /db_xref="taxon:3104385"
                     /geo_loc_name="India"
                     /collection_date="Dec-2024"
     gene            <1..>203
                     /gene="COX1"
     CDS             <1..>203
                     /gene="COX1"
                     /codon_start=1
                     /transl_table=5
                     /product="cytochrome c oxidase subunit I"
                     /protein_id="XQK06042.1"
                     /translation="STNHKDIGTLYILFGMWSGLVGTALSLLIRAELGQPGALLGDDQ
                     LYNVIVTAHAFVMIFFLVMPMMIG"
```

Lambis crocata voucher Fwd464_Moll52 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial

GenBank: PV335952.1

```
LOCUS       PV335952                 472 bp    DNA     linear   INV 24-MAR-2025
DEFINITION  Lambis crocata voucher Fwd464_Moll52 cytochrome c oxidase subunit I
            (COX1) gene, partial cds; mitochondrial.
ACCESSION   PV335952
VERSION     PV335952.1
KEYWORDS    .
SOURCE      mitochondrion Lambis crocata
  ORGANISM  Lambis crocata
            Eukaryota; Metazoa; Spiralia; Lophotrochozoa; Mollusca; Gastropoda;
            Caenogastropoda; Littorinimorpha; Stromboidea; Strombidae; Lambis.
REFERENCE   1  (bases 1 to 472)
  AUTHORS   Madhumita,R., Vasanthakumari,D. and Udhayan,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (19-MAR-2025) Centre for Wildlife Forensic Sciences,
            Advanced Institute for Wildlife Conservation (A Government of Tamil
            Nadu Institute), Vandalur, Chennai, TAMILNADU 600 048, India
COMMENT     ##Assembly-Data-START##
            Assembly Method       :: NGSpeciesID v. March-2024
            Sequencing Technology :: Nanopore sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
     source          1..472
                     /organism="Lambis crocata"
                     /organelle="mitochondrion"
                     /mol_type="genomic DNA"
                     /specimen_voucher="Fwd464_Moll52"
                     /db_xref="taxon:3104385"
                     /geo_loc_name="India"
                     /collection_date="Dec-2024"
     gene            <1..>472
                     /gene="COX1"
     CDS             <1..>472
                     /gene="COX1"
                     /codon_start=2
                     /transl_table=5
```

Lambis crocata voucher AIWC_16SBar1_Moll52 large subunit ribo
gene, partial sequence; mitochondrial

GenBank: PV395535.1

```
LOCUS       PV395535                 238 bp    DNA     linear   INV 01-APR-2025
DEFINITION  Lambis crocata voucher AIWC_16SBar1_Moll52 large subunit ribosomal
            RNA gene, partial sequence; mitochondrial.
ACCESSION   PV395535
VERSION     PV395535.1
KEYWORDS    .
SOURCE      mitochondrion Lambis crocata
  ORGANISM  Lambis crocata
            Eukaryota; Metazoa; Spiralia; Lophotrochozoa; Mollusca; Gastropoda;
            Caenogastropoda; Littorinimorpha; Stromboidea; Strombidae; Lambis.
REFERENCE   1  (bases 1 to 238)
  AUTHORS   Madhumita,R., Vasanthakumari,D. and Udhayan,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (27-MAR-2025) Centre for Wildlife Forensic Sciences,
            Advanced Institute for Wildlife Conservation (A Government of Tamil
            Nadu Institute), Vandalur, Chennai, TAMILNADU 600 048, India
COMMENT     ##Assembly-Data-START##
            Assembly Method       :: NGSpeciesID v. March-2025
            Sequencing Technology :: Nanopore sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
     source          1..238
                     /organism="Lambis crocata"
                     /organelle="mitochondrion"
                     /mol_type="genomic DNA"
                     /specimen_voucher="AIWC_16SBar1_Moll52"
                     /db_xref="taxon:3104385"
                     /geo_loc_name="India"
                     /collection_date="Dec-2024"
     rRNA            <1..>238
                     /product="large subunit ribosomal RNA"
ORIGIN
        1 ttattgagct ttaaaaaaat atttagagta aggttgcctt atttatagga catttaacta
       61 aatagaattt tggttggggc gactgaggaa caaagaaagc ttcctttaat ttaattaaaa
      121 cttttctagt tatgatccaa gctttattga tcaaaggaat tagttaccgt agggataaca
      181 gcataatctt ttttgagagc tcatatcgaa aaaaaggttt gtgacctcga tgttggac
//
```

Plate 9: Screenshots of sequences uploaded in NCBI.

11. Appendix 1

Codes for assembling nanopore sequences using De-Novo assembly using shell script.

Step 1: After basecalling, unzip the barcoded files using the command.

```
find /LOCATION/FILE NAME -type f -name "*.gz" -exec gunzip {} \;
```

Step 2: All the unzipped files are to be processed needs to be stored in a single folder along with a shell scrip file given below and saved as dec1,sh.

```
#!/bin/bash
for dir in */; do
  # Check if there are FASTQ files in the directory
  if ls "$dir"/*.fastq 1> /dev/null 2>&1; then
    # Concatenate all FASTQ files in the directory
    cat "$dir"/*.fastq > "$dir"/merged.fastq || { echo "merging FASTQ files in $dir"; continue; }
  else
    echo "No FASTQ files found in $dir"
    continue
  fi

  # Get the base name of the directory
  bn=$(basename "$dir")

  # Use NanoPlot to visualize the quality of the sequences
  NanoPlot --fastq "$dir"/merged.fastq -o "$dir"/NanoPlot_output -p sequencing_run || { echo "NanoPlot  in $dir"; continue; }

  # Use NanoFilt to filter sequences by quality
  NanoFilt -q 12 "$dir"/merged.fastq > "$dir"/filtered.fastq || { echo "NanoFilt  in $dir"; continue; }

  # Run NGSpeciesID on the filtered FASTQ file
  NGSpeciesID --ont --consensus --sample_size 1000 --m 200 --s 30 --medaka \
    --fastq "$dir"/filtered.fastq --outfolder "$dir"/NGSpeciesID_output || { echo "NGSpeciesID in $dir"; continue; }
  # Convert the NGSpeciesID output to FASTA format using write_fastq
```

```
  NGSpeciesID write_fastq --fastq "$dir"/NGSpeciesID_output/final_clusters.fastq \
    --fasta "$dir"/NGSpeciesID_output/final_clusters.fasta || {echo "Conversion to FASTA in $dir"; continue;}
echo "Processing completed for $dir"
done
```

Step 3: Go to terminal and change the directory using the command cd.

Step 4: use the following command

```
conda activate NGSpeciesID
bash dec1.sh
```

Step 5: Wait for the terminal to complete the files. Once it is completed, go to the individual sample folder, and check for the file NGSpeciesID_output folder. Inside which there is named “ConsensusXX.fasta”

Step 6: Try BLAST for the sequence. Check for the query cover and query length. Query length should match the parameters by adjusting --sample_size, --m, --s and –q.

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Project Summary

DNA barcoding is a reliant tool for protected marine mollusc species identification, but challenges arise with highly processed specimens.Conventional primers often fail due to poor DNA quality. To overcome three sets of Mini-barcodes were designed for successfully identifying and distinguishing between species. A total of 289 sequences representing 41 species and 65 individuals were sequenced and uploaded in NCBI including five protected mollusc species—*Cassis cornuta*, *Harpago chiragra*, *Lambis crocata*, *Pleuroploca trapezium*, and *Placuna placenta.*

To support rapid and on-site field sequencing, a portable Oxford Nanopore sequencer was procured and utilized for sequencing activities. For handling forensic samples, a reference-free assembly approach was identified and customized to generate consensus sequences without reliance on existing reference genomes. Outreach and awareness sessions on the identification of protected molluscs and the practical application of nanopore sequencing technology were conducted and delivered to a diverse group of stakeholders, including Forest Range Officers, legal professionals, and forensic personnel, throughout the project period.

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