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# Molecular Genetic Variation and Phylogenetic Relationships of Some Gastropod Species Using Cytochrome C Oxidase Subunit 1 Sequence

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## ABSTRACT

The present study aimed to assess the genetic variation and phylogenetic relationships among six gastropod species using cytochrome c oxidase subunit 1 (CO1) sequences. The nucleotide lengths obtained from sequencing the mitochondrial CO1 gene varied from 633 to 658 bp. The partial CO1 sequences were submitted to GenBank with the following accession numbers: PQ216357.1 to PQ216362.1. The mean A+T content was 61.28%, exceeding the C+G content. Additionally, the mean content of pyrimidine (C+T) bases was 56.53%, which was higher than that of purines. Two approaches were used for phylogenetic reconstruction: Neighbor Joining and Minimum Evolution. Both methods indicated that the species of Neritimorpha were divided into two main clusters. One cluster contained four species: the understudied *Nerita albicilla*, *Nerita sanguinolenta*, as well as *Nerita albicilla* (EU253101.1) and *Nerita sanguinolenta* (EU732306.1) from GenBank/NCBI. The other cluster included the remaining species. Furthermore, the results demonstrated a close genetic relationship among the understudied *Peronia verruculata* and both *Peronia verruculata* (ON524819.1) and *Peronia persiae* (MK312167.1) from GenBank/NCBI. Additionally, the analyses revealed that Neritimorpha is a sister group to Heterobranchia.

## INTRODUCTION

The Gastropoda, which is the largest class of the Mollusca phylum, is highly plentiful and exhibits a significant diversity among marine species **(Bouchet, 2006; Smith *et al*., 2011; Zapata *et al*., 2014; Al-Khafaji *et al*., 2021)**. The Gastropoda, due to its diversity and historical significance, has become one of the most extensively researched animal groups **(Smith *et al*., 2011)**. Gastropods are a very varied group of marine animals **(Appeltans *et al*., 2012)** and the only species of mollusks that have successfully inhabited terrestrial habitats. Gastropods exhibit a wide range of diversity, with tens of thousands of species that have been described. They also display a significant difference in their morphological characteristics, such as shell form, coloration, and size,

including snails, limpets, and slugs. Furthermore, they can be found in various ecologies and depths. Gastropods exhibit embryonic spiral cleavage, a variety of developmental modalities (including both direct and indirect development, with the presence of multiple types of larvae), and endure body torsion during their growth process **(Cunha & Giribet, 2019)**.

With a fossil record dated to the Middle Devonian, roughly 375 million years ago, but potentially as early as the Ordovician, the Neritimorpha (Neritopsina), a group of more than 450 extant species, are known. Besides, Neritimorpha possesses one of the most remarkable adaptive radiation mechanisms among gastropods. The group has a wide range of forms and has invaded freshwater, marine, and groundwater places **(Kano *et al.*, 2002)**.

Caenogastropods are the most extensive and varied group of living Gastropoda species. They include numerous ecologically and commercially significant marine families. Their remarkable capacity to adapt to many habitats resulted in significant ecological, morphological, and behavioral variety **(Ponder *et al.*, 2008)**. This group has a diverse range of shell morphologies, which often converge. These morphologies include coiled, uncoiled, elongate, globose, and limpet-shaped forms. Additionally, some species within this group have a reduced or, in rare cases, completely absent shell **(Colgan *et al.*, 2007)**.

It is believed that heterobranchs separated from other gastropods approximately 380 million years ago **(Dinapoli & Klussmann-Kolb, 2010; Jörger *et al.*, 2010)**. Heterobranchia is a clade of gastropod molluscs that is rich in species and includes a diverse range of freshwater, marine, and terrestrial snails and slugs **(Schrödl *et al.*, 2011; Schrödl & Stöger, 2014)**.

Since mitochondrial genes change ten fold faster than nuclear DNA, mitochondrial DNA sequences are extremely significant because they include an abundance of information that clarifies high taxonomic bounds (e.g., families). On the other hand, nuclear genes show minimal differences in their nucleotide locations at low taxonomic levels **(Brown *et al.*, 1979; Canapa *et al.*, 2000; An *et al.*, 2005)**.

Cytochrome c oxidase I, one of the 13 protein-coding genes included in the mt genome, has become especially well-liked for determining the relationships between closely related species **(Remigio & Hebert, 2003)**. Furthermore, compared to other mitochondrial genes, the gene that encodes protein cytochrome c oxidase subunit I (*COI*) exhibits a broader phylogenetic signal, evidenced by a greater number of parsimonious sites, and is considered a dependable evolutionary marker for delineating interspecific linkages **(Hebert *et al.*, 2003b; Hershler *et al.*, 2003; Remigio & Hebert, 2003; Grande *et al.*, 2004; An *et al.* 2005)**.

In a number of gastropod families, DNA barcoding has poriven to be an effective method for resolving the challenges associated with species identification and delimitation only by morphological features **(Barco *et al*., 2010; Johnson & Gosliner, 2012; Layton *et al*., 2014)**. Cytochrome oxidase subunit I (COI) is essential for cellular energy generation because it is a component of the mitochondrial cytochrome c oxidase protein (COX) **(Pentinsaari *et al*., 2016)**. These days, barcodes are an often used (and misused) marker, with several databases devoted to compiling barcode sequences for a variety of animal taxa **(DeSalle & Goldstein, 2019)**.

For these aforementioned reasons, we investigated the genetic variation and phylogenetic links of numerous gastropod species in our study using cytochrome c oxidase subunit 1 sequence.

## MATERIALS AND METHODS

### Ethics statement

The South Valley University Faculty of Science's research animal care ethical committee accepted all of the investigations in accordance with protocol: 002/02/24.

### Samples collection and species identification

Six gastropod species were collected from the Egyptian Red Sea, where three species (*Nerita albicilla*, *Nerita quadricolor* and *Nerita sanguinolenta*) belong to Neritimorpha, two species (*Pirenella cingulata* and *Tenguella granulata*) belong to Caenogastropoda, and one species *Peronia verruculata* belongs to Heterobranchia. The sample tissues were separated and kept in storage at -20°C for DNA extraction.

### DNA Extraction and PCR amplification

Utilizing the DNA Mini kit (Qiagen, Germany), the whole genomic DNA was extracted from the isolated tissues. We utilized primers as **Folmer *et al*. (1994)** to amplify the mitochondrial cytochrome c oxidase subunit 1 (*COI*) gene in the six gastropod species. The PCR reactions comprise of a final reaction volume of 50 and 1µL of genomic DNA, each primer, and 25µL of PCR master mix. The settings for the PCR cycling were as follows: a five-minute initial denaturation at 94ºC; thirty cycles of denaturation for sixty seconds at 94ºC, annealing for sixty seconds at 49°C, and an extension at 72°C for sixty seconds, with a post-cycling extension at 72ºC for five minutes. The PCR products were separated using a 1.5% agarose gel that had been stained with ethidium bromide.

### The sequencing of PCR products and phylogenetic tree construction

Every DNA sample was sequenced using the Sanger sequencing method at Macrogen, Seoul, South Korea. The *COI* gene sequences were sent to the National Center for Biotechnology Information (GenBank/NCBI) for the purpose of providing accession

numbers. Using MEGA software version 7.0 18, the sequences were aligned using CLUSTAL W **(Thompson *et al.*, 1994)** with the default parameters. Two approaches were used for phylogenetic reconstructions: Neighbor Joining and Minimum Evolution **(Kumar *et al.*, 2016)**. We employed 1000 bootstrap iterations of Kimura two-parameter distances **(Kimura, 1980)** to finalize the sequence divergences **(Felsenstein, 1985)**.

## RESULTS

### Genetic variation

Mitochondrial *CO1* gene sequencing in the six gastropod species yielded nucleotide length which varied from 633 to 658bp. The partial sequences of *CO1* were submitted into GenBank with the following accession numbers: PQ216357.1- PQ216362.1. The mean A+T content was 61.28%, exceeding the C+G content. Additionally, the mean content of pyrimidine (C+T) bases was 56.53, above that of purines. Additional information regarding pyrimidine contents, A+T contents, and nucleotide frequencies is provided in Table (1). The average nucleotide frequencies of adenine (A), cytosine (C), guanine (G), and thymine (T) are shown in Fig. (1).

**Table 1.** Accession numbers, nucleotide (%), pyrimidine contents, T+A contents and their averages of *CO1* gene in six gastropod species

| Species | Accession Number | Base Pair Length | Nucleotide (%) | | | | T+A Content% | T+C Content% |
|---|---|---|---|---|---|---|---|---|
| | | | T | C | A | G | | |
| *Pirenella cingulata* | PQ216357.1 | 648 | 35.96 | 20.83 | 24.54 | 18.67 | 60.5 | 56.79 |
| *Nerita albicilla* | PQ216358.1 | 658 | 40.88 | 16.72 | 19.91 | 22.49 | 60.79 | 57.6 |
| *Nerita quadricolor* | PQ216359.1 | 633 | 40.13 | 16.59 | 19.27 | 24.01 | 59.4 | 56.72 |
| *Nerita sanguinolenta* | PQ216360.1 | 634 | 39.59 | 17.82 | 20.03 | 22.56 | 59.62 | 57.41 |
| *Peronia verruculata* | PQ216361.1 | 643 | 39.66 | 15.40 | 25.19 | 19.75 | 64.85 | 55.06 |
| *Tenguella granulata* | PQ216362.1 | 648 | 37.81 | 17.75 | 24.69 | 19.75 | 62.5 | 55.56 |
| Avg. | - | 644 | 39.01 | 17.52 | 22.27 | 21.20 | 61.28 | 56.53 |

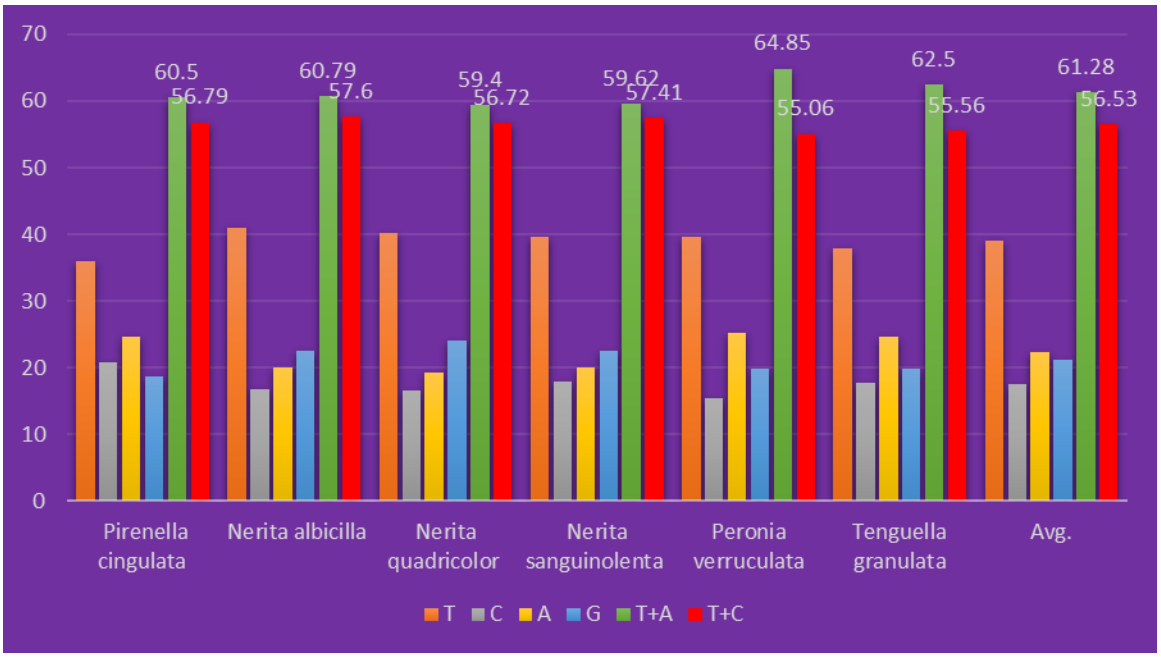


**Fig. 1.** The average nucleotide frequencies of partial sequence of the *CO1* gene in six gastropod species

**Phylogenetic analysis**

The *CO1* sequences from three species of *Neritimorpha* (*Nerita albicilla*, *Nerita quadricolor*, and *Nerita sanguinolenta*) were analyzed using BLAST/N at NCBI, revealing 11 related *Neritimorpha* species, along with the out-group species (*Cellana radiata*, *Cellana ornata*, and *Cellana sandwicensis*) from *Patellogastropoda*. Among these 11 related *Neritimorpha* species, the species genetically closest to the understudied *Nerita albicilla* was *Nerita albicilla* (EU253101.1), while the species most similar to *Nerita sanguinolenta* was *Nerita sanguinolenta* (EU732306.1). Additionally, the closest genetic match to *Nerita quadricolor* was *Nerita quadricolor* (EU732303.1).

To further elucidate the phylogenetic relationships, we employed two techniques: Neighbor Joining (NJ) and Minimum Evolution (ME). Both methods demonstrated nearly similar relationships with minor variations in support values. They also indicated that: (1) the out-group species formed a separate cluster, and (2) the species of *Neritimorpha* were divided into two main clusters. One cluster contained the understudied *Nerita albicilla* and *Nerita sanguinolenta*, along with *Nerita albicilla* (EU253101.1) and *Nerita sanguinolenta* (EU732306.1) from GenBank/NCBI, while the other cluster included the remaining species (Figs. 2, 3).

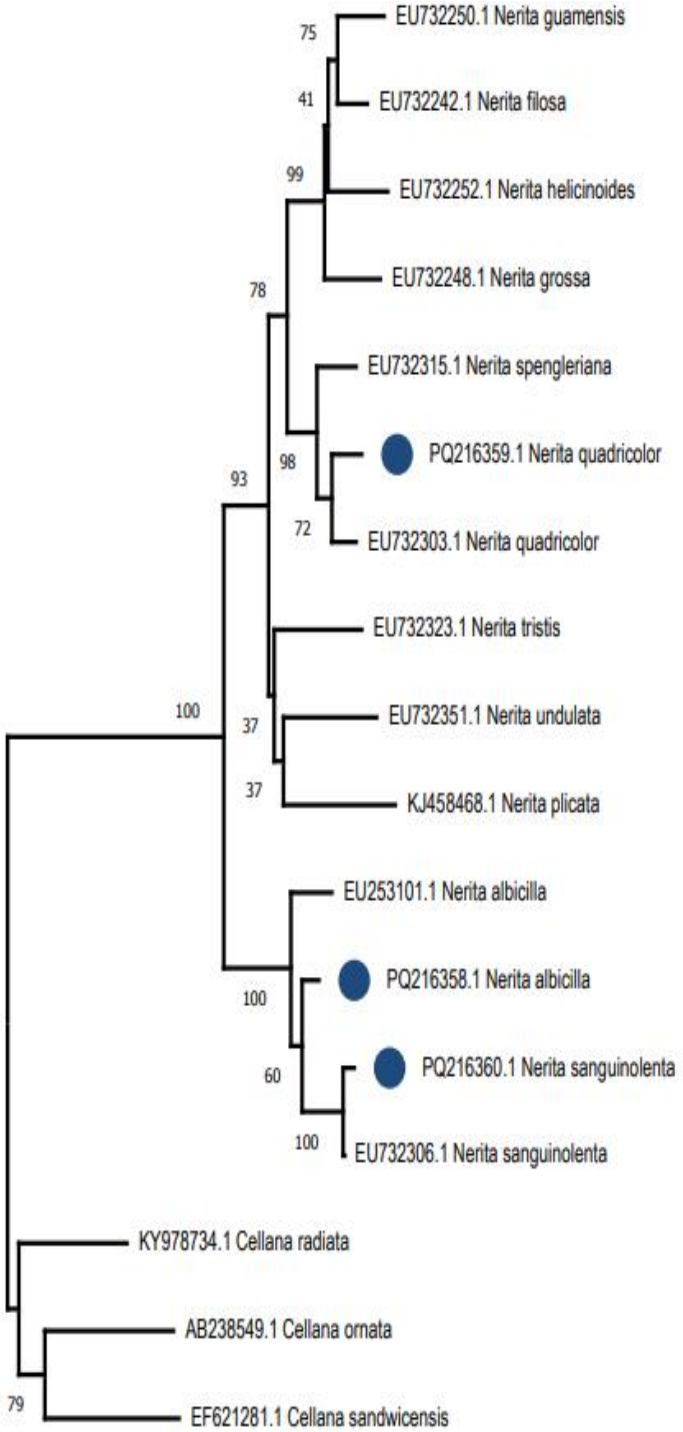


**Fig. 2.** Neighbor Joining phylogenetic tree among *Nerita albicilla*, *Nerita quadricolor* and *Nerita sanguinolenta* and their related Neritimorpha species; in addition, the outgroup using *COI* gene

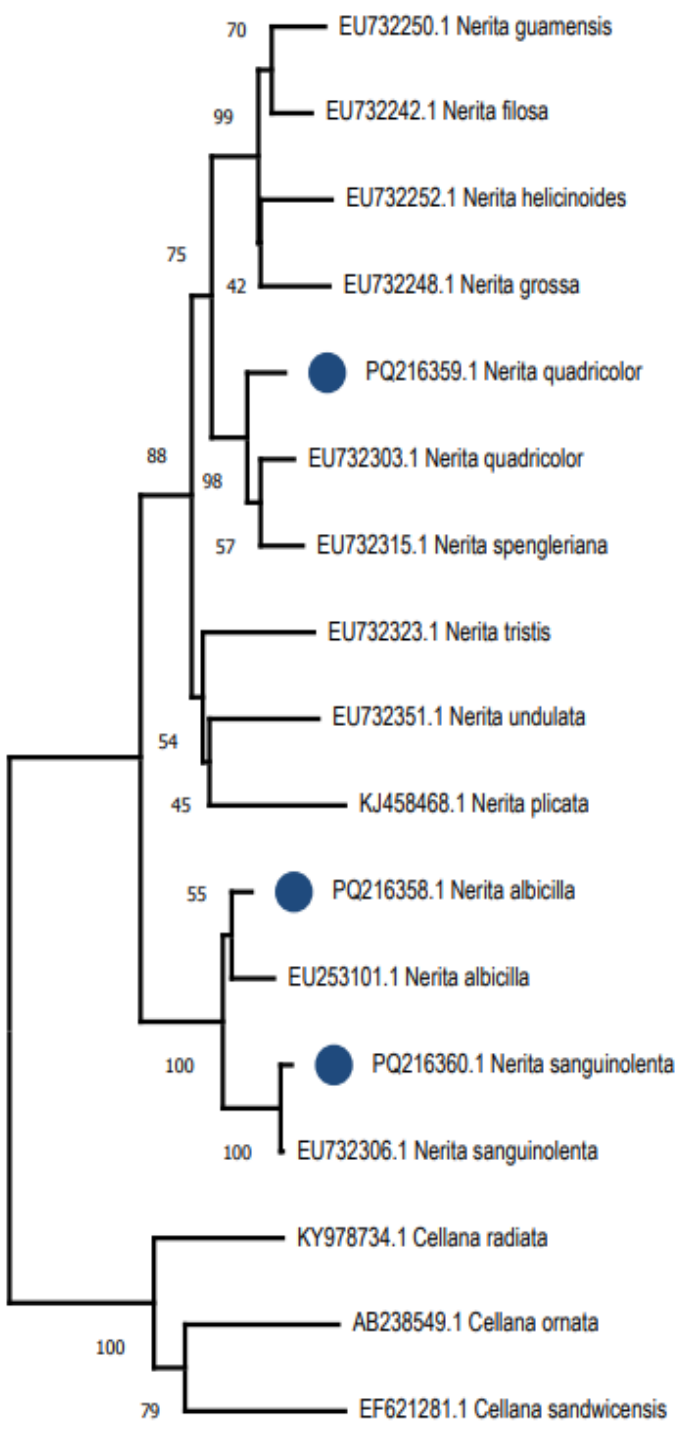


**Fig. 3.** Minimum Evolution phylogenetic tree among *Nerita albicilla*, *Nerita quadricolor* and *Nerita sanguinolenta* and their related Neritimorpha species; in addition, the outgroup using *COI* gene

The sequences of *CO1* in two species (*Pirenella cingulate* and *Tenguella granulata*) of caenogastropods were run with BLAST/N at NCBI, revealing 21 linked caenogastropods species in addition to the out-group species (*Jujubinus vexationis*, *Jujubinus exasperatus*, and *Jujubinus striatus*) of Vetigastropoda. Among all 21 related caenogastropods species, the genetically similar species to the understudied *Pirenella cingulate* was *Pirenella cingulate* MN389033.1, followed by *Cerithideopsilla cingulata* HE680396.1, and *Pirenella asiatica* MZ831985.1. The genetically similar species to the understudied *Tenguella granulate* was *Tenguella granulata* OK350737.1. We employed two phylogenetic techniques (Neighbor Joining and Minimum Evolution) to provide more illustrative phylogenetic relationships. The techniques demonstrated nearly identical relationships with minor variations in support values, and they also proved that (1) species of outgroup formed a separate cluster, and (2) species of genus *Pirenella* or *Cerithideopsilla* formed one main clade (Figs. 4 and 5).

The sequences of *CO1* in *Peronia verruculata* species were run with BLAST/N at NCBI, revealing ten linked Heterobranchia species in addition to the out-group species (*Patella caerulea*, *Patella candei*, and *Patella lugubris*) of Patellogastropoda. We employed two phylogenetic techniques (Neighbor Joining and Minimum Evolution) to provide more illustrative phylogenetic relationships. The methods demonstrated nearly similar relationships with minor variations in support values, and they also proved that (1) species of outgroup formed a separate cluster, and (2) among all ten related Heterobranchia species, the genetically related species to the understudied *Peronia verruculata* were *Peronia verruculata* ON524819.1 and *Peronia persiae* MK312167.1 (Figs. 6, 7).

Two phylogenetic techniques (Neighbor Joining and Minimum Evolution) were used to create the phylogenetic trees among the species of each of the Neritimorpha, Caenogastropods, Heterobranchia, and their related species, as well as the outgroup utilizing the *COI* sequences. The techniques proved highly similar correlations with slight differences in support values, and they additionally demonstrated that (1) species of outgroup formed a separate cluster and (2) species of Neritimorpha, Caenogastropods and Heterobranchia were separated according to their group; in addition, (3) Neritimorpha was a sister of Heterobranchia (Figs. 8, 9).

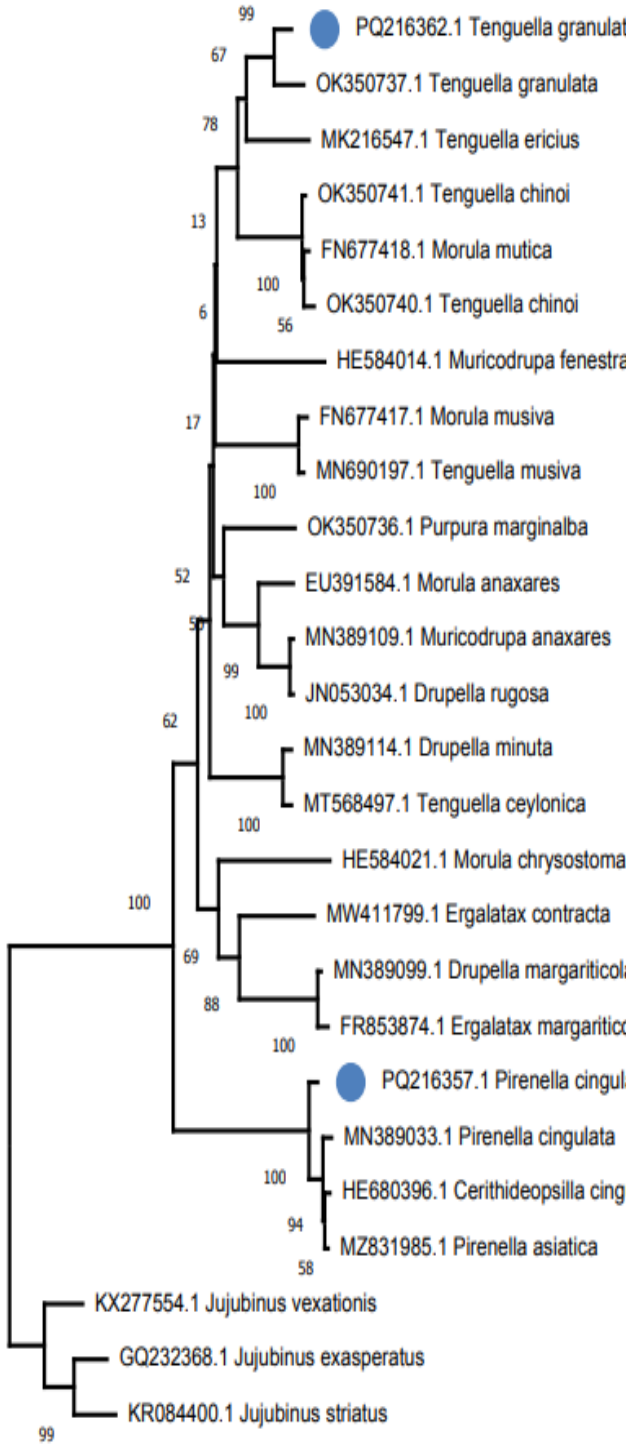


**Fig. 4.** Neighbor Joining phylogenetic tree among *Pirenella cingulate* and *Tenguella granulata* and their related Caenogastropods species; in addition, the outgroup using *COI* gene

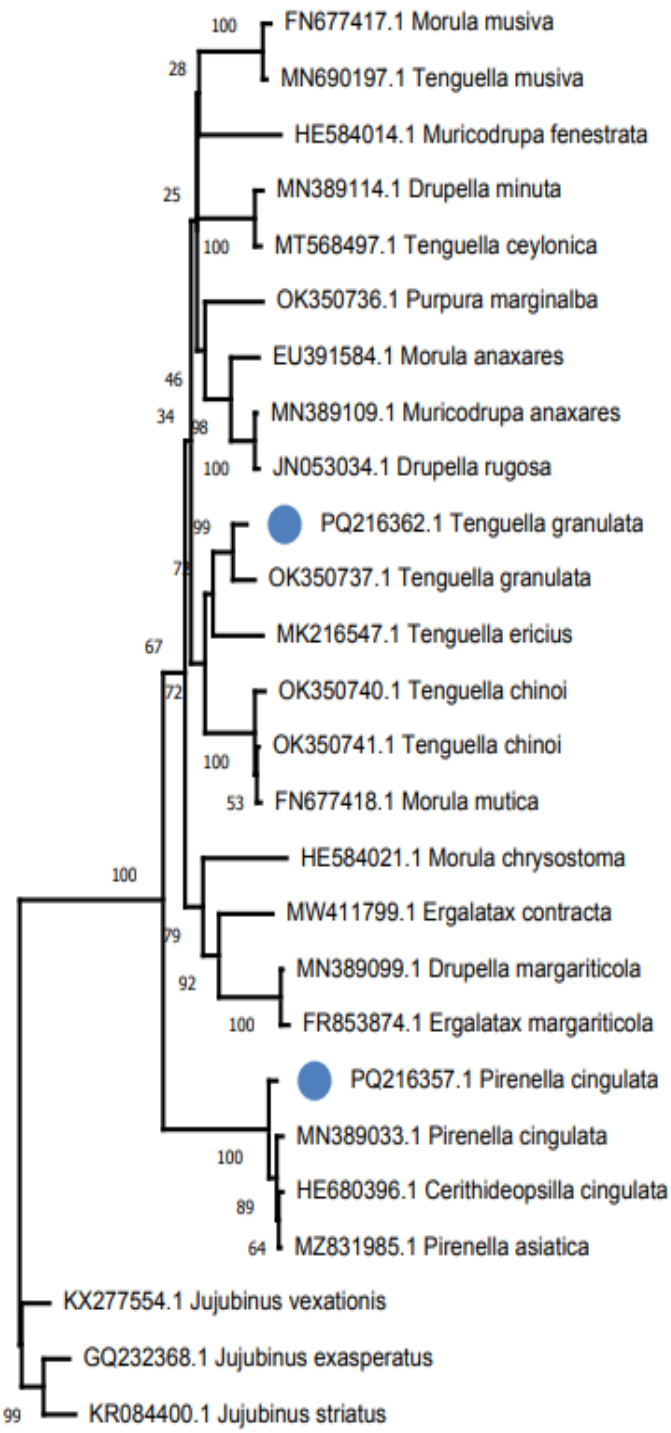


**Fig. 5.** Minimum Evolution phylogenetic tree among Pirenella cingulate and Tenguella *granulata* and their related Caenogastropods species; in addition, the outgroup using *COI* gene

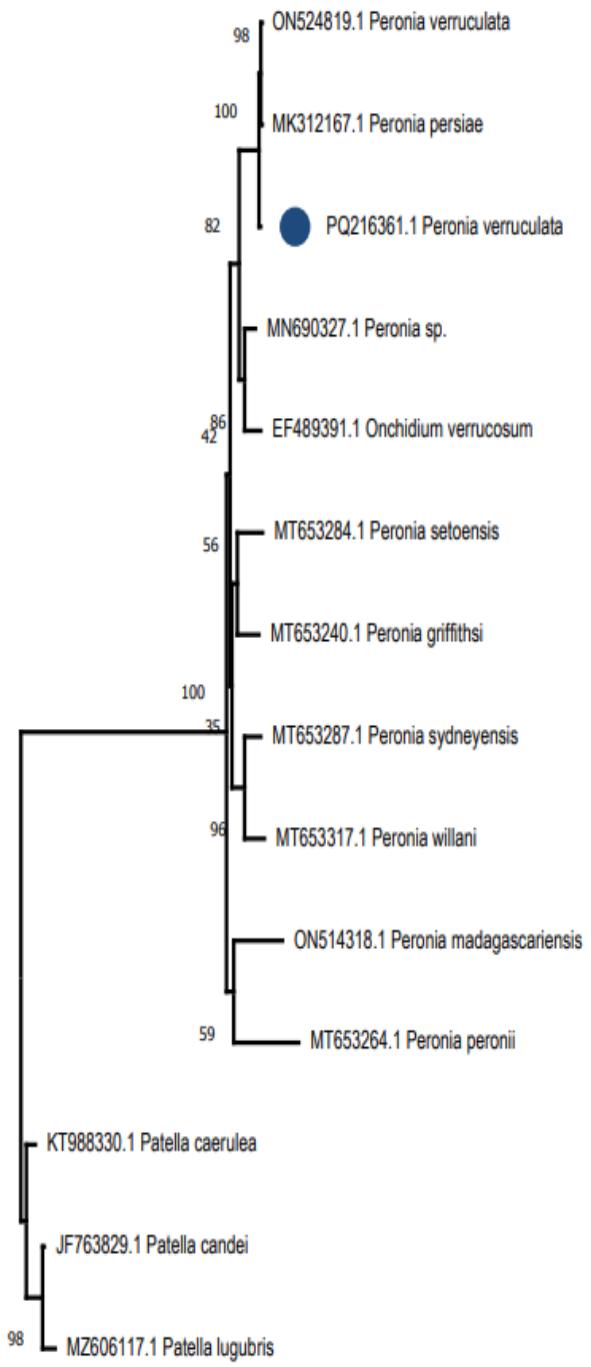


**Fig. 6.** Neighbor Joining phylogenetic tree among *Peronia verruculata* and its related Heterobranchia species; in addition, the outgroup using *COI* gene.

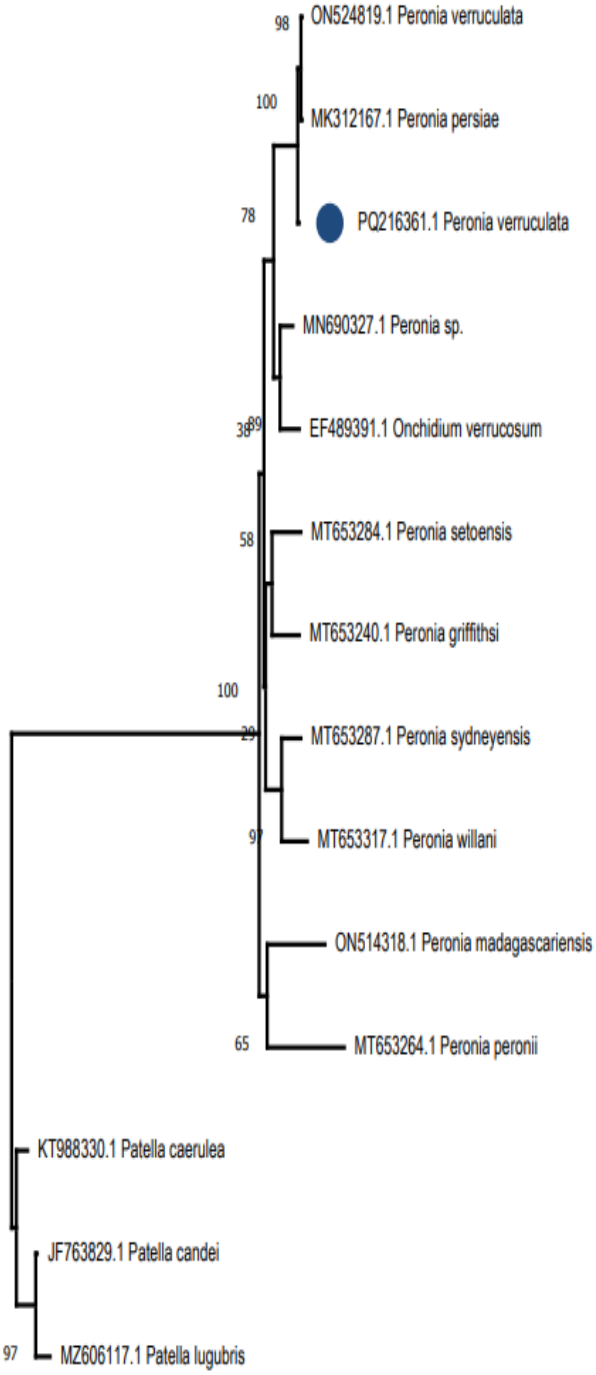


**Fig. 7.** Minimum Evolution phylogenetic tree among *Peronia verruculata* and its related Heterobranchia species; in addition, the outgroup using *COI* gene.

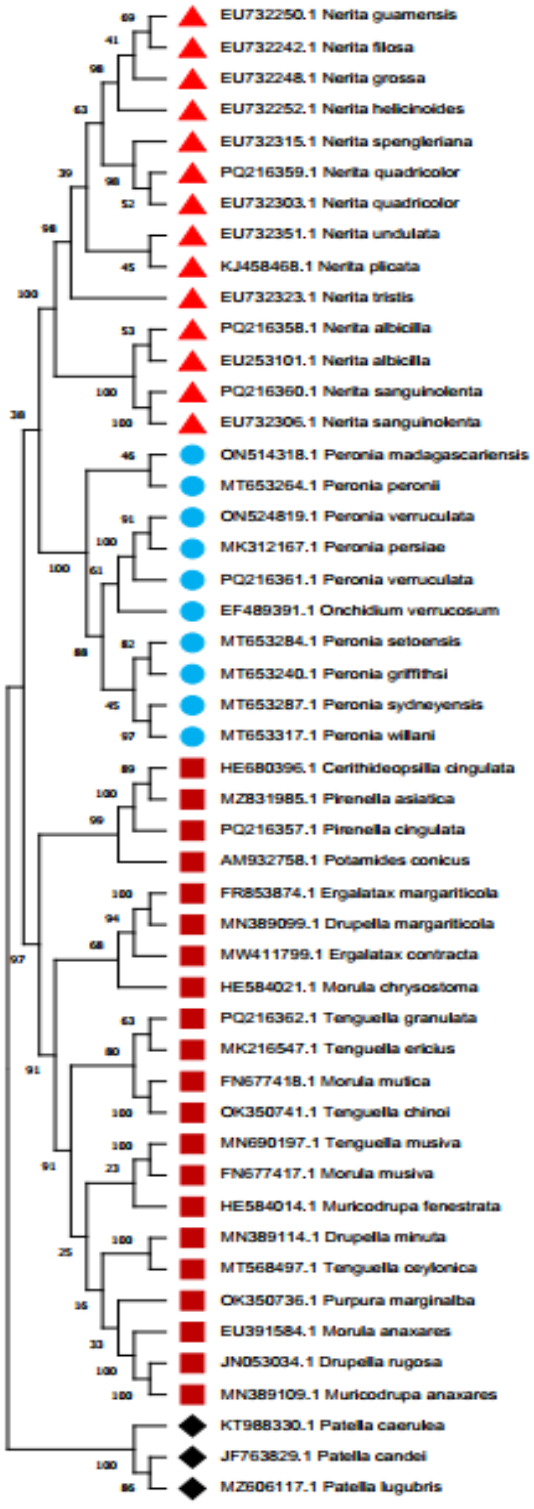


**Fig. 8.** Neighbor Joining phylogenetic tree among Neritimorpha ▲, Caenogastropods ■ and Heterobranchia ● species and their related species; in addition, the outgroup using *COI* gene.

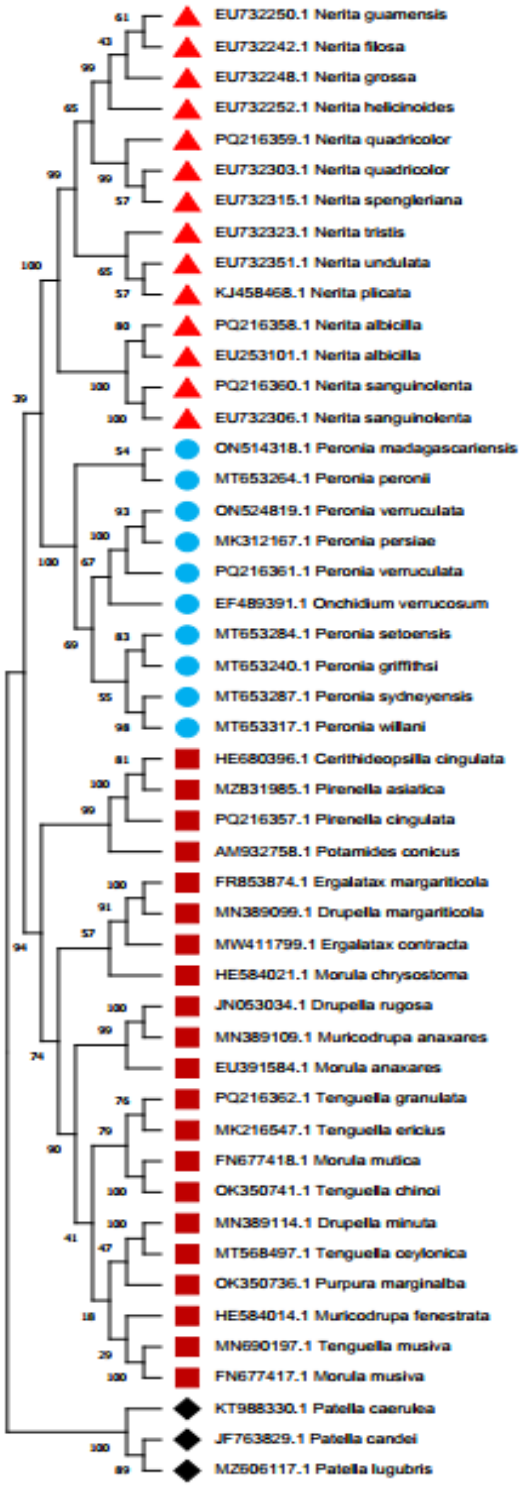


**Fig. 9.** Minimum Evolution phylogenetic tree among Neritimorpha ▲, Caenogastropods ■ and Heterobranchia ● species and their related species; in addition, the outgroup using *COI* gene .

## DISCUSSION

Several marine gastropods have been overexploited due to their significant commercial worth and rampant collection **(Schmidt *et al.*, 2002)**. Gastropods are remarkably a component of biodiversity **(Puillandre *et al.*, 2012; Modica *et al.*, 2014)**. Additionally, they serve as significant sources of aquatic animal protein for humans and possess considerable economic importance **(Leiva & Castilla, 2002)**. Marine gastropods provide a significant component of global invertebrate fisheries. Furthermore, gastropod fisheries significantly contribute to the national economies of numerous countries **(Keegan *et al.*, 2003; Cob *et al.*, 2009)**.

The DNA barcoding technology has effectively facilitated the identification of marine mollusks **(Sun *et al.*, 2012; Barco *et al.*, 2016)**. Conventional morphological identification techniques need an extensive experience and expertise, while the phenotypic plasticity of taxa may result in an erroneous identification **(Wang *et al.*, 2018)**. DNA barcoding technique is not overly dependent on an individual's skills and expertise and can accurately identify species, especially with specimens that are damaged, incomplete, or diverse at various growth stages **(Bingpeng *et al.*, 2018)**.

In addition to being a crucial component of taxonomic study, the categorization and identification of gastropods serve as a foundation for surveys of fisheries resources and assessments of natural resources. Owing to the deficiency of conventional morphological techniques and the decline of taxonomic specialists, it is imperative to develop a molecular approach for species identification **(Ran *et al.*, 2020)**.

In our samples, the A+T content ranged from 59.4 to 64.85 with an average of 61.28%, exceeding the C+G content. These agree with several studies such as **Quintero-Galvis and Castro (2013)**, who found that the mean A+T content was 60.8% in some gastropod species of family Neritidae. **Ran *et al.* (2020)** found higher A+T content in some gastropod species from Hainan island, China.

Representatives of the Neritidae family can be found in tropical and subtropical regions; they have adapted to many environments and display morphological changes in different habitats **(Holthuis, 1995; Kano *et al.*, 2002)**. The two used phylogenetic methods, Neighbor Joining and Minimum Evolution, showed that the species of Neritimorpha were devided into two main clusters; one contains four species; understudied *Nerita albicilla* and *Nerita sanguinolenta* species in addition to *Nerita albicilla* EU253101.1. and *Nerita sanguinolenta* EU732306.1, while the other cluster includes the remaining species.

The *COI* gene sequences were utilized as a barcode for species identification, with the expectation of species barcoding in order to achieve species identification **(Hebert *et***

***al.*, 2003a)**. Due to its great conservation within species and typical pattern of genetic variation amongst various species, the mitochondrial *COI* gene is typically utilized as a species barcode **(Hebert *et al.*, 2003a, b)**. In our study, species of genus *Pirenella* or *Cerithideopsilla* formed a separated clade from the rest species of family. This confirm the ability of *COI* gene to identify and classify the gastropod species. **Ran *et al.* (2020)** reported that, the same gastropod species were clustered together in monophyletic groups with great bootstrap levels, groups, suggesting that *COI* sequence-based DNA barcoding technology could effectively and precisely identify the gastropod species within the investigated taxa.

In the present study, the two phylogenetic techniques (Neighbor Joining and Minimum Evolution) demonstrated that species of each of Neritimorpha, Caenogastropods and Heterobranchia were separated according to their group. Furthermore, Neritimorpha was a sister group to Heterobranchia. **Castro and Colgan (2010)** reported that, Neritimorpha's relations with the other key categories varies between investigations. Molecular analyses indicate that *Neritimorpha* is the sister group to *Apogastropoda*, with moderate bootstrap support in certain analyses **(McArthur & Harasewych, 2003)**. It has also been shown to be related to *Vetigastropoda* in some analyses **(Harasewych & McArthur, 2000)** to *Caenogastropoda* **(Aktipis *et al.*, 2008)**. In some others, it was shown to be in relation with the sub-clade of *Caenogastropoda* **(Colgan *et al.*, 2000; Colgan *et al.*, 2003)**. The only integrated examination of morphological and molecular data also indicates that *Neritimorpha* is the sister group to *Apogastropoda* **(Aktipis *et al.*, 2008)**.

## CONCLUSION

Our results revealed the genetic variation and phylogenetic links of numerous gastropod species using cytochrome c oxidase subunit 1 sequence and confirmed the ability of *COI* gene to identify and classify the gastropod species.

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