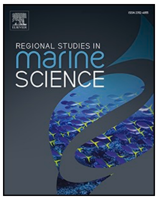
Regional Studies in Marine Science 67 (2023) 103205

Contents lists available at ScienceDirect

Regional Studies in Marine Science

journal homepage: www.elsevier.com/locate/rsma



# Genetic evidence of the unique identity of the West African Mangrove Oyster (*Crassostrea tulipa*) from the Gulf of Guinea

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ARTICLE INFO

*Keywords:*
COI gene
Phylogenetic analysis
Haplotypes
Population structure *Crassostrea tulipa*
Ghanaian Coastal Waters

## ABSTRACT

This research enabled the genetic identification of the West African mangrove oysters, *Crassostrea tulipa,* as well as establishing the evolutionary relationship between it and other Crassostrea species. Essentially, the study assisted in clearing up a long-standing confusion over this species' synonymy with *C. gasar*. Also, the population structure of 60 *C. tulipa* individuals, from three different ecotypes, was analyzed using the mitochondrial cytochrome oxidase I (COI) genes as a marker. Results provided the first genetic sequences for *C. tulipa* and deposited in the GeneBank. Optimal and consensus bootstrap Neighbor-Joining trees distinctively differentiated *C. tulipa* from other Crassostrea species and consistently formed a different clade with *C. gasar*, with no bootstrap value from either NJ, MPT, or UPG trees supporting their similarity. *C. tulipa* sequences occurred as different haplotypes from other Crassostrea sp, with a mutation value as high as 288 and a haplotype diversity of 0.893 between *C. tulipa* and *C. gasar* sequences. High estimates of genetic distance (1.40–1.55) and patristic divergence were recorded between *C. tulipa* and *C. gasar*, in the same range as with seven other species. The study thus reveals the unique identity of *C. tulipa* as genetically distinct from *C. gasar* and other *Crassostrea* species. Based on the population structure analyses from the neutrality test, a low to high haplotype diversity h (0.000–0.963) and low nucleotide diversity $\pi$ (0.00–0.378) were obtained. A negative mean Tajima's D (−0.65247), and a positive Fu's Fs (3.194), suggest rare variations or low-frequency polymorphisms. In addition to serving as the basis for phylogeny, the identification of *C. tulipa* and the recent data on its population structure also serve as the basis for conservation efforts and hatchery-based aquaculture.

## 1. Introduction

Oysters are a group of bivalves adapted to the harsh environment in intertidal zones characterized by strong variations in multiple abiotic factors (Li et al., 2018; Gracey et al., 2008). They are an important fishery resource distributed worldwide (Botta et al., 2020), and have provided food for humans for at least 100,000 years (Baily and Milner, 2008). Oysters are constituted of diverse species of high commercial value, including the mangrove oysters. These species are essential in marine ecosystems as well as in aquaculture programs. Accurate information on the specific identity and the understanding of oyster diversity and evolution are vital for conservation purposes and the effective management of this economically valuable and high-protein aquatic resource (Bay et al., 2017; Vargas et al., 2017). Identification of oysters is generally based on shell characteristics such as form, structure, color, and muscle scar (Ignacio et al., 2000). However, the error margin for this mode of identification is very high, basically because of the lack of distinctive morphological characters and disagreement as well as uncertainty regarding identification, which makes it difficult to identify oysters. In addition, the plasticity in shell morphology, that is, being strongly influenced by environmental conditions contributes to this difficulty (Kenkel and Matz, 2016; Kenkel et al., 2013; Pfennig et al., 2010). Nevertheless, the principal species of oysters of economic interest based on a combination of reproductive data, the presence/absence of a promial chamber and the morphology of the adult shell hinge, have been grouped into the genera Ostrea in the Western Atlantic Ocean (Ignacio

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https://doi.org/10.1016/j.rsma.2023.103205
Received 9 December 2022; Received in revised form 14 September 2023; Accepted 15 September 2023

et al., 2000). However, within the genus *Crassostrea*, there is still much debate as to the actual number of native species that occur on several coasts in South America and Africa (Brunetto et al., 2020; Osei et al., 2021; Morretes, 1949; Absher et al., 1989). A typical example is the *tulipa* species of *Crassostrea* which is believed to be the same as *Crassostrea gasar*.

The confusion surrounding the identity of *C. tulipa* is so immense that, some authors like Osei et al. (2021) in Ghana, and Brunetto et al. (2020) in Brazil, showed both *C. tulipa* and *C. gasar* as belonging to the same species and made it clear in their statements captured" *Crassostrea tulipa* (= *C. gasar*) (Lamarck, 1819)". This was to highlight the fact that, they were referring to the same species under different names. Also, according to FAO (1980), the most prevalent oysters on the West African coast are the mangrove oysters, *Crassostrea gasar* and *Crassostrea tulipa*, (FAO, 1980). This report also noted that "it is not yet clear whether they are different species or belong to the same species, as they show only local variations in taxonomic characters". Hence, it was advised to conduct research on the locally accessible *C. gasar*, which is likely equivalent to the *C. tulipa* described elsewhere on the West African Coast.

However, as of 2018, Guo et al. (2018) indicated that the Crassostreinae family contains roughly 26 species, some of which may be synonymous but have not yet undergone molecular confirmation. This included *Crassostrea tulipa,* and three other species: *Crassostrea cuttackensis, Crassostrea aequatorialis,* and *Crassostrea iredalei*. Thus, despite its exceptional palatability, commercial value, and ecological importance as an aquatic resource, the West African mangrove oysters (*Crassostrea. tulipa*), which are endemic to the coast along the Gulf of Guinea and primarily found on the coast of Ghana and the West African subregion (Asare et al., 2019; Anyinla et al., 2011; Ansa and Bashir, 2007; Yankson, 2004a,b; Afinowi, 1985), have never been genetically described nor confirmed as a species, as no data on this species from the West African coast has been reported.

According to Obodai (1997), Ghana, a West African country bordered on the south, by the Gulf of Guinea, with a coastal area stretching over 550 km, and covering four coastal regions namely Volta, Greater Accra, Central and Western Regions, thus representing the Southern part of Ghana, has 108 coastal water bodies. These comprises closed lagoons, open lagoons, and estuaries with their accompanied mangrove vegetation, mud/tidal flats, and marshes, which support the commercially important shellfisheries. To further tap into these locally significant resident stocks of shellfish, mainly the oysters, with appropriate conservation measures and oyster aquaculture, which is becoming imperative for food security for the region, accurate base information on species identity of local species of oysters, population structure, and species diversity is fundamental and crucial.

Towards achieving this, molecular methods are highly suitable for establishing such specific identity and status among oyster species (Wang et al., 2014; Gusmão et al., 2000). Molecular studies of living oysters have revealed high genetic diversity at species, population, and genome levels in recent studies (Guo et al., 2018). Genetic markers developed have been useful for the rapid and effective identification of oyster species (Klinbunga et al., 2003, 2005; Cordes et al., 2008), and have contributed to the understanding of the true distribution of oysters. Also, according to Hebert et al. (2003), from the "Consortium for the Barcode of Life" (Ratnasingham and Hebert, 2007), divergence in COI sequences consistently facilitates the discrimination of closely allied species in all animal phyla, except the Cnidaria. Thus, this study was undertaken to obtain the molecular identity of *C. tulipa* found in the coastal waters of Ghana using COI genes as a marker, to investigate the evolutionary history as well as establish the taxonomic relationship between it and other species of *Crassostrea*. The long-term objective is for the selection, breeding, and sustainable utilization of *C. tulipa* seed stocks.

## 2. Materials and methods

### *2.1. Study area*

A total of 60 samples, twenty each, were collected from three estuaries from three sites (coastal regions) in Ghana, namely the Densu, Nakwa and Whin (Fig. 1) in March, 2021. Sampling sites were selected based on a two-stage sampling criterion which included geographical isolation (Table 1), and the level of shell-fishing activities. Precisely, sampling locations are noted for oyster harvesting with fishing activities contributing over 50% as a primary occupation.

### *2.2. Sample collection and preparation*

Oysters were taken in a variety of methods at various times at each of the sampling sites due to changes in the substrate's characteristics and tides (but often on sandy-mud sediments, attached to the mangrove roots or other hard objects). Basically, this was done either by diving and hand-picking individual oysters from the lagoon floor, by wading in and cutting the roots of mangrove trees to remove oyster clusters, or mostly during low tides. Oysters were randomly selected from each of the three sampling locations, properly washed to eliminate sand particles, and then transferred to the laboratory (CSIR-Water Research Institute, Biomedical and Public Health Research Unit) on ice. They were kept at 4 °C while pending for further genetic testing. Samples were thawed out in the lab, where the individual weight, shell height, and length were all measured. Shells were split open at the posterior end and shucked into a clean glass beaker using a stainless-steel knife that had been cleaned and sanitized. The adductor muscles of each oyster were weighed, and approximately 10 g were taken for DNA extraction.

### *2.3. DNA extraction, PCR amplification and sequencing*

Genomic DNA extracts were obtained from the adductor muscle tissues of *C. tulipa* using the Quick-DNA Miniprep Plus kit 4068 (Zymo Research, USA). There were however slight modifications of the manufacturer's protocol, to equally obtain higher yield and DNA quality, which was determined by a spectrophotometer. The mitochondrial cytochrome c oxidase subunit I (COI) of *C. tulipa* was targeted for amplification and sequencing following standard protocols and using the forward primer, LCOC-CG-1490 (5′-TGTCAACAAATCATTTAGACATTGG-3′) and reverse HCOC-CG-2190 (5″TACTTGA CCAAAAACATAAGACATGA-3′) described previously by de Melo et al. (2010), and Folmer et al. (1994). Each PCR was performed in 10 μl reaction volume containing 5 μl Syber green master mix, 0.2 μl of each of the forward and reverse primer, 1.6 μl of Milli-Q water and 3 μl of template DNA. PCR was performed in a peQlab thermal cycler using the following amplification conditions. An initial denaturing at 95 °C for 3 min; 35 cycles of 1 min at 95 °C, 1 min at 45 °C and 90 s at 72 °C, followed by a final extension at 72 °C for 7 min. The PCR products were sequenced by Inquaba Company (Inquaba, South Africa) using Sanger sequencing. BioEdit was used to edit chromatographs of our sequencies to ensure accuracy. The sequence results were analyzed using BLAST in the NCBI server, and other oyster species were identified and retrieved according to the BLAST results of individual COI sequences.

### *2.4. Sequencing/Haplotype analysis*

Sequencing of the COI gene of *C. tulipa* was to highlight the phylogenetic relationship between it and the other *Crassostrea* species retrieved from GenBank. Inclusion of such sequences was based on their close identity to *C. tulipa,* and was set at a minimum identity of 95%. The scientific names and GenBank sequence accession numbers of oysters compared in the present study are described in Table 2.

**Fig. 1.** Location of oyster sampling sites included in this study.

**Table 1**
GPS locations of coastal sites where samples were collected.

| Population | Code | Location | Latitude | Longitude |
|---|---|---|---|---|
| Densu | Den | Greater Accra | 06°36.984′N | 000°10.724′E |
| Nakwa | Nak | Central Region | 06°41.203′N | 000°17.651′E |
| Whin | Whi | Western Region | 06°05.918′N | 000°09.019′E |

Two major sets of sequence analysis were carried out. For the analysis 1, 22 COI sequence of *Crassostrea* species, and 2 Ostrea sp. sequence which served as an outgroup, in addition to our five sequences from this study, were used. Thus, a total of twenty-seven sequences were analyzed. All sequences (apart from our *C. tulipa* sequences) were obtained from the NCBI GenBank platform. The 22 COI sequence of Crustacea species together with our sequences of *C. tulipa,* as well as that of the outgroups with their accession numbers from GenBank can be seen under Table 2. Analysis 1 was carried to ascertain the difference between our sequences and other important Crassostrea species from different sources and geographical locations. Analysis II was done to ascertain the genetic difference that exist between our sequences of *C. tulipa* and several other sequences of *C. gasar* species. The *C. gasar* sequences used were of the following accession numbers; FJ717611.1, HM003520.1, HM003503.1, HM003499.1 and HM003524.1 (Table 2).

Alignment of the sequences was done by ClustalW (Thompson et al., 1994) using Molecular and Evolutional Genetic Analysis software MEGA X (Kumar et al., 2018). Only distinct COI sequences of *C. tulipa* were aligned with sequences of other species obtained from the GenBank. The same software was used to generate phylogenetic tree from the nucleotide sequences. The best nucleotide substitutional method was determined by computing the minimum theoretical Akaike information criterion (AIC), corrected minimum theoretical Akaikei information criterion (AICc), Bayesian information criterion (BIC), and using the Hierarchical likelihood ratio test with a confidence level of 0.01 (Schwarz, 1978; Sakamoto et al., 1986; Frati et al., 1997; Huelsenbeck and Crandall, 1997; Posada and Crandall, 1998). Estimates of average evolutionary divergence over all sequence pairs were also estimated showing the number of base substitutions per site from averaging over all sequence pairs. Analyses were conducted using the Maximum Composite Likelihood model. This analysis involved 27 nucleotide sequences, with all ambiguous positions removed for each sequence pair (pairwise deletion option). The evolutionary analyses was inferred by using the Neighbor-Joining (NJ) method based on the Tamura 3-parameter model (Tamura and Nei, 1993) and conducted in MEGA X (Kumar et al., 2018). Bootstrap consensus trees by the Maximum Likelihood Tree (ML), UPGMA Tree method and Maximum Parsimony analysis (MP) of taxa were also used to obtain supporting bootstrap values on the relationship among sequences. Haplotype distribution pattern, neutrality test (Tajima, 1989) and polymorphic patterns amongst the *C. tulipa* populations and the other species retrieved were also generated using the DNA sequence polymorphism software (DNAsp 4.) and the POPART V1.7 software (Bandelt et al., 1999), (http://popart.otago.ac.nz), to further confirm the interrelationship among species. Haplotype networks were constructed based on minimum spanning network. The network estimation was run at 95% probability limit.

## 3. Results

### 3.1. *Morphological and general genetic information*

The sizes of the oysters used for this research work ranged from small to big, but were matured in all cases. A mean maximum shell height of 123 ± 0.20 mm and a minimum of 20 ± 0.05 mm was recorded and that of the shell length had a maximum of 79 mm and a minimum of 16 mm. Morphologically, the shells of *C. tulipa* samples obtained were quite variable depending on substrate, but mostly with roundish, or oval shape and elongated, with extensive fluting, and rough surface by irregular growth lamellae, as well as with irregular margins. Also, very

**Table 2**
GenBank accession numbers of COI sequences of species used in the analyses.

| Group | Species/description | GeneBank accession number | References | Location |
|---|---|---|---|---|
| Crassostrea | *Crassostrea rhizophorae* | JZ189401.1 | Americo et al. (2013) | Brazil |
| | *Crassostrea rhizophorae* | JZ189412.1 | Americo et al. (2013) | Brazil |
| | *Saccostrea_palmula* | FJ527304.1 | Gutierrez-Rivera et al. (2016) | Mexico |
| | *Saccostrea_palmula* | KT317603.1 | Raith et al. (2015a, b) | California (USA) |
| | *Crassostrea columbiensis* | KP455017.1 | Pagenkopp Lohan et al. (2015) | USA |
| | *Crassostrea madrasensis* | MN583310.1 | Suzana and Siti Azizah (2011) | Malaysia |
| | *Crassostrea iredalei* | JF915503.1 | Suzana and Siti Azizah (2011) | Malaysia |
| | *Crassostrea nippona* | LC005437.1 | Ozawa (2014) | Japan/Philippines |
| | *Crassostrea hongkongensis* | KP976208.1 | Shen et al. (2015) | China |
| | *Crassostrea belcheri* | JF915511.1 | Suzana and Siti Azizah (2011) | Malaysia |
| | *Crassostrea angulata* | KU933400.1 | Chiesa et al. (2016) | Portugal |
| | *Crassostrea ariakensis* | FJ743527.1 | Jung et al. (2009) | South Korea |
| | *Crassostrea dianbaiensis* | LC120781.1 | Hamaguchi et al. (2016) | Japan |
| | *Crassostrea gryphoides* | FJ262985.1 | Trivedi et al. (2008) | India |
| | *Crassostrea gigas* | KF644048.1 | Layton et al. (2008) | Canada |
| | *Crassostrea virginica* | KF644323.1 | Layton et al. (2014a, b) | Canada(eastern oyster) |
| Out group | *Ostrea sp.* | JF915514.1 | Suzana and Siti Azizah (2011) | Malaysia |
| | *Ostrea edulis* | KX713488.1 | Combosch et al. (2016) | USA |
| Our sequences | *Crassostrea tulipa*_Densu_7 | OM372500.1 | | |
| | *Crassostrea tulipa*_Nakwa_24 | OM372501.1 | Diyie and Armah (2022) | |
| | *Crassostrea tulipa*_Nakwa_36 | OM372502.1 | | |
| | *Crassostrea tulipa*_Whin _10 | OM372503.1 | | |
| | *Crassostrea tulipa*_Whin_11 | OM372504.1 | | |

similar to Crassostrea species, the adductor muscle impressions were as well mostly closer to the ventral margin than the hinge, with cupped lower valves.

A total of five sequences were obtained from the samples collected from three different geographical locations in Ghana along the Gulf of Guinea. Results from this study thus provide for the first time, genetic sequences for *Crassostrea tulipa* which has been deposited at the GeneBank under the accession numbers OM372500.1 (Crassostrea tulipa_-Densu_7), OM372502.1 (*Crassostrea tulipa*_Nakwa_36), OM372503.1 (*Crassostrea tulipa*_Whin _10), and OM372504.1 (*Crassostrea tulipa*_-Whin_11). These *C. tulipa* sequences formed one haplotype. The 27 different sequences of Crassostrea used in the study generated 22 haplotypes.

### 3.2. Multiple sequence alignment: nucleotide sequence analysis of *C. tulipa* and other Crassostrea species

An alignment of our sequence together with twenty-two other COI sequences retrieved from the NCBI platform, showed a clear and distinct difference in the nucleotide alignments among same species and between different species (Suppl. 1). Major similarities were found amongst the *C. tulipa* sequences. These similar sites were however well differentiated from the other sequences by the alignment pattern observed (Suppl. 1).

### 3.3. Estimates of evolutionary divergence between sequences

The genetic distances among all the samples ranged from 0.00 to 3.45, with an overall mean value of 1.08 (Table 3). Amongst the sequences for *C. tulipa,* no distances were observed at 0.00. Likewise, there was no or little divergence between *C. gasar* and *C. brasillinia* at 0.01 and 0.00. However, relatively high genetic distances were found between our sequences and the other sequences retrieved, ranging from 1.400 and 3.918. The genetic distances amongst the other sequences were also relatively shorter, between 0.02 and 0.358 than they were to the *C. tulipa* sequences (Table 3). Genetic distance between *C. tulipa* sequences and the 'supposedly' synonymous species *C. gasar* sequences ranged between 1.40–1.55, in the same range as with 7 other species (*C. madrasensis*, *C. nippona*, *C. dianbaiensis*, *C. angulata*, *C. virginica, C. iredalei*, and *C. brasiliana)*. The species with lowest genetic distance (0.28) from *C. tulipa* species was *C. gryphoides*

### 3.4. Molecular phylogenetics from COI gene sequences

The evolutionary history inferred by the Neighbor-Joining (NJ) method generated a dendogram (Fig. 2a), which showed three main clusterings. A bootstrap consensus tree (Fig. 2b), clearly showed the several subclusters under it, with *Ostrea sp* as true outgroups. The 1st cluster (Fig. 2a), at the top of the tree, consisted of almost all the other sequences and was further divided into four subclusters. The first subcluster was made of 7 species of Crassostrea, the second subcluster was made up of sequences; *C. dianbiansis, C. iredalei, C. madresensis.* The third subcluster consisted of two sequences of *C.gasar,* and *C. brasiliana,* on the same node with a bootstrap value of 83%, and together with *C. columbiensis* at 81% boostrap value. The fourth subcluster had 3 sequences; of the outgroups (*Ostrea sp., Ostrea edulis, Saccostrea palmula KT317603.1*), representing different genus from the true Crassostrea sp and diverged from all the true Crassostrea species at 81% (Fig. 2a). *C. virginica* also formed a divergent subcluster here at 63% bootstrap value. The mid or 2nd cluster consisted of our sequences; *C. tulipa* (Densu), *C. tulipa* (Nakwa 24), *C. tulipa* (Nakwa 36), *C. tulipa* (Whin 10) and *C. tulipa* (Whin 11), with *C. gryphoides* forming a divergent (79%) subclass under it. The other Crassostrea species sequences at the top cluster diverged from the *C. tulipa* sequences with a bootstrap value of 90%. The third cluster consisted of the 2 sequences of *C. rhizophora sp.* and *Saccostrea palmula* FJ527304.1. Sequences of *C. tulipa* from the various locations in Ghana, Nakwa, Densu and Whin forming a separate cluster from the other species suggests that the *C. tulipa* sequences are genetically similar to each other than they are to the GenBank downloaded sequences. Again, a booststrap concensus tree generated from three different evolutionary analysis, ML, UPGA and MP consistently differentiated between *C. tulipa* sequences and *C. gasar* as well as the other species, by concisely placing them on different clades, with no bootstrap values supporting their similarity (Supplementary 2). Among the *C. tulipa* sequences however, Densu and Whin 11 populations were more identical to each other, whereas Nakwa 36 and 24 were also close to Whin 10, than they were to Densu population. This was evident on the phylogenetic tree as the first two were on the same node, as a subcluster, with a bootstrap value of 87%, whiles Nakwa populations were on a different node with a divergent bootstrap value of 86% (Fig. 2a).

**Table 3**

Pairwise patristic distances between sequences of 27 COI sequences of oyster species.

| | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Crassostrea_Tulipa_Densu_7 | **0.00** | | | | | | | | | | | | | | | | | | | | | | | | |
| Crassostrea_Tulipa_Nakwa_24 | **0.00** | | | | | | | | | | | | | | | | | | | | | | | | |
| Crassostrea_Tulipa_Nakwa_36 | **0.00** | | | | | | | | | | | | | | | | | | | | | | | | |
| Crassostrea_Tulipa_Whin_10 | **0.00** | **0.00** | | | | | | | | | | | | | | | | | | | | | | | |
| Crassostrea_Tulipa_Whin_11 | **0.00** | **0.00** | **0.01** | | | | | | | | | | | | | | | | | | | | | | |
| Crassostrea_Gasar_FJ717611.1 | **1.55** | **1.55** | **1.53** | **1.55** | | | | | | | | | | | | | | | | | | | | | |
| Crassostrea_Brasiliana_FJ7176 | **1.55** | **1.55** | **1.53** | **1.55** | **0.00** | | | | | | | | | | | | | | | | | | | | |
| Saccostrea_Palmula_KT317603. | **1.85** | **1.85** | **1.85** | **1.85** | **1.71** | **1.71** | | | | | | | | | | | | | | | | | | | |
| Saccostrea_Palmula_KT317603. | **1.77** | **1.76** | **1.75** | **1.75** | **0.31** | **0.30** | **1.41** | | | | | | | | | | | | | | | | | | |
| Crassostrea_Sikamea_EU816012 | **1.56** | **1.56** | **1.56** | **1.56** | **0.23** | **0.23** | **1.50** | **0.32** | | | | | | | | | | | | | | | | | |
| Crassostrea_Columbiensis_KP45 | **1.63** | **1.61** | **1.61** | **1.60** | **0.18** | **0.19** | **1.56** | **0.30** | **0.26** | | | | | | | | | | | | | | | | |
| Crassostrea_Rhizophorae_JZ18 | **2.47** | **2.51** | **2.54** | **2.48** | **2.28** | **2.28** | **1.76** | **2.09** | **2.19** | **2.24** | | | | | | | | | | | | | | | |
| Crassostrea_Rhizophorae_JZ18 | **2.63** | **2.63** | **2.63** | **2.63** | **3.37** | **3.37** | **1.32** | **2.85** | **3.45** | **3.74** | **2.86** | | | | | | | | | | | | | | |
| Crassostrea_Madrasensis_MN58 | **1.48** | **1.47** | **1.47** | **1.46** | **0.26** | **0.26** | **1.48** | **0.30** | **0.19** | **0.27** | **2.06** | **3.02** | | | | | | | | | | | | | |
| Crassostrea_Iredalei_JF915503 | **1.51** | **1.50** | **1.50** | **1.50** | **0.26** | **0.26** | **1.43** | **0.29** | **0.18** | **0.27** | **2.03** | **3.07** | **0.02** | | | | | | | | | | | | |
| Crassostrea_Nipona_LC005437 | **1.55** | **1.55** | **1.55** | **1.52** | **0.26** | **0.26** | **1.32** | **0.32** | **0.16** | **0.27** | **2.19** | **3.07** | **0.17** | **0.17** | | | | | | | | | | | |
| Crassostrea_Gasar_HM00352 | **1.40** | **1.40** | **1.40** | **1.40** | **0.01** | **0.00** | **1.71** | **0.33** | **0.22** | **0.19** | **2.24** | **3.41** | **0.26** | **0.26** | **0.27** | | | | | | | | | | |
| Crassostrea_Hongkongensis_K | **1.67** | **1.66** | **1.66** | **1.63** | **0.26** | **0.26** | **1.56** | **0.29** | **0.15** | **0.27** | **2.06** | **2.92** | **0.18** | **0.17** | **0.12** | **0.27** | | | | | | | | | |
| Crassostrea_Belcheri_JF915511 | **1.63** | **1.61** | **1.61** | **1.61** | **0.28** | **0.28** | **1.56** | **0.30** | **0.16** | **0.28** | **2.30** | **3.26** | **0.17** | **0.18** | **0.18** | **0.29** | **0.18** | | | | | | | | |
| Crassostrea_Angulata_KU93340 | **1.53** | **1.53** | **1.53** | **1.53** | **0.26** | **0.26** | **1.43** | **0.29** | **0.11** | **0.29** | **2.14** | **3.12** | **0.18** | **0.18** | **0.17** | **0.27** | **0.14** | **0.18** | | | | | | | |
| Crassostrea_Irakensis_FJ7435 | **1.45** | **1.41** | **1.41** | **1.40** | **0.26** | **0.26** | **1.32** | **0.35** | **0.25** | **0.26** | **2.03** | **3.45** | **0.25** | **0.27** | **0.24** | **0.25** | **0.26** | **0.26** | **0.25** | | | | | | |
| Crassostrea_Dianbaiensis_LC12 | **1.55** | **1.54** | **1.54** | **1.52** | **0.27** | **0.27** | **1.48** | **0.32** | **0.16** | **0.27** | **2.28** | **3.02** | **0.18** | **0.17** | **0.15** | **0.27** | **0.14** | **0.17** | **0.17** | **0.29** | | | | | |
| Crassostrea_Gryphoides_FJ262 | **1.56** | **1.54** | **1.54** | **1.54** | **0.26** | **0.26** | **1.53** | **0.30** | **0.18** | **0.25** | **2.04** | **3.00** | **0.13** | **0.13** | **0.19** | **0.26** | **0.17** | **0.17** | **0.18** | **0.26** | **0.16** | | | | |
| Crassostrea_Gigas_KF644048.1 | **0.28** | **0.28** | **0.28** | **0.28** | **1.62** | **1.61** | **2.17** | **1.73** | **1.76** | **1.72** | **2.54** | **2.26** | **1.68** | **1.74** | **1.72** | **1.58** | **1.78** | **1.71** | **1.62** | **1.49** | **1.73** | **1.78** | | | |
| Crassostrea_Virginica_KF6443 | **1.62** | **1.61** | **1.60** | **1.60** | **0.26** | **0.26** | **1.41** | **0.30** | **0.11** | **0.29** | **2.18** | **3.13** | **0.18** | **0.18** | **0.16** | **0.27** | **0.13** | **0.17** | **0.03** | **0.25** | **0.15** | **0.19** | **1.72** | | |
| Ostrea_sp._JF915514 | **1.55** | **1.51** | **1.51** | **1.49** | **0.30** | **0.31** | **1.48** | **0.26** | **0.29** | **0.30** | **1.81** | **3.69** | **0.30** | **0.31** | **0.28** | **0.30** | **0.31** | **0.32** | **0.32** | **0.34** | **0.30** | **0.30** | **1.60** | **0.30** | |
| Ostrea_Edulis_KX713488.1 | **1.49** | **1.49** | **1.49** | **1.49** | **0.34** | **0.34** | **1.54** | **0.24** | **0.30** | **0.33** | **2.04** | **3.09** | **0.28** | **0.29** | **0.29** | **0.34** | **0.30** | **0.29** | **0.32** | **0.31** | **0.30** | **0.27** | **1.78** | **0.31** | **0.10** |

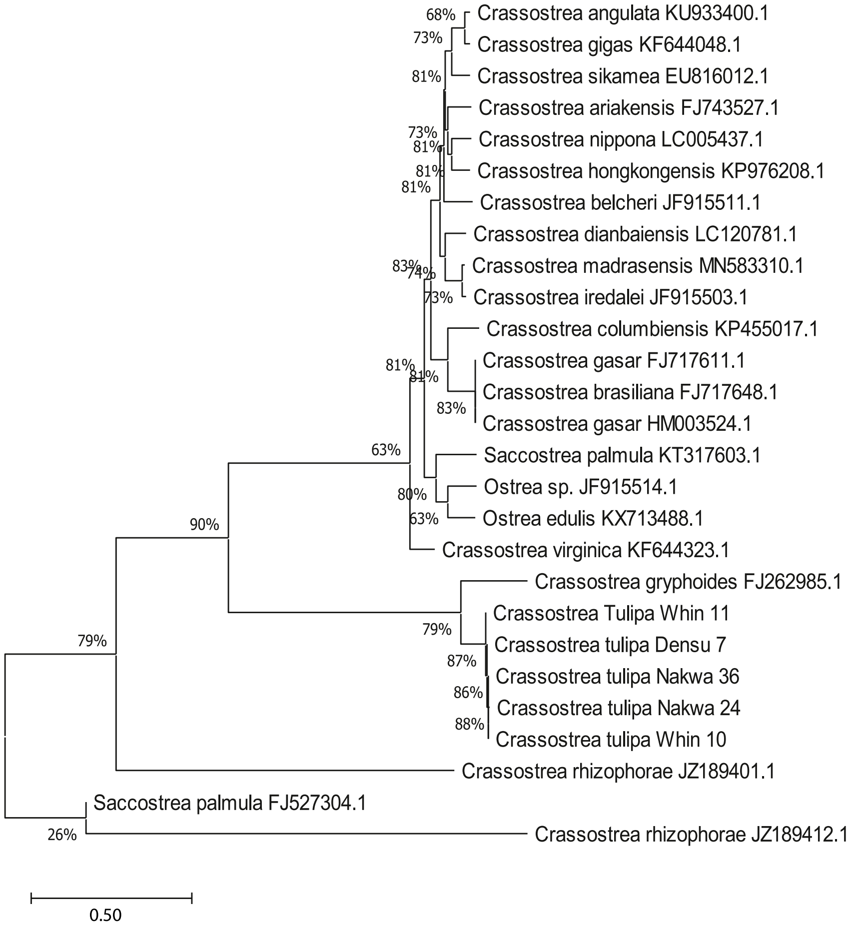

**Fig. 2a.** A Neighbor-Joining (NJ) phylogenetic tree of oysters constructed with 31 mitochondrion COI sequences to establish the evolutionary relationships. (Saitou and Nei, 1987).

## 3.5. *Haplotype analysis*

### 3.5.1. *Analysis 1: Haplotype network of C. tulipa and other Crassostrea species*

A haplotype network using minimum spanning network and Epsilon of 0 in Popart 1.7 (Bandelt et al., 1999), was produced from these analysis with each circle in the haplotype network (Fig. 3) corresponding to one haplotype and the size, proportional to its frequency among the samples. Colors of the circles correspond to each individual oyster sequence or sampling locations. Haplotypes 1 and 2 were shared by our five *C. tulipa* sequences whiles each of the other 22 sequences harbored one haplotype each. The number of haplotypes generated in this study was thus 21. The haplotype diversity *h,* recorded a mean value of 0.963. The mean of the Tajima's D analysis was −0.65247, whiles the Fu's F had a mean value of 3.194 (Table 4). The nucleotide diversity parameters recorded a total of 797 nucleotide sites, out of which 201 sites were viable (Table 5). The nucleotide diversity (Pi) $\pi$, and the total number of mutations (Eta) were 0.379 and 402 respectively (Table 5). The standard deviation and standard error of the nucleotide diversity were 0.04208 and 0.0017706 respectively, whiles the average number of nucleotide difference, k, was 87.111. Whereas the invariable monomorphic sites were very low recording a value of 3, with 20 Singleton variable sites, the Parsimony informative sites were very high at 181 and 179 from both DNAsp and popArt analysis respectively (Table 6). AMOVA showed high between population variations than within populations for *C. tulipa* populations, with *Fst* value showing no significant differentiation.

## 3.6. *Analysis II: Haplotype analysis of C. tulipa and C. gasar sequences*

A total of five more other COI nucleotide sequences of *C. gasar*; (*C. gasar* FJ717611.1 *C. gasar* HM003520.1 *C. gasar* HM003503.1 *C. gasar* HM003499.1 and *C. gasar* HM003524.1) were obtained from NCBI platform and aligned with 3 of our sequences, representing each sampling site; *C. tulipa* Den 1(OM372500.1), *C.* tulipa Nar2 (OM372502.1) and *C. tulipa* Whi1(OM372503.1), to ascertain if they could conceivably align with any of the published sequences of *C. gasar* from different geographical locations. The resulting haplotype network (Fig. 4), produced a total of six haplotypes with a haplotype diversity *h* of 0.893 and a nucleotide diversity $\pi$, of 0.298. The Tajima's D and Fu's F values were 2.099 and 7.708 respectively. The total number of mutations, Eta was 291 whiles the number of nucleotide sites were 762. Of the six haplotypes generated, one haplotype, Hap 6, was harbored solely by our sequences (*C. tupila* Densu, *C. tulipa* Nakwa and *C. tupia* Whin). Phylogenetic analysis showed few distinctions among all *C. tulipa* sequences, although not significant per AMOVA and *Fst* value. Since all sequences were not used in the 2nd analysis, those included formed one haplotype, thus, confirming *Fst* results of no significant differentiation. The other five haplotypes, Haps 1 to 5 where each occupied by one of the five *C. gasar* sequences. Specifically, Haplotypes 1, 2, 3 4 and 5 was shared solely by *C. gasar* (FJ717611.1), *C. gasar* (HM003520), *C. gasar* (HM03503.1), *C. gasar* (HM0034991-.1) and C. *gasar* (HM003524.1) respectively (Fig. 4). No single haplotype was shared by our sequences and the other *C. gasar* sequences. The mutations amongst the haplotypes were 1 and 288. The highest mutations of 288, were observed between Hap 6 (*C. tulipa* sequences) and Hap 1 (*C. gasar* FJ717611.1), and also between our sequences and Hap 2 (*C. gasar*

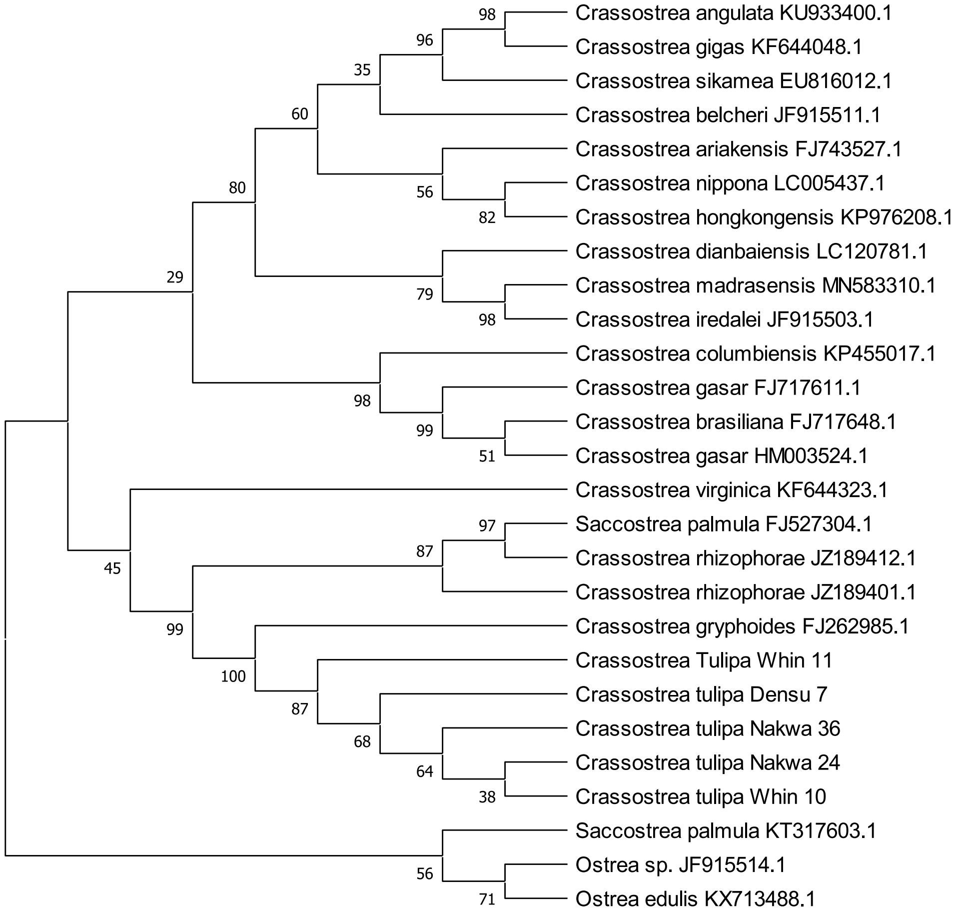

**Fig. 2b.** Bootstrap consensus tree by Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985).

**Table 4**
Haplotype neutrality tests of COI sequences of oysters.

| Haplotype parameters | All populations | *C. tulipa* populations |
|---|---|---|
| Number of haplotypes (h) | 21 | 1 |
| Haplotype diversity (Hd) | 0.9630<br>($P < 0.05$) | 0.00 |
| Variance of haplotype diversity | 0.00068 | |
| Standard deviation of haplotype diversity | 0.026 | |
| Fu's F statistic | 3.194 | |
| Tajima's D | −0.65247<br>($P < 0.05$) | |
| Strobeck's S statistic | 0.106 | |

**Table 5**
Nucleotide tests.

| Haplotype parameter | Mean (All populations) | Mean (*C. tulipa populations)* |
|---|---|---|
| Number of viable sites | 201 | |
| Number of total nucleotide sites | 797 | |
| Nucleotide diversity (Pi) from popart | 0.379 | 0.00 |
| Nucleotide diversity (Pi) from DNAsp | 0.427 | |
| Total number of mutations, Eta | 402 | |
| Total number of singleton mutations, Eta(s) | 124 | |
| Standard deviation of nucleotide diversity | 0.04208 | |
| Standard variance of nucleotide diversity | 0.0017706 | |
| Average number of nucleotide difference, k | 87.111 | |

**Table 6**
Polymorphic sites.

| Sites with alignment gaps or missing data | 593 |
|---|---|
| Invariable (monomorphic) sites | 3 |
| Singleton variable sites | 20 |
| Parsimony informative sites from DNAsp | 181 |
| Parsimony informative sites from popArt | 179 |
| **AMOVA** | |
| Fixation index (*Fst*) | 0 ($p > 0.05$) |

HM003520). However, among all the other *C. gasar* sequences, only a mutation value of one was recorded between them, suggesting strong similarity.

## 4. Discussion

### 4.1. Genetic identity and diversity

It is well recognized that environmental factors have a significant role in the difficulty of morphologically identifying Crassostrea species (Lam and Morton, 2003; Sheng et al., 2021). This, together with the enormous diversity and complex history of introduction of oysters and their seedlings, has led to numerous disputes regarding the identity of each species (Liu et al., 2021; Reece et al., 2008). The dominant and indigenous species are unsure if they are different or the same in certain areas. Therefore, molecular analysis was required to determine the

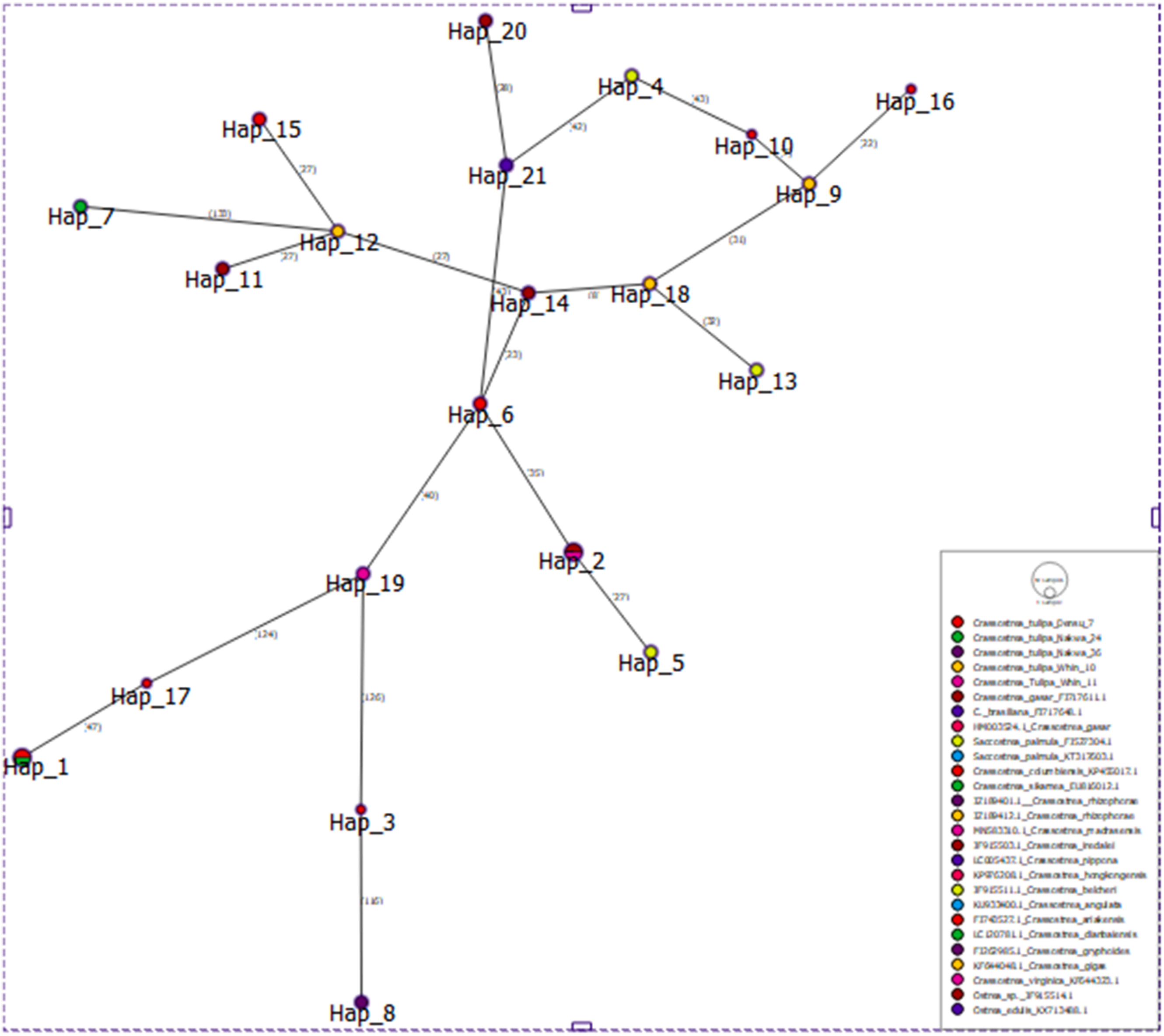

**Fig. 3.** Haplotype network of cytochrome oxidase 1 gene sequences. (Each circle in the network (Fig. 3), corresponds to one haplotype, and the size is proportional to its frequency among the samples. Colors of the circles correspond to oyster sampling locations and sequences downloaded from the NCBI platform). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

precise identity of the Ghanaian stocks of Crassostrea species and to compare them with other species of the same genus, in particular, to confirm the possibility of *C. tulipa* and *C. gasar* being different or synonyms due to their similar morphological characteristics. Thus, this study genetically describes the West African Mangrove Oysters, *C. tulipa*, endemic to the southern coast of Ghana.

Given that DNA sequencing is ideal for establishing species identity and phylogenetic analysis, it was the most reliable method utilized in this investigation (Wang and Guo, 2008), especially for the uncharacterized genetic *C. tulipa* species found on Ghana's coast. Additionally, since the cytochrome oxidase C subunit I (COI) gene is a highly-preferred genetic marker with highly conserved protein-coding genes in the mitochondrial genome of mammals, targeting it for sequencing was suitable (Folmer et al., 1994). They are frequently used to identify taxa and analyze the genetic diversity of mollusks (Hsiao et al., 2016; Sekino et al., 2016; In et al., 2017; Nowland et al., 2018; Özcan Gökçek et al., 2020; Tan et al., 2020; Melo et al., 2021). With the already existing sequences from earlier research that had been deposited at the NCBI database, sequence analysis also assisted with the financial and time benefits of having to collect samples from multiple geographic regions to incorporate in the analysis.

Blast analysis using sequenced results also helped in the discovery that sequences of the tulipa species of Crassostrea are not yet available on any genetic database and have no 100% identity to any other species bearing the same name. The lack of genetic information on these species along the West African Coast was therefore confirmed by this study. As a result, the sequences from this study have been registered and deposited on the NCBI website and could be retrieved under the accession numbers; OM372500.1, OM372501.1 OM372502.1 OM372503.1 for references.

ClustalW alignment of our sequences showed significantly distinct patterns in comparison with the other species of Crassostrea (Suppl. 1), retrieved from GenBank and selected for analysis based on their morphological similarity, economic importance, and geographical distributions. Particularly, the nucleotide alignment patterns of *C. tulipa* sequences were very different to those of *C. gasar* which is recorded as

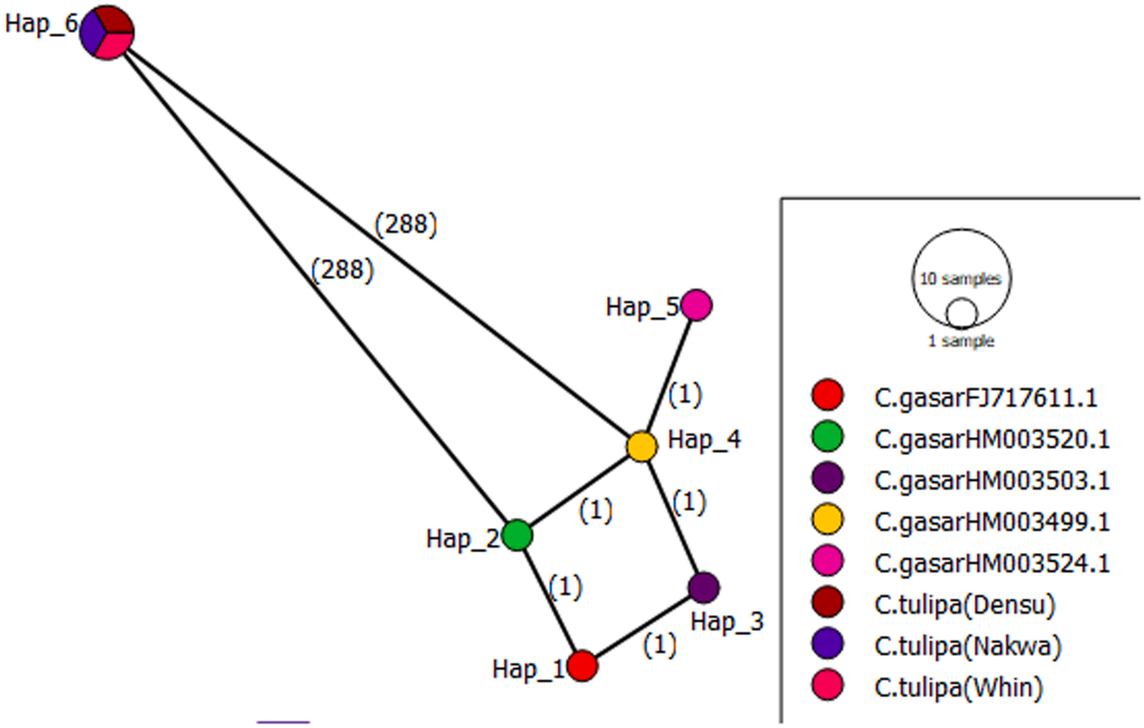

**Fig. 4.** Haplotype network of cytochrome oxidase 1 gene sequences. (Each circle in the haplotype network corresponds to one haplotype, and the size is proportional to its frequency among the samples. The number of perpendicular bars on branches represents the number of mutations between haplotypes. Popart 1.7 Bandelt et al., 1999).

the only mangrove oyster identified on the west coast of Africa, and a synonym of *C. tulipa*, Also, in further phylogenetic analysis, sequences of *C. tulipa* from this study formed a different clade (Figs. 2a and 2b) from other similar mangrove oysters from the same Crassostrea genus and 2 sequences from outgroup genus. The inclusion of the outgroup sequences, and their occurrence on a different clade in the phylogenetic tree from the reference *C. tulipa* species, with relatively lower bootstrap values of 80% and 26% (Figs. 2a and 2b) was to confirm the credibility of the analysis, as this is expected under ideal analytical conditions, where sequences aligned are diverse. This again indicates that *C. tulipa* has a unique identity and distinct from all known species of *Crassostrea*. Although *C. tulipa* is said to be synonymous with *C. gasar*, the topology of our trees showed that *C. gasar*, *C. brasiliana*, *C. columbiensis* and *C. virginica* are rather closely related in the broader clustering or grouping (Fig. 2a) forming a monophyletic group. The closeness of *C. sikemia, C. gigas* and *C. angulate* at the same node, and also that of *C. iredalei* and *C. madresensis.* in this study, is in agreement with the trees generated by de Melo et al. (2010), Lapègue et al. (2002), and Boudry et al. (2003). *Crassostrea gigas* in Japan (Kawamura et al., 2017), and the highly divergent species like, *C. virginica* in America as indicated in other studies (Thongda et al., 2018), was as well confirmed in this present study, as it formed a single divergent cluster in the phylogenetic analysis.

In a further analysis where the evolutionary divergence over all sequence pairs was estimated, the different sequences of *C. tulipa* species and on the other hand, the *C. gasar* and *C. brasiliana* sequences, recorded 0.00 genetic distance among themselves to confirm their similarity. However, a genetic distance as high as 1.40 to 1.55 was recorded between *C. tulipa* and *C. gasar* in a similar range as with some of the other Crassostrea species. The closest species to *C. tulipa* was rather *C gryphoides* with a genetic distance of 0.28, whiles the highest genetic distance was between *C. tulipa* and *C. rhizophora* sequences. Generally, populations with many similar alleles have small genetic distances, which indicates that they are closely related and have a recent common ancestor. Genetic distances as recorded in this study (Table 4) could not confirm the relatedness of *C. tulipa* and *C. gasar*

Haplotype analysis, that is, the study of a pattern of descent of a set of linked alleles occurring on the same chromosome and either preserved intact or separated by recombination over time, was also utilized as a critically important analytical tool in this study for the opportunities it offers in understanding the inheritance of polymorphic traits and their regulation. Generally, comparative analyses of haplotype sequences, allow many efficiencies in genetic studies (Lloyd et al., 2016), with the utmost being its potential in identifying identical-by-descent (IBD) regions that are shared between pairs of individuals (Gusev et al., 2009). Thus, in a haplotype analysis, only the sequences of *C. tulipa* oysters from this study shared one haplotype (Whin, Nakwa, Densu), while each of the other sequences from the NCBI database was found on a different haplotype (Figs. 3 and 4). This indicates that sequences of *C. tulipa* from the various ecological zones sampled are identical to each other but different from all the other species of the same genus. Most significantly, the haplotypes of *C. gasar* were rather much closer to that of *C. brasiliana* with just three (3) mutations and also, much closer to *the other Crassostrea species* (Figs. 3 and 4), than they were to *C. tulipa*. The inconsistency concerning *C. gasar* and *C. brasiliana* was clarified when a *C. brasiliana* rRNA 16S sequence deposited in the GenBank (DQ839413) was compared with that of *C. gasar* (AJ312937) by Lapègue et al. (2002) and found that they were identical, an indication that they are synonyms and belong to the same species. Thus, results from this study also confirm that *C. gasar* is a synonym of *C. brasiliana* as they occurred on the same haplotype. Accordingly, it was expected that other equally synonymous species would share the same haplotype with them however, the resulting haplotype network with only *C. gasar* and *C. tulipa* sequences (Fig. 4), produced a total of six different haplotypes instead of the expected one haplotype as was recorded among *C. tulipa* sequences or with a low mutation value between them, like the mutation of 1 recorded between the different *C. gasar* sequences. However, a mutation value as high as 288 with a haplotype diversity of 0.893 was recorded between *C. tulipa* and *C. gasar* sequences.

Lapègue et al. (2002), in their study on trans-Atlantuc distribution of

Crassostrea, where a phylogenetic tree was built with seven 16S sequences from Crassostrea and Saccostrea species, showed that *C. gasar* is intermediate between the American Crassostrea species (*C. virginica* and *C. rhizophorae*) and the Asian species (*C. gigas* and *C. ariakensis*). The study indicated among its conclusions that *C. gasar* was transported from Africa to America. Hence, against the background of *C. gasar* coming from Africa, it was expected that it would fall in the same group of COI sequence of *C. tulipa* which did not, although quite close, with low nucleotide diversity (0.289). This then suggests a unique identity for *C. tulipa* as a species.

The similarity between *C. tulipa* and *C. gasar* could therefore not be confirmed with any of the series of analyses, as they formed distinctly different nucleotide alignment patterns, consistently occurred on different clades and nodes either with Neighbor-Joining, ML UPGA, and MP phylogenetic analysis (Supl 2), in addition to the fact that bootstrap values did not support that similarity. They also occurred as different haplotypes and with relatively high estimates of genetic distance and divergence.

### *4.2. Genetic structure of C. tulipa in the coastal waters of Ghana*

The study also investigated the population structure of 60 samples from three different ecotypes of *C. tulipa*, utilizing mitochondrial cytochrome oxidase I (COI) genes as markers, for its importance in conservation and selective breeding programs. A low to high haplotype diversity (Table 4) was recorded amongst *C. tulipa* sequences and also with the other sequences in this study. The pattern of genetic variability with high haplotype diversity, but relatively low nucleotide diversity as recorded in this study suggests that the *C. tulipa* population analyzed from the various ecological zones has undergone population expansion. High genetic diversity often represents a greater opportunity for resiliency to climatechange, thus the genetic diversity in the present study is a likely adaptation to variable environments. However, the variations in the diversity indices indicate the need for conservation and positive traits for hatchery-based aquaculture development through selective breeding (Gjedrem, 2012; McAndrew and Napier, 2010). Analyses of all locations together from the haplotype neutrality test resulted in a negative mean Tajima's *D* that was significant and a positive Fu's *Fs* (Table 3). A negative Tajima's *D* indicates an excess of rare variations or low-frequency polymorphisms, a characteristic that is consistent with population growth. The positive Fu's *Fs* however indicates no excess alleles, expected from recent population growth bottleneck or directional selection under culture settings (Zainal et al., 2016; Ray et al., 2003). The overall negative and significant Tajima's D value obtained from the present analysis, is however, a distinctive characteristic of indigenous species implying that *C. tulipa* is expectedly an indigenous species.

Therefore, the development of hatchery protocols for this species could be regarded as a prerequisite for advancements and progress to commercial-scale hatchery productions as suggested by Southgate and Lee (1998). For a country like Ghana that is interested in the aquaculture of this species, especially in its hatchery production, the genetic confirmation of the geographic range of *C. tulipa* is important as shown by Utting and Spencer (1991), and Lucas (2012).

This study also drew further distinctions, thus mild structuring between all the sequences from the three ecological populations of *C. tulipa*, based on the phylogenetic and haplotype analysis, as two haplotypes (Hap 1 and 2), were shared among the five *C. tulipa* populations from analysis 1. However, *Fst* showed minimal and non-significant ($p > 0.05$) population differentiation which was also consistent with analysis 2. Nonetheless, this has implications for the proper management of wild harvested oysters in Ghana. Among the *C. tulipa* sequences, Densu and Whin, as well as Nakwa and Whin populations were more identical to each other, forming two subclusters. This could possibly be attributed to variations in water depth, salinity, and rate of tidal influence both from the sea and freshwater discharge, together with anthropogenic impacts and historical movements which tend to affect species adaptation and eventual modification and shaping of the genetic structure. This genetic information thus far suggests that balancing selection to include both subclusters could be essential to increase diversity and variability in genetic traits. Guo et al. (2018) highlighted on the importance of balancing selection as a major force in shaping genetic variation among oyster populations. High levels of genetic diversity can be achieved by strong balancing selection, which results from different life stages, long-distance dispersal potential, and fluctuating environmental conditions (Guo et al., 2018).

## 5. Conclusion

In this study, *Crassostrea tulipa* is genetically confirmed as occurring on the coastal waters of Ghana. It is also confirmed as genetically distinct from *C. gasar* and other Crassostrea species, as no single analysis undertaken in the present study confirmed their similarity. They consistently formed well-differentiated clades in the molecular phylogenies and also occurred as different haplotypes, and with a relatively high estimate of genetic distance and divergence.

The overall genetic structure of the Mangrove oyster, *C. tulipa* analyzed in this study also suggests that it is an indigenous species to Ghanaian coastal waters that has undergone population expansion, through adaptations to variable environmental conditions and with low to high haplotype diversity that informs a greater opportunity for resiliency to climate future change, and sustainable aquaculture development. The molecular information obtained from this study has implications for the proper management of wild-harvested oysters in Ghana.

## CRediT authorship contribution statement

**Rhoda Lims Diyie:** Conception and design of study, Acquisition of data, Analysis and/or interpretation of data, Writing – original draft, Writing – review & editing. **Samuel Addo:** Acquisition of data, Writing – review & editing. **Emmanuel Armah:** Acquisition of data, Analysis and/or interpretation of data, Writing – original draft. **Charles Mario Boateng:** Acquisition of data. **Mercy Oppong:** Writing – original draft. **Mike Y. Osei-Atweneboana:** Writing – review & editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

## Acknowledgments

The authors are grateful to the Biomedical and Public Health Research Unit of CSIR, Water Research Institute, Accra for permitting the use of the molecular laboratory, and supporting research with logistics. Technical support by Judith Wayo is duly acknowledged. All authors approved the version of the manuscript to be published.

### *Funding*

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

### *Ethics approval and consent to participate*

The work described herein has been carried out (where appropriate) in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments. All oysters were handled in accordance with the Northern Territory Governments animal ethics requirements and guidelines.

## Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.rsma.2023.103205.

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