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Molecular Identification of Strains of Caridean Species in a Tropical Lagoons of South-Western Nigeria

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Abstract

Species identification by morphological approach requires a high degree of experience, which is difficult and not practical enough for those interested in surveying a broad diversity of organisms. However, molecular or genetic approaches to identify species have been proposed and widely used by most scientists. DNA barcoding was used to identify *Macrobrachium* species occurring in three coastal lagoons (Badagry, Lagos and Epe) in South-western Nigeria. Samples of prawns for the DNA studies were collected from May to July 2015. The DNA of the *Macrobrachium* species was extracted using the phenol-chloroform protocol and Norgen tissue kit. Amplification and sequencing of the *Macrobrachium* species were carried out and further identification was done by comparing with sequenced data in the Genbank. New species of *Macrobrachium* were obtained in Badagry and Epe Lagoons having a close match with *M. asperulum* and *M. nipponense* at 85% and 84% respectively. *M. asperulum* and *M. nipponense* are reported in Badagry and Epe Lagoons for the first time.

Keywords: *Macrobrachium spp.*; Genomic DNA; South-western Nigeria lagoons.

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1. Introduction

The prawns, *Macrobrachium* species belonging to the family Palaemonidae are decapod crustaceans of high economic importance world-wide and have been subjected to intense aquacultural practices especially in Asia and the Americas [1,2]. *Macrobrachium* species occur in most inland waters including ponds, lakes, rivers and irrigation ditches, as well as in estuarine environment [3]. These prawns are found throughout the West African region. A total of about 200 species form the genus, in which only four species have been reported in Nigeria [4,5]. These include *Macrobrachium vollenhovenii* (African River

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prawn), *Macrobrachium macrobrachion* (Brackish water prawn), *Macrobrachium felicinum* (Niger River prawn) and *Macrobrachium dux* (Congo River prawn).

There are morphological similarities between *Macrobrachium olfersii* which is found in Central America and other species such as *M. faustinum* (Saussure, 1857), *M. crenulatum* [6], *M. digueti* (Bouvier, 1895), *M. hancocki* [6] and *M. acanthochirus* (Villalobos, 1967). Recently, *Macrobrachium birai* and *Macrobrachium holthuis* were considered junior synonyms of *M. olfersii* [7,8]. This however, depicts that sometimes morphological analysis alone is not sufficient to resolve the diversity of species complexes. The use of molecular data is an advancement and has proven very useful to elucidate the taxonomic relationships in morphologically variable groups of freshwater prawns [9-13]. It has been proposed [14,15] the use of cytochrome c oxidase subunit I (COI) as a standard method to help identify species, define species boundaries and aid in species delimitation. As part of the DNA Barcoding framework, the *COI* gene based identification system has proved superior within taxonomic groups of Crustacea [16]. R. Udayasuriyan *et al.* [17] used the mt-COI gene for the DNA barcoding of freshwater prawn species as the phylogenetic information obtained through the gene sequences are more conserved and less subjected to evolutionary forces, and thus, their species are genetically distinct.

The DNA barcoding is emerging as an essential supportive tool for morphology-based species identification [18] and the technique involves building a reference database (Barcode of Life Database, BOLD) where data about the specimens (photographic, geographic, and taxonomic including locations of the voucher specimens) are combined with molecular data [19]. Subsequently, sequenced DNA barcodes from unknown specimens can be compared against this reference library for proper identification. The method also helps with the discovery of new species and characterization of the taxonomic and genetic diversity of different geographic regions and help resolve cryptic species complexes [20-23]. Although a unified barcode region has not been reached on a single barcoding DNA segment chosen for taxological studies [24].

Macrobrachium species which appears in Southern Nigeria are adapted to freshwater conditions by using molecular (mtDNA) technique [25]. Very little documented works are available on these prawns in Western lagoons in Nigeria.

This study was carried out to identify the prawn species collected from three coastal lagoons at South-West Nigeria by molecular identification.

2. Materials and Methods

2.1. *Study area*

The Badagry Lagoon, with source in River Queme in the Republic of Benin to the west of Nigeria, is located in Lagos State (Southwest Nigeria) and opens into the Atlantic Ocean via the Lagos harbour. It lies between longitudes 3°54″ and 4°13″E and latitudes 6°25″ and 6°35″N [26]. Lagos Lagoon is located between latitudes 6º 26′ and 6º 39′N and

longitudes 3º 29′ and 3º 50′E [27] while Epe Lagoon lies between latitudes 6º29″N and 6º38″N; and longitudes 3º30″E and 4º05″E [28].

2.2. *Source of prawn species*

Fifty-seven (57) prawn species used for the DNA extraction was collected from May to July 2015 from Badagry, Lagos and Epe Lagoons (Fig. 1). The specimens from the set-traps in the lagoons were stored at -20 ºC prior to use.

2.3. *Morphometrics measurement*

The meristic characters observed were the rostrum teeth (dorsal and ventral) while the morphometric characters: total length, standard length, telson length, carapace length, carapace weight, rostrum length, flesh weight, left cheliped length and right cheliped length were investigated. The meristic character was determined by counting the number of spines on the dorsal and ventral side of the rostrum. The total length involves measuring the prawns from the tip of the rostrum to the end of the telson (to the nearest 0.1 cm) while the total weight was measured on an electronic weighing balance (Model: DT 1001A) to the nearest 0.01 g. The carapace length was determined by measuring the prawn from the eye socket to posterior end of the carapace. The length of the telson, rostrum, left cheliped and right cheliped were also measured. The sexes were determined according to the method adopted by [29].

2.4. *DNA extraction*

The DNA extraction was carried using the slightly modified phenol-chloroform method [30,31], Norgen cells and tissue genomic DNA isolation kit from Norgen Biotek Corporation in Canada (Cat. Number: 53100). Samples of prawn species were given an acronym for easy labelling and identification as shown below:
Emv - *M. vollenhovenii* from Epe Lagoon, Emm - *M. macrobrachion* from Epe Lagoon,
Bmv - *M. vollenhovenii* from Badagry Lagoon, Bmm - *M. macrobrachion* from Badagry Lagoon,
Lmv – *M. vollenhovenii* from Lagos Lagoon and Lmm – *M. macrobrachion* from Lagos Lagoon.

2.5. *Gel electrophoresis*

The quality of the DNA samples was checked on 2% agarose gel. The gel was run on 0.5 X Tris Borate EDTA (TBE) buffer at 75 V for 1.5 h, then visualized by staining with 10 mg mL^{-1} ethidium bromide under Ultra Violet (UV) light and photographed with the gel documentation system (UVitec, UK).

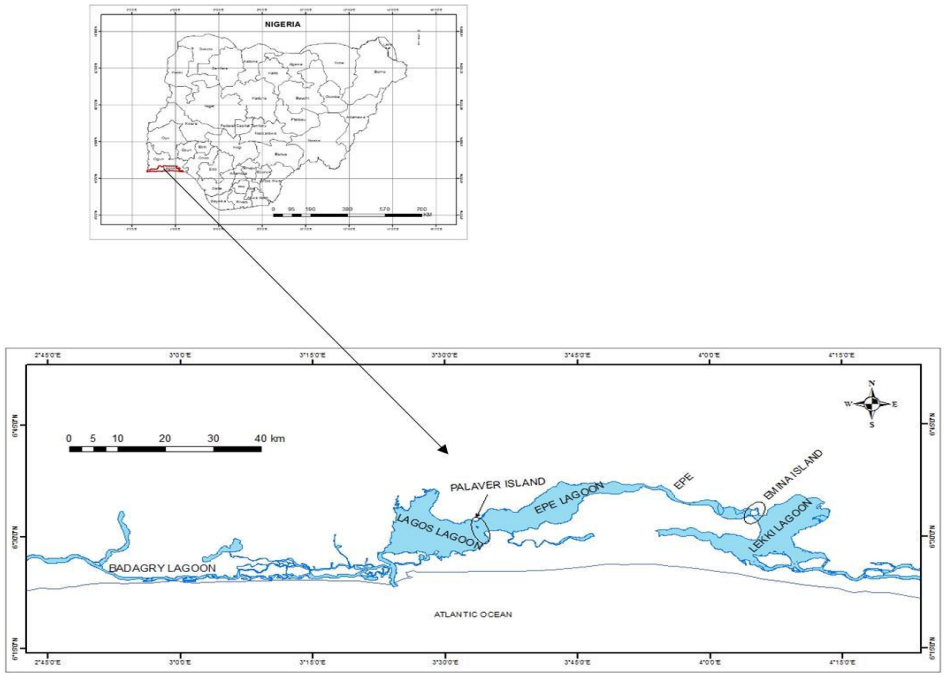

Fig. 1. Map of Badagry, Lagos and Epe Lagoons showing the sampling regions.

2.6. *Quantification of DNA samples*

The concentration of the DNA samples was determined using a spectrophotometer at 260 and 280 nm, respectively. The DNA concentration ranges between 22 and 544 ng/µL was suitable for PCR amplification (1.09 and 3.56).

2.7. *DNA amplification and sequencing*

The extracted DNA samples were taken to Macrogen Europe Laboratory Meibergdreef 31 1105 AZ Amsterdam, Netherlands for the DNA amplification and sequence analysis. Amplification of the DNA fragment was determined by the Polymerase Chain Reaction (PCR) using Cytochrome Oxidase sub-unit 1 (CO1). The 5' end of cytochrome c oxidase sub unit I gene region was amplified using the primer pair LCO1490 (forward reaction): 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198 (reverse reaction): 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' [32]. The PCR conditions were programmed at 94 °C for five min (Pre-denaturation) followed by 35 cycles at 94 °C for one min (Denaturation), at 55 °C for one min (Annealing) and at 72 °C for one min (Extension). It was finally terminated at 72 °C for ten min and stored at 4 °C [33]. The PCR products were gel checked and sequenced based on the standard protocols [34]. PCR products were sequenced using a standard cycle-sequencing protocol of the ABI Big-Dye

Ready Reaction Kit and analyzed on an Applied Biosystems automatic DNA sequencer (Model: 377, Applied Biosystems, CA, USA). The sequence data generated from the DNA extraction, PCR and sequences were further blasted in order to confirm their identities. Basic Local Alignment Search Tool (BLAST) was the program used to infer functional and evolutionary relationships between sequences as well as to help identify members of gene families. This was achieved by comparing nucleotide sequences data that were produced from the genomic DNA to sequences database at the Genbank.

2.8. *Statistical analysis*

Clean and clear unambiguous bands were scored for presence (1) and absence (0) of bands. The results for the quantification of the DNA samples were further analyzed using Microsoft excel (2007). The DNA sequences were automatically aligned using ClustalW [35] alignment algorithm under default parameters on Molecular Evolutionary Genetic Analysis (MEGA) 6.06 software [36]. Phylogenetic trees were derived using neighbor-joining algorithms [37]. The robustness of the topologies for the neighbor-joining trees was estimated through bootstrap analysis [38] based on 1000 re-sampling of the sequences.

3. Results

Two new species were identified from the collected prawn samples (Plate 1). These were amplified using PCR and further sequenced. The reverse primer of Emm generated a length of 712 base pair (bp) on the chromatograph and while the sequences generated were blasted on the Genbank, it had a close match with *Macrobrachium nipponense* (of length 644 bp) at 84% close identity. The forward primer of Emm reproducibly generated amplification products of length 697 base pair (bp) at 83% close match with *Macrobrachium nipponense* (of length 640 as illustrated in Table 1).

The reverse primer of Bmv generated a length of 768 base pair (bp) on the chromatograph, it had a close match with *Macrobrachium asperulum* (of length 658 bp) at 85% close identity. The forward primer of Bmv reproducibly generated amplification products of length 715 base pair (bp) at 85% close match with *Macrobrachium asperulum* (of length 658 bp) as shown in Table 1. There is no observation of unique haplotypes from the result and there are variations in each member of sequences generated.

The phylogenetic analysis of the sequenced data yielded a neighbor-joining (NJ) tree as shown in Fig. 2. The meristic and morphometric characters of the identified species are presented in Table 2 with some of the characters from Badagry Lagoon showing slight differences from those of Epe Lagoon. The sequences generated were deposited in the National Centre for Biotechnology Information (NCBI) database on the 10th of August, 2015 and these have been assigned Genbank accession numbers KT374065 – KT374068.

Plate 1. Identified prawns from the DNA barcode.
(a) *Macrobrachium asperulum* (Mag. x 0.8)
(b) *Macrobrachium nipponense* (Mag. x 0.7)

Table 1. Percentage closeness of the identified species in relation to GenBank sequence.

Query						Identities
Name	Length (base pair-bp)	Sequences	Gene	Length (base pair-bp)	Match	Pct. (%)
150305-32_E01/ME4/EmmHCO2198 **(KT374065)**	712	GCGGAAGATGCTGCGCATAGCGATTGTGGTCTCCCCCACCTGCCGGGTCAAAGAAAGAGGTGTTT	*Macrobrachium nipponense* mitochondrial CO1 gene for cytochrome c oxidase subunit 1, partial cds, isolate: MN1	644	533	84
150305-32_G01_ME4/EmmLCO1490 **(KT374066)**	697	CGAGGAATGTTGTCTTCGGAGCGTGAGCTGGGATAGTAGGCACATCTCTGAGACTCCTTATCCGGGC	*Macrobrachium nipponense* mitochondrion, complete genome	640	556	83
150305-32_I01_MB5/BmvHCO2198 **(KT374067)**	768	GGGAAAGTGTTGGAGGAGAATGGGGTCTCCCCCTCCTGCGGGGTCGAAGAAAGAGGTATTTAGG	*Macrobrachium asperulum* mitochondrial COI gene for cytochrome oxidase subunit I, partial cds, haplotype: TH-3	658	562	85
150305-32_K01_MB5/BmvLCO1490 **(KT374068)**	715	CCAATATTTTTTATCTGTCGTGAGCTTGAGCAGGAATAGTAGGCACATCCTCTAAGACTTCTTAAT	*Macrobrachium asperulum* mitochondrial COI gene for cytochrome oxidase subunit I, partial cds, haplotype: TH-3	658	562	85

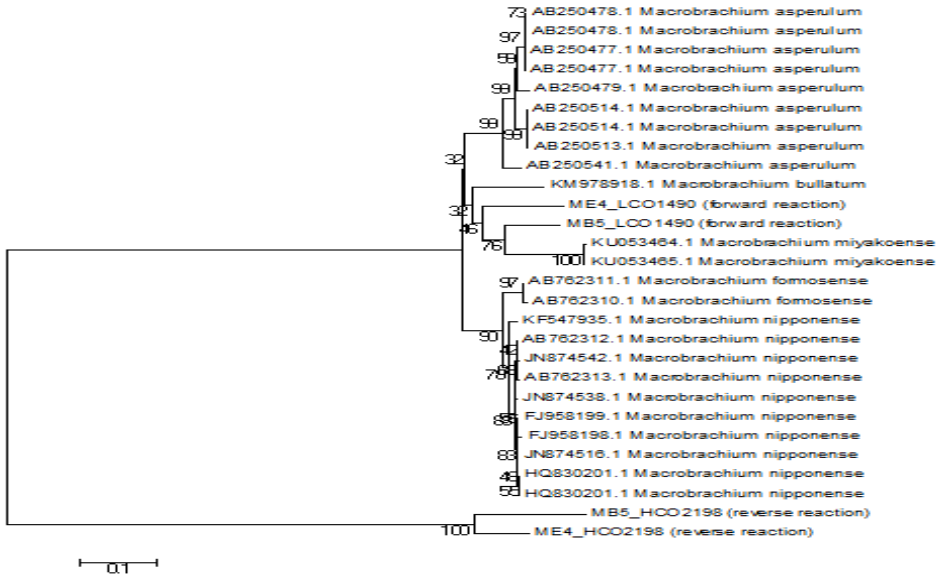

Fig. 2. Neighbor-joining (NJ) tree based on the COI sequenced data.

Table 2. Meristic and morphometric (morphomerics) characters of the identified species from the study area.

Species	TL (cm)	SL (cm)	TW (g)	Tel-L(cm)	CL (cm)	CW (g)	RL (cm)	RT dorsal	RT ventral	SEX	FW (g)	LCL	RCL
Badagry Lagoon													
MB5	10.1	8.7	16.0	1.4	3.0	0.6	3.0	15	4	M	7.2	8.8	5.4
Epe Lagoon													
ME4	7.5	6.5	5.5	1.0	2.2	0.3	2.1	13	4	M	3.2	10.4	12.5

Key:

TL = Total Length
SL = Standard Length
TW = Total Weight
Tel-L = Telson Length
CL = Carapace Length
CW = Carapace Weight
RL = Rostral Length
RT = Rostrum Teeth
FW = Flesh Weight
LCL = Left Cheliped Length
RCL = Right Cheliped Length

4. Discussion

The studies revealed that only two prawn samples were isolated for DNA sequenced while the remaining species could not be included in barcode analysis due to the fact that they were not successfully amplified with the universal primer (LCO1490 and HCO2198) used in this study. This may be due to several reasons right from the preservation of samples, handling of tissue samples to the steps involved in the DNA extraction and PCR amplification [23].

The result obtained from the morphometric characterization showed that both species had more rostrum teeth on the dorsal part than the ventral side. Though, MB5 from Badagry Lagoon had more teeth on the dorsal part of the rostrum than that of ME4 from Epe Lagoon while both species had the same number of spines on the ventral side of the rostrum. A significant differences was recorded in some of the morphometric characters (TL, TW, FW and RCL) from Badagry and Epe Lagoon, the observed variation from the two lagoons probably reflected environmental differences, Badagry and Epe Lagoons being low brackish waterbodies. Therefore, this environmental differences might be a factor responsible for the varied meristic and morphometric characters. On the contrary [39] mention that the species recorded 5 spines as the highest frequency of occurrence on the ventral side of *M. macrobrachion* and *M. vollenhovenii* respectively and on the dorsal side, 9 spines occurred in the most for *M. macrobrachion* while 9 and 10 spines occurred in most for *M. vollenhovenii*. However, S. D. Salman *et al.* [40] recorded a range of 11-14 and 1-3 spines on the dorsal and ventral rostrum teeth of *M. nipponense* respectively and the total length of the males ranged between 71.00 – 99.80 mm (7.10 – 9.98 cm). G. Ahmad [41] also observed 11 and 2 spines on the dorsal and ventral side of the rostrum teeth of *M. nipponense* respectively and a maximum total length of the male species as 75.4 mm (7.54 cm). According to the references [42,43], rostrum, teeth, morphology of the walking legs, palm and fingers have been viewed as useful and diagnostic taxonomic characters in prawns.

The DNA sequence data showed that *M. nipponense* and *M. asperulum* had 84% and 85% similarity with strains of *M. macrobrachion* and *M. vollenhovenii* from Epe and Badagry Lagoons respectively. Thus, it can be concluded that *M. asperulum* and *M. nipponense* can be found in Badagry and Epe Lagoons respectively (though they were of narrow gene pool) due to the relatively close match with sequences from the Genbank. The occurrence of *M. asperulum* and *M. nipponense* in the lagoons might be as a result of the low salinity of Badagry and Epe Lagoons during the rainy season. S. Shokita [44] reported that *M. asperulum* completes its life cycle only in freshwater. M. H. Ali [45] reported the occurrence of *M. nipponense* in Iraq, in waters with salinity range of 1.299 – 2.690 ‰. Q. A. Nguyen et al. [46] reported the occurrence of *M. nipponense* in Iran while A. Dimmock [47] found that environmental other than genetic factor could determine differences in morphological characters when identifying *Macrobrachium* species.

The use of molecular technique was employed to identify new species of *Macrobrachium* that were found during the period of collections. The PCR amplification and the universal decapods primer (LCO1490 and HCO2198) used in this study brought to light the emergence of new strains of *Macrobrachium* species from Epe and Badagry Lagoons, Nigeria. It is therefore important to employ the use of more molecular methods to investigate the possibilities of exploitations of these species in South-West Lagoons especially in Epe, Badagry Lagoons and adjacent waterbodies as it seems that the species are getting adapted to the new environment, though they constitute small gene pool.

5. Conclusion

In this study, new *Macrobrachium* species (*M. asperulum* and *M. nipponense*) were found in Badagry and Epe Lagoons using two protocols for the DNA extraction. It is therefore very important to adopt the use of more molecular methods to investigate the possibilities of exploitations of these species and also put into considerations several reasons right from the preservation of samples, handling of tissue samples to the steps involved in the DNA extraction and PCR, as these may interfere with the quality and quantity of the DNA samples.

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References

1. FAO, Food and Agriculture Organisation of the United Nations (Bangkok, Thailand, 2006), pp. 20.
2. L. A. Davassi, J. Fisher. Aquat. Sci. **6**, 649 (2011).
3. M. B. New, Food and Agriculture Organization Fisheries Technical Paper **428**, (2002).
4. I. E. Marioghae, Revue Zoologique Africaine. **96**, 3 (1982).
5. O. A. Bello-Olusoji, A. T. Omolayo, and A. Arinola, J. Food Agric. Environ. **2,** 280 (2004).
6. L. B. Holthuis, Siboga Expedition Monograph **39**, 1 (1950).
7. L. G. Pileggi and F. L. Mantelatto, Invertebrate Systematics **24**, 1 (2010). https://doi.org/10.1071/IS10009
8. L. G. Pileggi and F. L. Mantelatto, Iheringia Série Zoologia **102**, 4 (2012).
9. N. P. Murphy and C. M. Austin, Aust. J. Zool. **52**, 5 (2004). https://doi.org/10.1071/ZO03062
10. D. H. N. Munansinghe, J. National Sci. Found. Sri Lanka **38**, 3 (2010). https://doi.org/10.4038/jnsfsr.v38i1.1721
11. F. G. Vergamini, L. G. Pileggi, and F. L. Mantelatto, Contribut. Zool. **80**, 1 (2011).
12. K. V. Rintelen, T. J. Page, Y. Cai, K. Roe, B. Stelbrink, B. R. Kuhajda, T. M. Iliffe, J. Hughes, and T. Von-Rintelen, Mol. Phylogen. Evolut. **63**, 1 (2012). https://doi.org/10.1016/j.ympev.2011.11.018
13. L. S. Torati and F. L. Mantelatto, J. Crust. Biol. **32**, 4 (2012). https://doi.org/10.1163/193724012X635322
14. P. D. N. Hebert, A. Cywinska, S. L. Ball, and J. R. De-Waard - *Proceed. of the Royal Soc. of London, Series B: Biolog. Sci.* **270,** 1512 (2003).
15. Y. M. Saad and H. E. A. El-Sadek, Int. School. Sci. Res. Innov. **11**, 515 (2017).
16. T. Lefébure, C. J. Douady, M. Gouy, and J. Gibert, Mol. Phylogen. Evolut. **40**, 2 (2006). https://doi.org/10.1016/j.ympev.2006.03.014
17. R. Udayasuriyan, B.P. Saravana, and R. Kalpana, J. Gene Proteins **1**, 1 (2017).
18. K. Shantanu, R. Shibananda, T. Kaomud, C. Rajasree, and K. Vikas, Mitochondrial DNA **3**, 1 (2018). https://doi.org/10.1080/23802359.2017.1413288

19. P. D. N. Hebert, J. R. De-Waard, E. V. Zakharov, S. W. Prosser, J. E. Sones, J. T. McKeown, B. Mantle, and J. La-Salle, PLoS One **8**, 7 (2013). https://doi.org/10.1371/journal.pone.0068535
20. P. D. Hebert and R. T. Gregory, Systematic Biology **54**, 852 (2005). https://doi.org/10.1080/10635150500354886
21. F. O. Costa, J. R. De-Waard, J. Boutillier, S. Ratnasingham, R. T. Dooh, M. Hajibabaei, and P. D. Hebert, Can. J. Fish. Aquat. Sci. **64**, 272 (2007). https://doi.org/10.1139/f07-008
22. M. Hajibabaei, G. A. Singer, P. D. Hebert, and D. A. Hickey, Trends in Genetics **23**, 167 (2007).
23. G. Rajkumar, P. S. Bhavan, R. Udayasuriyan, and C. Vadivalagan, Int. J. Fish. Aquat. Stud. **2**, 4 (2015).
24. S. Lin, H. Zhang, Y. B. Hou, Y. Y. Zhuang, and L. Miranda, Appl. Environ. Microbiol. **75**, 5 (2009). https://doi.org/10.1128/AEM.00824-09
25. A. A. Jimoh, M. A. Anetekhai, S. Cummings, O. T. Abanikanda, G. F. Turner, C. V. Oosterhout, and B. Hanfling, Aquaculture **410**, 25 (2013). https://doi.org/10.1016/j.aquaculture.2013.06.013
26. A. O. Lawal-Are and K. Kusemiju, J. Sci. Res. Develop. **4**, 117 (2000).
27. P. E. Ndimele, M.Sc. Dissertation, University of Ibadan, Nigeria, 2003.
28. J. I. Agboola and M. A. Anetekhai, J. Appl. Ichthyol. **24**, 623 (2008). https://doi.org/10.1111/j.1439-0426.2008.01079.x
29. M. A. Anetekhai, Ph.D. Thesis, University of Ibadan, 1986.
30. J. Sambrook, E. F. Fritsch, and T. Maniatis, Molecular Cloning: A Laboratory Manual., 2nd Edition (Cold Spring Harbor Laboratory Press, 1989) **11**, pp. 2.
31. J. Sambrook and D. W. Russell, Molecular Cloning **1**-**3**, 2100 (2001).
32. O. Folmer, M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek, Mol. Marine Biol. Biotech. **3**, 294 (1994).
33. S. Nicole, E. Negrisolo, G. Eccher, R. Mantovani, T. Patarnello, D. L. Erickson, W. J. Kress, and G. Barcaccia, Food Tech. Biotech. **50**, 4 (2012).
34. K. S. Ajmal, P. S. Lyla, J. B. Akbar, K. C. Prasanna, S. Murugan, and K. C. Jalal, Biotech. **9**, 3 (2010).
35. J. D. Thompson, D. G. Higgins, and T. J. Gibson, Nucl. Acids Res. **22**, 22 (1994). https://doi.org/10.1093/nar/gni186
36. K. Tamura, G. Stecher, D. Peterson, A. Filipski, and S. Kumar, Mol. Biol. Evolut. **30**, 12 (2013). https://doi.org/10.1093/molbev/mst197
37. K. Strimmer and A. Von-Haeseler, Mol. Biol. Evolut. **13**, 7 (1996). https://doi.org/10.1093/oxfordjournals.molbev.a025664
38. J. Felsenstein, Evolution **39**, 4 (1985). https://doi.org/10.1111/j.1558-5646.1985.tb00420.x
39. A. A. Jimoh, E. O. Clarke, O. O. Whenu, M. A. Anetekhai, and P. E. Ndimele, Asian J. Biol. Sci. **5**, 126 (2012).
40. S. D. Salman, J. P. Timothy, D. N. Murtada, and G.Y. Ama'al, Aquatic Invasions. **1**, 3 (2006). https://doi.org/10.3391/ai.2006.1.3.2
41. G. Ahmad, Adv. J. Biol. Sci. Res. **1**, 003 (2013).
42. P. Naiyanetr, Crustaceana **74**, 609 (2001). https://doi.org/10.1163/156854001505541
43. N. P. Murphy and C. M. Austin, Zoologica Scripta. **34**, 187 (2005). https://doi.org/10.1111/j.1463-6409.2005.00185.x
44. S. Shokita, Annotations Zoologicae Japonenses. **50**, 110 (1977).
45. M. H. Ali, S. D. Salman, and A.Y. Al-Adhub, Scientia Marina. **59**, 1 (1995).
46. Q. A. Nguyen, D. P. Phan, T. L. Phan, T. T. Nguyen, and B. Le-Phoc – *Proc. of the 6th Technical Symp. on Mekong Fisheries* (Pakse, Lao PDR., 2003) **26.**
47. A. Dimmock, L. Williamson, and P. B. Mather, Aquacult. Int. **12**, 435 (2004). https://doi.org/10.1023/B:AQUI.0000042140.48340.c8