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New observations of the enigmatic West African *Cellana* limpet (Mollusca: Gastropoda: Nacellidae)

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Abstract

Background: Identification of limpets is often hampered by highly variable within-species shell morphologies and colour patterns. Since pre-Linnean times this has produced complex taxonomies with confusing nomenclatorial histories and uncertain distribution patterns. This is the case for a complex of taxa associated with *Cymbula safiana* (Lamarck, 1819) and with the rejected name *Patella nigra*. We DNA sequenced limpets from Nigeria that were originally identified as *C. safiana*. Comparisons with available cox1 data of patellogastropods show that the specimens actually belong to the genus *Cellana* Adams, 1869 which has been recorded only once before in the Atlantic Ocean with the finding of specimens from Ghana.

Results: We are reporting findings of *Cellana* sp. from the Gulf of Guinea for the second time. Specimens from Nigeria are 100 % similar to previously published cox1 sequences from Ghana. Due to variable shell characteristics we suspect that this species may have been confused with *Cymbula safiana* (Lamarck, 1819) in previous records. Inspection of the radula sack and radula demonstrates clear similarities with other *Cellana* species and contrasting differences in the organization of the teeth in *Cymbula*.

Conclusions: Because *Cellana* is a possible candidate of invasive species in West Africa and *Cymbula* is considered as endangered, is seems particularly important to be able to distinguish between the two without being dependent on DNA analysis. When shell morphology seems to be of questionable diagnostic value, examination of the radula will help in future mapping and monitoring of these two species. A cox1 gene tree with Nigerian sequences included is in line with findings of previous authors and restates the need for taxonomic revision of the species clustering with *Cellana toreuma* (Reeve, 1854) and parts of a polyphyletic *Cellana radiata* von Born, 1778.

Keywords: Nigeria, Patellogastropoda, Limpets, DNA-barcode, *Cellana*

Background

Knowledge about West African limpets is relatively poor (Ridgway et al. 1998; Nakano and Espinosa 2013). Identification of limpets is often hampered by highly variable shell morphologies and colour patterns within species (Nakano and Sasaki 2011). This has produced complex taxonomies and nomenclatorial histories that are expressed in sometimes lengthy lists of synonyms and misidentified records (Christianens 1974; Gofas 2016). Since the advent of molecular techniques the traditional conchological taxonomies are under considerable revision. A striking example of a confused taxonomic state is seen in the case of *Patella nigra*, which until recently was referred to the authorship of da Costa, 1771 (Christianens 1974), but now is rejected as an unavailable name because it was published under a non-binominal pre-Linnean taxonomic regime (Petit 2013). However, the "*nigra*" name is still present in a nexus of combinations in diverse literature and the actual taxonomic status of many of these records is uncertain. According to MolluscaBase (Gofas 2016), the accepted name for the species that *P. nigra* refers to is *Cymbula safiana* (Lamarck, 1819). Still, a picture of shells on the same web site is somewhat confusingly called *Cymbula nigra* (da Costa, 1771). A popular conchological book on West African sea shells (Ardovini and Cossignani 2004)

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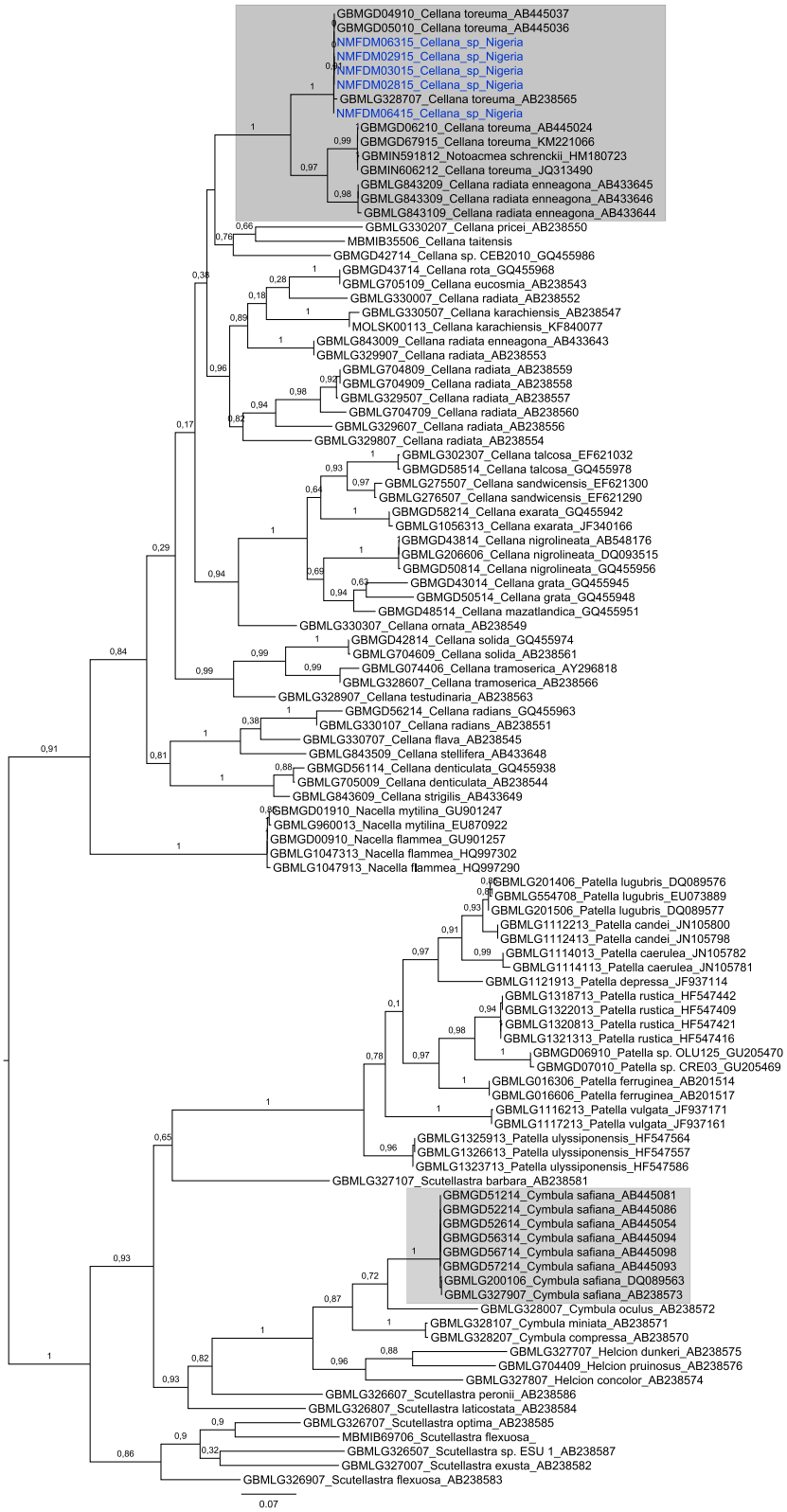

Fig. 1 Partial cox1 gene tree for selected DNA sequences of families Patellidae and Nacellidae with position of Nigerian *Cellana* sp. in upper shaded area. Lower shaded area indicates *Cymbula safiana,* which may have been confused with the former in historical records

has a photograph of *Patella safiana* on page 60 and of *Patella nigra* on page 61, implying with no further explanation that these are two different species, but provides no clues on how these species can be told apart. Powell (1973) also considered *P. nigra* and *P. safiana* as different species (Nakano and Espinosa 2013). However, although *Cymbula safiana* is regarded as an endangered species in Europe, it is still listed as being distributed all the way from the Iberian Peninsula to Angola (Espinosa et al. 2011). Molecular evidence for this was found in the close similarity of 16S sequences from Angola and Spain (Koufopanou et al. 1999).

Christianens (1974) described two morphotypes of *P. nigra*: *Patella nigra plumbea* (Christianens 1974), which he considered a "typical" form from Sénégal, and *Patella nigra ghananis* which he described as a "*nov. var.*" based on specimens from Ghana (Christianens 1974). When Nakano and Espinosa (2013) made the surprizing discovery from DNA-sequencing that two of their specimens from Ghana clustered with a species of *Cellana* Adams, 1869, it was the first record of the latter genus in the Atlantic Ocean and because the most similar sequence to the pair was a *Cellana toreuma* (Reeve, 1854) specimen collected from Java, they speculated that the African individuals could belong to a relatively recently introduced species from the Indo-Pacific. However, they also referred to Christianens' (1974) distinctions between *"plumbea"* and *"ghananis"* based on apparent differences in shape and size and suggested that their *Cymbula nigra* specimens that genetically cluster with other Patellidae sequences are Christianens'*"plumbea"* (= *Cymbula safiana*) whereas *Cymbula nigra var. ghananis* is actually a *Cellana* species.

As a result of an initiative to produce DNA-barcodes for Nigerian molluscs via BOLD (Ratnasingham and Hebert 2007) we discovered that mitochondrial cytochrome subunit 1 sequences from five Nigerian specimens initially identified as *Cymbula safiana* had 100 % nucleotide similarity with the African *Cellana* sequences discussed by Nakano and Sasaki (2011; Christianens 1974). We report these findings to supplement the discovery of Nakano and Espinosa (2013) with additional information that will hopefully contribute to more accurate records of limpet species in West Africa.

Results and discussion

Genetic data

We compared cox1 sequences from the Nigerian specimens with publicly available data from related taxa and produced a gene tree that generally corresponds well with phylogenetic results from previously published work (Koufopanou et al. 1999; Colgan et al. 2003; Nakano and Ozawa 2007; Nakano and Sasaki 2011; Bird et al. 2011; Nakano and Espinosa 2013). Detailed inspection of the topology indicates several cases of putative misidentifications that are beyond the scope of discussion in the present paper. However, two points are of particular interest here, firstly the placement of *Cymbula safiana* (Fig. 1, shaded area) with other Patellidae sequences and, secondly (Fig. 1, shaded area; Table 1), the

Table 1 Percentage of identical nucleotide COX1 positions

Bold access Name GenBank access	AB433645	AB433645	AB433646	JQ313490	HM180723	KM221066	AB445024	AB238565	AB445036
GBMLG843109 *C. radiata enneagona* AB433645		99.70	99.70	94.97	94.53	94.66	94.65	91.93	92.20
GBMLG843209 *C. radiata enneagona* AB433645	99.70		100.0	95.14	94.83	94.96	94.95	91.78	92.05
GBMLG843309 *C. radiata enneagona* AB433646	99.70	100.0		95.14	94.83	94.96	94.95	91.78	92.05
GBMIN606212 *C. toreuma* JQ313490	94.97	95.14	95.14		100.0	100.0	100.0	91.79	92.13
GBMIN591812 *N. schrenckii* HM180723	94.53	94.83	94.83	100.0		99.85	99.85	91.17	91.44
GBMGD67915 *C. toreuma* KM221066	94.66	94.96	94.96	100.0	99.85		100.0	91.28	91.59
GBMGD06210 *C. toreuma* AB445024	94.65	94.95	94.95	100.0	99.85	100.0		91.28	91.59
GBMLG328707 *C. toreuma* AB238565	91.93	91.78	91.78	91.79	91.17	91.28	91.28		99.69
GBMGD05010 *C. toreuma* AB445036	92.20	92.05	92.05	92.13	91.44	91.59	91.59	99.69	
GBMGD04910 *C. toreuma* AB445037	92.20	92.05	92.05	92.13	91.44	91.59	91.59	99.69	100.0
NMFDM02915 *C.* sp Nigeria	92.17	92.01	92.01	92.13	91.40	91.55	91.55	99.69	100.0
NMFDM06315 *C.* sp Nigeria	92.09	91.93	91.93	92.00	91.32	91.48	91.48	99.58	99.89
NMFDM06415 *C.* sp Nigeria	92.91	92.91	92.91	92.91	92.91	92.91	92.91	99.48	100.0
NMFDM02815 *C.* sp Nigeria	92.06	91.90	91.90	92.13	91.28	91.43	91.43	99.69	100.0
NMFDM03015 *C.* sp Nigeria	92.17	92.01	92.01	92.13	91.40	91.55	91.55	99.69	100.0

Table 1 Percentage of identical nucleotide COX1 positions *(Continued)*

Bold access Name GenBank access	AB445037	NMFDM02915	NMFDM06315	NMFDM06415	NMFDM02815	NMFDM03015	Sample origin
GBMLG843109 *C. radiata enneagona* AB433645	92.20	92.17	92.09	92.91	92.06	92.17	Pacific: Japan
GBMLG843209 *C. radiata enneagona* AB433645	92.05	92.01	91.93	92.91	91.90	92.01	Pacific: Japan
GBMLG843309 *C. radiata enneagona* AB433646	92.05	92.01	91.93	92.91	91.90	92.01	Pacific: Japan
GBMIN606212 *C. toreuma* JQ313490	92.13	92.13	92.00	92.91	92.13	92.13	Pacific: China
GBMIN591812 *N. schrenckii* HM180723	91.44	91.40	91.32	92.91	91.28	91.40	Pacific: Korea
GBMGD67915 *C. toreuma* KM221066	91.59	91.55	91.48	92.91	91.43	91.55	Pacific: China
GBMGD06210 *C. toreuma* AB445024	91.59	91.55	91.48	92.91	91.43	91.55	Pacific: Japan
GBMLG328707 *C. toreuma* AB238565	99.69	99.69	99.58	99.48	99.69	99.69	Indonesia: Java
GBMGD05010 *C. toreuma* AB445036	100.0	100.00	99.89	100.0	100.0	100.0	Atlantic: Ghana
GBMGD04910 *C. toreuma* AB445037		100.00	99.89	100.0	100.0	100.0	Atlantic: Ghana
NMFDM02915 *C.* sp Nigeria	100.0		99.88	100.0	100.0	100.0	Atlantic: Nigeria
NMFDM06315 *C.* sp Nigeria	99.89	99.88		100.0	99.88	99.88	Atlantic: Nigeria
NMFDM06415 *C.* sp Nigeria	100.0	100.00	100.00		100.0	100.0	Atlantic: Nigeria
NMFDM02815 *C.* sp Nigeria	100.0	100.00	99.88	100.0		100.0	Atlantic: Nigeria
NMFDM03015 *C.* sp Nigeria	100.0	100.00	99.88	100.0	100.0		Atlantic: Nigeria

complete sequence match of our Nigerian specimens with the specimens (AB445036, AB445036) previously published from Ghana as *Cellana toureuma* or *Cymbula nigra* var. *ghananis* (Nakano and Espinosa 2013). Again we observe (Table 1) that the African specimens differ by only 2–3 nucleotides from the *C. toreuma* specimen (AB238565) from Java, Indonesia. The relationships of three clades comprised by Indonesian and West African *C. toreuma*, with sisters from China, Japan and Ogasawara Islands have also been indicated previously (Nakano et al. 2009; Bird et al. 2011). These clusters include some, but not all, of the sequences identified as *C. radiata enneagona*. The *C. toreuma* clade also presents a misidentified *Notoacmea schrenckii* (HM180723) from Korea. The gene tree clearly shows polyphyly of various *C. radiata*, as also remarked by Nakano and Sasaki (2011). Subspecies of *C. radiata* such as *C. radiata capensis* (AB238552) may have to be resurrected to full species status.

Anatomy

There is considerable phenotypic variation among the shells of specimens of different sizes (Fig. 2a, b, d, e), initially leading the authors to presume that there were several species represented in the samples. The shell variability among genetically identical individuals suggests that this *Cellana* species may have been confused with *Cymbula* in shell-based identifications and reported in previous records either as *C. safiana, C. nigra,* or as *Patella nigra* or as some other nominal species of *Patella* that have been judged as synonyms of *P. nigra* at some point (Christianens 1974; Ridgway et al. 1998). At present *Cymbula safiana* is regarded as having a distribution from the Iberian Penninsula to Angola (Espinosa et al. 2011). Even the specimen figured by Nakano and Espinosa (2013) as *Cymbula nigra* from Ghana does not seem to display obvious differences from our *Cellana* specimens. In order to discriminate between these two quite unrelated West African species without the help of DNA data, one may have to examine internal organs. According to Ridgway et al. (1998), the radula sack in *Cymbula* penetrates the visceral mass and is not visible from dorsal view while in *Cellana* it is situated beneath the digestive glands (visceral mass) and the gonads. The latter seems to be the case in our specimens (Fig. 2f) but unfortunately this is also stated as a characteristic for *Patella* (Ridgway et al. 1998).

But the radula itself (Fig. 3a–f) is very different from that of *Cymbula safiana* (Ridgway et al. 1998) and clearly more similar to the radula described for *Cellana toreuma* by Lu et al. (1995) based on specimens from Taiwan. They described the radula as "*..each row of teeth has eight cusps (3 marginal teeth, 1 lateral tooth, 0 rachidian tooth, 1 lateral tooth, 3 marginal teeth, and the tooth formula is 3-1-0-1-3) and several base pieces..*". We suspect that this description of a tooth formula refers to the number of cusps or denticles and not to the number of teeth, because we observe two laterals and one marginal tooth in our specimens (Fig. 3a, d) and this seems to be the case also in Lu et al.'s figures (1995). In a

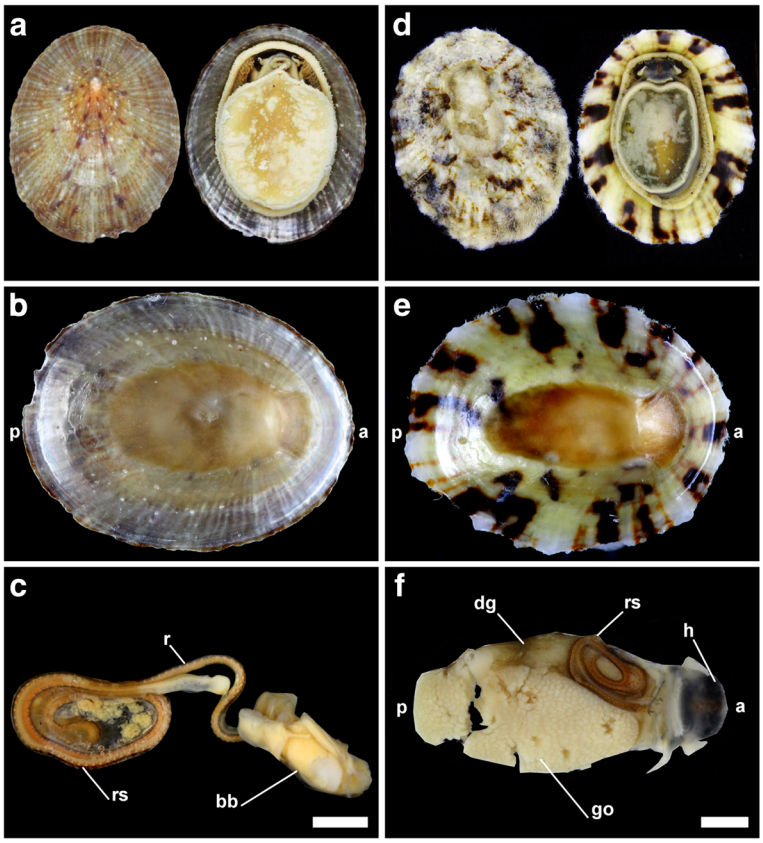

Fig. 2 **a**, **b**, **c** specimen MNFDMOL029, length: 31.8 mm, width 25.5 mm: **d**, **e**, **f** specimen MNFDMOL030, length: 12.9 mm, width: 10.2 mm. **a** complete animal, left image dorsal view, right image ventral view. **b** ventral view of shell, **c** left lateral view of buccal bulb, dorsal view of radula sack. Scale bar: 2 mm. **d** complete animal, left image dorsal view, right image ventral view. E, ventral view of shell. **f** ventral view of visceral mass and head, Scale bar: 1 mm. Abbreviations: a: anterior. p: posterior. r, radula. rs, radula sack. bb, buccal bulb. dg, digestive glands. go, gonads. h, head

description of *Cellana nigrolineata* (Reeve, 1854) (Nakano et al. 2010) the radula formula is given as 3.2.1.2.3 and declared as *"..a typical Cellana.."*. It seems clear from their pictures (Nakano et al. 2010) and description details in writing that the number 3 refers to marginal teeth that are fused basally and that there are 2 pairs of lateral teeth.

C. nigrolineata is also reported to have a very small, narrow vestigial rachidian. We believe that this is the state in the Nigerian specimens too, and perhaps even in Lu et al.'s (1995) picture of *C. toreuma*. The inner laterals are simple and hook-like with one acutely pointed cusp. The outer laterals have three prominent cusps and a smaller inner denticle. This configuration of the laterals is a good match with the pictures referred to above (Lu et al. 1995; Nakano et al. 2010). We were not able to see the type of three-cusped marginal teeth that *C. nigrolineata* has, but one simple marginal tooth is also present in *Cellana* sp. This suggests a radula formula of 1.2.1.2.1 for the Atlantic *Cellana* species. The radula had only slight variations between the examined specimens (Fig. 3a, d) but there was quite significant differences between the more newly formed teeth (Fig. 3c) and the more mature teeth (Fig. 3a, d, e).

Conclusions

The discovery of *Cellana* in the Gulf of Guinea could be interpreted as a recent anthropogenic introduction (Nakano and Espinosa 2013), but there is also a possibility that the species has simply been overlooked in the past due to misidentification and confusion with other species. The taxonomic history *Cymbula safiana* makes it a candidate for misidentification with *Cellana* sp. (Nakano and Espinosa 2013). *Cymbula safiana* is known as an endangered species in Europe (Espinosa et al. 2011). Although it has been recorded from Angola (Koufopanou et al. 1999), very little is known about the status for this species in the Gulf of Guinea. Accurate ecological data on the biology of *C. safiana* and *Cellana* sp. in this region is certainly dependent on credible species identifications. DNA-analyses may often be required, at

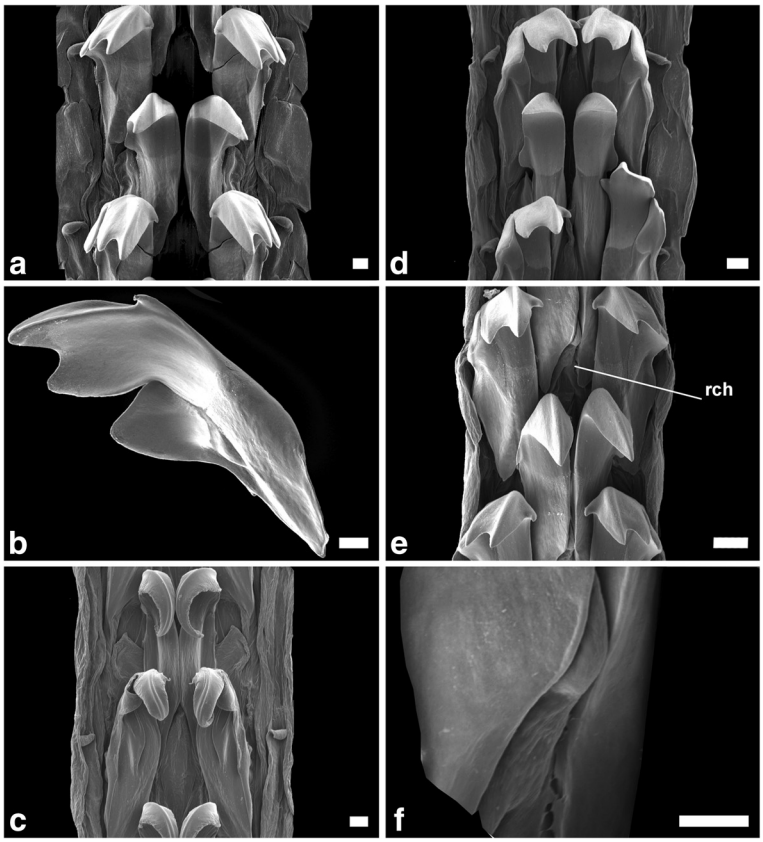

Fig. 3 **a**, **b**, **c** specimen MNFDMOL029, **d**, **e**, **f** specimen MNFDMOL030. **a** Radula, scale bar: 20 μm. **b** lateral view of outer lateral tooth, scale bar: 20 μm. **c** Distal part of radula (from radula sack), scale bar: 20 μm (**d**) Radula, scale bar: 20 μm. **e** Radula displaying possible rachidian teeth, scale bar: 20 μm. **f** possible rachidian tooth, scale bar: 10 μm. Abbreviations: rch, rachidian tooth

least as an initial approach to sort out the natural evolutionary units of a lesser well known regional fauna, particularly now that globalized DNA-data are putting traditional taxonomies to test. However, we have pointed out here that microscopy of the radula will probably be sufficient to discriminate between *C. safiana* and *Cellana* sp. in West Africa. This holds a promise of more accurate ecological data on African limpets in the future. Combined with additional knowledge about the Indo-Pacific relatives the species status of the African *Cellana* should be resolved and a better understanding of its biogeographic history and population biology can be obtained.

Methods

Collecting

The specimens were found on the rocky shores of Takwa Bay area bordering the mouth of the western side of Lagos Harbour, Nigeria. The harbour mouth is protected by heavy igneous boulder rocks on both the western and eastern part to reduce sand deposition and allow for easy passage of vessels into the harbour. These rocks receive direct splashes of ocean waves and are partly covered during high tide. Sample collection was done at low tide when the animals were exposed. The samples were preserved in ethanol.

Identification and DNA barcoding

For a comparative genetic analysis, we selected and downloaded sequences of Nacellidae and Patellidae from Boldsystems.org and NCBI.nlm.nih.gov based on previous studies (Sá-Pinto et al. 2005; Giribet et al. 2006; Nakano and Ozawa 2007; Sá-Pinto et al. 2008; de Aranzamendi et al. 2009; Nakano et al. 2009; Gonzalez-Wevar et al. 2010; Nakano et al. 2010; Sá-Pinto et al. 2010; Espinosa et al. 2011; Gonzalez-Wevar et al. 2011; Sanna et al. 2011; Munoz-Colmenero et al. 2012; Sá-Pinto et al. 2012; Dong et al. 2012; Kim et al. 2012; Nakano and Espinosa 2013; Lin et al. 2015) (Additional file 1). We did not change any of the taxa names in the downloaded sequence data. Tissue samples of approximately 3 mm^3 were cut from the foot of the Nigerian specimens in preparation for DNA extraction and sequencing at the Canadian Centre of DNA Barcoding (CCDB) in Guelph following protocols and procedures of the BOLD system (Ratnasingham and Hebert 2007). The PCR primer pairs BivF4_t1 and BivR1_t1 were used for PCR and primers M13F - M13R for used for Sanger

sequencing (see BOLD primer database (Ratnasingham and Hebert 2007)). The CCDB standard PCR for invertebrates is initial denaturation at 94 °C for 2 min, 5 cycles of 94 °C for 30 s, annealing at 45 °C for 40 s, and extension at 72 °C for 1 min, 35 cycles of 94 °C for 30 s, annealing at 51 °C for 40 s, and extension at 72 °C for 1 min. Finally extension at 72 °C is for 10 min. Assembly of forward and reverse sequences resulted in five high quality gene fragments, 384–658 (mean 599) nucleotides long. Four of these are considered as barcode compliant according to the criteria of the BOLDSYSTEM. Voucher specimens for these observations are stored in the Invertebrate Collections of the University Museum of Bergen, Norway, with the collection codes ZMBN106650, 106651, 106652, 106685, 106686. Sequences with voucher pictures and metadata are available from the Boldsystems.org web site with the following accession codes: NMFDMOL028, NMFDMOL029, NMFDMOL030, NMFDMOL063, NMFDMOL064. They have automatically been assigned to BIN AAI7334 in the BOLD database.

A total of 104 sequences were assembled with the software package Geneious (version 9.0.4) (Kearse et al. 2012) and aligned with the MAFFT plugin (Katoh et al. 2002; Katoh and Standley 2013). We used FastTree2 ver. 2.1.5 (Price et al. 2010) with the GTR and gamma model to estimate an approximated gene tree from the sequences. FastTree2 computes support values for nodes with the Shimodaira-Hasegawa (1999) test and 1000 bootstrap replicates.

Anatomical and scanning electron microscopy work

Radulae from two of the most morphologically divergent specimens, one relatively large and one smaller, were dissected, photographed with a Cannon EOS6D camera and cleaned with proteinase K-solution (Holznagel 1998) obtained from Qiagen DNeasy® Blood and Tissue Kit (https://www.qiagen.com). The radula was placed in 180 µl buffer with 20 µl Proteinase K-solution and incubated at 56 °C for approximately 15 min. The cleaned radulae were partitioned and mounted on metallic SEM stubs with carbon sticky tabs for scanning electron microscopy (SEM). The stubs were then coated with gold-palladium and images taken with a SEM (Zeiss Supra 55VP) at the Laboratory for Electron Microscopy at the University of Bergen. Picture graphics was prepared with the GIMP software (Natterer and Neumann S 2013).

Additional file

Additional file 1: Accession codes with links to BOLD and Genbank sequences used in inference of the gene tree in Fig. 1. Numbers in brackets refer to reference list. (PDF 274 kb)

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Availability of data and materials

DNA sequence data are available in BOLD or Genbank as in Additional file 1. Direct access to the BOLD data including sequence trace files is provided by the following URL: http://dx.doi.org/10.5883/DS-NMFDCELL. The studied specimens are kept the Invertebrate Collections of the University museum of Bergen, Norway.

Authors' contributions

ABW collected the specimens and prepared samples for DNA-barcoding. TRO dissected the specimens and prepared the anatomy and Scanning Electron Microscopy pictures. EW analysed the genetic data and wrote the paper with input from ABW and TRO. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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