J. Black Sea/Mediterranean Environment
Vol. 26, No. 1: 112-118 (2020)

SHORT COMMUNICATION

Genetic confirmation of the first Mediterranean record of *Holacanthus africanus* Cadenat, 1951

Alan Deidun[1], Teresa Maggio[2], Luca Castriota[2], Manuela Falautano[2], Giulio Franzitta[1,3*]

[1] Department of Geosciences, University of Malta, Msida MSD 2080, MALTA
[2] ISPRA (Istituto Superiore per la Protezione e la Ricerca Ambientale), Lungomare Cristoforo Colombo 4521 (Ex Complesso Roosevelt), 90149 Palermo, ITALY
[3] Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Naples, ITALY

ORCID IDs: A.D. 0000-0002-6919-5374; T.M. 0000-0003-1832-4161; L.C. 0000-0002-3349-9501; M.F. 0000-0001-7844-665X; G.F. 0000-0003-4162-3506

***Corresponding author:** giulio.franzitta@szn.it

Abstract

The first Mediterranean record of the pomacanthid *Holacanthus africanus*, caught within the Maltese waters, was assigned based on morphological and meristic characters. However, molecular and genetic analyses are required to confirm the taxonomic determination and avoid misidentification given the abundance of closely-related Pomacanthidae species and the biogeographic significance of this record for the Mediterranean. At the species level, the analyzed specimens gave a 99.7% identity match with *H. africanus*. This study represents yet another example of molecular analyses supplementing the conclusions of conventional morphological identification exercises.

Keywords: Guinea angelfish, taxonomic identification, genetic analyses

Received: 14.11.2019, **Accepted:** 03.03.2020

Taxonomic misidentification, especially of closely-related species, is a very real prospect and an insidious hazard to rigorous biogeographical assessments. Taxonomic exercises relying exclusively on morphological assessments are increasingly being supported by the results/outcomes of molecular techniques, including DNA barcoding, based on the cytochrome *c* oxidase subunit I (COI - Ratnasingham and Hebert 2007), and genetic markers involving other mitochondrial DNA (mtDNA) regions, such as cytochrome *b* and 12S/16S rDNA. The latter are increasingly being deployed in the identification of fish species, including non-indigenous species (NIS – Hamner *et al.* 2007). Such techniques are particularly useful when i) the individuals to be identified at

species level are not in pristine condition, so that diagnostic characters are not easily detectable, or ii) the same individuals have lost some phenotypic characters (e.g., the body coloration fading after capture) helpful for species identification, or iii) characters may be misleading for identification down to species level (e.g., some pomacanthid fish species exhibit very similar liveries during their juvenile stage, when they are hardly distinguishable, while they undergo extraordinary changes in color patterns with growth, acquiring their distinctive coloration when they are adults) (Bailly 2016). In the case of non-indigenous species, molecular analyses may also help in reconstructing their invasion pathways or in corroborating hypotheses on their introduction vectors (Cristecu 2015).

Deidun *et al.* (2017) ascribed two individuals of Pomacanthidae caught within the precincts of a shipping port in the Maltese Islands to *Holacanthus africanus* Cadenat, 1951, on the basis of morphological attributes and meristic characters, which were mostly consistent with those described for the species by Bailly (2016). Given the significance of this record, the first Mediterranean one for the species, the authors embarked on a subsequent molecular study to further confirm the taxonomic identity of the two *H. africanus* individuals recorded in Maltese waters.

Muscle tissue samples from two specimens of *H. africanus* caught in Maltese coastal waters (Deidun *et al.* 2017) were stored in EtOH 90% for subsequent molecular analyses. Genomic DNA was extracted through the use of PureLink Genomic DNA Kits (Invitrogen). Amplification of mtDNA cytochrome *b*, 12S rDNA, 16S rDNA, and control region segments was performed using the universal primers 28FOR-34REV, 12SAL-12SBH, 16SAR-16SBR, and CRA-CRE, respectively (Palumbi *et al.* 1991; Lee *et al.* 1995). PCR was performed following these conditions: hot start for 2 min at 94 °C, 30 cycles of 94 °C for 30 s, 56-48 °C for 30 s and 72 °C for 45 s, with a final 72 °C extension stage for 7 min. The resultant PCR fragment was visualized on 1% agarose gel and subsequently purified and sequenced using an ABI Prism 373 automated sequencer.

Sequences were checked on BLASTn and subsequently aligned using the MUSCLE plugin within the MEGA 6 software (Tamura *et al.* 2013). The sequences from the two Maltese samples were also aligned with those originating from different *Holacanthus* species cited in Alva-Campbell *et al.* (2010) and available in GenBank for taxonomic identification purposes (by comparing the genetic distances obtained). This alignment was performed in order to reconstruct phylogenetic relationships already investigated by these authors. Genetic distance was calculated using the Kimura's 2-parameter model (Kimura 1980).

Phylogenetic reconstructions were performed based on the Neighbour-Joining and Maximum Likelihood methods generated through the MEGA software version 6. Genbank sequences of *Pomacanthus* sp. represented the out group (Genbank Accession Number KC845386, KC845323, KC845344, and FJ447595). In order to estimate support for the nodes, 1000 bootstrap replicates were performed, with only the node-supporting values accounting for more than 50% of the same bootstrap replicates being retained.

These primers successfully amplified the cytochrome *b*, 12S rDNA, 16S rDNA, and Control Region regions for the two specimens of *Holacanthus* caught in Maltese waters analyzed. For cytochrome b, 12S rDNA and 16S rDNA, the two sequences were identical. This result was expected, given the highly conserved status of these three mitochondrial genes. The control region sequences reported five variable sites out of a total of 339 base pairs (see Supplementary file 1). All the genes confirmed the genus with high identity matches via BLASTn.

The alignment with *Holacanthus* sp. GenBank sequences exercise resulted in 389, 344, 545, and 414 base pairs for cytochrome b, 12S, 16S and Control Region, respectively. For cytochrome b, 267 base pairs were conserved and 122 base pairs were polymorphic, with 80 informative and 42 singleton substitutions; for the 12S region, 317 sites were conserved and 27 were variable, of which 22 were informative and 5 were singleton; for the 16S region, 470 base pairs were conserved and 71 were variable, of which 30 informative and 41 singleton; for the Control Region, 218 base pairs were conserved and 121 were variable, of which 118 were informative and 3 were singleton.

The NJ and ML methods reported a similar tree topology for the four molecular markers used. The tree topology obtained was identical to that cited in Alva-Campbell *et al.* (2010), with the two Maltese *Holacanthus* sp. specimens (indicated in the figure as “Holacanthus Med”) clustering within the *H. africanus* group, supported by very strong bootstrap support for all the molecular markers utilized (Figure 1).

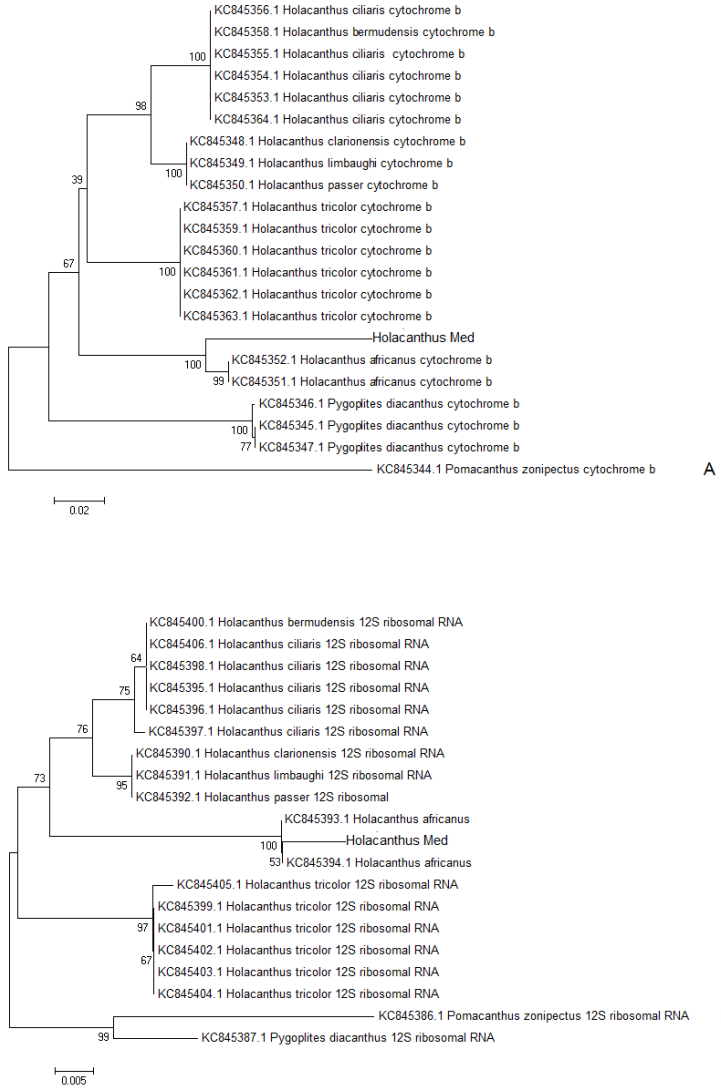

Figure 1. Phylogenetic tree constructed using the Neighbour-Joining method for cytochrome b (A), 12S rDNA (B), 16S rDNA (C) and Control Region (D) of *Holacanthus* spp. The percentage of clustering within each replicate tree for each bootstrap test (1000 replicates) is shown on each branch. The resulting evolutionary distances were computed using the Kimura's 2-parameter model and are in the units of the number of base substitutions per site.

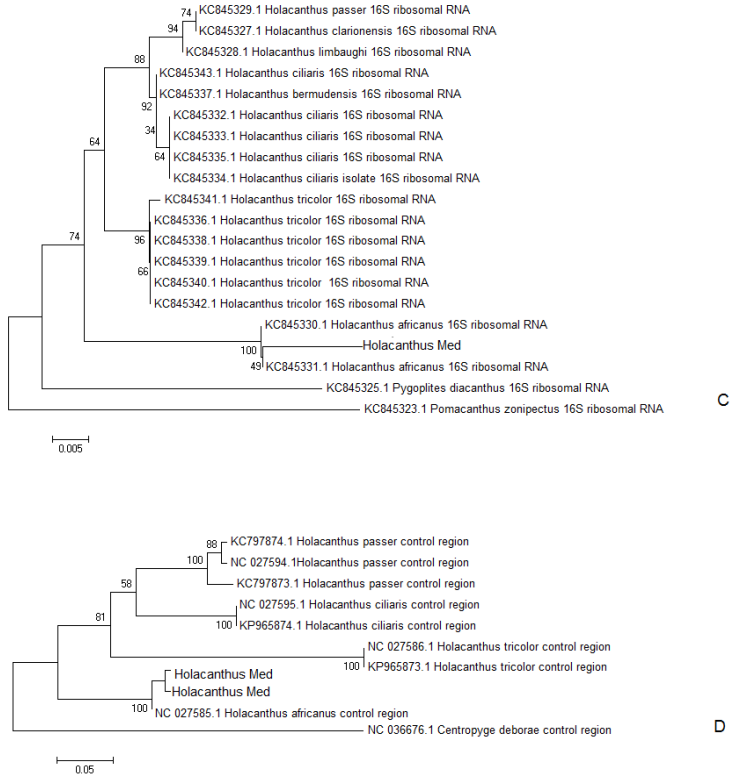

Figure 1. Continued.

At species level, the current specimens had 99.7% identity match with *H. africanus* KC845330 (Alva-Campbell *et al.* 2010) and KP898264.1 (Shen *et al.* 2016) at 16S; and 99.7 to 99.2% matches with KC845394 (Alva-Campbell *et al.* 2010) and KP898264 (Shen *et al.* 2016), respectively at 12S; and 92.7% for KC845351 (Alva-Campbell *et al.* 2010) and KP898264 (Shen *et al.* 2016) at cytb; and 94.6% for KP898264 (Shen *et al.* 2016) at the control region.

Our results confirmed the taxonomic identification of Deidun *et al.* (2017), conducted solely on morphological criteria. It is interesting to note that, in Alva-Campbell *et al.* (2010), the *H. africanus* samples originating from Cape Verde and Sao Tome were not genetically distinct, despite the considerable (approximately 4,000km) geographical distance between these two localities, suggesting extensive gene flow between the two populations and, consequently, a high degree of dispersal by the species. A Mediterranean introduction for *H.*

africanus mediated by the entry of an oil rig/platform, as postulated by Deidun *et al.* (2017) is reinforced by the fact that the species was not recorded from Mediterranean locations in closer proximity to the Atlantic point of entry than the Maltese Islands.

The need for the current molecular study is justified in terms of the preserved nature and state of the two *H. africanus* individuals recorded in Maltese waters, which might have led to the loss/deterioration of diagnostic phenotypic features and a consequent mid-identification of the same individuals, as well in terms of the similar livery of different pomacanthid fish species. Integrated identification studies, combining both conventional (i.e. based on morphological features) and molecular tools, are becoming more common, allowing for an unambiguous assignment of taxonomic identity.

References

Alva-Campbell, Y., Floeter, S.R., Robertson, D.R., Bellwood, D.R., Bernardi, G. (2010) Molecular phylogenetics and evolution of *Holacanthus* angelfishes (Pomacanthidae). *Molecular Phylogenetics and Evolution* 56: 456-461.

Bailly, N. (2016) Pomacanthidae. In: The Living Marine Resources of the Eastern Central Atlantic, (eds., Carpenter, K.E., De Angelis, N.), Volume 4: Bony Fishes Part 2 (Perciformes to Tetradontiformes) and Sea Turtles. FAO Species Identification Guide for Fishery Purposes, FAO, Rome, pp. 2674-2679.

Cristescu, M.E. (2015) Genetic reconstructions of invasion history. *Molecular Ecology* 24: 2212-2225.

Deidun, A., Castriota, L., Falautano, M., Maggio, T. (2017) Yet another angelfish species for the Mediterranean - the first record of *Holacanthus africanus* Cadenat, 1951 from Maltese waters, central Mediterranean. *Bioinvasions Records* 6: 373-376.

Hamner, R.M., Freshwater, D.W., Whitfield, P.E. (2007) Mitochondrial cytochrome b analysis reveals two invasive lionfish species with strong founder effects in the western Atlantic. *Journal of Fish Biology* 71: 214-222.

Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16: 111-120.

Lee, W.J., Conroy, J., Howell, W.H., Kocher, T.D. (1995) Structure and evolution of teleost mitochondrial control regions. *Journal of Molecular Evolution* 41: 54.

Palumbi, S.R., Martin, A.P., Romano, S.L., McMillan, W.O., Stice, L., Grabowsky, C. (1991) The simple fool's guide to PCR. Special Publication of the Dept. of Zoology, University of Hawaii, Honolulu, U.S.A.

Ratnasingham, S., Hebert, P.D. (2007) BOLD: The Barcode of Life Data System (http://www. barcodinglife. org). *Molecular Ecology Notes* 7: 355-364.

Shen, K.N., Chen, C.H., Hsiao, C.D. (2016) Complete mitogenomes of Guinean angelfish (*Holacanthus africanus*) and rock beauty (*Holacanthus tricolor*) (Teleostei: Pomacanthidae). *Mitochondrial DNA Part A* 27: 2769-2770.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. (2013) Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6. *Molecular Biology and Evolution* 30: 2725-2729.