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Unveiling the Hidden Identities of *Botrylloides niger* Herdman, 1886 in Tunisian Marinas Using DNA Barcoding

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ABSTRACT

During our monitoring of non-indigenous species in four Tunisian marinas between May and December 2019, we discovered a colonial ascidian with striking orange zooids arranged in irregular and elongated rows within a transparent tunic. This species caught our attention due to its vibrant coloration and morphology, which closely resembled that of *Botrylloides violaceus* and *B. leachii*. It was found to predominantly grow on fouling invertebrates, solitary ascidians, and other fouling organisms. The species was later identified as *Botrylloides niger* Herdman, 1886. We provide herein a molecular analysis of *B. niger*, using the mitochondrial Cytochrome oxidase (COI) gene as a DNA barcode. Sequences comparison of Tunisian *B. niger* with those deposited in GenBank belonging to different countries showed a percentage of similarity ranging from 80.63% to 100%. Phylogenetic analysis suggested that Tunisian *B. niger* might have been introduced from the Gulf of Mexico.

INTRODUCTION

Botrylloides niger Herdman, 1886 (Class: Ascideacea, Family: Styelidae) is a colonial tunicate species commonly found in various marine environments, including marinas. This sessile organism grows up to several centimeters in diameter and is discovered on various surfaces including invertebrates (personal observations), mussel farms (Della Sala *et al*., 2022), as epiphytes on seaweed (Virgili *et al*., 2022), oyster banks (Rocha *et al*., 2019), and in diverse habitats including lakes and lagoons (Della Sala *et al*., 2022; Virgili *et al*., 2022), and marinas (according to personal observations and Png-Gonzalez *et al*., 2021). They are attached to variety of substrates, including rocks, debris, moorings, ropes, and other artificial structures (Virgili *et al*., 2022; Png-Gonzalez *et al*., 2021). Temiz *et al*. (2023) classified this species as invasive having originating from the West Atlantic region. This filter-feeding has been observed in various locations worldwide, including temperate regions along both coasts of North America (Sheets *et al*., 2016), the coast of Israel (Brunetti, 2009; Reem *et al*., 2018), and around the Suez Canal (Halim and Messeih, 2016). Most recently, Temiz *et al*. (2023) reported sightings of this species along the coasts of the north-eastern Mediterranean Sea within the Antalya, Mersin and Hatay regions as well as in the Fusaro Lake in Italy (Della Sala *et al*., 2022). It was first reported in 2019 in Tunisia's marinas including those in Gammarth, Port El Kantaoui and Cap Monastir marinas (Mnasri-Afifi *et al*., 2024). However, it has not yet been reported in other coasts of Mediterranean Sea or might be identified as another species due to its strong morphological similarity with different species such as *B. leachii* (Brunetti, 2009; Reem *et al*., 2018; Temiz *et al*., 2023). According to Pérès (1958) who initially identified *B. niger* as *Metrocarpa nigrum*, the first specimen of *B. niger* was sampled in Israel in 1952. It

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would have been the first known instance of the species in the southern region of the Mediterranean Sea.

The morphological identification is a crucial first step in the species identification. In the case of *B. niger*, it can be challenging because colonial tunicates are known for their high levels of morphological convergence and plasticity (Rinkevich *et al*., 1993; Blanchoud *et al*., 2018), making them difficult to distinguish between closely related species based solely on their morphological features (Temiz *et al*., 2023). Additionally, many colonial tunicates can change their morphological appearance in response to environmental conditions (Brunetti, 2009), further complicating the process of morphological identification.

Monniot and Monniot (1997) described this species as *Botryllus niger* which evolved to *Botrylloides niger* (Van Name, 1945; Herdman, 1886). Then numerous authors used different taxonomic nomenclature for *B. niger*, including reclassification into different genera, or synonyms with other species. The complexity of the taxonomic identification of *B. niger* and other closely related species was due to the difficulty of distinguishing between species based on only morphological features (Stefaniak *et al*., 2012), such as colony shape, size, and zooids arrangement; these characteristics were often unreliable and leading to numerous misidentifications and taxonomic confusion cited above (Temiz *et al*., 2023).

Recently, advances in molecular techniques have provided a more reliable approach for species identification, and DNA barcoding has become a powerful tool for resolving the taxonomic confusion of *B. niger* and other similar species (Rubinstein *et al*., 2013; Bariche *et al*., 2015; Karahan *et al*., 2017; Alie *et al*., 2018; Viard *et al*., 2019; Salonna *et al*., 2021; Temiz *et al*., 2023). DNA barcoding uses a short, standardized segment of DNA to discriminate species. One of the most widely used DNA barcoding region is the mitochondrial Cytochrome C oxidase subunit I (*COI*) gene (Muirhead *et al*., 2008; Iyappan *et al*., 2016; Kumaran *et al*., 2017; Mastrototaro *et al*., 2019). The *COI* gene is an ideal barcoding region because it evolves rapidly and accumulates differences between species, allowing a clear distinction between closely related species. By comparing the *COI* sequences of specimens, researchers can identify the species affiliation with high accuracy; this is especially useful for organisms lacking morphological specific features or for species exhibiting a significant morphological plasticity.

Our aim is to identify *B. niger* using molecular data based on DNA barcoding and investigate the similarities between *B. niger* collected in Tunisia and those from different countries using phylogeographic assessments aiming to identify the possible origin of Tunisian specimens.

MATERIALS AND METHODS

Sampling

Colonies were obtained from four recreational boating marinas distributed along the Tunisian coastline, namely Gammarth in the north-east, Port El Kantaoui and Cap Monastir in the center-east during May and December 2019, and Djerba in the south in December 2022 (Fig. 1). These colonies of *B. niger* were collected from artificial structures as ropes, buoys, docks and boat hulls. Samples were then relaxed with menthol crystals in seawater for approximately four hours and preserved in a 5% formaldehyde solution in seawater for 48h. A subsample of each colony was also preserved in 99% ethanol for DNA extraction (Ramos-Esplá, 1988; Chebbi *et al*., 2010).

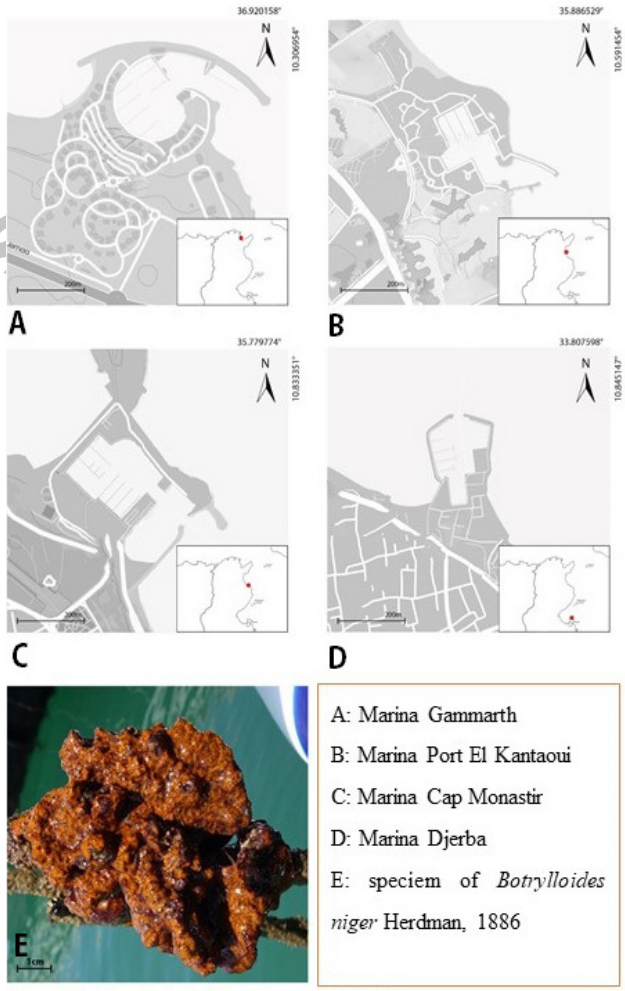

Fig. 1. Sampled marinas along the Tunisian coasts.

Morphological analysis

Over 10 colonies were collected from artificial

substrates in four marinas. Morphological analyses were carried out on the colonies characteristics (e.g., shape, colour, zooids arrangement and features (eg., number of anal lobes), tentacles, presence of sand within or only on the tunic surface (Van Name, 1945; Brunetti, 2009).

The mitochondrial COI *1 analysis*

Total DNA of *B. niger* colonies was extracted from 6-7 zooids sampled at marina Gammarth and preserved in 99% ethanol. Genomic DNA was extracted using two methods to have a good DNA quality: CTAB method (Stefaniak *et al*., 2009) and the DNeasy Blood and Tissue® kit (Wizard® Genomic DNA Purification Kit) following the producer's protocol. DNA quality and quantity were measured using Qubit 3 fluorometer. To successfully amplify and identify the target species, 2 sets of primers with their corresponding *COI* barcoding regions, namely LCO1490 and HC02198 from Folmer *et al*. (1994), and mlCOIintF and jgHCO2198 from Leray *et al*. (2013) were tested (Table I). PCR amplifications were performed with a final reaction volume of 25 µl containing: 10 µM of each primer, TAKARA Taq polymerase (5 U), 50 mM Mg^{2+}, 10 µM dNTP and 100 ng of genomic DNA. "Touchdown" PCR was carried out for 16 initial cycles: denaturation for 10s at 95°C, annealing for 30s at 62°C (-1°C per cycle) and extension for 60s at 72°C, followed by 25 cycles at 46°C.

Table I. COI primers used in this study.

Primer	Sequences 5`→3`
LCO1490	GGTCAACAAATCATAAAGATATTGG
HC02198	TAAACTTCAGGGTGACCAAAAAATCA
MlCOIintF	GGWACWGGWTGAACWGTWTAYCCYCC
jgHCO2198	GGRGGRTASACSGTTCASCCSGTSCC

*Note: R, S, W, T are degenerate nucleotides with R= G/ A; S=G/C; W= A/ T; Y= T/ C

To check PCR reactions success, PCR products were separated on 1% agarose gel and detected by staining with Ethidium Bromide under UV light. The obtained amplicons were purified using spin columns (Wizard PCR Preps, Promega) following the manufacturer's protocol and then sequenced for both directions (forward and reverse) by an ABI-373 automated DNA sequencing system.

Obtained sequences were manually edited using BioEdit version 7.7 software and deposited in the GenBank database. To conduct comparative analyses, a search was performed on January 30th, 2023 for homologous sequences of *Botrylloides* genus within the non-redundant nucleotide database of the NCBI (National Center for Biotechnology Information). Employing BLASTn (Altschul *et al*., 1990) using our *B. niger* sequences as the query.

The MEGA 11 software (Tamura *et al*., 2021) was used to calculate uncorrected pairwise distances with the Kimura-2-parameter (K2P) distance model, and to generate Phylogenetic trees. The consensus tree was evaluated from 500 bootstrap replications. The evolutionary distances were computed using the maximum composite likelihood method and the units of the number of base substitutions per site. The proportion of sites where at least one unambiguous base is present in at least one sequence for each descendent clade is shown next to each internal node in the tree (Pereira *et al*., 2022)

RESULTS

Morphological analysis

All colonies of *B. niger* were collected from the four sampling marinas during May and December. Morphologically, this species shares similar colony color and zooids aspect with *B. leachii*. The specimens exhibit zooids arranged in a ladder-like configuration, a characteristic observed in multiple *Botrylloides* species, as a precaution in the absence of discernable eggs. We identify this species at the genus level as *Botrylloides* sp.

COI sequence comparisons

The mt *COI* gene fragment was successfully amplified from *B. niger* DNA, resulting in a single PCR product of 319 bp. The obtained sequence was deposited in the GenBank database and showed 100% BLAST similarity with *B. niger* from other worldwide specimens deposited into GenBank. A search in the nt-nr database identified 100 sequences belonging to six distinct genera with similarities ranging from 80.63% to 100%. Among these sequences, 42 sequences were classified as *B. niger*, or inaccurately labelled as *B. nigrum*. Table II displays the mean inter-species pairwise uncorrected distances calculated only for the confirmed *Botrylloides* sequences with a representative specimen of each species. The divergence among sequences ranged from 0.001 to 1.09. The highest distance was reported between *Botrylloides cf. anceps* and the other *Botrylloides*. *B. aff. leachii* (MG0095791), *B. nigrum* (NC_021467.1), and *B. niger* (OM866151.1) database sequences diverged less than 1% from the present sequences. Phylogenetic analysis of *B. niger* (OQ920906) from Tunisia clustered with all other *B. niger* strains in GenBank and was compared to other *B. niger* sequences from different regions, revealing some intraspecific variations (Fig. 2). The sequence obtained was remarkably grouped with

Table II. Pairwise uncorrected distances for inter-species comparisons within the genus *Botrylloides*. Square brackets contain the number of sequences analysed for each species. Min and max values are in bold. "*B. niger* (1)" is the sequence analysed in this study, while the label "Public *B. niger*" represents the sequences of *B. niger* present in the nt-nr database. AC number: 1 (OQ920906); 2 (NC_021467.1); 3 (ON053355.1); 4 (MT873573.1); 5 (LS992551.1); 6 (LS992546.1); 7 (ON098245.1); 8 (OM866151.1); 9 (MG009579.1).

Percentage values	*B. niger* (1)	*B. nigrum* (2)	*Botrylloides sp.* (3)	*B. cf. anceps* (4)	*B. perspicuus* (5)	*B. simoensis* (6)	*B. cf. lentus* (7)	*Public B. niger* (8)	*B. aff. leachii* (9)
B. niger (1)	-								
B. nigrum (2)	-	-							
Botrylloides sp. (3)	0,15	0,15							
B. cf. *anceps* (4)	1,09	1,09	1,07						
B. perspicuus (5)	0,17	0,17	0,15	1,21					
B. simodensis (6)	0,17	0,17	0,14	1,33	0,08				
B. cf. *lentus* (7)	0,17	0,17	0,16	1,25	0,18	0,15			
Public B. niger (8)	-	-	0,13	1,09	0,14	0,14	0,14		
B. aff. *Leachii* (9)	0,01	0,01	0,15	1,09	0,16	0,16	0,15	0,01	-

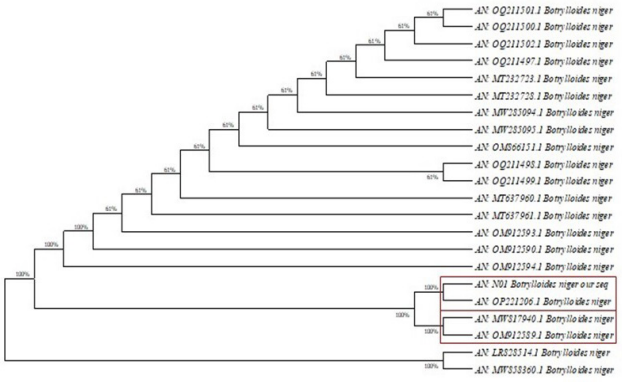

Fig. 2. ML phylogenetic tree of the sequences based on COI nucleotide sequences of all deposited *B. niger* in GenBank. *B. niger* (Our seq) is the sequence analysed in this study.

B. niger sequences previously reported in Turkey, Italy, Mexico, Florida, Honolulu, Brazil, Panama, and Puerto Rico. The highest similarity was reported to *B. niger* strains isolated from Mexico (OP221206.1), Honolulu (MW817940.1), Hawaii (MW817940.1), Tunisia (OQ920906) and Italy (OM912589.1) sharing 100% nucleotide identity (Table III). Sequences of Tunisia and Italy clustered together and also with those of Mexico and Honolou suggesting an introduction of this species into the Mediterranean, particularly in Tunisia and Italy, via the Gulf of Mexico. *B. niger* from Turkey (OQ211502.1) is distant from the two sequences found in Tunisia and Italy which could indicate that there may be more than one clade in the Mediterranean.

DISCUSSION

B. niger identification can be challenging due to its morphological similarity with other *Botrylloides* species. Therefore, it is likely that specimens of *B. leachii* collected in the south part of the Mediterranean Sea since 1960s onwards are *B. niger* (pers. com. Prof. Ramos-Espla). One of the main specific criteria is the coloration; however, in some cases, the misidentification may be due to a lack of self-collection and loss of coloration as result of preservation techniques that leading the degradation of morphological characteristics. It's the case of many ascidians not easily identifiable with morphological methods. Molecular data, such as DNA barcoding has been used to avoid taxonomic confusion of this species and other closely related species (Lambert, 2009; Stefaniak *et al.*, 2012; Brunetti *et al.*, 2015; Montesanto *et al.*, 2022). The *COI* gene has also gained popularity in recent years as a barcoding marker, especially for *Botrylloides* (Reem *et al.*, 2018; Viard *et al.*, 2019; Temiz *et al.*, 2023) due to its higher level of resolution for species-level identification. Samples of *B. niger* colonies from marinas are not collected in a sterile environment. As a result, the isolated DNA is often composite DNA, including genetic material from other organisms such as polychaete worms, algae, bacteria, and nearby organisms that are either attached to the same substrate or attached to the colony itself, such as amphipods. This is the main barcoding difficulty we encountered due to the presence of mixed DNA. The 658 bp barcoding region of the *COI* gene is commonly employed for *B. niger* identification (Streit *et al.*, 2021; Della Sala *et al.*, 2022; Virgili *et al.*, 2022) (Table III). However, in our case, these primers failed to amplify

Table III. COI sequences of the nt-nr database (NCBI, 19 January 2023) analysed in this study with identity percent > 80.50% to our sequence.

AC number	Species description	bp	Locality	Year of submission	Reference
OQ920906	*Botrylloides niger*	319bp	Tunisia	2023	This study
OQ211497.1	*Botrylloides niger*	538 bp	Turkey	2023	Karahan unpub
OQ211502.1	*Botrylloides niger*	514 bp	Turkey	2023	Karahan unpub
OQ211498.1	*Botrylloides niger*	512 bp	Turkey	2023	Karahan unpub
OQ211499.1	*Botrylloides niger*	512 bp	Turkey	2023	Karahan unpub
OQ211501.1	*Botrylloides niger*	512 bp	Turkey	2023	Karahan unpub
OQ211500.1	*Botrylloides niger*	512 bp	Turkey	2023	Karahan unpub
OM912589.1	*Botrylloides niger*	795 bp	Italy	2022	Virgili *et al*., 2022
OM912594.1	*Botrylloides niger*	777 bp	Italy	2022	Virgili *et al*., 2022
OM912590.1	*Botrylloides niger*	771 bp	Italy	2022	Virgili *et al*., 2022
OM912593.1	*Botrylloides niger*	768 bp	Italy	2022	Virgili *et al*., 2022
OM866151.1	*Botrylloides niger*	602 bp	Italy	2022	Della Sala *et al*., 2022
OP221206.1	*Botrylloides niger*	870 bp	Mexico	2022	Palomino-Alvarez unpub
MW858360.1	*Botrylloides niger*	836 bp	Florida	2021	Nydam unpub
MW817940.1	*Botrylloides niger*	854 bp	Honolulu	2021	Nydam unpub
LR828514.1	*Botrylloides niger*	846 bp	Brazil	2020	Gissi unpub
MW285094.1	*Botrylloides niger*	592 bp	Florida	2020	Nydam unpub
MT232728.1	*Botrylloides niger*	553 bp	Panama	2020	Nydam unpub
MT232723.1	*Botrylloides niger*	553 bp	Panama	2020	Nydam unpub
MW285095.1	*Botrylloides niger*	600 bp	Florida	2020	Nydam unpub
MT637961.1	*Botrylloides niger*	596 bp	Puerto Rico	2020	Streit *et al*., 2021
MT637960.1	*Botrylloides niger*	596 bp	Puerto Rico	2020	Streit *et al*., 2021
ON053355.1	*Botrylloides* sp.	859 bp	Saudi Arabia	2022	Nydam unpub
ON098245.1	*Botrylloides* cf. *lentus*	681 bp	Japan	2022	Nydam unpub
MT873573.1	*Botrylloides cf. anceps*	856 bp	Australia	2020	Salonna *et al*., 2021
LS992551.1	*Botrylloides perspicuous*	844 bp	Australia	2018	Gissi unpub
LS992546.1	*Botrylloides simodensis*	856 bp	Japan	2018	Gissi unpub
MG009579.1	*Botrylloides* aff. *Leachii*	467 bp	Israel	2017	Reem *et al*., 2018
NC_021467	*Botrylloides nigrum*	14427 bp	Israel	2012	Rubinstein *et al*., 2013
MW285096.1	*Botryllus* sp.	689 bp	Florida	2020	Nydam unpub
KT693191.1	*Botryllus schlosseri*	625 bp	India	2015	Jaffar ali unpub
KU360789.2	*Botryllus aster*	515 bp	India	2015	Jaffar ali unpub
KU360787.2	*Botryllus arenaceus*	543 bp	India	2015	Jaffar ali unpub
MT840166.1	*Pyura herdmani*	1368 bp	Moroco	2020	Dinoi *et al*., 2021
MH011447.1	*Polycarpa sp.*	487 bp	France	2018	Alie *et al*., 2018
MN138378.1	*Cnemidocarpa finmarkiensis*	658 bp	Washington	2019	Leray unpub

the target COI region which could be due to mutation in binding region leading to PCR failure, compared to 319 bp barcoding region described by Leray *et al*. (2013) were successfully used in our analysis. We report herein the first molecular identification of the non-indigenous *B. niger* in Tunisia. Similarities reported between sequences support a Gulf of Mexico origin. The hypothetical introduction of this species from the Gulf of Mexico to the Tunisian

coasts, either directly or through secondary spread via Italy, could have occurred via multiple pathways. One probable scenario involves maritime shipping and global trade networks. This assertion is based on personal observations of this species in multiple Tunisian marinas, especially in significant numbers post summer season. These marinas act as hotspots for non-indigenous species (NIS) as a result of their composition of artificial substrates, which provide an ideal habitat for NIS in general (Ferrario *et al*., 2016, 2017; Ulman *et al*., 2019a, b) and *B. niger* in particular (Png-Gonzalez *et al*., 2021). Furthermore, it has been suggested by Della Sala *et al*. (2022) that mussel farms are likely be the primary source of introduction for this species. Additionally, Temiz *et al*. (2023) have conducted research which suggests that the clade of *B. niger* present in Turkey differs from the clades found in our study area and Italy. This indicates that the Turkich clade may have been introduced from the Red Sea. However, further sequences will be required to investigate the potential occurrence of this species in Tunisia and the wider Mediterranean region to confirm its origin.

Finally, in the phylogenetic tree *B. niger* clustered with its closest species *B. leachi* confirming the previous morphological identification. It is crucial to identify of *B. niger* in order to fully comprehend its biology and particularly its impact as an invasive species in various regions of Tunisia and globally. A trough examination requiring detailed morphological descriptions and molecular investigations on a larger number of samples is acknowledged as necessary. In order to gain a comprehensive understanding, conducting a more in-depth morphological description and conduct molecular analyses on a greater number of samples is necessary.

DECLARATIONS

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Statement of conflict of interest

The authors have declared no conflict of interest.

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