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Morphological and Molecular Characterization of the Invasive Pestiferous Land Snail *Macrochlamys indica* Godwin-Austen, 1883 (Gastropoda: Ariophantidae) from Saudi Arabia

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Abstract: Many terrestrial gastropod species have been dispersed as a result of anthropogenic activities and have adapted to new habitats where they are considered as alien or invasive species. Several synanthropic gastropods are invasive in urban and agricultural environments worldwide. In Saudi Arabia, increased human activities have accelerated the introduction of terrestrial gastropod species, increasing the need to study its gastropod fauna. Our preliminary survey disclosed the presence of ariophantid snails in a number of agricultural nurseries. Based on morphological features of shell, body, and reproductive system, we report the first record of *Macrochlamys indica* from Saudi Arabia. The phylogenetic analysis obtained from DNA sequences of the mitochondrial COI and 16S regions confirmed the identity of *M. indica*. One third of the investigated nurseries were infested with this snail, however, we did not find any specimens of *M. indica* in natural habitats or open agricultural fields. The snail-infested nurseries were distributed all over Saudi Arabia. The occurrence of one haplotype of partial mitochondrial COI sequence from these nurseries suggests that the introduction of *M. indica* is likely very recent. Based on the obtained results, it is recommended that control measurements must be implemented in order to eradicate or at least restrict the dissemination of *M. indica* from nurseries to natural habitats or agricultural fields.

Keywords: terrestrial snails; *Macrochlamys indica*; invasive species; COI; 16S

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1. Introduction

Mollusks are the second largest phylum and one of the most important groups of the animals on earth, forming a major part of the world fauna. Gastropoda is the most diverse class of the phylum Mollusca, both in habit and shape [1]. Around 35,000 terrestrial gastropod species (snails and slugs) are recognized with an expected 11,000 to 40,000 cryptic species needing to be well identified and characterized [1,2]. Moreover, gastropods have successfully conquered the land and been able to inhabit terrestrial extreme environments, such as deserts, and arctic and alpine habitats [3–5].

Dozens of terrestrial mollusk taxa have been widely dispersed as a result of anthropogenic activities and have adapted to new habitats where they are considered as exotic species [6,7]. Synanthropic gastropods are among these 'traveling species' and are invasive in urban and agricultural environments worldwide [8]. Accordingly, very distant regions may have similar terrestrial mollusk fauna of exotic species [8,9]. In Saudi Arabia, as in several other countries, increased human activities have accelerated the introduction of mollusk species, demanding the need to study its mollusk fauna. Moreover, the terrestrial gastropod fauna of Saudi Arabia and its importance for agricultural and environmental issues has been largely ignored throughout the history of malacological studies.

Herbivorous terrestrial gastropods are important agricultural pests worldwide. They are causing economic damage to many agricultural crops, including vegetables, fruit trees, field crops, ornamentals, and medicinal plants [10,11]. By virtue of their abundance and biomass, they deposit considerable amounts of mucus and fecal materials on the infested crops decreasing their value and quality. They make wounds in plants facilitating the entrance of pathogenic microbes to plants [12,13]. Furthermore, numerous land gastropod species are intermediate hosts for parasites and pathogens posing a health risk to both livestock and humans [14]. Many gastropod species have been shown to be highly invasive and disruptive in zones of conservation attention [15].

Macrochlamys Gray, 1847 is a potentially invasive genus and includes more than 100 species extensively distributed in India and Southeast Asia [16–19]. Ueshima [20] suggested that *Macrochlamys* may have been introduced from Southeast Asia and distributed through horticulture in Japan. One of the most common *Macrochlamys* species is *M. indica* Godwin-Austen, 1883 which is native to India [16,21]. The horn tail snail *M. indica* is a voracious plant eater and able to totally devour young seedlings [22]. It is a widespread pest in vegetables and ornamental plants in India and Bangladesh [17,23,24]. In the USA, *M. indica* is listed as one of the quarantine plant pests posing a paramount danger to agriculture [25]. Singh et al. [22] reported the occurrence of *M. indica* on citrus and guava nursery plants in Punjab, India, feeding on tender and succulent plant leaves and stems. In the forest nurseries of Rajasthan, India, *M. indica* has been described as a serious pest of neem (*Azadirachta indica*) causing 10–65% mortality to neem seedlings [26,27]. Our preliminary survey in this study disclosed the presence of ariophantid snails in different regions of Saudi Arabia. Therefore, we aimed to collect more snail specimens to confirm their identity using morphological and molecular data, as well as explore their habitats in different regions of Saudi Arabia.

2. Materials and Methods

2.1. Sampling and Morphological Characterization

Snail samples were collected by direct visual searching and hand picking from several sites throughout Saudi Arabia including Abha, Al-Ahsaa, Al-Madinah, Al-Qassim, Jazan, Riyadh, Tabouk, and Taif regions from 2020 to 2021. The survey included nurseries, gardens, parks, agricultural fields, and natural habitats. The coordinates of collection sites were recorded using GPS (GPSmap 64S model) as shown in Table 1. The distribution map was generated using ArcGIS v.10.3. The shells and living snails were photographed using a Canon 70D DSLR camera. Individual images were stacked via HeliconFocus v6.22 software and calibrated scale bars (mm) were added using Adobe Photoshop CS5. Most of the collected specimens were hand-picked from beneath plant pots and leaf litters as well as from plant leaves. The following shell morphological measurements were taken using vernier caliper (Raider Pro, RD VC500): shell height (SH), shell width (SW), aperture height (AH), aperture width (AW), height of spire (HSP), and height of the body whorl (HBW) as shown in Figure 1 [28]. In addition, the number of whorls of the shell were counted.

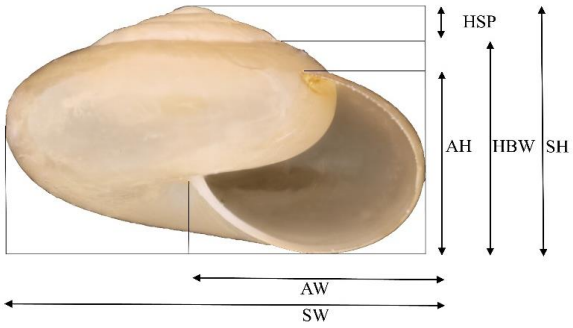

Figure 1. Shell measurements used for characterizing *Macrochlamys indica*. Abbreviations: SH, shell height; SW, shell width, AH, aperture height; AW, aperture width; HSP, height of spire, and HBW, height of the body whorl.

Table 1. Sampling data and GenBank accession numbers for *Macrochlamys indica* specimens collected from different locations in Saudi Arabia.

Region	Site	Specimen ID	Collection Date	GenBank Accession Number		Latitude	Longitude	Altitude
				COI	16S			
Tabouk	Al-Yosif Nursery	SATN2a SATN2b SATN2c SATN2d SATN2e SATN2f SATN2g	29.X.2020	OK559635 OK559636 OK559637 OK559638 OK559639 OK559633 OK559634	OK559650 OK559651 OK559652 OK559653 OK559654 OK559648 OK559649	N28.429333	E36.62695	739
	Magic Rose Nursery	SATN18a SATN18b	6.VI.2021	OK559640 OK559641	OK559655 OK559656	N28.428683	E36.6120166	757
Riyadh	Al-Hair Nursery	SARN2a SARN2b	11.IV.2021	OK559629 OK559630	OK559644 OK559645	N24.520694	E46.7771120	657
	Nursery 10	SARN3a SARN3b	11.IV.2021	OK559631 OK559632	OK559646 OK559647	N24.587879	E46.7227594	668
	Al-Habry Nursery	SARN7a SARN7b	5.II.2022	ON469564 ON469565	ON468668 ON468669	E24.7724166	N46.667567	662
Taif	Bostan Al-Zohor Nursery	SAFN16a SAFN16b	2.X.2021	ON469568 ON469569	ON468672 ON468673	N21.269283	E40.4070333	1679
	Wardet Al-Soltan Nursery	SAFN17a SAFN17b	2.X.2021	-	-	N21.340683	E40.4401166	1609
	Rose City nursery	SAFN24a	2.X.2021	-	-	N21.280333	E40.403112	1659
Jazan	Alam Domiat Nursery	SAJN9a	28.XI.2020	OK559642	OK559657	N17.83765	E42.38015	255
Al-Ahsaa	My Garden Nursery	SAHN23a SAHN23b	4.XI.2021	ON469566 ON469567	ON468670 ON468671	N25.34842	E49.57065	140
Abha	Abeer Alworod Nursery	SAAN4a SAAN4b	2.III.2022	ON469570 ON469571	ON468674 ON468675	N18.58080	E42.70048	2150
Al-Madinah	Al-Wafy Nursery	SAMN1a SAMN1b	30.III.2022	ON469572 ON469573	ON468676 ON468677	N24.5468	E39.57833	617
Al-Qassim	Al-Fatah Nursery	SAQN1a	13.V.2022	-	-	N26.385945	E44.064817	650

2.2. Morpho-Anatomy of the Reproductive System

Twenty live specimens were euthanized [29]. Afterwards, the specimens were preserved in 70% ethanol. The specimens were dissected and the pattern of reproductive system morphology were characterized under a Labomed® Luxeo 6z stereo microscope and photographed using a Labomed® Vega 6MP microscope digital camera (Labo America, Inc., Fremont, CA, USA). The terminology of the reproductive organs was used according to Roy [30].

2.3. Molecular Characterization

Genomic DNA was extracted from 24 snail specimens preserved in 95% ethanol, using QIAamp® DNA Mini kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. Approximately 10 mg of foot tissue was used for DNA extraction. DNA quantity and quality were checked by NanoDrop 2000/2000c spectrophotometer (Thermo Scientific™, Waltham, MA, USA) and 1% agarose gels in 1× TBE buffer, stained with 1 µg/mL acridine orange and visualized under the Gel Documentation In Genius system, (Syngene, Cambridge, UK). DNA concentrations were adjusted to 10 ng/µL for further molecular work.

Two mitochondrial regions were PCR-amplified: cytochrome c oxidase subunit 1 (COI) and ribosomal 16S (16S) subunit using the primer-pairs LCO1490/HCO2198 and 16SarF/ 16SbrR, respectively (Table 2) [31,32]. PCR reactions were performed in 30 µL volume containing 15 µL 5× Promega Master Mix (Promega Corporation, Madison, WI, USA), 0.5 µM of each forward and reverse primers, 3 µL 10 ng/µL DNA, and appropriate volume of molecular biology grade water. The optimum annealing temperatures for COI and 16S primer-pairs were initially determined by gradient PCR using DNA samples of two different snails. The PCR conditions were as follows: an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of a denaturation step at 94 °C for 30 s, an annealing step at 40–58 °C for 30 s and an extension step at 72 °C for 1 min, then a final extension step at 72 °C for 5 min in the Mastercycler® nexus gradient PCR machine (Eppendorf AG, Hamburg, Germany). After determining the optimum annealing temperature for COI and 16S primer-pairs, PCR reactions were performed in a final volume of 30 µL as described in Table 2. The PCR products were analyzed and visualized using 1% agarose gels as previously mentioned.

Table 2. The Primer-pairs and PCR conditions used for amplifying the COI and 16S rRNA regions from *Macrochlamys indica*.

Gene	Primer-Pairs (5′-3′)	Cycling Conditions	Reference
COI	LCO1490: GGTCAACAAATCATAAAGATATTGG HCO2198: TAAACTTCAGGGTGACCAAAAAATCA	94°: 5 min; 94°: 30 s, 42°: 1 min, 72°: 1 min, 35 cycles; 72°: 5 min	Folmer et al. 1994 [32]
16S	16SAR: CGCCTGTTTATCAAAAACAT 16SBR: CCGGTCTGAACTCAGATCACGT	94°: 5 min; 94°: 30 s, 50°: 1 min, 72°: 1 min, 35 cycles; 72°: 5 min	Palumbi et al. 1991 [33]

The PCR products of COI and 16S markers were directly sequenced using the same primers used for PCR amplification at Macrogen sequencing facility (Macrogen, Seoul, Korea). Raw DNA sequences were edited and annotated using the BioEdit v7.0.9.0 [33]. Cleaned sequences obtained from Saudi specimens were searched against the NCBI-GenBank database using BLAST algorithm (http://www.ncbi.nlm.nih.gov accessed on 5 March 2022) for identifying their counterpart's homologous sequences. Based on the sequence homology, the Saudi specimens were recognized to the species level. The pairwise p-distances of COI sequences among different snail species were calculated by using MEGA-X version 10.1.7 [34]. Phylogenetic trees were also constructed using MEGA-X. The evolutionary history was inferred by using the Maximum Likelihood method based on Kimura 2-parameter model [35]. Phylogenetic tree branches were supported with bootstrap values using 1000 replicates.

3. Results

3.1. Distribution of M. indica in Saudi Arabia

The conducted survey revealed that *M. indica* is found only in plant nurseries and distributed in nearly all Saudi provinces: Tabouk in the north, Al-Ahsaa in the east, Taif and Al-Madinah in the west, Jazan and Abha in the south, and Riyadh and Al-Qassim in the middle region (Figure 2). Moreover, *M. indica* was found at different altitudes ranging from 140 to 2150 m above the sea level. We collected 110 specimens from all surveyed nurseries.

3.2. Systematics

Class Gastropoda Cuvier, 1795
Order Stylommatophora A. Schmidt, 1855
Superfamily Helicarionoidea Bourguignat, 1877
Family Ariophantidae Godwin-Austen, 1883
Subfamily Macrochlamydinae Godwin-Austen, 1883
Genus Macrochlamys Gray, 1847
Macrochlamys indica Godwin-Austen, 1883

Materials examined: **SAUDI ARABIA**: **Tabouk**: Al-Yosuf nursery, 29.X.2020, Magic Rose nursery, 6.VI.2021; **Jazan**: Alam Domiat nursery, 28.XI.2020; **Riyadh**: Al-Hair nursery, Nursery 10, 11.IV.2021, Al-Habry Nursery, 5.II.2022; **Taif**: Bostan Al-Zohor nursery, Rose City nursery, Wardet Al-Soltan nursery, 2.X.2021; **Al-Ahsaa**: My Garden nursery, 4.XI.2021; **Abha**: Abeer Alworod nursery, 2.III.2022; **Al-Madinah**: Al-Wafy nursery, 30.III.2022; **Al-Qassim**: Al-Fatah nursery, 13.V.2022; coll. Yasser Abobakr, Ali Al-Sarar, Ali Alzabib (King Saud University).

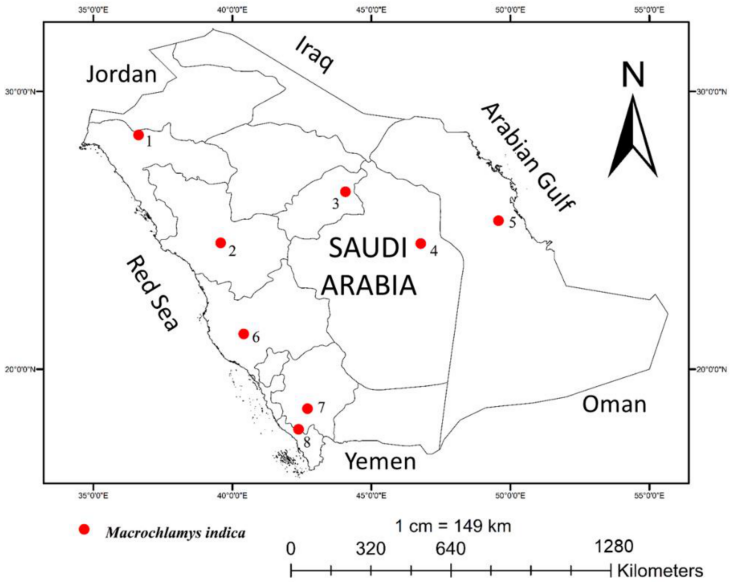

Figure 2. Distribution map of *Macrochlamys indica* in Saudi Arabia. 1. Tabouk, 2. Al-Madinah, 3. Al-Qassim, 4. Riyadh, 5. Al-Ahsaa, 6. Taif, 7. Abha, and 8. Jazan.

3.3. Morphological Description

Shell characters: medium (width up to 17.32 mm; height up to 9.76 mm), depressed, dextral, slightly convex above, pale brownish, translucent, thin but not brittle, surface shiny and smooth with fine growth lines; spire conoid, little raised; suture somewhat impressed; aperture widely lunate and a bit oblique; peristome thin; columellar edge thickened to some extent, oblique, curved, and slightly reflected above the narrowly open umbilicus, number of whorls five to six; the last whorl rounded at the periphery (Figure 3; Table 3).

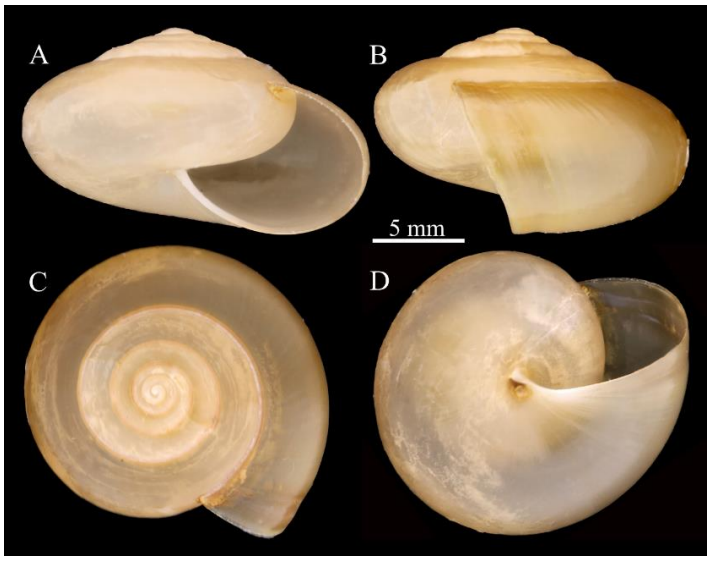

Figure 3. The shell of Macrochlamys indica of SAFN17a specimen collected from Wardet Al-Soltan nursery, Taif, Saudi Arabia in apertural view (**A**), lateral view (**B**), apical view (**C**), and basal view (**D**).

Table 3. Morphometric characteristics of *Macrochlamys indica*.

Characters	Means ± SD	Ranges
Number of shell whorls	5.42 ± 0.26	5.00–6.25
Shell width SW (mm)	17.32 ± 1.78	11.35–22.65
Shell height SH (mm)	9.76 ± 1.02	8.00–12.20
Aperture width AW (mm)	9.21 ± 0.91	7.30–12.55
Aperture height AH (mm)	7.82 ± 0.64	6.60–8.65
Height of spire HSP	1.38 ± 0.25	1.00–1.95
Height of the body whorl HBW (mm)	6.22 ± 0.63	5.00–7.75
SH/SW ratio	0.57 ± 0.08	0.39–0.81
AH/AW ratio	0.85 ± 0.08	0.86–1.14
AW/HSP ratio	6.91 ± 1.36	10.04–4.40
HBW/AH ratio	0.80 ± 0.09	1.01–0.63

Body features: sole tripartite; skin is reticulated and pale to dark gray, becoming lighter near foot sole and darker at the head and eye stalks; caudal horn raised and caudal foss large; mantle lobes dark gray and well developed, dorsal lobes large and broad while shell lobes large and long. Animals secrete yellowish-green mucus when bothered (Figure 4).

Genital organs: the genital opening is on the right side of the head. The genital atrium (at) is a muscular structure elongated to some extent and is wider than the vagina (v) (Figure 5A). The vagina is found between the dart apparatus and the complex of the penial sheath. It opens into the genital atrium and hence the genital opening. The penis (p) is a muscular prolonged structure enclosed by a semi-transparent sheath (Figure 5A,E). The epiphallus (e) is a slender structure longer than the penis. The epiphallus has three distinguished parts; the proximal epiphallus (pe), epiphallic caecum (ec), and distal epiphallus (de) (Figure 5A,D). The epiphallic caecum (ec) is a small circular sac. It is coiled for about two circles, located at one third the length of the epiphallus and attached with the penial retractor muscle (prm) (Figure 5A,D). The vas deferens (vd) is a narrow, long, tubular structure connected between the free oviduct and distal end of epiphallus (Figure 5C). The flagellum (fl) is also long and slender (Figure 5B,D). Its free distal end is slightly less in diameter than that of the proximal end. The free oviduct (fo) is a tubular structure that is extended proximally to vagina (Figure 5A).

A muscular brownish tissue which developed at the junction of the gametolytic organ, vagina, and free oviduct is termed the vaginoviducal capsule (vo) (Figure 5A,C). The spermoviduct is a structure consisting of a folded oviducal channel (ov) and prostate gland (pr) (Figure 5B,C). The oviducal channel comprises a pile of plate-like lamellae which are arranged against the prostate gland. The albumen gland (ag) is a creamy white, moderately elongated tongue-shaped structure near the concave surface of the digestive gland (Figure 5A,F). The hermaphrodite duct (hd) is a convoluted tubular creamy white structure that joins the ovotestis to the spermoviduct (Figure 5F). The gametolytic organ is an elongated, tubular structure comprised of a gametolytic duct (gd) and a cylindrical gametolytic sac (gs) (Figure 5A). The dart apparatus is a well-developed, elongated, whitish, and S-shaped tubular structure, and opens into the genital atrium. It consists of dart bag (db), dart neck (dn), median dart shaft (mds), and dart retractor muscle (drm) (Figure 5A,B).

3.4. Molecular Characterization

To confirm the identity of *M. indica*, two fragments of COI and 16S mitochondrial genes were amplified and sequenced from 24 snail samples representing ten populations collected from agricultural nurseries in different locations/regions of Saudi Arabia.

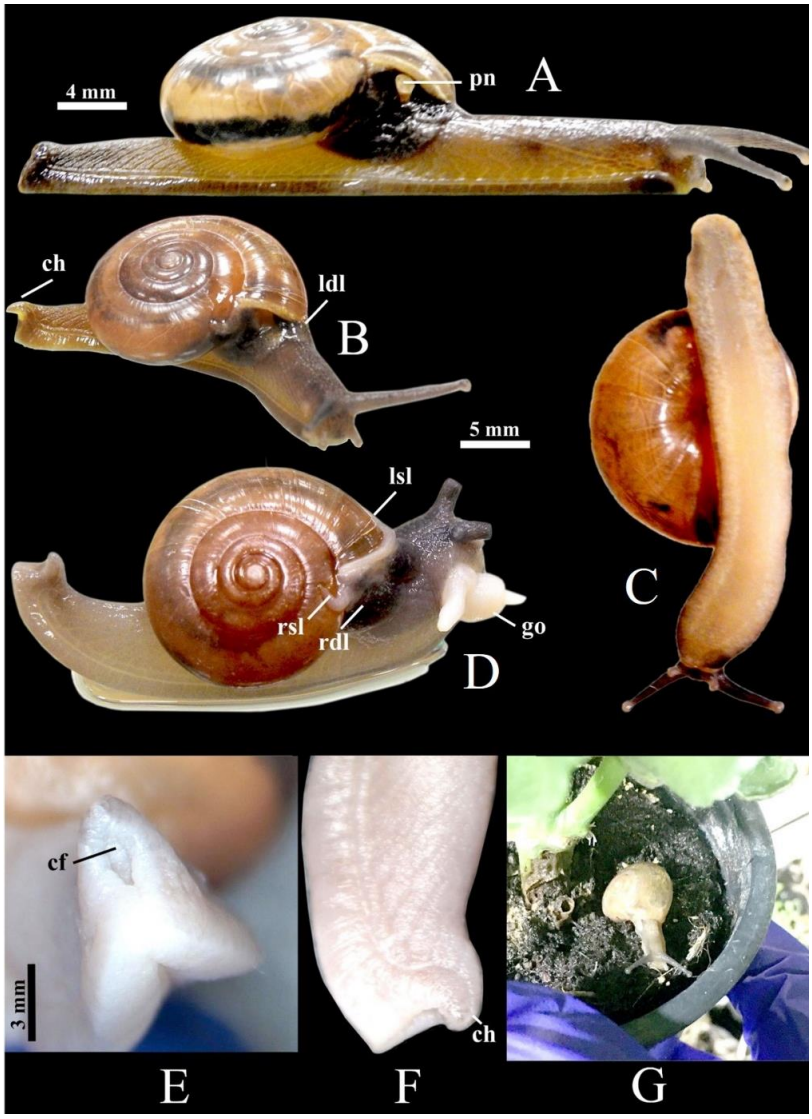

Figure 4. External morphology of Macrochlamys indica. (**A**–**C**) living snail collected from Al-Yosif nursery, Tabouk ((**A**) lateral view; (**B**) dorsal view; (**C**) ventral view); (**D**) preserved snail collected from Magic Rose nursery, Tabouk; (**E**) caudal foss; (**F**) caudal horn; (**G**) living snail found on an ornamental plant in Al-Habry nursery, Riyadh. Abbreviations: cf, caudal foss; ch, caudal horn; go, genital organs; lsl, left shell lobe; ldl, left dorsal lobe; rdl, right dorsal lobe; rsl, right shell lobe; pn, pneumostome.

3.4.1. COI

After removing the LCO1490 and HC02198 primers, the size of the amplified COI fragment generated from 24 *M. indica* snails was 655 bp and represented one haplotype. The 24 COI sequences were deposited in NCBI-GenBank under the accession numbers OK559629–OK559642 and ON469564–ON469573 (Table 1). When the Saudi COI haplotype of *M. indica* was searched against NCBI-GenBank, six sequences (accession numbers: MH819411, MH819412, EF015438, MT803095, LC365425, and MF476193) showed >99% nucleotide sequence identity with the Saudi COI sequence. These six sequences were recovered from different countries, namely India (accession numbers: MH819411, MH819412, and MF476193), Thailand (accession number: MT803095), Japan (accession number: LC365425), and Australia (accession number: EF015438). Five accession numbers were identified as either Macrochlamys sp. (Japan and Australia) or *M. indica* (India), whereas the sixth one was identified as Sarika sp. (Thailand). The alignment of these COI homologous sequences with the Saudi COI haplotype showed six synonymous single nucleotide polymorphisms (SNPs), with no change in their translated amino acid sequence

(Figure 6). The detected synonymous SNPs resulted from either C/T or A/G transitions. We suggest that all the SNPs detected in MF476193 are not real and, most probably, they are artifacts because all of them are G nucleotides and upon their translation, they produced amino acids different from their closely related taxa (Figure 6). For MT803095 identified as Sarika, we suggest that it is misidentified and it needs to be revised. The phylogenetic analysis confirmed the identity of the Saudi snails as *M. indica* where the Maximum Likelihood tree branch, containing its COI sequence along with its homologous ones, received 100% bootstrap value (Figure 7). The very low values (range 0–0.2%, excluding MF476193) of pairwise p-distances of COI sequences also confirmed the identity of *M. indica* from different countries (Table 4). However, the pairwise p-distances of COI between *M. indica* and other snail species were generally above 12%, supporting the uniqueness of *M. indica*.

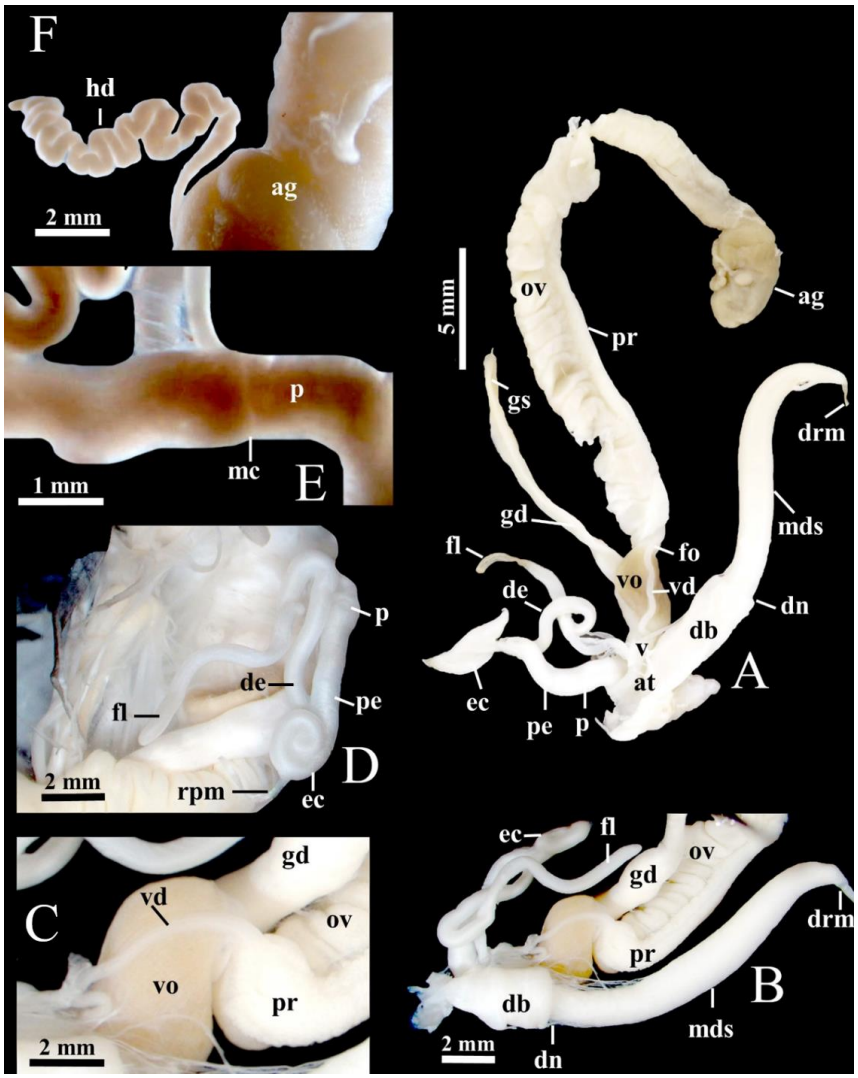

Figure 5. Reproductive system of Macrochlamys indica collected from My Garden nursery, Al-Ahsaa. (**A**) general view of genitalia; (**B**) enlarged view of proximal portion of the genitalia; (**C**) magnified view of prostate gland, vas deferens, and vaginoviducal capsule; (**D**) enlarged view of epiphallus and epiphallic caecum; (**E**) magnified view of penis; (**F**) hermaphrodite duct and albumen gland. Abbreviations: ag, albumen gland; at, genital atrium; db, dart bag; de, distal epiphallus; dn, dart neck; drm, dart retractor muscle; ec, epiphallic caecum; fl, flagellum; fo, free oviduct; gd, gametolytic duct; gs, gametolytic sac; hd, hermaphrodite duct; mc, median constriction; mds, median dart shaft; ov, oviducal channel; p, penis; pe, proximal epiphallus; pr, prostate gland; rpm, penial retractor muscle; vd, vas deferens; vo, vaginoviducal.

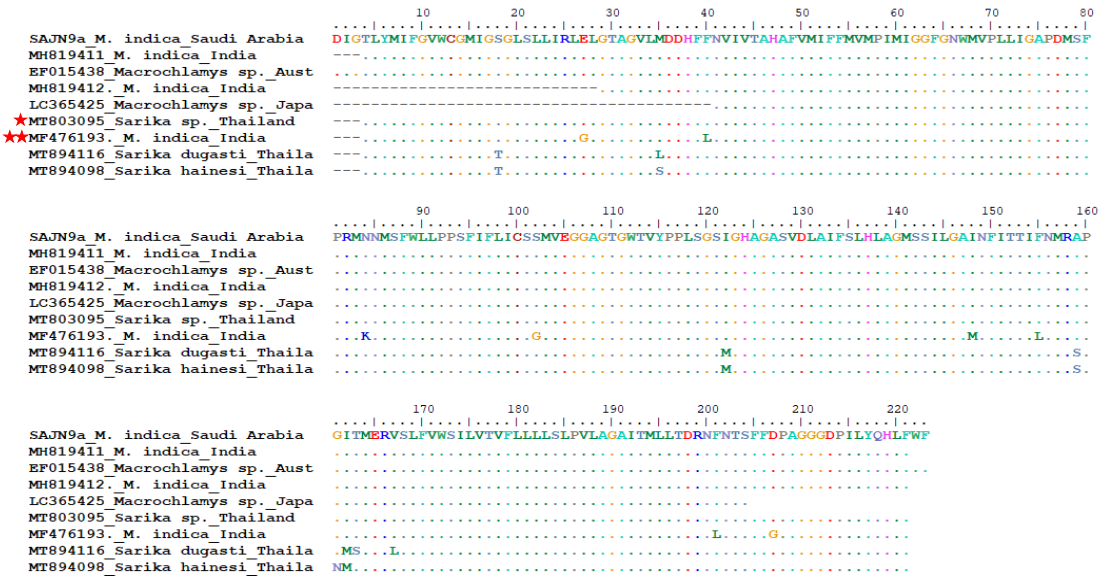

Figure 6. An alignment of amino acid sequences deduced from COI DNA sequences of different snail specimens collected from Saudi Arabia (SAJN9a, *M. indica*) and other closely related homologous sequences retrieved from the NCBI-GenBank database. Dashes refer to missing positions and dots indicate that amino acid residues are identical at these positions. The one star indicates that the COI sequence of MT803095 accession number is misidentified and the two stars indicates that the COI sequence of MF476193 accession number has many artifact DNA nucleotides as all of them are "Gs" and they produce different amino acids compared with their closely related taxa.

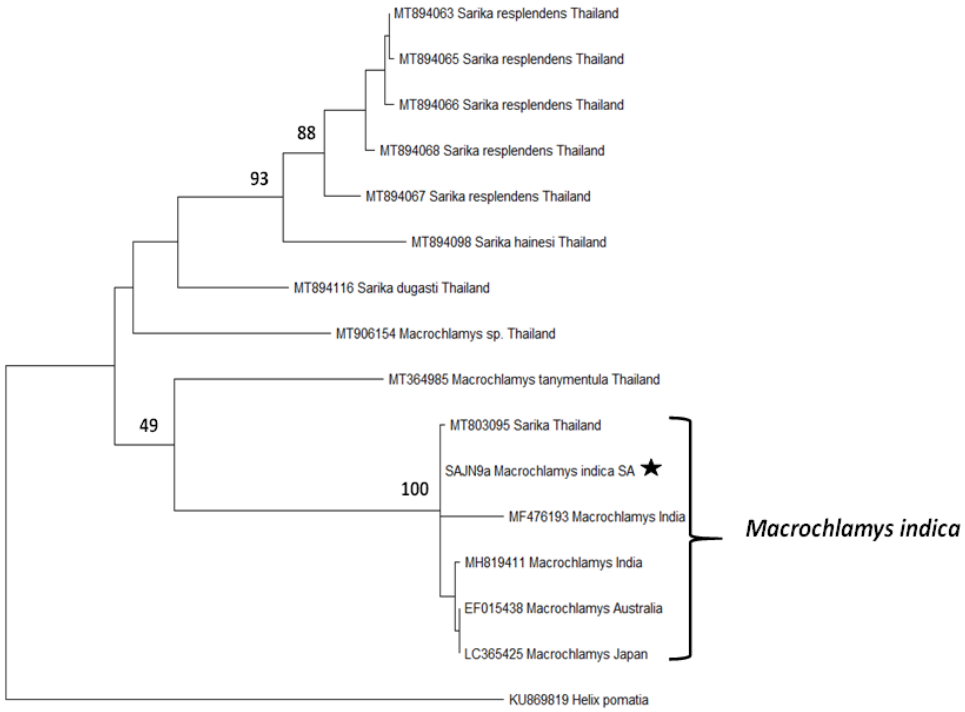

Figure 7. Maximum Likelihood tree obtained from aligned COI sequences from *Macrochlamys* and *Sarika* snails. The star-labeled SAJN9a COI Saudi haplotype represents 24 sequences obtained from 10 snail populations collected from different locations in Saudi Arabia (SA). The other homologous COI sequences were retrieved from the NCBI-GenBank database. *Helix pomatia* (accession number KU869819) was used as an outgroup taxon. Bootstrap values (1000 replicates) are given on the tree branches.

Table 4. Pairwise p-distances calculated from COI sequences from *Macrochlamys* and their closely relative snails.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
MH819411 *Macrochlamys* India (1)															
EF015438 *Macrochlamys* Australia (2)	0.002														
LC365425 *Macrochlamys* Japan (3)	0.002	0.000													
MT803095 *Sarika* Thailand (4)	0.008	0.006	0.006												
MF476193 *Macrochlamys* India (5)	0.028	0.026	0.026	0.024											
SARN2a *Macrochlamys indica* SA (6)	0.006	0.004	0.004	0.002	0.022										
MT364985 *Macrochlamys tanymentula* Thailand (7)	0.127	0.129	0.129	0.131	0.149	0.129									
MT906154 *Macrochlamys* sp. Thailand (8)	0.141	0.143	0.143	0.139	0.160	0.141	0.115								
MT894116 *Sarika dugasti* Thailand (9)	0.127	0.129	0.129	0.127	0.139	0.125	0.107	0.093							
MT894098 *Sarika hainesi* Thailand (10)	0.121	0.123	0.123	0.121	0.133	0.119	0.113	0.105	0.091						
MT894066 *Sarika resplendens* Thailand (11)	0.127	0.129	0.129	0.127	0.141	0.125	0.117	0.107	0.079	0.071					
MT894063 *Sarika resplendens* Thailand (12)	0.125	0.127	0.127	0.125	0.139	0.123	0.117	0.109	0.079	0.069	0.002				
MT894068 *Sarika resplendens* Thailand (13)	0.127	0.129	0.129	0.127	0.139	0.125	0.111	0.105	0.077	0.067	0.012	0.014			
MT894067 *Sarika resplendens* Thailand (14)	0.125	0.127	0.127	0.125	0.139	0.123	0.109	0.095	0.083	0.048	0.036	0.038	0.032		
MT894065 *Sarika resplendens* Thailand (15)	0.127	0.129	0.129	0.127	0.141	0.125	0.119	0.111	0.081	0.071	0.004	0.002	0.016	0.040	
KU869819 *Helix pomatia* (16)	0.214	0.216	0.216	0.216	0.236	0.216	0.206	0.200	0.194	0.192	0.198	0.198	0.194	0.190	0.200

3.4.2. 16S

The amplified 16S fragment was 415 bp, excluding the 16SarF and 16SbrR primers, generated from the Saudi *M. indica* snails. As in the case of COI, no SNPs were detected in 16S fragments. When the Saudi 16S haplotype of *M. indica* was searched against the NCBI-GenBank, three homologous sequences were 100% identical with the Saudi haplotype. The accession numbers of these three sequences were LC365393 (Macrochlamys sp., Japan), MT741772 (Sarika sp., Thailand), and MW685665 (Sarika bocourti, Bangladesh). Based on the morphology description and COI data, 16S sequences of Macrochlamys recovered from Saudi Arabia were deposited in NCBI-GenBank as *M. indica* under the accession numbers OK559644–OK559657 and ON468668–ON468677 (Table 1).

4. Discussion

In the present study, we have reported, for the first time, the occurrence of *M. indica* in Saudi Arabia. In our survey, we did not find any specimens of *M. indica* in natural habitats or open agricultural fields, however, it was found in many plant nurseries. One third of the investigated nurseries were infested with this snail. The snail-infested nurseries were distributed all over Saudi Arabia. El-Alfy et al. [36] reported the occurrence of *M. indica* in the State of Qatar in the Arabian Peninsula. They collected this snail from different ornamental nurseries in Doha city and they thought that the snail was introduced from India with ornamental plants. This Indo-Asian ariophantid snail is native to Bangladesh, India, and Nepal [13,18,22,37–40]. It is proposed to be introduced to the Andaman Islands and Sri Lanka [16,39], Pakistan [41], Peninsular Malaysia [42], and Sabah and adjacent islands [43]. Recently, Agudo-Padrón and Luz [44] and Kudo et al. [19] reported the occurrence *M. indica* as an alien species in southern Brazil and Japan, respectively. *Macrochlamys indica* is apparently an invasive species adversely affecting natural ecosystems and human health [45]. Nandy et al. [46] reported that *M. indica* was linked with several habitats in India, and strong interaction was with tree trunks and leaves. Collectively, it is very probable that *M. indica* was introduced to Saudi Arabia through importing ornamental plants from its native regions.

The type species of *Macrochlamys* was, for a long time, identified as *Helix vitrinoides* Deshayes, 1831, from an unknown locality. The name *M. indica* and the complete description of this species was given by Godwin-Austen [47]. The shell and body morphological characters of *M. indica* in this study agree with those reported by Chanda and Mandal [44]. Taxonomic ambiguity in ariophantid snails has emerged because the species recognition was mainly dependent on shell morphology that is very similar among species [48]. Therefore, other morphological traits are needed for accurate species identification, e.g., snail's reproductive structures that display significant differences among genera and species [49,50]. In the present study, we described morphological characteristics of the reproductive system of *M. indica*. The morphology of *Macrochlamys* penial structures is useful for species-level taxonomy [47,50]. In agreement with Roy [30], the penis of *M. indica* contains a distinct median constriction (Figure 5E). This constriction is weak in *M. coleus* [49] and absent in other *Macrochlamys* species [17,50,51]. The gametolytic organ of *M. indica* consists of an extended proximal gametolytic duct and a distal gametolytic sac (Figure 5A) which is a genus-specific distinctive trait of *Macrochlamys* [30,50]. The elongated epiphallus with a spirally coiled epiphallic caecum (Figure 5D) is a distinctive feature of *Macrochlamys* that distinguishes this genus from *Sarika* which has a straight epiphallic caecum [47]. The dart apparatus of *M. indica* is a major auxiliary copulatory organ possessing four distinct regions and described for the first time by Roy [30]. A protruding dart lip separates the dart from the rest of the dart body. The dart is a non-disposable structure, so the dart retractor muscle may aid the dart eversion and retraction during copulation. The development of a thick, brownish tissue on the free oviduct was described in different *Macrochlamys* species [50]. In the present study, the examination of an *M. indica* reproductive system showed a brownish, muscular tissue that is developed at the connection of the vagina, free oviduct, and gametolytic organ (Figure 5C). Based on its position between the vagina and

the free oviduct, this brownish tissue in *Macrochlamys* was termed as the vaginoviducal capsule [30]. In an earlier study on some helicarionid snails, Hyman et al. [52] characterized a variously-shaped capsular gland on the distal part of the free oviduct. This gland may have structural relationships with the vaginoviducal capsule of *Macrochlamys* and needs further investigation [30]. The structural development of the vaginoviducal capsule along with genitalia may be a species-specific trait in *Macrochlamys* [30]. Nevertheless, this vaginoviducal capsule is poorly-developed in *M. kelantanensis* [50] and totally absent in *M. petrosa* [51].

The integration of molecular data with morphological characteristics has helped in resolving many discrepancies of closely related snails [48,53]. For example, Pholyotha et al. [48] used phylogenetic analysis of COI, 16S, and 28S sequences to recognize *Taphrenalla* Pholyotha & Panha gen. nov., formerly described as *Macrochlamys*, as a new and closely related genus to its sibling genus *Macrochlamys*. In this study, the phylogenetic analysis obtained from DNA sequences of the mitochondrial COI and 16S regions confirmed the identity of *M. indica* collected for the first time from Saudi Arabia. The closest COI and 16S homologous sequences to *M. indica* were either recognized as *Macrochlamys* or *Sarika*, closely sister genera [48]. The average genetic divergence within COI sequences was 0.4%, confirming the identity *M. indica*. Generally, the intra-specific genetic divergences of COI within the same snail species range from 0–2.4% [48]. However, inter-specific genetic divergences among species of the same genus range from 3.0–7.7% [48,54]. For 16S, the same Saudi haplotype was recovered from Japan (LC365393; *Macrochlamys* sp.), Thailand (MT741772; *Sarika* sp.), and Bangladesh (MW685665; *Sarika bocourti*). Consequently, we expect that *M. indica* is becoming a problematic invasive species, especially after Kudo et al. [19] proposed its introduction to and spread in Japan from Bangladesh and West Bengal. To be able to distinguish *M. indica* from other closely related sails, DNA-based markers should be used. In addition, more studies using morphological and molecular markers are needed to investigate *Macrochlamys* in its native habitats.

5. Conclusions

The occurrence of the alien snail *M. indica*, a representative of the family Ariophantidae, is confirmed for the first time in Saudi Arabia (13 localities in 8 regions). We have not found any specimens of *M. indica* in either natural habitats or open fields, however, the infested nurseries can be focal points for spreading this invasive pestiferous snail and may impose great threats on crops in Saudi Arabia. We recommend that the Saudi agricultural authorities implement control measurements in order to eradicate or at least restrict the dissemination of *M. indica* from nurseries to natural habitats and agricultural fields.

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