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DNA reveal new invasive species of tiger shrimp *Penaeus monodon* (Penaeidae) along the world's largest mangrove region in the Brazilian Blue Amazon

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Bioinvasions represent a major environmental issue, particularly when they take place in biodiversity hotspots, such as mangrove ecosystems that serve as shelter for many marine species and fisheries resources. In this work, we used an integrative approach based on DNA and morphological analyses to identify individuals and the putative presence of cryptic diversity in the invasive tiger prawn (*Penaeus monodon*) along a mangrove area on the northern coast of Brazil, referred to as "Blue Amazon". A fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene was selected for DNA Barcode and associated with distance-based (ABGD—Automatic Barcode Gap Discovery) and probabilistic (GMYC—Generalized Mixed Yule Coalescent and bPTP—Bayesian Poisson tree processes) species delimitation methods. Furthermore, the maternal origin of collected specimens was tracked. The molecular analyses recovered two genetically divergent lineages (7.7%) within the tiger prawn, indicating the occurrence of two species of this bioinvader on the northern coast of Brazil. Even though no differences in external morphology were detected, both lineages could be differentiated by their internal structures. The molecular traceability of the origin of samples showed that lineages I and II shared haplotypes with specimens from 11 and nine countries, respectively, including a shrimp breeding center in Vietnam. This is the first record of two species of tiger prawn along the Brazilian continental shelf. These findings are useful to the development of effective management policies in a region of particular biological relevance.

Biological invasions or bioinvasions represent a major threat to local biodiversity, considered one of the main causes of population decline in native species[1]. The tiger prawn (*Penaeus monodon*, Fabricius, 1798) is a native crustacean from Indo-Pacific region[2] that has invaded numerous countries[3–6], including records of established invasive populations in some regions[4,7]. In general, these invasions are related to improper management in aquaculture systems that allow cultivated specimens escaping into natural aquatic habitats[8]. Accordingly, the tiger prawn is an important fisheries resource that has been intensively cultivated in many countries, particularly India, Indonesia, Australia, Philippines, Taiwan, Thailand, Vietnam, Sri Lanka, Malaysia, Mozambique, South Africa, Saudi Arabia and Singapore[2].

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In Brazil, the first report of bioinvasion by *P. monodon* dates to 1987 in a coastal site of Maranhão, northeastern Brazil[9]. Ever since, this species has been recorded in several localities along northern and northeastern Brazil, such as the continental shelf of the state of Pará[10], at the mouth of the Oiapoque River in the state of Amapá[11], and the coastal zones of the states of Maranhão[12] and Rio Grande do Norte[13]. Species invasion can lead to both ecological[14] and economic[15] losses, including the dissemination of diseases and competition with native species[16]. In fact, the tiger prawn is recognized as a potential vector of several pathogens[17,18]. Furthermore, the high fecundity rate[19], the aggressive behavior and the non-specialist carnivorous habits of this invasive species would increase the harmful impacts over native crustaceans.

The tiger prawn belongs to the family Penaeidae, which comprises of 27 genera, 226 species and 13 subspecies[20]. Nonetheless, the taxonomy of penaeids remains poorly understood, with cases of species complexes of species complexes and evidence of cryptic diversity[21,22]. In this context, DNA-based tools represent an efficient approach to providing a precise identification of crustacean species, such as the sequencing of the first portion of the cytochrome c oxidase subunit I (COI) gene, referred to as DNA Barcode[23,24].

The DNA Barcode based on COI allows comparative analyses with public databases such as the BOLD Systems, assuming a minimum threshold of 2% in the genetic divergence to discriminate species[25]. In addition, these data can be analyzed by distinct algorithms of molecular species delimitation based on distance or coalescence methods[26–28] to identify accurately distinct lineages of many taxonomic groups, including crustaceans[22,29].

Furthermore, when possible, integrative taxonomic methods are highly recommended to be performed by associating, for instance, molecular and traditional morphological data. This approach should provide reliable inferences about the species delimitation, including the unambiguous identification of cryptic diversity and a detailed documentation of biodiversity.

The correct discrimination of species is essential to provide a deep knowledge of regional biodiversity and invasive species, thus being useful to effective management plans focused on the conservation of ecosystems and native biota. Therefore, the goal of this study was to validate the occurrence of the giant tiger prawn in coastal and mangrove areas of the eastern "Blue Amazon" using DNA Barcode tool. This area is of relevant interest because it has a high biodiversity, in addition to presenting pressure from human actions on habitats and species that are threatened with extinction in almost all of their extension[30], and the invasion of tiger shrimp has been frequently reported in this region[9,10,12,13]. Following an integrative approach, we tested the putative occurrence of cryptic diversity in the tiger prawn and the traceability of the maternal origin of invasive specimens along the northern coast of Brazil.

Material and methods
Ethics declarations
The specimens were collected dead from artisanal fishermen who practice subsistence fishing, thus dispensing with the approval of ethics in the use of animals, according to Law n°. 11,794/2008.

Sampling
The collection of samples was carried out in distinct sites from the largest continuous mangrove ecosystem in the world along the "Blue Amazon", northern Brazil. Most of them were located within areas of environmental protection, including the extractive reserves (RESEX) of Araí-Peroba, Gurupi-Piriá, Caeté-Taperaçú, and Canelas Island (Fig. 1).

In the RESEX of Caeté-Taperaçú, the specimens were collected by using fixed traps (fishing corral), while the samples from the remaining localities were obtained by trawl nets (20 mm of mesh size). In all collection expeditions, we were assisted by artisanal fishermen from the local communities. The number of samples per locality and their respective identification codes are described in Table 1.

After collection and preliminary in-field identification, the specimens were transported to the Laboratory of Applied Genetics (LAGA) at the Institute of Coastal Studies (IECOS), UFPA, Bragança—PA. Samples of muscle tissue were obtained from the specimens identified as *P. monodon* based on the specialized literature[31] and stored in 90% ethanol at −20 °C. Voucher specimens were fixed in 10% formaldehyde, washed and then transferred to 70% ethanol[32] to be deposited in the Fisheries Zoological Collection from LAGA.

DNA sequencing and alignment of COI sequences
The total DNA was isolated using the Wizard Genomic DNA (Promega) kit according to the manufacturer's instructions. Afterwards, the DNA samples were quantified in a NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher).

The COI fragment was amplified via polymerase chain reaction (PCR) using the primers LCO-1490 and HCO-2198[33]. The PCR conditions were optimized as follows: 2.4 μL of dNTPs (1.25 mM), 1.4 μL of buffer (10x), 0.5 μL of $MgCl_2$ (50 mM), 0.6 μL of each primer (50 ng/μL), 0.1 μL of Taq DNA polymerase (U/ μL), 15 to 30 ng of template DNA and ultrapure water to a final volume of 15 μL. The PCR steps encompassed a first denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 45 s, annealing at 45 °C for 45 s and extension at 72 °C for 45 s, plus a final extension at 72 °C for 5 min.

The amplicons were purified in polyethylene glycol (PEG 8000) as reported by[34] and then sequenced by the dideoxy method[35] using the Big Dye 3.1 (ABI Prism TM Dye Terminator Cycle Sequencing Ready Reaction, Thermo Fisher) kit, according to the manufacturer's instructions. Then, the precipitated products were sequenced in an ABI 3500 XL automatic sequencer (Thermo Fisher).

The electropherograms were individually inspected using the software BioEdit v. 7.2.5[36] to evaluate sequence quality and correction when necessary. Subsequently, the sequences were automatically aligned using the ClustalW tool[37,38] available in BioEdit.
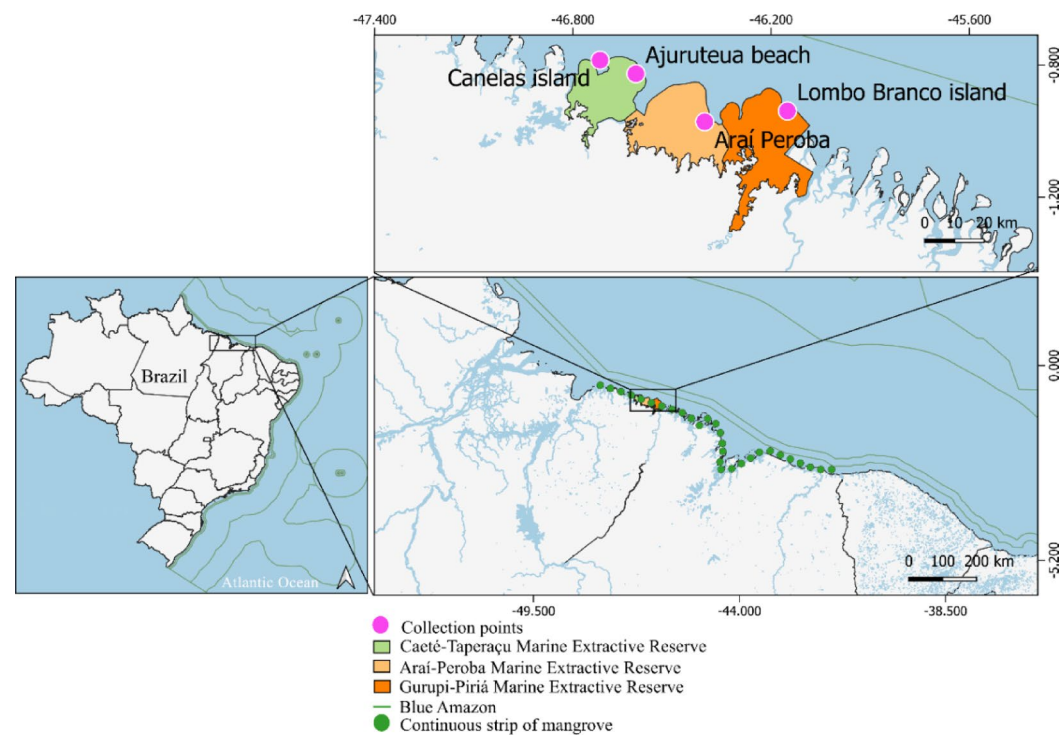
Fig. 1. Map of the Brazilian coast highlighting the continuous mangrove area and the collection sites of *Penaeus monodon* specimens along "Blue Amazon" in the northern coast of Brazil. The map was prepared by Charles Ferreira using freely accessible QGIS software.

Collection site	Code	Number of samples	RESEX
Ajuruteua beach	PmoAJ	10	Caeté-Taperaçu
Canelas island	PmoIC	2	–
Araí peroba	PmoAR	3	Araí-Peroba
Lombo branco island	PmoLB	2	Gurupi-Piriá
Total of samples		17	

Table 1. Collection sites of *Penaeus monodon* with their respective codes and number of sampled specimens. *RESEX* extractive reserves.

Molecular identification and species delimitation analyses

The haplotypes in the sequence dataset were identified using the software DNAsp v6[39]. After the first screening, a final dataset was generated based on a single representative from each haplotype to provide a refined search. Each haplotype was compared separately to the following public databases: GenBank (National Center for Biotechnology Information—http://www.ncbi.nlm.nih.gov) using BLAST (Basic Local Alignment Search Tool) in the Nucleotide BLAST (BLAST-N) window, and BOLD (Barcoding of Life Database—http://www.barcoding life.org) system[40].

Besides the similarity analyses, a neighbor-joining (NJ) tree was built assuming the Kimura-2 parameter (K2P) model[41] and 1,000 pseudoreplicates of bootstrap[42] in the software MEGA v. X[43]. The NJ tree was composed of the sequences from the present study as well as 16 public COI sequences available in BOLD systems, represented by two specimens per species from eight genera of Penaeidae.

The species delimitation tests were performed assuming the HKY + G evolutionary model as recommended based on the Bayesian Information Criterion (BIC) by the jModelTest2 available in ACCESS 2.1.6[44,45], using the CIPRES Science Gateway v3.3 platform[46]. A Bayesian-inference (BI) tree was generated in the software BEAST v. 1.8.4[47,48], following a strict clock and Yule speciation prior as defaults. The support values of posterior probabilities were determined based on 100 million generations and a burn-in of 10%. The log files were checked in Tracer v. 1.5, to evaluate the adequacy of burn-in and the chain convergence, assuming as appropriate only those chains with ESS (effective sample size) values above 200. The tree generated in BEAST was summarized in TreeAnnotator, v. 1.10.4.[47,48], visualized in FigTree v.1.4.4[49] and edited in the software Inkscape v. 1.3.2. The edition of the BI tree was based on the putative groups indicated by the delimitation species tests.

Three species delimitation algorithms were used being based on single-locus (COI) trees and genetic distances, as follows: (i) ASAP (Analysis of Species by Automatic Partitioning) using the web ASAP version

available at https://bioinfo.mnhn.fr /abi/public/asap/asapweb.html[50] based on .FASTA database and assuming the Kimura (K80) TS/TV evolutionary model; (ii) GMYC (Generalized Mixed Yule-Coalescent) using the Splits package available in the (software R v. 3.2[51]) and a summarized ultrametric tree generated by BEAST as input; c) PTP (Poisson Tree Process) carried out on the PTP website (https://species.h-its.org/ptp/;[26]) following the standard defaults available using a maximum likelihood tree as input in the software IQ-TREE v. 2.1.3. and assuming the GTR + F model and 1000 replications[52].

The genetic distance values within and among groups were calculated based on the K2P models[41]. The verification of polymorphic sites and putative stop codons was performed in the software MEGA v. X[43].

Traceability of the maternal origin in tiger prawn samples from the northern coast of Brazil

To trace back the maternal origin of the haplotypes from the present study, we downloaded from BOLD systems the 100 more similar sequences to each haplotype. Therefore, a dataset composed of 517 COI sequences was assembled and inspected using the software DNAsp to verify the shared haplotypes, while the screening of these sequences was performed using WordPad. Some sequences were removed after this step according to the following criteria: (I) repeated sequences, (II) presence of degenerated bases, (III) fragments comprising less the 590 bp, (IV) sequences lacking the origin of samples, and (V) sequences obtained in samples from the same country. A BI tree was built following the abovementioned methodology. The best evolutionary model was HKY + G, while the posterior probabilities were estimated from 100 million generations, assuming a burn-in of 10% and ESS values higher than 200.

Morphological and morphometric identification

The morphological analyses were carried out in the Laboratory of Carcinology at the Institute of Coastal Studies (IECOS), UFPA. The samples were dissected using a scalpel and ultrafine needles (BD Ultra-Fine®, 12.7 × 0.33 mm) onto glass slides, under a binocular stereoscopic microscope (model Q7740SZ). The samples analyzed were the specimens PmoAj07 and PmoLB11, which represent, respectively, lineages I and II. The morphological characteristics measured were the pair of mandibles and the maxilla of each specimen, fundamental structures for the correct identification and discrimination of shrimp. These measurements were performed by a specialist in morphology, with experience in the field of carcinology.

Results

Identification of public databases and species delimitation tests

The 17 COI sequences generated in this study encompassed 591 bp, 42 polymorphic sites and five haplotypes, being invariably similar (> 99%) to those referred to as *P. monodon*. However, these haplotypes were clearly divided into lineage I, comprising three haplotypes (H1, H2, and H3), and lineage II, formed by two haplotypes (H4 and H5). The most frequent haplotypes in the final dataset were H2 (64.7%) and H4 (17.64%) (Table 2). The genetic distance between and within these groups was equal to 7.7% and 0%, respectively. The topology of the NJ tree separated both lineages of the tiger prawn in two highly supported reciprocal monophyletic clusters (Fig. 2), as usually observed for distinct species.

The species delimitation methods recovered a different number of candidate taxa. While the GMYC separated the samples into three clusters, both ASAP and PTP indicated the presence of two species (Fig. 3).

Maternal origin of the tiger prawn samples from northern coast of Brazil

The dataset used in the traceability of the maternal origin of collected samples was composed of 35 sequences standardized to 590-bp fragments. Both lineages of the tiger prawn shared haplotypes with specimens from, at least, 15 countries (Australia, Vietnam, Thailand, Philippines, Egypt, Germany, United States of America, China, Indonesia, India, Taiwan, Bangladesh, South Africa, Mozambique, and Brazil). The lineage I is widespread across 11 countries while the lineage II is found in nine countries, including cases of sympatry (Brazil, Germany, Egypt, India, and China) (Fig. 4).

Morphological analyses

Description of samples from lineage I

Mandibles: asymmetrical with a distinct gap between the incisive and molar processes. Left mandible presents incisive process with four slightly prominent teeth. Molar process with protuberances, being more robust than the incisive. Right mandible presents incisive process with tree few prominent teeth. Molar process with protuberances, being more robust than the incisive (Fig. 5, @1A). Maxilla: Scaphognatite with numerous

Lineages	Haplotypes	Similarity (%)	BIN	Frequency (%)
I	H1	99.82	AAD1869	5.88
I	H2	100	AAD1869	64.7
I	H3	99.66	AAD1869	5.88
II	H4	100	ABA2373	17.64
II	H5	100	ABA2374	5.88

Table 2. List of haplotypes from both lineages of giant tiger prawn collected along the northern coast of Brazil, with their respective similarity percentage in relation to public COI sequences available on BOLD and frequencies. *BINbarcode index number.*
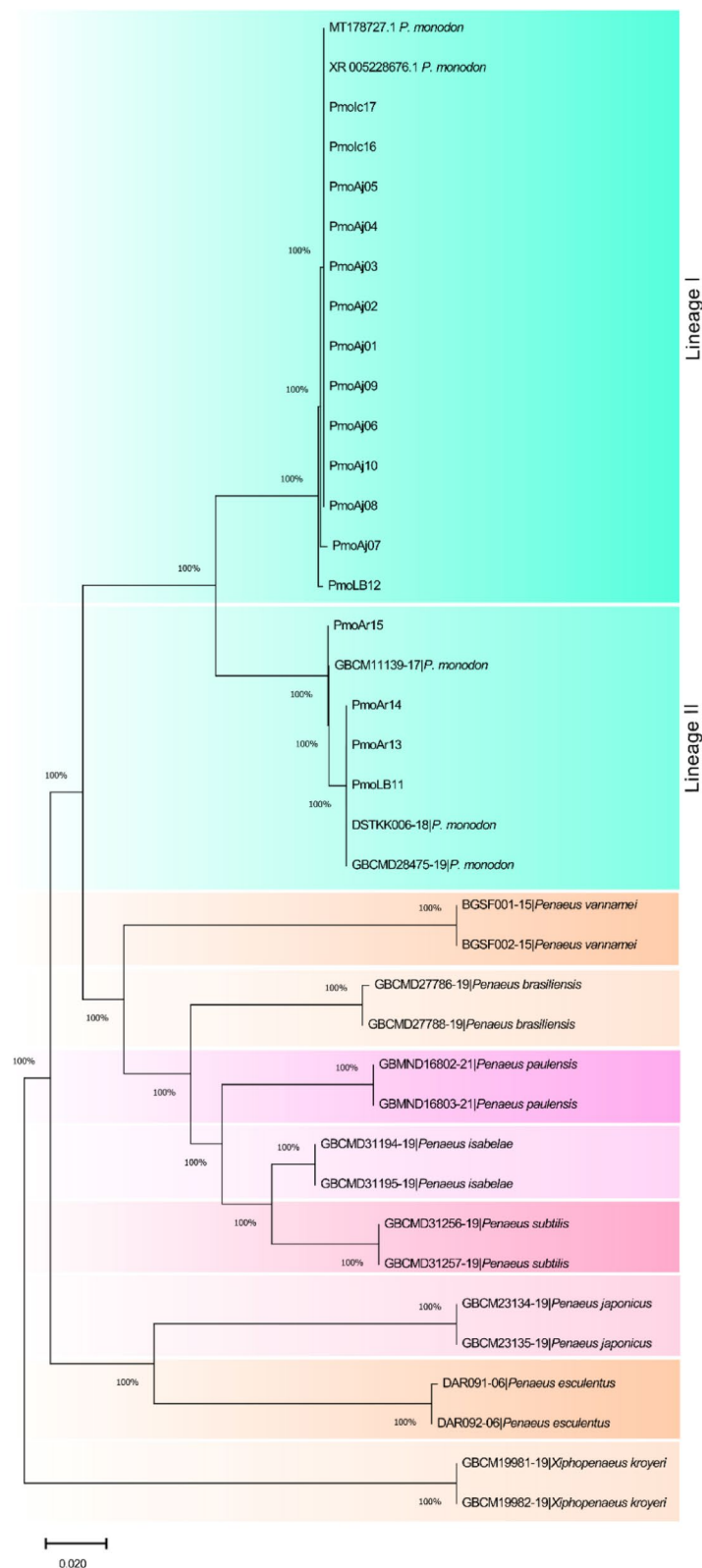
Fig. 2. Neighbor-joining (NJ) tree based on the COI sequences of *Penaeus monodon* collected along the Brazilian Amazon coast and from public databases. The percentages refer to bootstrap values. The color patterns highlight the clades that represent species. This art was created in the Inkscape software.
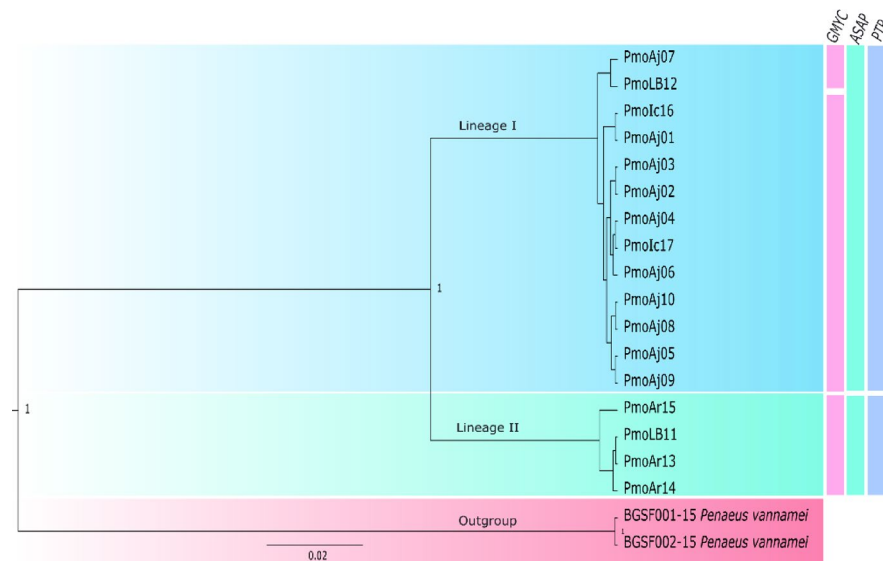
Fig. 3. Bayesian inference (BI) tree evidencing the presence of two giant river prawn lineages along the northern coast of Brazil (the number on branches refer to the posterior probabilities). The bars on the right indicate the species delimitation results from each tested algorithm. (PmoAj-*Penaeus monodon* Ajuruteua; PmoLB *Penaeus monodon* Lombo Branco; PmoIc-*Penaeus monodon* Canelas Island; PmoAr- *Penaeus monodon* Arai; GMYC- Generalized mixed Yule–coalescent; ASAP- Poisson tree processes Assemble; PTP-Species by automatic partitioning. This art was created in the Inkscape software.
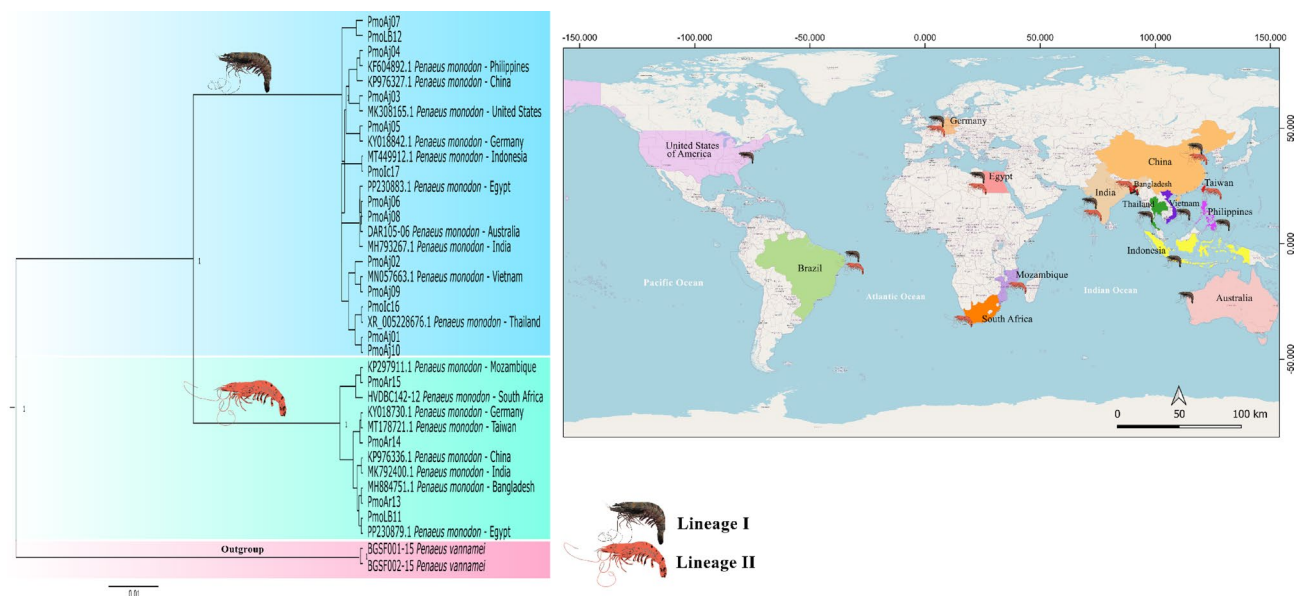

Fig. 4. Bayesian inference (BI) tree based on COI sequences of *Penaeus monodon* indicating the maternal origin of both lineages in the invasive tiger prawn from Brazil and other localities. The map was prepared by Charles Ferreira using the free access QGIS software. The images of the specimens are merely illustrative of the fact that there are two distinct lineages and do not correspond to the actual coloration. This art was created in the Inkscape program.

marginal plumose setae; distal and proximal lobes of the basal and coxal endites with numerous distal plumose setae, with the proximal lobe of the coxal endite being smaller with a few distal setae (Fig. 5 1B). Non-segmented endopod with two distal spines (Fig. 5 1C).

Description of samples from lineage II
Mandibles: Palpi present. Asymmetrical mandibles with a distinct gap between the incisor and molar processes. Left mandible presents incisive process with four well-developed teeth. Molar process with protuberances, being
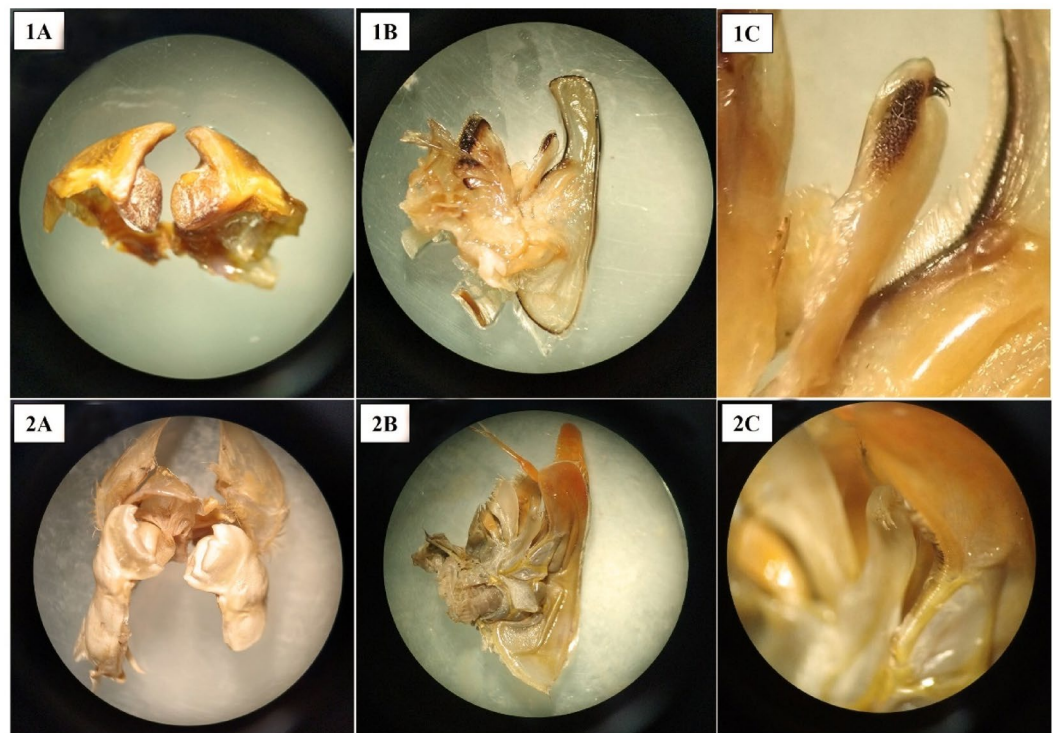
Fig. 5. Internal structures of lineages I (1A-1C) and II (2A-2C) of *P. monodon* from the northern coast of Brazil. (1A- mandible pair, 1B- maxilla, 1C- non-segmented endopod with two distal spines; 2A- mandible pair, 2B- maxilla, 2C- non-segmented endopod with five distal spines). This art was created in the Inkscape program.

more robust than the incisive. Right mandible presents incisive process with two wide teeth. Molar process with protuberances, being more robust than the incisive (Fig. 5 2A). Maxilla: Scaphognatite with numerous marginal plumose setae; distal and proximal lobes of the basal and coxal endites with numerous distal plumose setae, with the proximal lobe of the coxal endite being smaller with a few distal setae (Fig. 5 2B). Non-segmented endopod with five distal spines (Fig. 5 2C).

Discussion
Cryptic diversity in the invasive tiger prawn P. monodon along the largest mangrove area worldwide

Two well-defined, reciprocally monophyletic and highly divergent lineages (genetic distance of 7.7% in COI) of the tiger prawn *P. monodon* were identified along protected areas of the "Blue Amazon" in northern Brazil. In fact, previous reports have already suggested the *P. monodon* should encompass cryptic species. For example,[53] indicated the presence of two lineages of the tiger prawn based on analyzes of COI data in samples collected in Southeastern Pacific, Southwestern Atlantic and Southeastern Indian oceans. Recently,[22] recovered three Molecular Operational Taxonomic Units (MOTUs) in *P. monodon* after screening 1680 COI sequences available in the public database from BOLD systems. Additionally, these authors established a minimum value of 1.8% in genetic distances as a threshold to distinguish between intra and interspecific variation within the tribe Peneaini. Therefore, samples exhibiting values above this threshold would refer to unique MOTUs[22]. However, none of these reports have reported sympatric lineages or have analyzed the invasive populations of *P. monodon* in Brazil.

The species delimitation tests provided contrasting results, since both ASAP and PTP methods identified two candidate species while the GMYC indicated the existence of three clusters. Even though the GMYC and PTP algorithms are based on coalescent approaches[54], the latter usually recovers smaller numbers of putative groups than the GMYC[53]. Accordingly, some reports have demonstrated that GMYC tends to determine an overestimated division of lineages[55,56]. Therefore, we consider that the presence of two cryptic species in the giant river prawn from northern Brazilian coast would be more likely, as suggested by ASAP and PTP methods and corroborated by the phylogenetic inferences and values of genetic distance as well.

In addition to the genetic differences identified, the lineages exhibit clear differences in their internal morphologies, in the structures of the pair of mandibles and maxillae. This was the first study to report morphological differences between the lineages, which is very important, as it reinforces the idea that they may represent distinct species beyond genetic evidence[22].

In fact, molecular species delimitation methods are highly effective in detecting hidden diversity of several groups[22,55,56]. In the case of Penaeidae, most of their diversity remains largely overlooked and associated with evidence of cryptic forms, thus hindering a reliable species discrimination[53]. On the other hand, the DNA

barcode has proved to be a reliable tool for the identification of cryptic species, as recently reported in taxa of Penaeini[22], one of the largest tribes in Penaeidae that includes the giant river prawn[57]. In addition,[22] proposed that genetic distance values equal or above 1.8% would be compatible with the presence of distinct molecular operational taxonomic units or MOTUs in this group of crustaceans.

The two invasive lineages/species of tiger prawn identified in this study are sympatrically widespread throughout the largest continuous mangrove zone in the world[58]. Moreover, a recent study in the same area also recorded the bioinvasion by the rainbow shrimp *Mierspenaeopsis sculptilis*, a native species from the Indo-Pacific region[59]. Considering the importance of mangroves as nursery habitats for the marine fauna[60], the presence of bioinvaders in these ecosystems are potentially harmful, as already reported in mangroves of Bengal, India[61].

As a matter of fact, the mangrove zones along the Amazon coast have been recognized as priority areas for conservation by the Brazilian legislation that defined six Extractive Marine Reserves (RESEX) along this region. Two of them (RESEX Filhos do Mangue and RESEX Viriandeua were implemented recently[62,63]. Thus, genetic information about the diversity of bioinvaders is useful to design proper monitoring policies for the management and conservation of these ecosystems.

Maternal origin of invasive populations in the tiger prawn from northern coast of Brazil and the effects of aquaculture activities

Both lineages or species of the tiger prawn identified along the northern coast of Brazil are also present in, at least 16 countries, revealing multiple origins and invasion routes. A similar finding was reported for alien populations of *P. monodon* in Colombia[64]. As a matter of fact, the tiger prawn is one of the main crustaceans used in aquaculture, ranking the fourth most cultivated species worldwide in 2020. Furthermore, crustaceans represent the second most produced resource in aquaculture, particularly in Asia, followed by Americas, Europe, Oceania and Africa[2]. Following this trend, Brazil has imported two major stocks of *P. monodon* for local shrimp farm from the Philippines, in the late 1970s[65], and Taiwan, in 1984[66]. It should be highlighted that the Philippines remained as the largest producer of giant river prawn in the 1990s[67].

Accordingly, the identification of shared haplotypes between the invasive Brazilian populations and specimens from the Philippines and Taiwan suggests that the imported stocks for shrimp farming activities contributed to the bioinvasion of *P. monodon* in natural Brazilian habitats, as previously suggested by other authors[9,68]. Furthermore, one of the haplotypes observed in lineage I from this study has already been recovered in a shrimp breeding center from Vietnam[69], reinforcing the present hypothesis of invasion routes.

This is the first report about the presence of two invasive lineages of the tiger prawn along the "Blue Amazon" in Brazil. We provided robust evidence that these lineages refer to two species of *P. monodon* distributed along the northern coast of Brazil. We also showed that both species have multiple mitochondrial origins and have already spread to 19 countries. This information is essential to design proper management and conservation policies of these areas. Based on the molecular data, we confirmed that aquaculture activities, particularly the importation of specimens for shrimp farming, have been the main vector of bioinvasion.

Data availability
The sequences obtained were deposited in GenBank, with the following access codes: lineage_I: PQ433754-PQ433766 and lineage_II: PQ433767-PQ433770.

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Author contributions

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Declarations

Competing interest

The authors have no relevant financial or non-financial interests to disclose.

Additional information

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