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Genetic variability of the Amazon River prawn *Macrobrachium amazonicum* (Decapoda, Caridea, Palaemonidae)

Fernanda G. Vergamini[1], Leonardo G. Pileggi[1], Fernando L. Mantelatto[1, 2]
[1] *Laboratory of Bioecology and Crustacean Systematics, Postgraduate Program in Comparative Biology, Department of Biology, Faculty of Philosophy, Science and Letters of Ribeirão Preto (FFCLRP), University of São Paulo (USP), Bandeirantes Av., 3900, 14040-901, Ribeirão Preto, SP, Brazil*
[2] *E-mail: flmantel@usp.br*

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Abstract

The freshwater prawn *Macrobrachium amazonicum* is widely distributed in South America, and occupies habitats with a wide range of salinities. Several investigations have revealed the existence of wide intraspecific variability among different populations, although the understanding of this variability is still fragmentary and incomplete. We compared and characterized inland and coastal populations of *M. amazonicum* from Brazil, using molecular data (16S and COI mtDNA) to describe the degree of variability, structure, and relationships among them. Genetic divergence rates among populations showed variability at the intraspecific level. All the analyses evidenced significant genetic divergence among populations, structuring them in three groups: I- inland waters of the Amazonian Hydrographic Region (HR); II- Paraná/Paraguay HR; and III- coastal systems of northern and northeastern Brazil. Phylogenetic reconstructions revealed that the populations form a single monophyletic clade, which supports their characterization as a single species. Clade I was a sister clade of that formed by clades II and III, which were themselves sister clades. Populations from Sertãozinho/Miguelópolis and Avaré, introduced into the state of São Paulo, may have originated from natural populations in the states of Mato Grosso do Sul and Pará, respectively. Geographical isolation probably contributed to the observed variation, and if this isolation continues, *M. amazonicum* may undergo speciation within its broad geographical distribution. The sequences obtained here can be used as name-tags for population identification, and the DNA barcodes are useful to identify the origin of specimens used in different freshwater-prawn cultures or introduced populations of unknown origin.

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Introduction

Many species of the 'freshwater' prawn genus *Macrobrachium* Bate, 1868 require access to the sea during their larval development (Short, 2004). The members of this genus have three types of reproductive strategies: the first type has extended larval development that depends on marine access; the second includes species with distributions including inland and coastal waters, and their larval development is more or less extended; and the third type includes species with abbreviated larval development that are independent of marine influence and are restricted to inland waters (Williamson, 1973; Magalhães and Walker, 1988; Bueno and Rodrigues, 1995; Alekhnovich and Kulesh, 2001). The Amazon River prawn *Macrobrachium amazonicum* (Heller, 1862) is of the second type (Magalhães and Walker, 1988; Alekhnovich and Kulesh, 2001), and occupies a wide range of salinities, from fresh water (Gamba, 1984; Magalhães, 1985; Bialetzki *et al*., 1997; Gamba, 1997; Porto, 1998; Magalhães, 2000; Hayd and Nakagaki, 2002; Magalhães *et al*., 2005) to estuaries (Barreto and Soares, 1982; Vega-Pérez, 1984; Lobão *et al*., 1986; Odinetz-Collart and Rabelo, 1996; Peixoto, 2002; Silva *et al*., 2007).

Macrobrachium amazonicum is endemic to South America, with a wide geographical distribution including the Amazon and Orinoco river basins and rivers

between these basins (Holthuis, 1952; Odinetz-Collart and Rabelo, 1996), as well as rivers and estuaries in the Guyanas, Venezuela, Colombia, and the northern and northeastern coasts of Brazil (Holthuis, 1952; Melo, 2003; Valencia and Campos, 2007). Inland populations have been recently reported from the Upper Paraná and Paraguay basin in Brazil (Bialetzki *et al*., 1997; Magalhães, 2000; Hayd and Nakagaki, 2002; Melo, 2003; Magalhães *et al*., 2005; Anger *et al*., 2009), Panama and Peru (FLM and LGP, pers. obs.), Bolivia, Paraguay (Melo, 2003), and Argentina (Pettovello, 1996). The presence of this species in Central America (Nicaragua and Costa Rica) has been conjectured by local people and researchers during a field trip by one of us (FLM), but no material is presently available for analysis.

The presumptive natural distribution of *M. amazonicum* includes the Orinoco, Amazon, and Paraguay/Lower Paraná river basins (Magalhães *et al*., 2005). The species probably evolved in one of these regions and then dispersed across these paleobasins after subsequent geological events shifted their boundaries (Magalhães *et al*., 2005 for review). Accordingly, the presence of *M. amazonicum* in northeastern and eastern Brazil and in the Upper Paraná River basin is considered to be unnatural and probably a result of human-mediated dispersal, either accidentally or for aquaculture (Coelho, 1963; Pinto, 1977; Magalhães *et al*., 2005). *Macrobrachium amazonicum* may have been introduced into the state of São Paulo between 1966 and 1973 together with *M. jelskii* (Miers, 1877) in the CESP (Companhia Energética de São Paulo) fish-farming stations, as part of the process of transplanting the fish *Plagioscion squamosissimus* (Heckel, 1840) from reservoirs in northeastern Brazil (Torloni *et al*., 1993). Some small fish were reported to have escaped to natural environments, and the prawns could have followed the same dispersal route (Magalhães *et al*., 2005).

Macrobrachium amazonicum could also have been transplanted to some localities in São Paulo from natural populations occurring in the Pantanal, in the state of Mato Grosso do Sul. The prawns may have been accidentally transported together with some fish species caught in natural environments, to stock ponds

Fig. 1. Collection localities in Brazil for *Macrobrachium amazonicum* samples used in this study. Locations of sampling sites (city and state, respectively): 1: Tapauá, Amazonas; 2: Itacoatiara, Amazonas; 3: Santana, Amapá; 4: Abaetetuba, Pará; 5: Belém, Pará; 6: Santa Bárbara, Pará; 7: Aquiraz, Ceará; 8: Miguelópolis, São Paulo; 9: Sertãozinho, São Paulo; 10: Avaré, São Paulo; 11: Aquidauana, Mato Grosso do Sul; 12: Miranda, Mato Grosso do Sul; 13: Corumbá, Mato Grosso do Sul. Hydrographic Regions in the Brazilian territory: A: Amazonian; B: Tocantins/Araguaia; C: Western North-east Atlantic; D: Parnaíba; E: Eastern North-east Atlantic; F: São Francisco; G: Eastern Atlantic; H: Paraguay; I: Paraná: J: South-eastern Atlantic; K: Uruguay, and L: South Atlantic.

and reservoirs used in sport fishing, where people pay per weight of fish caught. This sport is widespread in the state of São Paulo (Magalhães *et al*., 2005). Another likely reason for the establishment of *M. amazonicum* in the Upper Paraná River basin is the inundation of the Guaíra Falls after the formation of the Itaipu Reservoir in 1982. The removal of this barrier made it possible for several aquatic species to travel upstream into the upper basin (Magalhães *et al*., 2005).

Knowledge of variability among populations of *M. amazonicum* inhabiting different environments has accumulated in recent years. Inland populations show different reproductive strategies from estuarine populations (A.L. Meireles, W.C. Valenti, and FLM, unpublished data); the egg size seems to increase as distance from the ocean increases (Odinetz-Collart and Rabelo, 1996); independent populations show considerable variation in the osmoregulatory and survival capability of larval and adult stages (Augusto *et al*., 2007); the maximum size attained by adults differs between populations from rivers and lakes (Odinetz-Collart, 1987; Odinetz-Collart and Moreira, 1993; Odinetz-Collart and Magalhães, 1994); there is variability among some inland and coastal populations from northern Brazil (Peixoto, 2002); a morphometric analysis suggested the partition of populations from Brazil into two different species (Porto, 2004); and larval morphology differs among some populations (Anger *et al*., 2009). However, the entire life cycle of this species is still under investigation.

This extensive intraspecific variability may be due to genetic isolation of populations, and possibly an incipient speciation process (Anger *et al*., 2009). This condition makes *M. amazonicum* an ideal candidate for comparative studies on population features and evolution throughout its geographical distribution.

In heterogeneous or geographically isolated environments, a single species may have genetically diversified and structured populations. Molecular markers can be useful in delimiting boundaries between lineages and/or species, as well as in studies of intra- and interspecific relationships (Liu *et al*., 2007; Baker *et al*., 2008). As far as we are aware, knowledge of the genetic variability of *M. amazonicum* is restricted to the unpublished thesis by Peixoto (2002), which examined the cytochrome c oxidase subunit I gene - COI) for a small number of populations from northern Brazil.

Considering that *M. amazonicum* has a good potential in Brazilian freshwater prawn aquaculture (Moraes-Riodades and Valenti, 2001, 2004; Maciel and Valenti, 2009 for review) and that knowledge of its life history remains fragmentary (Anger *et al*., 2009; Maciel and Valenti, 2009), the need is evident for complementary studies evaluating the degree of variability among the diversified populations of this species. This led us to evaluate the level of genetic variability and structure among several inland and coastal populations of *M. amazonicum*, covering a wide geographical range in Brazil, using mtDNA data (16S and COI). We also investigated the phylogenetic relationships among these populations and whether they constitute a monophyletic clade.

Material and methods

Sample collection

We used specimens from most of the coastal and inland regions of Brazil where this species has been reported to date. Thirteen populations of *M. amazonicum* were analyzed, from throughout the country (Fig. 1). The populations were classified according to the Brazilian National Hydrographic Division (Brasil, 2003) and the influence of brackish water (coastal: those restricted to river systems close to the seacoast, with brackish water influence; and inland: those found in inland river systems with no connection to the coast).

Coastal populations covered the following Hydrographic Regions (HR): Amazonian, Tocantins-Araguaia, and Eastern/Northeast Atlantic (Fig. 1). The inland populations were divided in two groups: Amazonian HR, and Paraguay and Paraná HR (Fig. 1). Inland populations sampled in the state of São Paulo and along the northeastern Brazilian coast were classified as introduced, because of their unnatural distributions (Magalhães *et al*., 2005).

Some specimens were obtained from field collections, carried out in compliance with current applicable state and federal laws of Brazil (DIFAP/IBAMA, 126/2005; permanent license for collection of Zoological Material No. 11777-1 MMA/IBAMA/SISBIO). These specimens were incorporated into the Crustacean Collection of the Biology Department (CCDB) of the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto (FFCLRP) and the University of São Paulo (USP) (Appendix). Complementary specimens were acquired by donation or loan from crustacean collections, or were collected and sent to us by collaborating researchers from several institutions in Brazil (Appendix). Donated material was preserved directly in 80% ethanol and deposited in the CCDB.

The identifications were based on the diagnostic morphological traits of *M. amazonicum* (Heller, 1862; Holthuis, 1952; Gomes-Corrêa, 1977; Melo, 2003).

Based on the proposed phylogeny for *Macrobrachium* by Pileggi and Mantelatto (2010), we identified the species that are more closely related and reliably distant from *M. amazonicum*, to compose the outgroup in our analyses (Appendix).

DNA extraction, amplification, and sequencing

All sequences used in this study were generated from our own extractions for this project. When possible, the analyses used three to ten specimens from each collection site, in order to limit the chance of misidentifications and variability. Genetic vouchers, from which tissue samples were obtained, were deposited in appropriate collections (Appendix). All procedures followed Mantelatto *et al.* (2007, 2009a) and Pileggi and Mantelatto (2010), with appropriate modifications. Total genomic DNA was extracted from the abdomen or from the pereiopod muscle tissue.

A polymerase chain reaction (PCR) was conducted in a Thermo® PxE 0.2 Thermal Cycler, using the universal primers for invertebrates: 16Sar (5′-CGCCTGTTTATCAAAAACAT-3′) and 16Sbr (5′-CCGGTCTGAACTCAGATCACGT-3′) (Palumbi *et al.*, 1991) for the 16S rRNA (the large subunit of the ribosomal

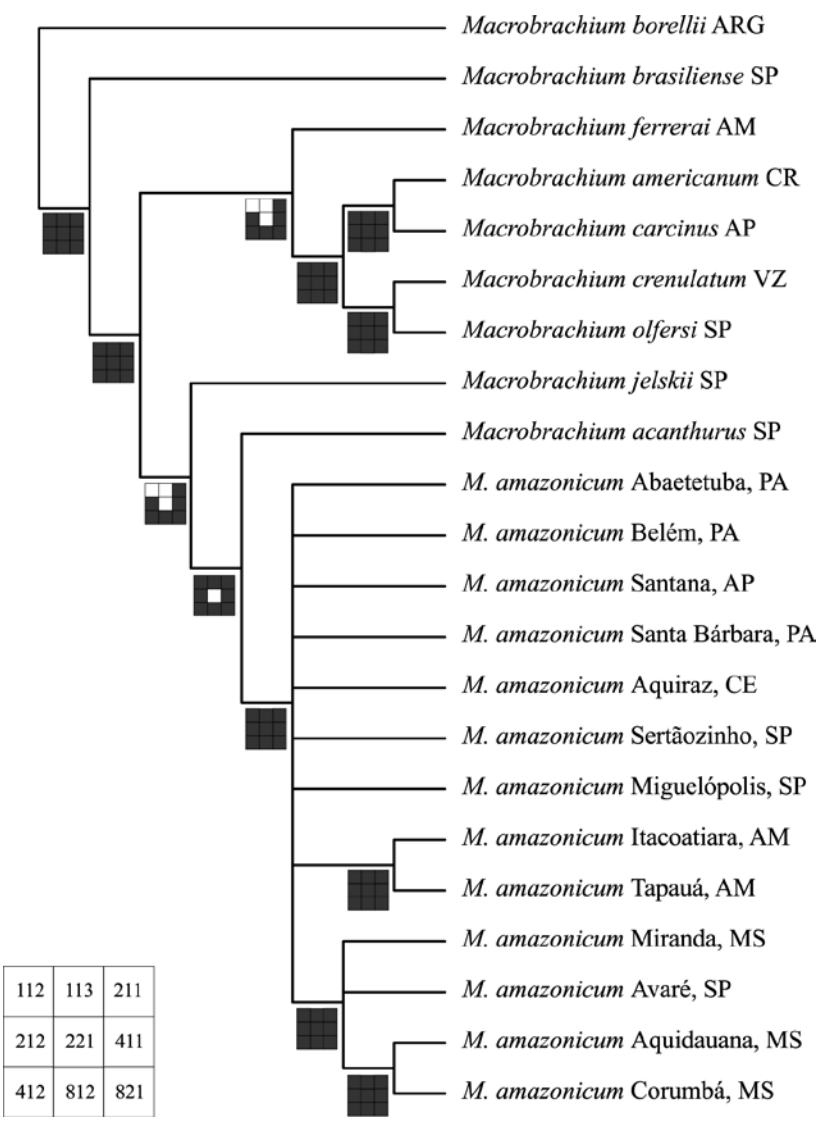

Fig. 2. Phylogenetic tree for populations of *Macrobrachium amazonicum* from Brazil based on direct optimization analysis of 16S rRNA data sets. The box on the left/down side indicates the parameter sets used in the sensitivity analysis. Filled boxes correspond to the parameters under which the clade was stable. ARG: Argentina. CR: Costa Rica. VZ: Venezuela. Brazilian states: AM: Amazonas; AP: Amapá; CE: Ceará; MS: Mato Grosso do Sul; PA: Pará; SP: São Paulo.

rRNA), and COI-a (5'-AGTATAAGCGTCTGGGTAG TC-3') and COI-f (5'-CCTGCAGGAGGA GGAGAC-CC-3') (Palumbi and Benzie, 1991) for the COI gene. PCR products were purified using Microcon 100® filters and a SureClean Plus kit, and were sequenced with the ABI Big Dye® Terminator Mix in an ABI Prism 3100 Genetic Analyzer® following Applied Biosystems protocols. All sequences were confirmed by sequencing both strands. The consensus sequence for the two strands was obtained using BioEdit Version 7.0.7.1 (Hall, 1999). Sequences were edited using BioEdit and aligned in Clustal W (Thompson *et al*., 1994) with interface in BioEdit, with default parameters. All sequences were submitted to GenBank (Appendix).

Phylogenetic analyses

It is recommended that, at least preliminarily, the phylogenetic relationships that delimit a monophyletic group be resolved, so that an analysis can be undertaken with only one segment of this group (Amorim, 2002). Considering *Macrobrachium* as a natural group (Murphy and Austin, 2005; Lui *et al*., 2007; Pileggi and Mantelatto, 2010), our phylogenetic analysis focusing on *M. amazonicum* populations can be considered relevant and justified.

The gaps from the 16S mtDNA sequences, which are due to real gaps in the alignment, were removed in order to obtain non-aligned sequences. No gaps were found in the alignment of COI sequences. These sequences were analyzed in POY Version 4.0 (Varón *et al*., 2007) using the direct optimization method, with parsimony as the optimality criterion (Wheeler, 1996). This methodology has given consistent results in recent molecular phylogenies of crustaceans (Mantelatto *et al*., 2009b; Pileggi and Mantelatto, 2010). Topologies were constructed through random addition sequence, followed by a combination of refinement parameters. Sensitivity analysis was carried out using different cost matrices, as suggested by Wheeler (1995). All data sets for the parsimony analysis were analyzed under 10 parameter sets for a range of indels, transition, and transversion ratios. The matrix digits (111, 112, 113, 211, 212, 221, 411, 412, 812, and 821) correspond to the ratio of indel/transversion/transition values, respectively.

Distance analyses

Distance analyses were carried out by the static alignment procedure for both gene sequences. Ambiguous regions of the sequences were removed. Substitution models used in distance matrix calculations were previously selected under the Akaike Information Criterion (AIC) (Posada and Buckley, 2004) among 56 available alternatives of the program ModelTest Version 3.7 (Posada and Crandall, 1998). Matrix data were grouped by Neighbor Joining (NJ) (Saitou and Nei, 1987) in PAUP Version 4.0 beta10 (Swofford, 2003) using the maximum-likelihood distance correction set. The consistency of topologies was measured by the bootstrap method (Felsenstein, 1985) with 1000 replicates; only confidence values > 50% were reported. In order to estimate intra- and interspecific divergence rates, genetic distances were also calculated in PAUP using the p distance. All positions were compared directly for each pair of sequences, one at a time.

Population analyses

In this analysis, we considered COI sequences from coastal and inland populations of *M. amazonicum*. The haplotype number was calculated in DnaSP Version 4.10.9 (Rozas and Rozas, 1999). The haplotype and nucleotide diversities were calculated for each population using Arlequin Version 3.1 (Excoffier *et al*., 2005).

Haplotype networks were constructed by the statistical parsimony method in TCS (Version 1.21) (Clement *et al*., 2000) and by the Median-Joining method in Network (Bandelt *et al*., 1999), with data preparation in DnaSP. Networks were constructed in two phases. First, introduced populations with an unnatural distribution (Appendix, Fig. 1) were not included because their origins are unknown, and the results could be skewed or masked by their presence in the analysis. In a second phase, when the genetic variability among natural populations had been estimated, an analysis with all populations was carried out so that the probable origin of the introduced populations could be inferred.

Series of analyses of molecular variance (AMOVA) (Excoffier *et al*., 1992) were computed in Arlequin to examine the distribution of genetic variation. Analyses were run based on haplotype frequencies with no hierarchical structure (all populations in a single group) and with regional subdivisions defined according to the results of the haplotype networks. The significance was tested using a nonparametric permutation procedure (Excoffier *et al*., 1992), incorporating 10,000 permutations.

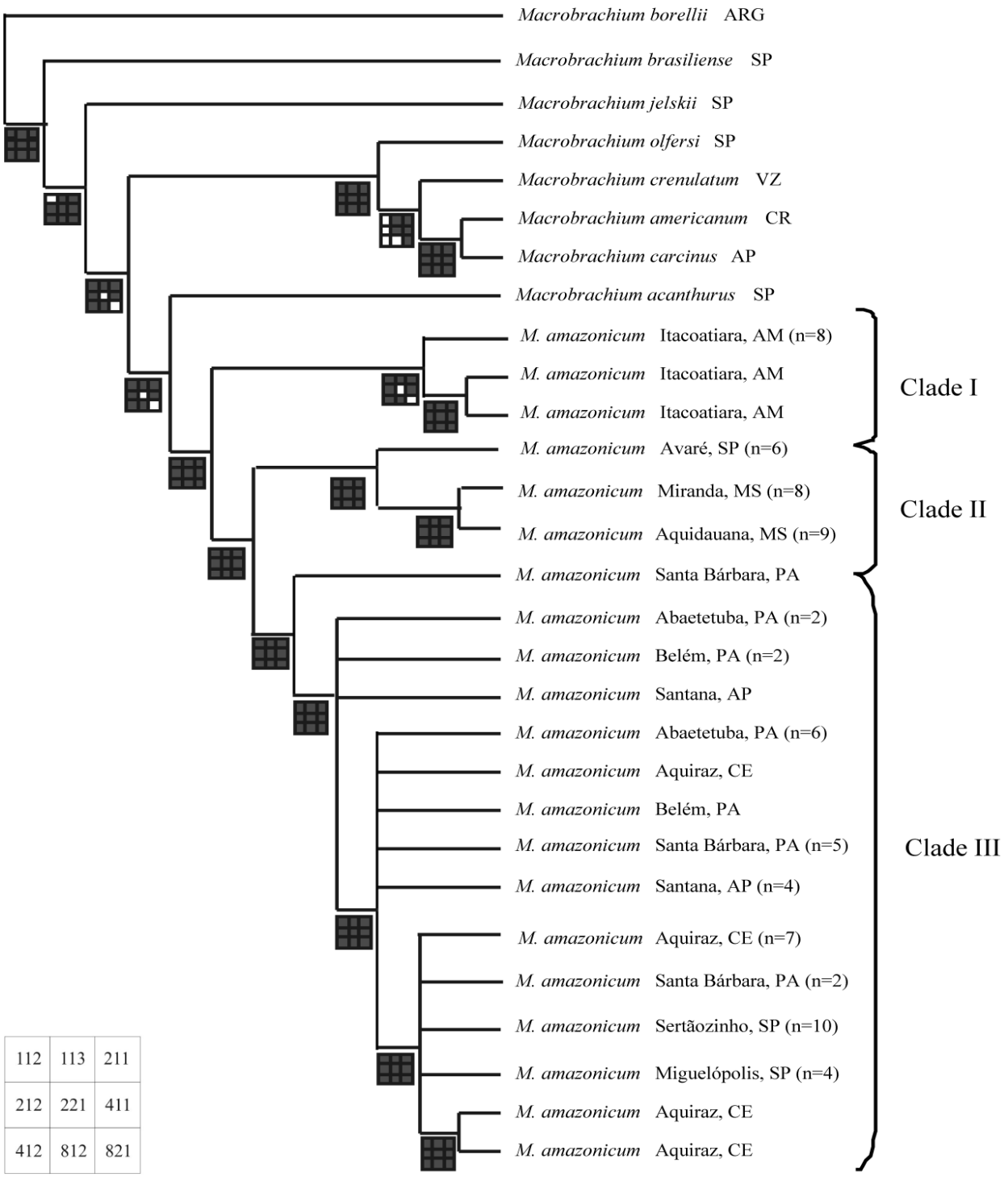

Fig. 3. Phylogenetic tree for populations of *Macrobrachium amazonicum* from Brazil based on direct optimization analysis of COI data sets. The box on the left/down side indicates the parameter sets used in the sensitivity analysis. Filled boxes correspond to the parameters under which the clade was stable. ARG: Argentina. CR: Costa Rica. VZ: Venezuela. Brazilian states: AM: Amazonas; AP: Amapá; CE: Ceará; MS: Mato Grosso do Sul; PA: Pará; SP: São Paulo. Clades: I - inland population from Amazonian Hydrographic Region (HR); II - inland populations from Paraná/Paraguay HR; III - coastal populations from northern and north-eastern Brazil.

Results

Phylogenetic and distance analyses

We acquired 16S rRNA gene partial sequences with 540 aligned base pairs (bp) from 22 specimens, of which 13 were *M. amazonicum* (each representing a different population) and 9 were other *Macrobrachium* species (outgroup) (Appendix). The COI sequences were 569 bp in length, obtained from 89 specimens, 81 of which were *M. amazonicum* from 11 sites in different regions of Brazil, and 8 from the outgroup (Appendix).

The analyses of different methodologies (distance and parsimony methods) and different mitochondrial genes (16S and COI) resulted in similar tree topologies with several clades, which were found in all cases (Figs 2-5). *Macrobrachium amazonicum* formed a distinct group, with *M. acanthurus* (Wiegmann, 1836) and *M. jelskii* as the closest clades, respectively.

In the phylogenetic analyses, of the 10 parameter sets used in the direct optimization analysis, the set that produced the shortest trees had 1:1:1 indels/transition/transversion ratio (Matrix 111), for both 16S and COI sequences. The 16S sequences yielded two parsimonious trees of length 270, while for the COI sequences, four trees of length 485 were found. In our parsimony analyses, *M. amazonicum* was consistently found to be monophyletic in all trees during the sensitivity analysis (Figs 2- 3).

In distance analyses, the optimal model for the 16S data set, selected under AIC, was the transversional model of sequence evolution (Posada and Crandall, 1998) plus gamma distributed rate heterogeneity (TVM+G) with the following parameters: assumed nucleotide frequencies A = 0.3038, C = 0.1142, G = 0.2205, T = 0.3616; proportion of invariable sites I = 0; the variable sites followed a gamma distribution, with shape parameter = 0.1593. For the COI data set, the chosen model was the Hasegawa, Kishino, Yano 85 model of sequence evolution (Hasegawa *et al*., 1985) plus gamma distributed rate heterogeneity with a significant proportion of invariable sites (HKY+I+G), with the following parameters: assumed nucleotide frequencies A = 0.2463, C = 0.1256, G = 0.3036, T = 0.3245; proportion of invariable sites I = 0.5410; the variable sites followed a gamma distribution, with shape parameter = 0.4135.

The 16S phylogeny showed that specimens of *M. amazonicum* from Itacoatiara and Tapauá, located in the state of Amazonas in the Amazonian HR, were closely related. Similarly, specimens from Aquidauana and Corumbá in Mato Grosso do Sul (Paraguay HR) constituted a distinct group (Fig. 2). In general it was not possible to identify the relationships among the populations because of the large number of unsolved steps within the *M. amazonicum* clade (Fig. 2).

Considering the NJ dendrogram based on 16S sequences, we identified three small subgroups, which are closely related to each other (Fig. 4): specimens from the Paraná/Paraguay HR (sample sites 10-13, Fig. 1); specimens from the Amazonian HR (sites 1-2, Fig. 1); and from coastal and São Paulo populations (sites 6-7 and 8-9, respectively, Fig. 1). It was not possible to assess the genetic distance between these subgroups and the other analyzed specimens. The close similarity between sequences reflected the unsolved steps in the dendrogram (Fig. 4).

On the other hand, both the COI phylogeny and the NJ dendrogram clearly evidenced three distinct clades (Figs 3, 5): I- inland population from the Amazonian HR (sample site 2, Fig. 1); II- inland populations from the Paraná/Paraguay HR (sites 10-12, Fig. 1), and III- coastal populations from northern and northeastern Brazil (sites 3-7, Fig. 1) and two populations from the state of São Paulo (sites 8- 9, Fig. 1). Clade I was a sister group of that formed by clades II and III, which themselves formed sister groups. The relationships within each group were not well resolved in the phylogeny (Fig. 3).

We observed that, for 16S, interspecific distance among *Macrobrachium* ranged from 4.8-14.7%, whereas the intraspecific (among *M. amazonicum* populations) ranged from 0-1.1%. For COI sequences, the interspecific distance varied from 13.2-19.9%, whereas the intraspecific distance ranged from 0-3.3%.

Population analyses

We acquired sequence data for 81 specimens of *M. amazonicum* collected from 11 different sites. Based on a 569 bp COI fragment of unambiguous sequence, we identified 13 haplotypes (H), of which 4 (30.4%) represented single individuals. Of the 569 bp sequenced, 29 (5.1%) were polymorphic. Substitution patterns favoured transitions (Ts) over transversions (Tv), and the Ts:Tv ratio was high (28:1). Only specimens from coastal populations and two populations from the state of São Paulo shared haplotypes. Although total haplotype diversity (0.8488) was relatively high, four populations showed null nucleotide and haplotype diversities, with all individuals sharing the same haplotype (see Table 1).

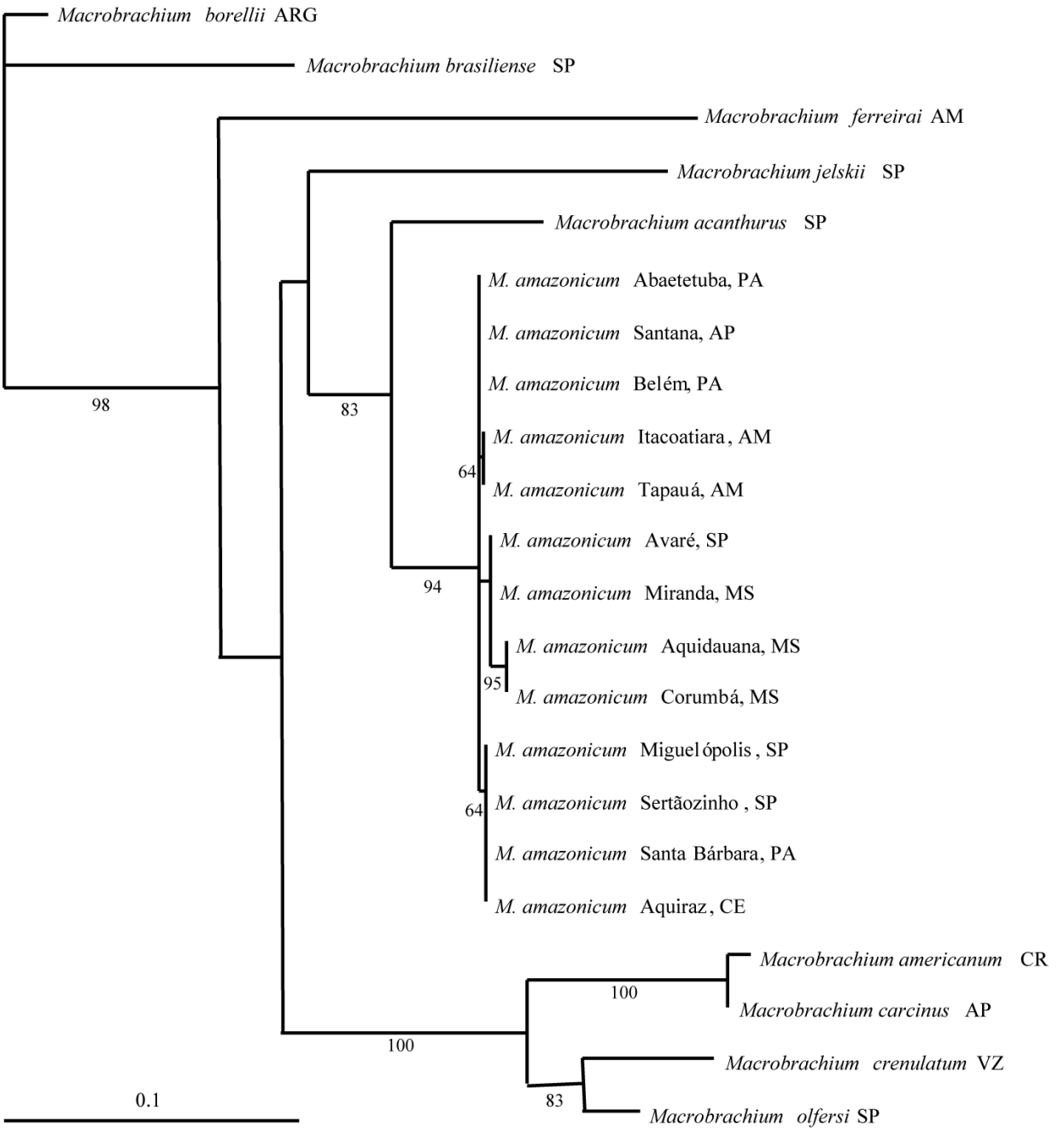

Fig. 4. Dendrogram for *Macrobrachium amazonicum* populations from Brazil based on Neighbor-Joining analysis of 16S rRNA gene sequences. Numbers above are significance values for 1000 bootstraps; values ≤ 50% are not shown. ARG: Argentina. CR: Costa Rica. VZ: Venezuela. Brazilian states: AM: Amazonas; AP: Amapá; CE: Ceará; MS: Mato Grosso do Sul; PA: Pará; SP: São Paulo.

Whether or not the introduced populations were included, it was evident, by both methods of network construction, that the haplotype network was divided into three groups (Fig. 6), exactly the same ones revealed by parsimony analysis with COI (Fig. 3). No haplotype was shared between the groups.

Table 1. Distribution of haplotypes detected in *Macrobrachium amazonicum* from Brazil.

Populations	Haplotype													N	Hd	Nd + Sd
	1	2	3	4	5	6	7	8	9	10	11	12	13			
2 / Itacoatiara, AM										2	1	1	6	10	0.64	$2.42 \times 10^{-3} \pm 1.84 \times 10^{-3}$
3 / Santana, AP	4	1												5	0.40	$0.70 \times 10^{-3} \pm 0.89 \times 10^{-3}$
4 / Abaetetuba, PA	6	2												8	0.43	$0.75 \times 10^{-3} \pm 0.86 \times 10^{-3}$
5 / Belém, PA	1	2												3	0.67	$1.17 \times 10^{-3} \pm 1.46 \times 10^{-3}$
6 / Santa Bárbara, PA	5		2		1									8	0.61	$1.63 \times 10^{-3} \pm 1.42 \times 10^{-3}$
7 / Aquiraz, CE•	1		7	2										10	0.51	$0.98 \times 10^{-3} \pm 0.98 \times 10^{-3}$
8 / Miguelópolis, SP•			4											4	0.00	0.00 ± 0.00
9 / Sertãozinho, SP•			10											10	0.00	0.00 ± 0.00
10 / Avaré, SP•							1		5					6	0.33	$0.59 \times 10^{-3} \pm 0.77 \times 10^{-3}$
11 / Aquidauana, MS						9								9	0.00	0.00 ± 0.00
12 / Miranda, MS							8							8	0.00	0.00 ± 0.00
Total	17	5	23	2	1	9	8	1	5	2	1	1	6	81	0.85	$16.97 \times 10^{-3} \pm 8.71 \times 10^{-3}$

Populations: the numbers before the sample site name correspond to the ones used in Fig. 1. N: number of analyzed individuals in each population. Hd: haplotype diversity. Nd: nucleotide diversity. Sd: standard deviation. • Introduced populations. Brazilian states: AM: Amazonas; AP: Amapá; CE: Ceará; MS: Mato Grosso do Sul; PA: Pará; SP: São Paulo.

Regarding populations introduced in the state of São Paulo, specimens from Sertãozinho and Miguelópolis shared haplotypes with specimens from group III, specifically with individuals from Aquiraz and from the state of Pará; whereas specimens from Avaré showed haplotypes closely related to those found in group II. Specimens from Aquiraz, probably also an introduced population, shared haplotypes with individuals from coastal populations in the state of Pará (Fig. 6).

Analysis of molecular variance without hierarchical structure indicated that the highest percentage of variation (95.74%) was among *M. amazonicum* populations, whereas the variation within each population was extremely low (4.26%). When populations were structured according to the groups indicated by all the analyses (phylogenetic, distance, and haplotype network), significant levels of genetic variation were detected. Variations among populations within groups and within populations were very low (see Table 2).

Discussion

The present investigation, based upon analysis of a partial fragment of mtDNA genes, is the first to describe the phylogenetic position and genetic variability of *Macrobrachium amazonicum* from a wide geo-

Table 2. Analysis of molecular variance in Brazilian *Macrobrachium amazonicum*.

AMOVA structure	Source of variation	Percentage	F_{st} / F_{ct}	P
Without	among populations	95.74	0.957	< 0.001
	within populations	4.26		
Coastal, Amazonian Hydrographic Region (HR) and Paraná/ Paraguay HR (introduced populations excluded)	among groups	93.27	0.933	< 0.001
	among populations within groups	3.29	0.488	< 0.001
	within populations	3.44	0.965	< 0.001
Coastal, Amazonian HR and Paraná/ Paraguay HR (introduced populations grouped following the haplotype networks results)	among groups	92.26	0.922	< 0.001
	among populations within groups	4.99	0.645	< 0.001
	within populations	2.75	0.972	< 0.001

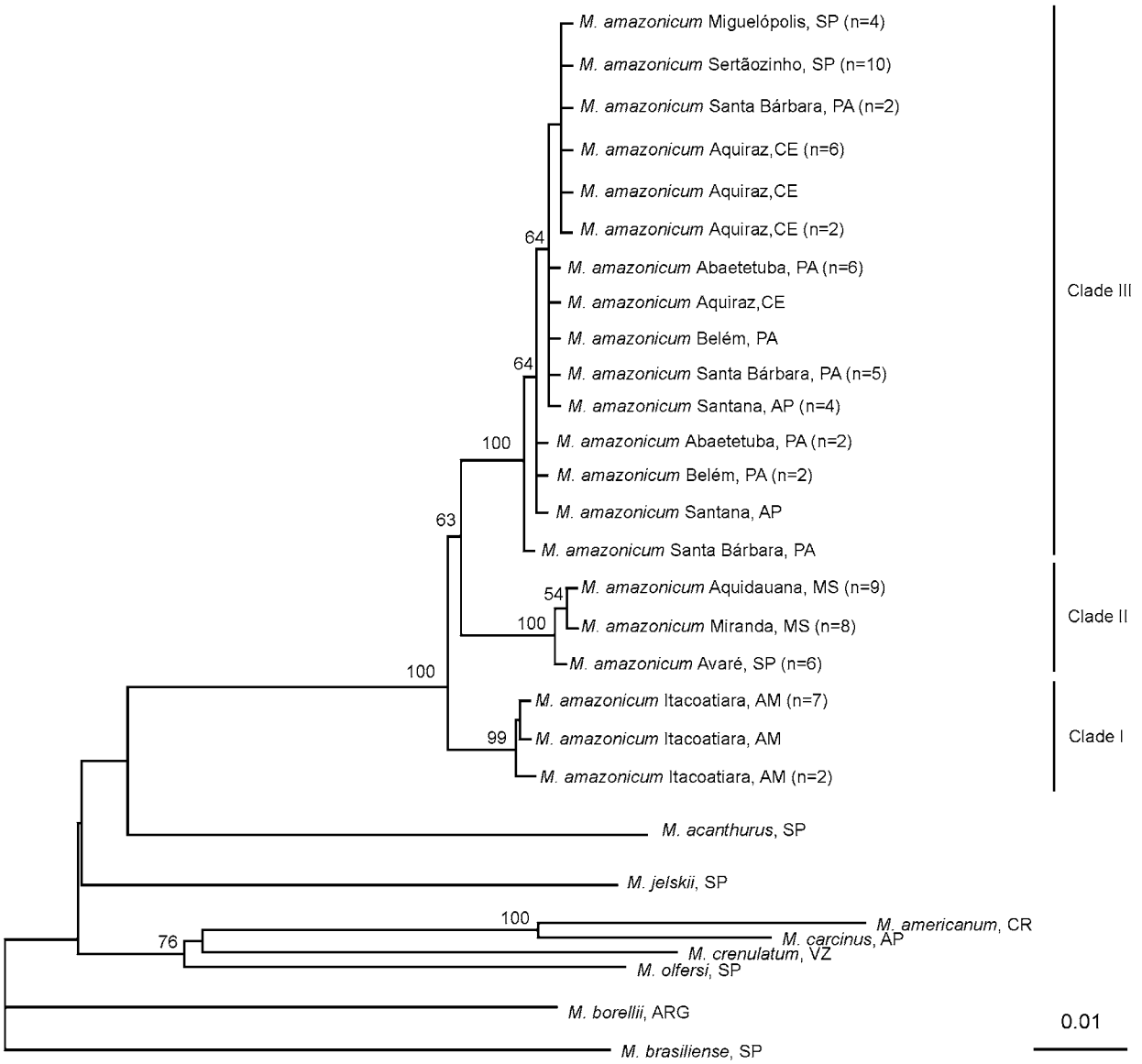

Fig. 5. Dendrogram for *Macrobrachium amazonicum* populations from Brazil based on Neighbor-Joining analysis of COI gene sequences. Numbers above are significance values for 1000 bootstraps; values ≤ 50% are not shown. ARG: Argentina. CR: Costa Rica. VZ: Venezuela. Brazilian states: AM: Amazonas; AP: Amapá; CE: Ceará; MS: Mato Grosso do Sul; PA: Pará; SP: São Paulo. Clades: I - inland population from Amazonian Hydrographic Region (HR); II - inland populations from Paraná/Paraguay HR; III - coastal populations from northern and north-eastern Brazil.

graphical range of inland and coastal sites. Our findings revealed important aspects of the evolutionary history of the species, especially regarding natural versus unnatural and inland versus coastal populations.

The first important information was that these populations of *M. amazonicum* composed one monophyletic clade, which ranks them as a single species according to the Phylogenetic Species Concepts *sensu* Mishler and Theriot (2000). Organisms are grouped into species rather than at some higher level because they are the least inclusive taxon recognized in a formal phylogenetic classification, and because they are the smallest monophyletic group deemed worthy of formal recognition (Mishler and Theriot, 2000). The positioning of *M. amazonicum* in the genus concords

with a previous phylogenetic analysis of *Macrobrachium* (Pileggi and Mantelatto, 2010), in which *M. acanthurus* and *M. jelskii* appear as related taxa. A morphologically based analysis by Pileggi (2009) indicated that *M. jelskii* shares significant similarities with inland populations of *M. amazonicum* from the Upper Paraná and Paraguay basin in Brazil, and that *M. acanthurus* shares significant similarities with coastal populations of *M. amazonicum*.

Phylogenetic analyses with COI sequences revealed that an ancestral population originated the inland population of *M. amazonicum* from the Amazonian HR (clade I), and another ancestral population gave rise to the inland populations of the Paraná/Paraguay HR (clade II) and the coastal populations from northern and northeastern Brazil (clade III). Populations from clades II and III shared common ancestry, and were more closely related to each other than to individuals from clade I. Distance analyses showed the same structure described above. More detailed information about the relationships among the populations could not be obtained because the COI gene was not variable enough to provide sufficient resolution.

The inclusion of specimens from other localities than those used here, especially from the Amazonian HR owing to its immense geographical extent, would make possible a more profound reconstruction of the origin, life history, and phylogenetic relationships of *M. amazonicum* populations. It is also essential to add specimens from other countries in South and Central Americas.

Analyses with 16S rRNA gene sequences were not informative concerning phylogenetic and distance relationships among the populations, because of the small variation among the specimens (genetic divergence from 0 to 1.1%). Therefore, the 16S gene was not variable enough to evidence any structure in *M. amazonicum*. This gene is conservative and has a low rate of evolution, which means that it is more precise in discriminating between species than within species. Variation in 16S sequences is low or null between sequences from specimens belonging to the same species (see Francisco and Galetti Junior, 2005 for a review). Thus, the homogeneity found in *M. amazonicum* seems to be related to the conservative nature of this gene.

Previous studies on the systematics of *Macrobrachium* (Liu *et al*., 2007; Pileggi and Mantelatto, 2010) have estimated interspecific divergences ranging from 5.5 to 17.5% for 16S and 15.1 to 25.5% for COI. The intraspecific divergence ranged from 0 to 3.2% for 16S and 0 to 12.6% for COI. The maximum values (1.1% for 16S and 3.3% for COI) found in the populations of *M. amazonicum* fall within the range of intraspecific variation described for the genus. The degree of variation in the COI sequences concords with that found by Peixoto (2002). Consequently, the genetic variability found in our study seems to indicate variation at a population level rather than at a species level.

Our results indicated that because specimens of *M. amazonicum* from Sertãozinho and Miguelópolis (state of São Paulo) share haplotypes and morphological patterns (FGV, LGP, FLM, unpublished results) with coastal populations from northern and northeastern Brazil, they probably originated from these regions and were introduced into São Paulo as part of the process of transplanting the fish *P. squamosissimus* from reservoirs in northeastern Brazil (see Introduction for details).

Considering that specimens from Avaré share haplotypes and morphological traits (FGV, LGP, FLM, unpublished data) with inland populations from the state of Mato Grosso do Sul in the Paraguay HR, they were probably accidentally introduced into São Paulo or dispersed naturally upstream (Magalhães *et al*., 2005).

In the 1940s, the National Department of Anti-Drought Construction (Departamento Nacional de Obras Contra as Secas - DNOCS) introduced *M. amazonicum* from the Amazon basin into several reservoirs of northeastern Brazil, as a forage species for carnivorous fishes (Coelho, 1963; Pinto, 1977; Bragagnoli and Grotta, 1995; Paiva and Campos, 1995 *apud* Da Silva *et al*., 2004). Specimens from Aquiraz in the state of Ceará may have been introduced for the same reason from natural coastal populations in the state of Pará in northern Brazil, as was revealed by the network haplotypes and morphological revision (FGV, LGP, FLM, unpublished data).

All the inferences concerning the possible origin of the populations from the state of São Paulo and northeastern Brazil were based on the presumptive natural distribution of *M. amazonicum* suggested by Magalhães *et al*. (2005). However, this presumptive distribution may not be complete, because of a possible lack of records of *M. amazonicum* (under-sampling) in other regions of the country, as well as in other parts of its range in the Americas.

Haplotype networks and AMOVA evidenced genetic structure between populations of *M. amazonicum* in the same three groups that were revealed by the parsimony and distance analyses. Apparently, the degree of genetic variability found among populations

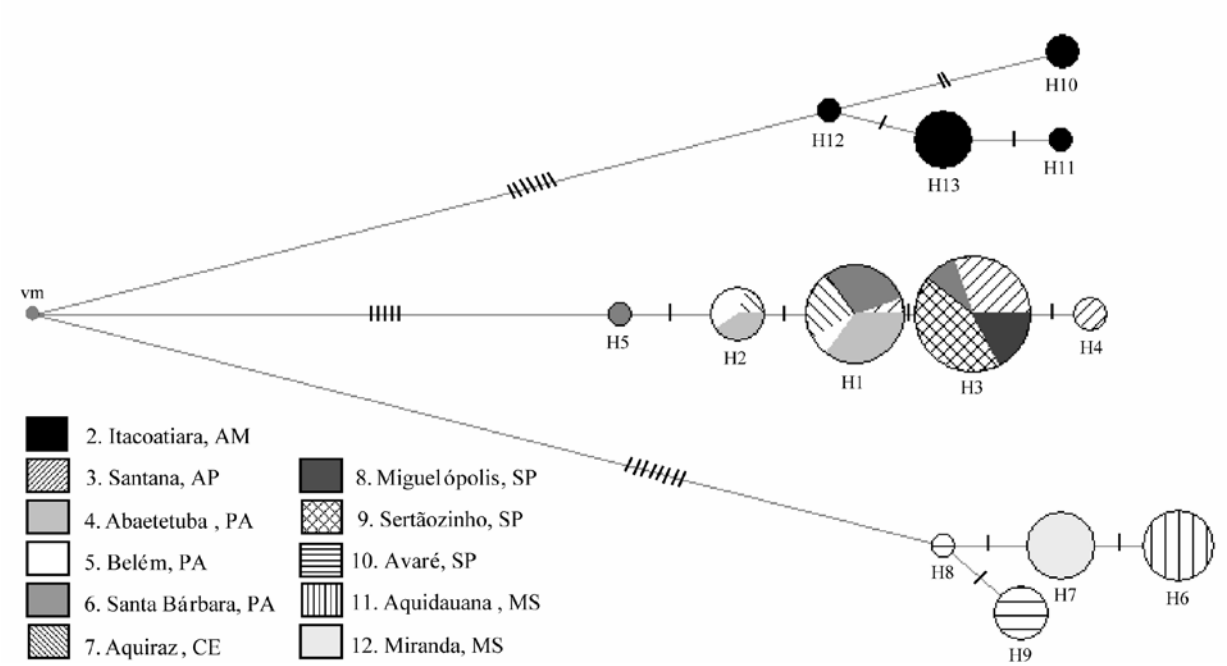

Fig. 6. Haplotype network based on Median-Joining analysis indicating the distribution of each haplotype (H) found in *Macrobrachium amazonicum*. The haplotype identification is below each circle. Circle size for each haplotype is proportional to the overall frequency in our sample. Each small trace represents a mutational step. vm: median vector. The numbers before the sample site name in the legend boxes correspond to the ones used in Fig. 1. Brazilian states: AM: Amazonas; AP: Amapá; CE: Ceará; MS: Mato Grosso do Sul; PA: Pará; SP: São Paulo. Groups: I - inland population from Amazonian Hydrographic Region (HR); II - inland populations from Paraná/ Paraguay HR; III - coastal populations from northern and north-eastern Brazil.

reflects their geographical distance and habitat fragmentation. The absence of a shared haplotype between groups supports this inference. The geographical isolation and the lack of gene flow (lack of migration and dispersal) between groups are also corroborated by the low levels of genetic variation found within the populations and among the populations within groups. The loss of genetic variability in other populations of freshwater crustaceans is mostly a result of high levels of inbreeding (García-Dávila, 2002; Carini and Hughes, 2004).

Movements of freshwater species are strictly limited by the physical nature and arrangement of the river system. Species with apparently good dispersal abilities frequently show unexpectedly high levels of population subdivision (Carini and Hughes, 2004 for review). Populations of *M. amazonicum* were divided into three groups, which correspond to geographically different environments: inland areas in the Amazonian HR, inland areas in the Paraná/Paraguay HR, and coastal areas in northern and northeastern Brazil. Dry land areas may form an insuperable barrier preventing dispersal and connectivity among aquatic populations, which can cause isolation and genetic divergence in freshwater populations inhabiting separate drainage basins (Carini and Hughes, 2004).

Genetic diversity can enhance adaptation to a particular environment and also expand colonization and distributional boundaries, enabling a species to survive in a wide variety of conditions (Carvalho, 1993). As a result, high levels of genetic variability between populations of the same species may be related to its ecological versatility (Walker, 1992; Leuzzi *et al.*, 2004). This seems to be the case for *M. amazonicum*, whose populations can be found in habitats with a wide range of salinities (see introduction for references), demonstrating its capability of colonizing different habitats (Odinetz-Collart, 1991a,b). In conclusion, all the arguments presented here lead us to conjecture that variations in the *M. amazonicum* life-cycle phenotypes, including differences in reproductive strategies, egg size, osmoregulatory and survival capability, adult size, and larval and adult morphology (see Introduction for references), are related to its

great ecological plasticity developed in response to different environmental conditions (near or far from the sea).

Assessments of intraspecific genetic diversity and population genetic structure provide information of biological and evolutionary interest, and are essential to the success of studies on conservation and maintenance of biological diversity (McMillen-Jackson and Bert, 2004). *Macrobrachium amazonicum* is heavily exploited by Brazilian artisanal fisheries, particularly in northern Brazil (Odinetz-Collart and Moreira, 1993; Maciel and Valenti, 2009), and is a notable and promising species in freshwater prawn aquaculture in Brazil (Moraes-Riodades and Valenti, 2001, 2004). In this context, we strongly recommend that each group of the *M. amazonicum* populations should be considered as a distinct genetic stock in any conservation strategy and should be separately managed in order to guarantee the sustainability and maintenance of the genetic resources of the species in Brazil. Furthermore, the existence of genetic structure among *M. amazonicum* populations should be taken into consideration during the selection of matrices for aquaculture purposes, in order to improve knowledge of the levels of genetic variability among populations.

In conclusion, specimens of *M. amazonicum* from Brazil showed significant intraspecific variability, in addition to other kinds of variability previously reported (see Introduction for references). Populations were structured in three distinct groups: specimens from inland areas in the Amazonian HR, inland areas in the Paraná/Paraguay HR, and coastal areas in northern and northeastern Brazil. This structure probably results from geographical isolation between them, precluding dispersal and connectivity. If this isolation continues, *M. amazonicum* may possibly begin a speciation process within its extensive geographical distribution.

Some inferences for *M. amazonicum* populations can be extracted, but are limited by the nature of our analysis, which was based on two molecular markers (16S and COI mtDNA). At this time, in combination with morphological data (FGV, LGP, FLM, unpublished data), the sequences obtained here can be used as nametags for population identification, and the DNA barcodes are useful to identify the origin of specimens used in different freshwater prawn cultures or of introduced populations of unknown origin. However, we continue efforts to confirm and refine these results, especially in terms of new genes (mitochondrial and nuclear) and more variable molecular markers (microsatellites). We also continue to add coverage at the population level, particularly to elucidate the reasons for the wide distribution of this species in the Americas.

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Appendix

List of species used during the analyses

Species	Sample site and date	N° ind. COI	Source	N° ind. 16S	Source	Distribution	Catalogue n°
Macrobrachium amazonicum	1 / Tapauá, Amazonas, Brazil, 2007 (04°30'19''S; 62°03'19''W)	-	-	1	GU929462	South America	CCDB 2312
	2 / Itacoatiara, Amazonas, Brazil, 2007#	10	GU929542 to GU929551	1	GU929460		CCDB 2085
	3 / Santana, Amapá, Brazil, 2005#	5	GU929500 to GU929504	1	GU929452		CCDB 1965
	4 / Abaetetuba, Pará, Brazil, 2007#	8	GU929471 to GU929478	1	GU929450		CCDB 2084
	5 / Belém, Pará, Brazil, 2005#	3	GU929489 to GU929491	1	GU929451		CCDB 1963
	6 / Santa Bárbara, Pará, Brazil, 2008# (01°14'30'S; 48°19'52''W)	8	GU929492 to GU929499	1	GU929453		CCDB 2119
	7 / Aquiraz, Ceará, Brazil, 2007#•	10	GU929479 to GU929488	1	GU929454		CCDB 1973
	8 / Miguelópolis, São Paulo, Brazil, 2007#• (20°06'02,48''S; 47°56'45,53''W)	4	GU929538 to GU929541	1	GU929459		CCDB 2015
	9 / Sertãozinho, São Paulo, Brazil, 2006#• (21°06'46,7''S; 48°03'18,2''W)	10	GU929505 to GU929514	1	GU929455		CCDB 1953
	10 / Avaré, São Paulo, Brazil, 2006#•	6	GU929532 to GU929537	1	GU929458		CCDB 2081
	11 / Aquidauana, Mato Grosso do Sul, Brazil, 2007#	9	GU929515 to GU929523	1	GU929456		CCDB 1970
	12 / Miranda, Mato Grosso do Sul, Brazil, 2007#	8	GU929524 to GU929531	1	GU929457		CCDB 1971
	13 / Corumbá, Mato Grosso do Sul, Brazil, 1990	-	-	1	GU929461		CCDB 2313
Outgroups							
M. acanthurus	Ilha de São Sebastião, São Paulo, Brazil, 2006	1	GU929470	1	GU929449	South/Central America	CCDB 2134
M. americanum	South Pacific, Costa Rica, 2005	1	GU929552	1	GU929463		CCDB 1731
M. brasiliense	Serra Azul São Paulo, Brazil, 2006	1	GU929468	1	GU929446	South America	CCDB 2135
M. borellii	San Antonio de Areco, Buenos Aires, Argentina, 2003	1	GU929467	1	GU929445		UFRGS 3669
M. carcinus	Santana, Amapá, Brazil, 2005	1	GU929553	1	GU929464	South/Central America	CCDB 2122
M. crenulatum	Cerro Copei, Isla Margarita, Venezuela, 2006	1	GU929555	1	GU929465		CCDB 2124
M. ferreirai	Manaus, Amazonas, Brazil, 2005	-	-	1	GU929447	South America	CCDB 2125
M. jelskii	Pereira Barreto, São Paulo, Brazil, 2006	1	GU929469	1	GU929448		CCDB 2129
M. olfersi	Ilha de São Sebastião, São Paulo, Brazil, 2006	1	GU929554	1	GU929466	South/Central America	CCDB 2138

Sample site and date (the numbers before the sample site name correspond to the ones used in Fig. 1). N° ind: number of individuals from which DNA was obtained and amplified with each molecular marker (mtDNA). Source of sequences: genetic database accession numbers at GenBank). Catalogue n°: number in Crustacean Collections. #: Specimens used in population analyses. • Introduced populations. CCDB: Crustacean Collection of the Biology Department of the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo. UFRGS: Federal University of Rio Grande do Sul.