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Gracilaria caudata (Gracilariales, Rhodophyta) is reproductively compatible along the whole Brazilian coast

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Abstract

Gracilaria spp. are economically important for the production of agar. The distribution of ecotypes of *Gracilaria caudata* all along the Brazilian coast raises questions regarding whether they are undergoing speciation and are possibly reproductively incompatible. Therefore, in the present work, we selected different female and male gametophytes of *G. caudata* from three populations along an extended Brazilian coastline (from 3° to 23° S) to perform crossing tests and observe possible reproductive barriers. In addition, we tested post-zygotic isolation, by following meiosis, the production of haploids, and the reproduction of haploids, in the progeny of these inter-population crosses. DNA sequences of the mitochondrial cytochrome *c* oxidase subunit 1 gene (COI) were used to determine relationships among all gametophytes employed in the crosses and to understand inheritance of mitochondria. The crosses showed interfertility between all populations tested. Neither pre- nor postzygotic isolation was seen in *G. caudata* from Brazil. Individuals with haplotypes that differ by 1–5 bp (0.15–0.79% divergences) in COI were reproductively compatible. In conclusion, the exchange of genetic material among populations from a wide geographic distribution allows consideration of *G. caudata* as a biological species.

Keywords Crossing test · Mitotype · Mitochondrial inheritance · COI · *Gracilaria* · Rhodophyta

Introduction

Biological species are defined as groups of interbreeding natural populations that are reproductively isolated from other such groups (Mayr 1992). This is the most widely used species definition and emphasizes pre- and postzygotic isolating mechanisms (Allendorf et al. 2013). This concept seems to fit many Rhodophyta, since species-attempted hybridization has shown reproductive isolation (e.g., West et al. 1978; Zuccarello et al. 2002).

The accurate identification of a taxon is especially important when economically important species are involved, essentially as accumulated knowledge about a resource can improve management and economic exploitation. In particular, species of *Gracilaria* are broadly distributed worldwide and commercially exploited for agar production, a phycocolloid widely used in the food and pharmaceutical industries (Bixler and Porse 2011; Porse and Rudolph 2017). In *Gracilaria,* crossing tests are a very useful and powerful tool for distinguishing cryptic species (Plastino and Oliveira 1988, 1996, 1997). Recent studies have found a total of 24 species of *Gracilaria* along the Brazilian coast (Lyra et al. 2015), and two of them are exploited for agar production: *G. birdiae* Plastino & E.C. Oliveira and *G. caudata* J. Agardh (Plastino and Oliveira 1997; Oliveira and Miranda 1998; Oliveira et al. 2000). They are usually harvested from natural beds on the northeast coast (Carneiro et al. 2011), but concerns were raised when their availability began to decrease. To overcome this problem, artisanal mariculture has increased in the past few years (Hayashi et al. 2014).

On the Brazilian coast, *G. caudata* is found between Maranhão (2°S) and Santa Catarina States (28° S, southern limit). Its wide geographical distribution and simple terete morphology contribute to frequent difficulty in distinguishing it from other species of the genus (Plastino and Oliveira 1997). *Gracilaria caudata* from distinct populations have been cultivated under

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laboratory control conditions and physiological analyses have been performed on growth, photosynthesis, and pigment content, contributing to a better understanding of intraspecific diversity (Araújo et al. 2014; Faria and Plastino 2016; Faria et al. 2017). Strains of *G. caudata* from a location closer to the equator (Ceará State, CE) showed higher growth rates (GR) than strains from an area closer to the Tropic of Capricorn (São Paulo State, SP) when cultivated under control conditions at artificial ultraviolet B radiation (Araújo et al. 2014). Strains from a geographically intermediate population (Bahia State, BA) were included in a new study in addition to the CE and SP strains (Faria et al. 2017). Under high irradiance (150 μmol photons m^{-2} s^{-1}), strains from CE presented the highest GR when compared to strains from SP and BA, the latter with the lower GR. CE strains also had the highest maximum photosynthesis. These results indicate the occurrence of physiological ecotypes of *G. caudata* along the Brazilian coastline.

Recently, the use of distinct molecular markers such as the mitochondrial cytochrome *c* oxidase subunit 1 gene (COI) and microsatellites, has shown the existence of high genetic diversity among populations of *G. caudata* along the Brazilian coast (Ayres-Ostrock 2014; Ayres-Ostrock et al. 2016). Thus, the existence of genetic variation for local adaptations and regional physiological ecotypes allowed to question if these strains from different populations would still be able to reproduce among themselves.

Gracilaria caudata has a triphasic life-history ("*Polysiphonia*" type) which includes two free-living isomorphic stages of different ploidy levels, a diploid (tetrasporophyte) stage and a haploid (mostly dioicous gametophyte) stage, as well as a diploid carposporophyte stage, which develops on the female gametophyte (Oliveira and Plastino 1984). Therefore, to understand the reproductive compatibility of populations or putative species of algae with complex life history, it is important to follow all generations. This allows observation of both syngamy and meiosis, and confirms the viability of their descendants (Montecinos et al. 2017). There are still few studies on reproductive isolation in red algae looking for prezygotic and post-zygotic isolation (Maggs et al. 2011).

The aim of this work was to know if ecotypes of *G. caudata* are reproductive isolated. Therefore, we selected different female and male gametophytes of *G. caudata* from three populations along an extended Brazilian coastline (from 3° to 23° S) to perform crossing tests and observe possible reproductive barriers. Moreover, we observed putative incompatibility caused by mechanisms that prevent meiosis in the subsequent generations. DNA sequences of the mitochondrial cytochrome *c* oxidase subunit 1 gene (COI) were used to determine relationships among all gametophytes employed in the crosses and to understand mitochondrial inheritance. It is expected that this study will provide further information on intraspecific diversity of *G. caudata* along the Brazilian coast and shed light on whether its populations constitute a potentially single interbreeding species, thus fitting the biological species concept.

Materials and methods

Biological material

Female and male gametophytes and tetrasporophytes of *Gracilaria caudata* were collected from three different localities along the Brazilian coast (Supplementary Fig. S1; Table 1). Female gametophytes were recognized in the field by presence of cystocarps. Male gametophytes and tetrasporophytes were recognized using stereoscopic microscope. Male gametophytes showed spermatangia in deep conceptacles, and tetrasporophytes had tetrasporangia scattered on the thallus.

Apical segments of sample plants were transported to the laboratory, and unialgal non-axenic cultures were established (Plastino and Oliveira 1990). Gametophytes used in attempt crossings (three males and three females from each population, Fig. 1) were obtained directly from the field or from tetraspores released under laboratory conditions from tetrasporophytes sampled in the field (Table 1). Plants were kept in the Gracilariaceae Germplasm Bank of the University

Table 1 Localities, sampling dates, and biological material of *Gracilaria caudata* used in the crossings. Haplotypes present in each population were also showed. *F* female gametophyte, *M* male gametophyte, *T* tetraesporophyte

	Ceará	Espírito Santo	São Paulo
Localities/coordinates	Pedra Rachada Beach, Paracurú (03.24° S, 39.01° W)	Church Beach, Marataízes (21.2° S, 40.49° W)	Cigarras Beach, São Sebastião (23.55° S, 46.20° W)
Sampling date	February 2012	March 2012	March 2015
Biological material used in the crossings (3 F and 3 M)	1 F and 1 M originated from each of the 3 T collected in the field	1 F and 1 M collected in the field; and 1 F and 1 M originated from each of the 2 T collected in the field	2 F and 2 M collected in the field; and 1 F and 1 M originated from 1 T collected in the field
Haplotypes	CE1 + CE2	ES3	SP4

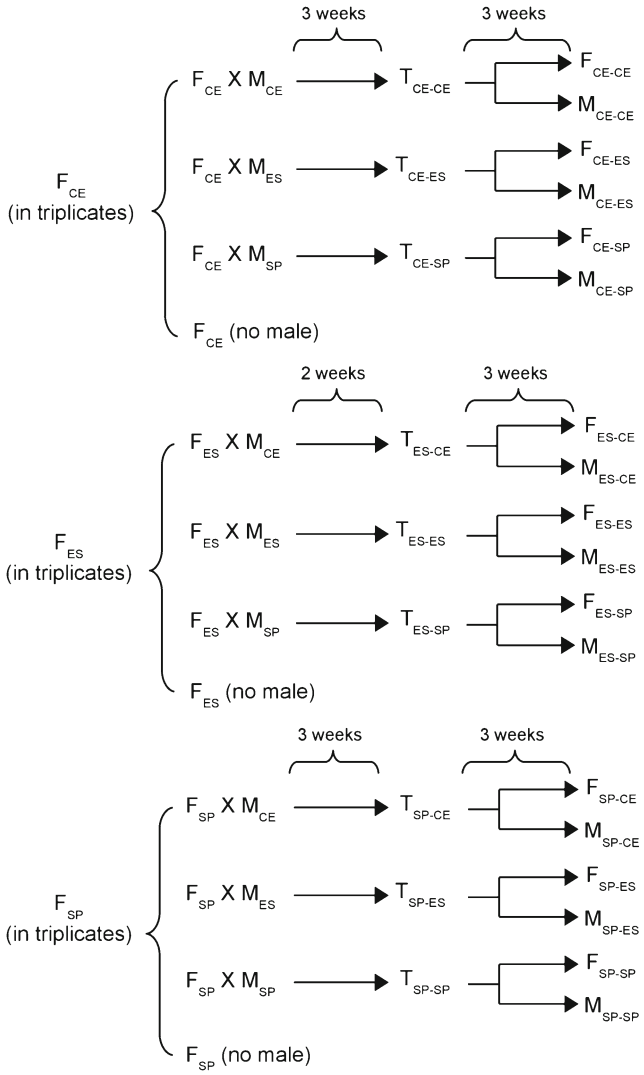

Fig. 1 Experimental design of the crossing tests between female and male gametophytes of *Gracilaria caudata* from Ceará (CE), Espírito Santo (ES), and São Paulo (SP) States and derived generations. Each of the crossings and the isolated female gametophytes were repeated in triplicates, as well as the subsequent generations. Each triplicate was cultivated in separated flask. F female gametophyte, M male, T tetrasporophyte, c carpospores, t tetraspores. The period for each step is shown in the figure in weeks. Life history was completed in 12–13 weeks

of São Paulo (Costa et al. 2012). Voucher specimens were deposited in the herbarium of the Bioscience Institute at the University of São Paulo (CE, SPF-57843; ES, SPF-58089; and SP, SPF-58096).

General culture conditions

Apical segments of *G. caudata* were maintained in von Stosch-enriched seawater (Ursi and Plastino 2001) diluted to 25% (salinity at 32). The algae were kept in a temperature-controlled room at 25 ± 1 °C with a photoperiod of 14 h (14 h light/10 h dark). Photosynthetically active radiation (PAR) was 70 μmol photons m^{-2} s^{-1} provided by Osram 40-W daylight fluorescent tubes, and PAR was measured with a Quantameter (Li-COR model L1-185). Cultures were aerated for 30 min h^{-1}, and the medium was renewed weekly.

Experimental design

Crossings were attempted among gametophytes of *G. caudata* from the three different populations: Ceará (CE), Espírito Santo (ES), and São Paulo (SP) States. From each location, four apical segments were obtained from each of three female gametophytes (a total of 12), and three apical segments were obtained from each male gametophyte (a total of 9), resulting 63 apical segments (Fig. 1). All apical segments had the same mass (0.3 mg) at the beginning of the experiment. Apical female branches were kept isolated from male branches for at least 3 weeks to assure absence of fertilized carpogonia before attempted crossings.

Control conditions were established according to the following protocol. First, one of the four apical segments from a single female gametophyte was cultivated in the presence of one apical segment of a male gametophyte from the same population. Second, one of the three apical segments was cultivated without any male gametophyte as a control for parthenogenesis or hermaphroditism. The two other female gametophytes were cultivated in different flasks with one apical segment of a male gametophyte from the other two populations (Fig. 1). This was repeated for each of the three individuals of each population and for the three populations ($n = 3$). Each flask contained two apical segments, one male and one female, except for the hermaphroditism control (total of 36 flasks).

Cultivation was performed in 500 mL Erlenmeyer flasks, containing 450 mL enriched seawater under general culture conditions. Gametophytes are hereinafter designated as follows: F_{CE} and M_{CE} (female gametophytes and male gametophytes from Ceará State); F_{ES} and M_{ES} (female gametophytes and male gametophytes from Espírito Santo State); and F_{SP} and M_{SP} (female gametophytes and male gametophytes from São Paulo State). Development of cystocarps and release of viable carpospores were considered positive fertilization results. To estimate viability and fertility of the offspring, inclusive fitness, carpospores obtained from each crossing were cultivated in different flasks (Fig. 1) until plantlets formed. Three of them were randomly selected from each cross and these young tetrasporophytes were cultivated until they produced tetraspores. The latter were cultivated until gametophytes reproduced and females produced cystocarps (Fig. 1).

Apical segments of the six gametophytes from the three different populations (nine female and nine male gametophytes) used in the crosses were selected for sequencing. Additional sequences were obtained from the progenies produced in four out of six crosses: $F_{CE} \times M_{SP}$, $F_{ES} \times M_{CE}$, $F_{ES} \times M_{SP}$, and $F_{SP} \times M_{CE}$.

DNA extraction and sequencing

DNA was extracted using the NucleoSpin 96 Plant II kit (Macherey-Nagel, Germany). For each sample, approximately 8 mg of silica-dried apical fragments of *G. caudata* were powdered using mechanical tissue disruption with 3-mm diameter steel beads. Extraction followed the manufacturer's instructions, except for incubation in cell lysis buffer during which tissue samples were left at room temperature overnight rather than heated at 65 °C for 30 min.

PCR amplification of the mitochondrial cytochrome *c* oxidase subunit 1 gene (COI) was performed in a total volume of 20 μL. PCR mix contained 4 μl of DNA template, 250 μM dNTPs (Thermo Fisher Scientific, USA), 2 μM of each primer, 1 U Taq DNA polymerase (5 U μL^{-1}, Invitrogen, USA), 6.5 mM $MgCl_2$, 1× Taq Buffer (without Mg^{+2}). Primer pair GazF1 and GazR1 were used for amplification and PCR cycling conditions followed the protocols described in Saunders (2005).

The amplified COI fragments were purified using the K7000-01 TOPO Shotgun Subcloning Kit (Thermo Fisher Scientific). Sequences were edited, aligned, and compared using ClustalW/Bioedit v 7 (Hall 1999) and Mega v 5 (Tamura et al. 2011). Maximum likelihood (ML) phylogenetic trees were constructed with the IQ-TREE software using 2000 iterations for both the optimal-tree search and as bootstrap pseudo replicates (Minh et al. 2013; Nguyen et al. 2014; Kalyaannamoorthy et al. 2017). Models of evolution were selected according to the Bayesian Information Criterion (BIC) using the IQ-TREE software. Phylogenetic trees were visualized in Figtree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). A haplotype network was reconstructed for the COI sequences using TCS 1.2.1 (Clement et al. 2000) to calculate the minimum number of mutational steps by which the sequences can be joined with > 95% confidence.

Results

Crossing experiments

Crossing results indicated that female gametophytes of *Gracilaria caudata* were fertilized by male gametophytes from the same and different populations, implicating the exchange of genetic material by sexual reproduction (Table 2). All gametophytes used in the crosses formed spermatangia (male) or cystocarps (female) in all triplicates. Only one female gametophyte (F_{CE}, control condition) produced spermatangia, resulting in self-fertilization; all other plants were dioicous.

The period taken to observe cystocarps by naked eye was of about 7 days for the ES female gametophytes and about 14 days for the CE and SP female gametophytes, regardless of the crosses (Fig. 1). Although the exact number of cystocarps was not counted, they were numerous in all triplicate branches as well as in the three populations (CE, ES, and SP), regardless of the crosses. A large number of carpospores was released for each of the fertilized female gametophytes, regardless of the crosses. These carpospores germinated and produced tetrasporophytes that showed their thalli replete of tetrasporangia, and all of them released many tetraspores that gave rise to gametophytes. Therefore, complete reproductive compatibility between and within populations was observed.

The life history of *G. caudata* was completed in 12–13 weeks (Fig. 1). The period required to complete the life history in which the ES female gametophytes was included ($F_{ES} \times M_{CE}$, $F_{ES} \times M_{ES}$, and $F_{ES} \times M_{SP}$) was reduced by 1 week (12 weeks), because the period required for producing mature cystocarps was shorter when compared to CE and SP female gametophytes.

Table 2 Crossing tests between female and male gametophytes of *Gracilaria caudata* from Ceará State (CE), Espírito Santo State (ES), and São Paulo State (SP). (+) positive crossing (all replicates with female and male gametophytes presented numerous cystocarps regardless of population). (−) negative crossing

		♀			
		ES	SP	CE	Control (no males)
♂	ES	+	+	+	−
	SP	+	+	+	−
	CE	+	+	+	[a]

[a] Occurrence of cystocarps in one of the three repetitions of controls

Mitochondrial marker COI

In this study, 22 mitochondrial sequences were obtained. All analyzed progenies (tetrasporophytes) had maternal COI sequence.

An alignment of 630 base pairs (bp) built for the COI sequences, revealed five polymorphic sites and four mitotypes. In addition to the sequences produced in this work, 28 published COI sequences of *G. caudata* from GenBank were used for the construction of the phylogenetic tree. The Hasegawa-Kishino-Yano (HKY, Hasegawa et al. 1985) model of evolution was selected as the best-fit model. Maximum likelihood (ML) tree reconstruction shows the presence of four haplogroups distributed in distinct regions (Supplementary Fig. S2). The sequences produced in this study for female and male gametophytes from CE and tetrasporophyte derived from $F_{CE} \times M_{SP}$, plus sequences from GenBank of individuals sampled in CE, Rio Grande do Norte, and Pernambuco States formed two groups: CE1 and CE2. The third group corresponds to the sequences of female and male gametophytes from ES and tetrasporophytes derived from $F_{ES} \times M_{CE}$ and

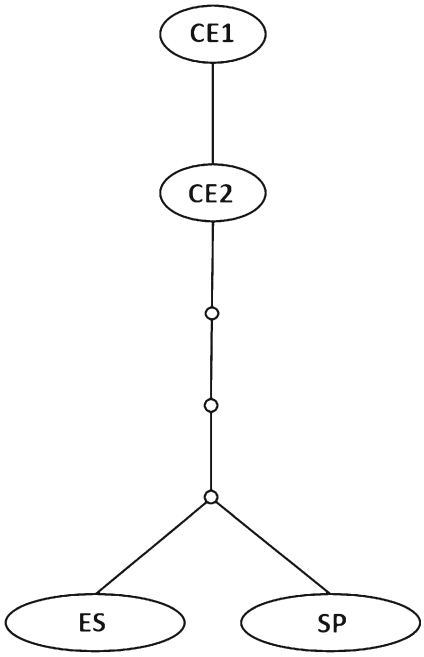

Fig. 2 Statistical parsimony networks for COI haplotypes of *G. caudata*. Lines represent parsimonious connections between haplotypes with a probability higher than 95%, with each representing one mutational step, and the small circles represent hypothetical un-sampled haplotypes. The size of oval corresponds to the haplotype frequency. CE1 (three sequences, only female gametophytes) and CE2 (three sequences, only male gametophytes) in Ceará State, ES is found only in Espírito Santo State (six sequences, three male and three female gametophytes), and SP only in São Paulo State (six sequences, three male and three female gametophytes)

$F_{ES} \times M_{SP}$, plus GenBank sequences from ES. A fourth group includes female and male gametophytes from SP, tetrasporophytes derived from $F_{SP} \times M_{CE}$, plus GenBank sequences from SP, ES, Bahia, and Santa Catarina States.

The haplotype network for the COI sequences produced in this work shows four haplotypes differing by 1–5 bp. Two mitotypes were found in CE, CE1 (three female gametophytes) and CE2 (three male gametophytes), one in ES (three male and three female gametophytes), and one in SP (three male and three female gametophytes) (Fig. 2; Table 1).

Discussion

The results of the crossing tests of distinct populations of *Gracilaria caudata* from northeastern to southeastern Brazil showed complete reproductive compatibility in all samples, including control intrapopulational crossings. Cystocarps were previously observed in crossings between female and male gametophytes of two other populations of *G. caudata* (Plastino and Oliveira 1996, as *Gracilaria* sp_1 and *Gracilaria* sp_2); however, no information on subsequent generations was provided. In our work, neither reproductive barriers against the formation of diploid hybrid genotypes nor postzygotic isolating mechanisms were observed in *G. caudata* since subsequent generations could be followed. Previously, postzygotic barriers were observed in populations of cryptic species of *Ectocarpus* (Phaeohyceae), an alga with a complex sexual haploid-diploid life history (Montecinos et al. 2017). These results emphasize the importance of following the different phases of sexual reproduction in algae with alternate generations, as we did with *G. caudata*.

The cystocarps observed in one of the three F_{CE} apical segments of *G. caudata* cultivated without any male were probably the result of self-fertilization, since this gametophyte was identified as hermaphroditic (bearing both cystocarps and spermatangia). However, male reproductive structures were not observed on any other female gametophyte, and no other control crosses were positive. Monoicous gametophytes were already occasionally observed in other species of *Gracilaria* that usually are dioicously (Kain and Destombe 1995).

Ceará and São Paulo (SP) populations of *G. caudata*, even though separated by an extended distance, were interfertile and produced fertile and viable descendants. Previous studies had already showed remarkable physiological differences between these populations (Araújo et al. 2014; Faria et al. 2017). However, there is no information on physiological aspects of the Espírito Santo population. This population is located between CE and SP, an upwelling area subjected to periods of enhanced nutrient availability and low temperature (Guimarães and Coutinho 2000; Rodrigues and Lorenzetti 2001). Nevertheless, gametophytes from ES were interfertile with CE and SP producing fertile and viable descendants. Global climate change in the oceans is already affecting species distribution (Poloczanska et al. 2013). Latitudinal range shifts as a response to sea-surface warming have already been observed in marine brown algae (Assis et al. 2014). Thus, the successful interbreeding among physiological ecotypes might ensure the subsistence of the species in the face of global climate change. Therefore, future studies should be conducted with the aim of investigating the physiological characteristics of the ES population.

The shorter time needed to complete the life history of *G. caudata* (12–13 weeks), when compared to previous study with the species (9 months, Oliveira and Plastino 1984), can be explained by the most suitable method of cultivating gracilarioid algae. *G. caudata* grew very well and reached reproductive maturity quickly, irrespective of life history phases, whether gametophytes or tetrasporophytes. The success in obtaining fertile plants in a short time makes the species a candidate for future studies related to the selection of strains with characteristics more suitable for mariculture. One possibility would be to evaluate the performance of the generations resulting from crosses between different populations.

Despite their reproductive compatibility, gametophytes derived from the three populations of *G. caudata* sampled in this work presented distinct COI sequences. Sequence divergence

values of *G. caudata* ranged from 1 to 5 bp (0.15–0.79%), which showed higher intraspecific diversity when compared to other *Gracilaria* species. In *Gracilaria changii* (B.M.Xia & I.A.Abbott) I.A.Abbott, J.Zhang & B.M.Xia, from Peninsular Malaysia, intraspecific nucleotide differences ranged from 0 to 3 bp (0.33%) over 923 bp (Yow et al. 2011). Although Saunders (2005) suggests that intraspecific differences for red algae usually range from 0 to 0.3% divergence, our study also showed higher results. Similarly, in *Bostrychia intricata* (Bory) Montagne, genetic divergence among sampled population around New Zealand ranged from 0.2 to 12.8% for COI sequences (Muangmai et al. 2015). Our results further demonstrate the usefulness of mitochondrial DNA in inferring the relationship within populations of red algae as previously observed (Robba et al. 2006).

The mitotypes found on the tetrasporophytic offspring of *G. caudata* were consistent with those of the maternal parents, proving that the mitochondrial genomes were maternally inherited in all crosses. These results corroborate previous findings for red and brown algae (e.g., Zuccarello et al. 2002; Choi et al. 2008; Destombe et al. 2010; Li et al. 2016).

The biological species concept (BSC) defines species as interbreeding natural populations (Mayr 1992). In order to ascertain whether this was true for *G. caudata* along the Brazilian coast, we used crossing tests and mitochondrial DNA sequencing. We demonstrated a potential capability of gene exchange among three populations, even though they were located up to 3000 km apart from each other. This exchange of genetic material indicates that *G. caudata* fits the BSC concept. However, our data does not allow to confirm that this gene exchange is occurring in nature. Considering the extensive distribution and ample physiological variation of *G. caudata*, we can presume the occurrence of putative distinct genetic lineages with geographically structured distributions. The spatial variability of these lineages is currently under investigation, and these studies will clarify the actual gene exchange between the populations along the Brazilian coast.

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