

All non-indigenous species were introduced recently? The case study of *Cassiopea* (Cnidaria: Scyphozoa) in Brazilian waters

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Upside-down jellyfish (genus Cassiopea) can be found in tropical coastal waters worldwide. Until now reports of the genus from Brazilian waters have been scant. We report here medusae and scyphistomae collected from Cabo Frio, Rio de Janeiro state. Although we could not unambiguously identify the material using morphological criteria, genetic sequence data (COI) indicate that the Brazilian jellyfishes are genetically similar to those from Bermuda, Hawaii and Florida, which are related to specimens from the Red Sea (Cassiopea andromeda). We hypothesize that the presence of C. andromeda in Brazil is due to an invasion event, as the scyphistomae were found growing over the known invasive ascidian Styela plicata. Estimation of divergence time between Brazil (Cabo Frio) and Florida/Bermuda populations is that it occurred at the beginning of ship movement to South America.

Keywords: Invasive species, marine, rhizostome, jellyfish, blooms

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INTRODUCTION

Invasive alien species are the second greatest threat to global biodiversity loss (Sala *et al.*, 1999). In the case of the marine environment, these invasive species may be escapees following intentional introduction, but are more usually the unintended consequence of global shipping and maritime transport (Carlton, 1987; Williams *et al.*, 1988; Carlton & Geller, 1993; Sax *et al.*, 2007; Rodríguez-Labajos *et al.*, 2009; Briski *et al.*, 2012; Guo *et al.*, 2012). Although most alien taxa that are introduced into new environments following ballast-water containment or hull-fouling fail to establish viable populations in their new environment (Carlton, 1996), a sufficiently large number do to warrant international programmes of prevention and control.

Although jellyfishes (Cnidaria: Medusozoa) have a number of biological features that allow them to be potentially invasive (Graham & Bayha, 2007; Bayha & Graham, 2014), there are relatively few species (five out of ~201; Bayha & Graham, 2014) with proven reports of invasion worldwide, although with many records distributed in different ocean basins (Bayha & Graham, 2014). Part of the reason for this must reflect an incomplete understanding of the biology, taxonomy and distribution of most jellyfishes (Dawson, 2005a), many of which have a number of cryptic species (Dawson, 2003; Holland *et al.*, 2004), which was estimated as 7% (Appeltans *et al.*, 2012). In Brazilian waters (along 9230 km of coast)

only one (*Phyllorhiza punctata* von Lendenfeld, 1884; Moreira, 1961; Haddad & Nogueira, 2006) of the 22 recorded species of scyphozoans (Morandini *et al.*, 2005; Lopes *et al.*, 2009) can be considered as exotic to date.

The genus *Cassiopea* is widely distributed across the globe in shallow tropical waters (Kramp, 1961). Although a total of 10 species are considered valid (Kramp, 1961; Hummelinck, 1968; Thiel, 1975), a number of genetically distinct lineages have been identified (Holland *et al.*, 2004). Species of the genus can be abundant in disturbed/eutrophicated environments (Arai, 2001). Members of this genus have been considered invasive in the Mediterranean (Galil *et al.*, 1990; Çevik *et al.*, 2006; Schembri *et al.*, 2010). Holland *et al.* (2004) also mapped the distribution of some populations and attributed the 'unusual' pattern observed to maritime transport.

The first report of the genus *Cassiopea* Péron & Lesueur, 1810 in Brazil was published in 2002 (Migotto *et al.*, 2002), although a number of sightings and grey-literature accounts pre-date that, but unfortunately without any voucher specimens.

Here we report specimens of *Cassiopea* from along the Brazilian coast using both morphological and molecular data and we compare our results with published information in order to try and determine their origin. The application of molecular (genetic) data to define and identify populations and species in marine research is now a well-recognized venture (Schander & Willassen, 2005; Radulovici *et al.*, 2010; De Broyer *et al.*, 2011). Together with approaches such as morphology and ecology, this information let us analyse historical aspects such as time of possible invasion based on the divergence of Brazilian jellyfishes compared with the available data in the literature (Holland *et al.*, 2004).

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MATERIALS AND METHODS

Field sampling

Jellyfish specimens were sampled by hand at a small branch of the Itajuru Channel, a connection of the Araruama Lagoon to the open sea in Cabo Frio county, Rio de Janeiro state ($22^{\circ}52'32''\text{S}$ $42^{\circ}01'07''\text{W}$) (Figure 1) during the period November 2008 to October 2012 (weekly intervals from 2008–2009 and every 3 months from 2010–2012). Regular measurements of salinity, dissolved oxygen concentrations and temperature (recorded with an YSI 85 probe) indicate that salinity varied from 0–36, water temperature from 26.6 – 28.7°C , and dissolved oxygen from 7.01 – $15.56\ \mu\text{g L}^{-1}$ in a period of 6 h (from high to low tide).

Medusae were preserved either in ethanol 95 or 4% formaldehyde solution in seawater. Two hundred freshly collected specimens were examined in the laboratory facilities to measure bell diameter, count number of rhopalia, lappets, mouth arms, define shape of the oral arm appendages and the organization of the canal system (with injection of dye). Morphological comparisons were conducted with descriptions in the literature as well as with specimens of *Cassiopea frondosa* (Pallas, 1774) and *Cassiopea* cf. *xamachana* Bigelow, 1892 that had been collected in February 2004 at Laguna Golf Club, Varadero (Cuba), and deposited at the Museu Nacional da Universidade Federal do Rio de Janeiro (MNRJ 5837 and 5838, respectively).

Scyphistomae morphologically resembling *Cassiopea* scyphistomae (Hofmann *et al.*, 1978; Straehler-Pohl, 2009; Heins *et al.*, 2015) were collected at the same place that

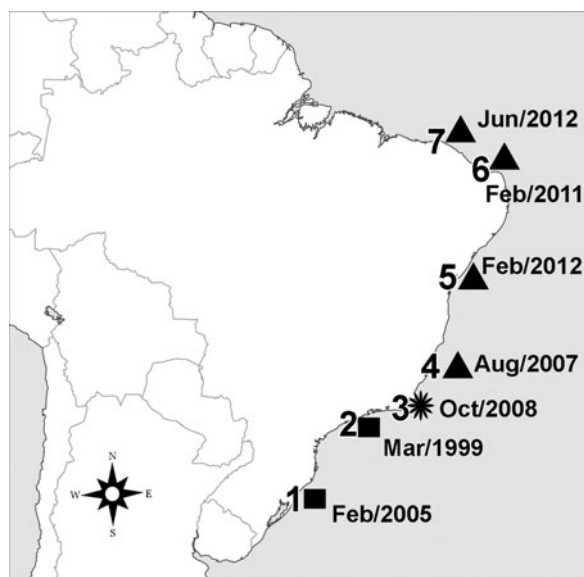


Fig. 1. Map of Brazil, showing records of *Cassiopea* Péron & Lesueur, 1810 specimens. Squares refer only to polyp records (1–2), triangles refer to medusa photographic records (4–7), star refers to sampling site at Cabo Frio (3), numbers after each symbol refer to date of first sighting in each area: (1) Imbé ($29^{\circ}58'26''\text{S}$ $50^{\circ}08'18''\text{W}$; February 2005), Rio Grande do Sul state; (2) São Sebastião ($23^{\circ}49'41''\text{S}$ $45^{\circ}25'22''\text{W}$; March 1999), São Paulo state; (3) Cabo Frio ($22^{\circ}52'32''\text{S}$ $42^{\circ}01'07''\text{W}$; October 2008), Rio de Janeiro state; (4) Aracruz ($19^{\circ}50'07''\text{S}$ $40^{\circ}03'28''\text{W}$; August 2007), Espírito Santo state; (5) Salvador ($12^{\circ}57'26''\text{S}$ $38^{\circ}31'50''\text{W}$; February 2012), Bahia state; (6) Macau ($5^{\circ}03'53''\text{S}$ $36^{\circ}30'18''\text{W}$; February 2011), Rio Grande do Norte state; (7) Itarema ($02^{\circ}52'48''\text{S}$ $39^{\circ}54'15''\text{W}$; June 2012), Ceará state.

medusae were found. The scyphistomae were found growing on a variety of substrata, including plastic, wood and rubber shoes as well as on the exotic ascidian *Styela plicata* (Lesueur, 1823). Ten scyphistomae (Figure 2) were measured in the laboratory following the parameters proposed by Straehler-Pohl *et al.* (2011).

Voucher specimens of medusae and scyphistomae were deposited at the Museu de Zoologia da Universidade de São Paulo (MZUSP, 1944, 1945).

Molecular protocols

DNA was extracted from the oral arms removed from three specimens of medusae using an Agencourt DNAdvance[®] kit (#A48708); DNA extraction from scyphistomae was not successful. Partial gene sequences were amplified using PCR, Universal PCR primers for the cytochrome oxidase I gene (COI) were used to amplify ~500 base pairs of this protein coding mitochondrial gene, using the original PCR program (forward LCO1490 and reverse HCO2198 primers; Folmer *et al.*, 1994). The PCR products were purified with the Agencourt AMPure[®] kit (#A63881) and this amplified DNA sequences were prepared to sequencing using the Applied Biosystems BigDye[®] Terminator v3.1 kit (#4337455), with the same primers and temperature conditions from the PCR's reactions. The sequencing procedure was carried out on a Hitachi ABI PRISM[®] 3100 genetic analyser.

DNA analysis

Sequences were assembled and edited using Geneious[™] 5.4.4 (Drummond *et al.*, 2011), and new sequences were deposited in GenBank (KC464458–KC464459). COI sequences were compared to those of the other *Cassiopea* specimens available in GenBank (see table 1 in Holland *et al.*, 2004): sequence alignment was made using the MUSCLE plugin in Geneious[™] 5.4.4 (default parameters) (Edgar, 2004). Kimura's two-parameter model of base substitution and *p*-distance was used to calculate genetic distances in MEGA 6.05 software (Tamura *et al.*, 2011). The Maximum Likelihood analysis was conducted via PHYML 3.0.1 beta with a more inclusive model, the general time reversible model with gamma values estimated directly from data (GTR + GAMMA) (Guindon *et al.*, 2010). The Maximum Parsimony analysis was conducted via MEGA 5.05 (Tamura



Fig. 2. *Cassiopea andromeda* (Forskål, 1775) scyphistoma just after collection, note long stalk and planuloid budding at the base of calyx; polyp ~5 mm long.

et al., 2011): optimal tree was obtained by the search of optimal tree space using the Close-Neighbour-Interchange (CNI) algorithm (see more in Stampar *et al.*, 2012). Support estimation was assessed with two non-parametric methods: bootstrap (500 pseudoreplicates) and SH-aLRT test (Anisimova *et al.*, 2011) in PHyML 3.0.1 beta (Figure 3).

RESULTS AND DISCUSSION

The morphological and range meristic characters determined from the 200 specimens collected at Cabo Frio are shown in Table 1 in comparison with data from the other presently recognized species in the genus. It should be noted that all inspected specimens were males, suggesting that the population is perhaps maintained through asexual reproduction at the scyphistoma stage. The morphological and meristic data indicate that, with the possible exception of *Cassiopea frondosa*, it is not possible to distinguish between the different

species (see Table 1) using the selected character set because of the high level of overlap between species (see Mayer, 1910; Kramp, 1961; Hummelinck, 1968; Thiel, 1975; Holland *et al.*, 2004). The exception, *Cassiopea frondosa*, can be separated from the other recognized species by the number of rhopalia (12, although some variation is also found) and it is clear that the Brazilian material is not of this species.

Collected scyphistomae were also measured (N = 10): oral disc diameter 0.65–1.5 mm; total length 2–3.5 mm; stalk length 1.15–2.5 mm; calyx length 0.35–0.5 mm; hypostome length 0.2–0.7 mm; number of tentacles 27–36. These measurements are in accordance with the available data for the genus (Ludwig, 1969; Straehler-Pohl, 2009). The scyphistomae were kept in laboratory conditions, and produced ephyrae (by monodisc strobilation) similar to the young medusae found in the field.

The COI data obtained from the three sequenced specimens were identical. The phylogenetic analysis (Figure 3)

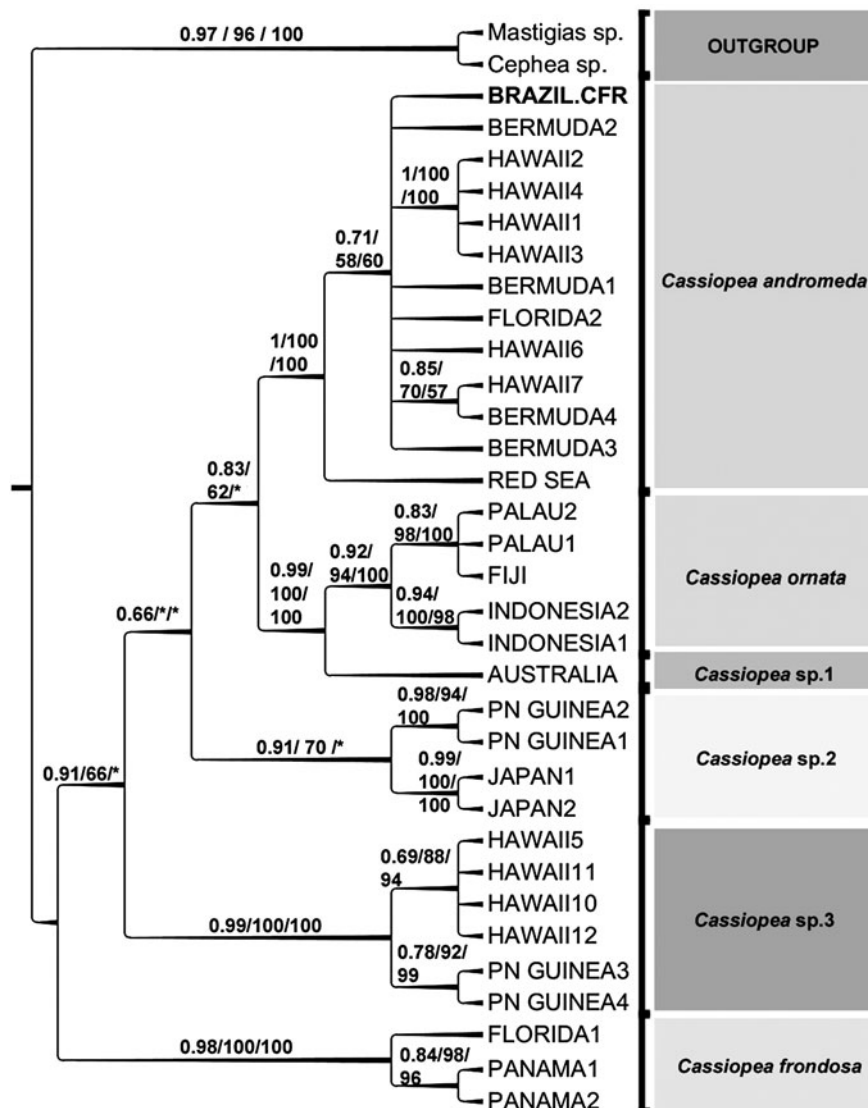


Fig. 3. Left panel – Cladogram representation for maximum likelihood tree (ML) of *Cassiopea* species with support values on branches (as in figure order: SH-aLRT/maximum parsimony's tree bootstrap result/maximum likelihood bootstrap) * = less than 50; in cases where figure permits, ML bootstrap values are below their respective branch. Right panel: phylogram representation only for same result (ML phylogram).

Table 1. Comparison of available characters from the literature of the 10 valid/nominal species of the genus *Cassiopea* Péron & Lesueur, 1810 (species list from Mayer, 1910; Kramp, 1961; Gershwin *et al.*, 2010). *n* = several appendages, not specified on the papers. For mouth arms we are considering number of branches, type of branching and general shape, according to the descriptions.

Species	Size cm	Rhopalia	Lappets	Mouth arms	Appendages	Type locality
<i>C. andromeda</i> (Forskål, 1775)	10–12	16?	variable	4–6 branches	<i>n</i> small, 5–more large/arm	Red Sea
<i>C. depressa</i> Haeckel (1880)	10–12	16?	144	6–8 branches	<i>n</i> small	Madagascar, Mozambique
<i>C. frondosa</i> (Pallas, 1774)	12–26	12	60		30–40 small	West Indies, Caribbean
<i>C. maremetens</i> Gershwin, Zeidler & Davie (2011)	2–20	19	57 (3/rhopalia)	4–6 branches	1–2 central, 1 base arm, 1 tip arm	Lake Magellan, Australia
<i>C. medusa</i> Light (1914)	26	17	119 (7/rhopalia)	<i>n</i> branches (3 main distal)	<i>n</i> small/large	Culion Bay, Philippines
<i>C. mertensi</i> Brandt (1835)	10–12	16?	128	<i>n</i> branches	<i>n</i> large	Kosrae Island, Micronesia
<i>C. ndrosia</i> Agassiz & Mayer (1899)	5	18–22	64 (4/rhopalia)	?	<i>n</i> leaf	Suva, Fiji
<i>C. ornata</i> Haeckel (1880)	10–12	16?	80 (5/rhopalia)	?	<i>n</i> small (only)	Palau, Papua New Guinea
<i>C. vanderhorsti</i> Stiasny (1922)	17	14–18	48–80 (3–5/rhopalia)	dichotomous	<i>n</i> small/large	Caracas Bay, Curaçao
<i>C. xamachana</i> Bigelow (1892)	15	16?	80 (5/rhopalia)	Triangular 10–15 alternate branches	<i>n</i> large ribbon	Kingston Harbor, Jamaica
<i>C. andromeda</i> (from Cabo Frio)	1–20	16–22	48–80 (3–5/rhopalia)	3–10 (different sizes)	<i>n</i> small/large	Cabo Frio, Rio de Janeiro, Brazil

including the new Brazilian specimens, and terminals (=sequences) already presented in a previous study (Holland *et al.*, 2004) clustered the Brazilian *Cassiopea* sequences group with the *Cassiopea andromeda* samples (from Florida, Bermuda and Hawaii) as defined by Holland *et al.* (2004). The molecular distance between the Brazilian material and Florida and Bermuda specimens is 0.54% based on Kimura 2-parameter model and 0.005 on *p*-distance (3 to 6 nucleotide variation). A comparison of the molecular divergence between the *Cassiopea* species considered by Holland *et al.* (2004) is shown in Table 2, and it reveals that intraspecific variation (from 0.6–10%) is much smaller than interspecific variation (11–26%). It is estimated that the *C. andromeda* specimens sampled here from Cabo Frio diverged from those in Florida and Bermuda ~500 years ago, which is relatively soon after the ‘discovery’ of Brazil in 1500. In an alternative method (Dawson, 2005b) to estimate the divergence time between species the equation $T = d/2\lambda$ (where *d* is the nucleotide distance between populations and the mutation rate $\lambda = 4.87 \times 10^{-6}$) was applied. Based on this equation the Cabo

Frio specimens diverged from Florida and Bermuda ~554 years ago.

Whilst early ships did not have the ballast capacity of modern vessels, and their ballast would invariably have been rocks or other heavy solid material, their hulls would have inevitably been subject to heavy fouling (see more in Carlton & Hodder, 1995). An introduction into Brazil via the fouling route is perhaps more likely than by ballast water – although the scyphistomae of *Cassiopea* can produce a number of planuloids asexually on a daily basis (Hofmann *et al.*, 1978), which could persist within ballast tanks – because scyphistomae were observed on artificial substrates as well as on another invasive species, *Styela plicata* (Barros *et al.*, 2008). Given that genetic evidence (same haplotypes) indicates that *Styela plicata* populations off Florida and Brazil are similar (Barros *et al.*, 2008), it is possible that populations of both species were introduced into Brazilian waters from Florida.

Cassiopea andromeda clearly has a number of ecological characteristics that predispose it to invasion success, chiefly

Table 2. Estimates of average evolutionary divergence over sequence pairs within and between ‘species’ as defined by Holland *et al.* (2004). Analyses were conducted using the Kimura 2-parameter model. The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). All positions containing gaps and missing data were eliminated (total positions in the final dataset: 554). Evolutionary analyses were conducted in MEGA5 (Drummond *et al.*, 2011). n/c = not possible to estimate evolutionary distances. Figure 3 lists the individuals used in the comparison as well as their location.

	<i>C. andromeda</i> (%)	<i>C. frondosa</i> (%)	<i>C. ornata</i> (%)	<i>C. sp1</i> (%)	<i>C. sp2</i> (%)	<i>C. sp3</i> (%)
<i>C. andromeda</i>	1.6	25	23	23	23	22
<i>C. frondosa</i>		1.3	26	25	24	22
<i>C. ornata</i>			1.8	11	22	23
<i>C. sp1</i>				n/c	20	23
<i>C. sp2</i>					10	21
<i>C. sp3</i>						0.6

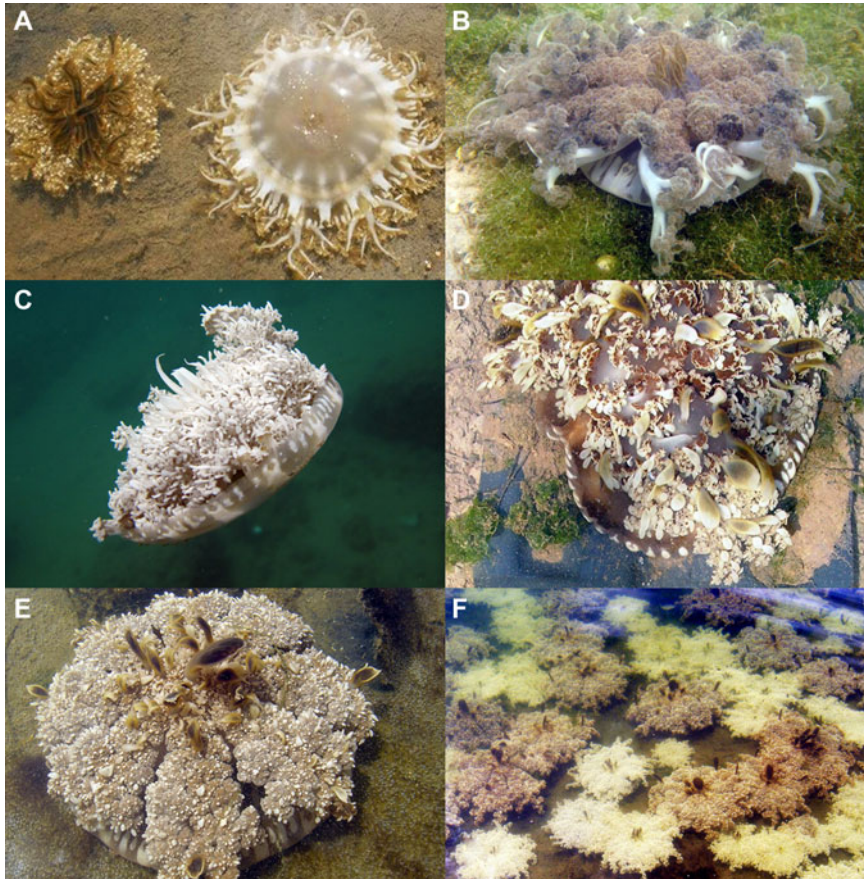


Fig. 4. Photographic records of *Cassiopea* Péron & Lesueur, 1810 specimens from Brazilian coast (A–F). (A) Itarema, Ceará state (photo Carlos H.P. Marques & Yara G. Oliveira); (B) Macau, Rio Grande do Norte state (photo Thelma L.P. Dias); (C) Salvador, Bahia state (photo Claudio L.S. Sampaio); (D) Aracruz, Espírito Santo state (photo Oberdan J. Pereira); (E–F) *Cassiopea andromeda* (Forskål, 1775) from Cabo Frio, Rio de Janeiro state (photos by André C. Morandini and Sergio N. Stampar).

the ability of its scyphistomae to produce planuloids at a high rate (personal observation). Off Brazil, it is clearly tolerant to quite wide variations in salinity and temperature (salinity 0–36, water temperatures up to $\sim 29^{\circ}\text{C}$), and this allows it to be an opportunistic species (Fofonoff *et al.*, 2003; Devin & Beisel, 2007; Zerebecki & Sorte, 2011). It is known that *Cassiopea* species can be tolerant to salinity fluctuations (Goldfarb, 1914). Several other reports of *Cassiopea* medusae exist from the coast of Brazil based on photographic records (Figures 1 & 4), as they do for scyphistomae (states of Rio Grande do Sul and São Paulo; Figure 1). Although we do not at this stage have genetic data to confirm their identity to species, it is clear that the genus *Cassiopea* is finding suitable habitats along the Brazilian coast and that in the future they may be much more common.

The detection of non-indigenous species in Brazilian waters is important (Rocha *et al.*, 2013), because lists of such species shall serve as elements for conservation policies and priorities. Even as exhaustive biological surveys provide the best methods for detecting alien species, the appearance of unusual taxa in large numbers is often the first evidence of an invasion event. This is the case of the present species, with blooms of *C. andromeda* (Figure 5) in the Araruama Lagoon since 2008. But not all blooms of unusual species need be similarly interpreted owing to the lack of comprehensive species maps, the scarcity of long-term data on jellyfish blooms (Condon *et al.*, 2012), and because jellyfish naturally

exhibit episodic and seasonal interannual variability (Mills, 2001). We are aware that our genetic data only suggest the hypotheses of possible appearance of this species as following an invasive event in the Brazilian shoreline. Furthermore, whilst *C. andromeda* may have been resident in Brazilian waters for ~ 500 years, only now are populations (in some areas) blooming. The reasons for this are not yet understood, neither are the impacts on local biological communities, but to date there appear to be no obvious economic or social consequences of the bloom. Regardless, further work on the species in Brazil is now needed to define a broad and complete picture on present and past evolution of the species *C. andromeda*.

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Fig. 5. Medusae of *Cassiopea andromeda* (Forskål, 1775) at a branch of Itajuru Channel (Cabo Frio, Rio de Janeiro State, Brazil) during December 2008. Note first author as scale (~1.65 m).

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