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ORIGINAL ARTICLE

It's not what it looks like: molecular data fails to substantiate morphological differences in two sea hares (Mollusca, Heterobranchia, Aplysiidae) from southern Brazil

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Abstract Species of sea hares have been recognized traditionally based on morphological traits, mainly the radula, external coloration, and reproductive anatomy. However, recent studies have shown substantial color variation in some sea slug species. Molecular data have been successfully used to differentiate morphologically similar species of "opisthobranchs" and resolve questions on the taxonomic value of color. The objective of this paper is to use molecular data in an attempt to elucidate whether specimens of *Aplysia brasiliana* with distinct colorations and morphologies are actually the same species. To this end, DNA from 14 specimens of *A. brasiliana* was extracted, including five specimens identified as a distinct morphotype from typical *A. brasiliana.* Although the two morphotypes have consistent differences in their external morphology and radula, the molecular data confirmed that there are no significant genetic differences between them. This is another example of the need to re-evaluate taxonomic decisions based on morphology in light of molecular evidence.

Keywords *Aplysia* · CO1 · 16S · Morphological variation

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Introduction

Species of sea hares have been recognized traditionally based on morphological traits, mainly the radula, external coloration, and reproductive anatomy (Eales 1960). The original descriptions of most species of *Aplysia* were based solely on external morphological characteristics, body and shell shape and particularly color pattern (see Eales 1960). Marcus and Marcus (1958) and Eales (1960) were the first authors to described species of *Aplysia* including illustrations of the radula and the reproductive anatomy, but always stressing color differences between species for taxonomic decisions.

The wide range of variation in the coloration of some species of *Aplysia* has been the source of considerable confusion as how to differentiate species (Winkler 1959). MacMunn (1899) and Winkler (1959) have shown that in *Aplysia* the normal skin pigment is basically made up of degradation products of the tetrapyrrole molecule of chlorophyll, in other words, there should be a close connection between diet and coloration. However, new species names for *Aplysia* and other "opisthobranch" molluscs have been introduced based on color differences with closely related species. Recent studies have shown substantial color

variation in some "opisthobranch" species (Valdés et al. 2013) including sea hares (Alexander and Valdés 2013). The implication of these findings is that in *Aplysia* color differences among individuals may have limited taxonomic value.

In recent years, molecular data have been successfully used to differentiate morphologically similar species of "opisthobranch" sea slugs and to resolve taxonomic questions on the significance of color variation for systematics (Medina et al. 2001, 2005; Ornelas-Gatdula et al. 2011; Alexander and Valdés 2013). In this paper, we study two different morphotypes of *Aplysia brasiliana* (Rang, 1828) collected from Southern Brazil. These two color forms display consistent morphological differences, but the two morphotypes are found sympatrically, in the same habitat during the same time of the year. We use molecular data in an attempt to elucidate whether specimens with distinct colorations and morphologies are actually the same species.

Materials and methods

Source of material

DNA from 14 specimens of *A. brasiliana* was extracted (Table 1). Five specimens were identified as a distinct morphotype from typical *A. brasiliana*, here referred to as *Aplysia* aff. *brasiliana.* The specimens were collected, from November 2011 to January 2013, in Ponta da Praia, Santos, São Paulo, Brazil (23°59′29″S; 46°18′15″W) and in São Sebastião, São Paulo, Brazil (23°49′43.06″S; 45°25′21.83″W). Additionally, sequences of *A. brasiliana* from Florida, *Aplysia parvula* Mörch, 1863 from the Bahamas, *Aplysia kurodai* (Baba, 1937) from Japan, *Aplysia dactylomela* (Rang 1828) from the Bahamas and *Aplysia argus* Rüppell and Leuckart 1828 from the Hawaiian Islands ware included in this study for comparison using sequences available in GenBank. *Phyllaplysia taylori* Dall 1900 was used as the outgroup (Table 1).

All the specimens from Brazil are deposited at the Museu de Zoologia Universidade de São Paulo (MZUSP). Additional material was obtained from the collections of the Department of Biological Sciences of the California State Polytechnic University, Pomona (CPIC) and Museu Nacional Universidade Federal do Rio de Janeiro (MNRJ).

Morphological study

All the specimens of *A. brasiliana* and *A.* aff. *brasiliana* were photographed alive in the field using a Nikon P100 digital camera. Several traits were recorded photographically, including the shell, the opening of the siphon and the gill, and the anterior end of the animal. All sea hares were photographed with the parapodia opened and closed. The specimens were taken to the laboratory at MZSP. All the individuals were euthanized by asphyxiation (kept in water without oxygenation) and preserved in 70 % EtOH. Under a stereomicroscope, the buccal mass was dissected from two specimens of each morphotype (*A. brasiliana:* MZSP 109978, MZSP 103251 and *A.* aff. *brasiliana:* MZSP 114920, MNRJ 18228). Tissue surrounding the radula and jaws was dissolved in 10 % sodium hydroxide for 2 days. The radulae were rinsed in water, dried, mounted on stubs, and coated with a gold–palladium alloy for scanning electron microscopy. The samples were observed under a scanning electron microscope (SEM) Zeiss LEO-440 at the Laboratório de Microscopia Eletrônica do Museu de Zoologia, Universidade de São Paulo.

DNA extraction

DNA was extracted from a small foot sample using a hot Chelex® protocol with modifications. The tissue was rinsed and rehydrated using 1.0 mL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) for 20 min. A 10 % (w/v) Chelex® 100 (100–200 mesh, sodium form; Bio-Rad) was prepared using TE buffer. After rehydration, the mixture was then centrifuged, 975 mL of the supernatant was removed, and 175 mL of the Chelex® solution was added. Samples were then heated in a 56 °C water bath for 3 h, then heated in a 100 °C heating block for 8 min, and the supernatant was used for PCR.

Primers

Palumbi's universal 16S rRNA primers (16S ar-L 5′-CGCC TGTTTATCAAAAACAT-3′, 16S br-H 5′-CCGGTCTGA ACTCAGATCACGT-3′) (Palumbi 1996), Folmer's universal cytochrome oxidase subunit 1 primers (LCO1490 5′-GGTCAACAAATCATAAAGATATTGG-3′, HCO2198 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al. 1994) and Colgan's universal Histone-3 primers (H3 AF 5′-ATGGCTCGTACCAAGCAGACGGC-3′, H3 AR 5′-ATATCCTTGGGCATGATGGTGAC-3′ developed by Colgan et al. 1998) were used to amplify the regions of interest for all specimens.

The internal primers 16Sar-FAP 5′-AAAGACGAGA AGACCCTTAGAGTTTT-3′ and 16Sbr-FAP 5′-AAAACT CTAAGGGTCTTCTCGTCTTT-3′ (Ornelas-Gatdula et al. 2011) were used to amplify 16S in the São Sebastião specimens (MZSP103233; MZSP103234; MZSP103250) and only partial sequences were obtained.

Table 1 List of specimens included in this study Including Locality, Voucher Numbers and GenBank Accession Numbers

Species	Locality	Voucher Numbers	GenBank accession Numbers		
			16S	CO1	H3
Aplysia aff *brasiliana*	São Sebastiao, São Paulo, Brazil	MZSP 103234	KM272281*	KM272290	KM272296
Aplysia aff *brasiliana*	São Sebastiao, São Paulo, Brazil	MZSP 103233	KM272282*	KM272291	KM272298
Aplysia aff *brasiliana*	São Sebastiao, São Paulo, Brazil	MZSP 103250	KM272283*	KM272292	KM272299
Aplysia aff *brasiliana*	Ponta da Praia, Santos, São Paulo, Brazil	MZSP 109978	KM272287	–	–
Aplysia aff *brasiliana*	Ponta da Praia, Santos, São Paulo, Brazil	MZSP 109973	–	–	–
Aplysia aff *brasiliana*	Ponta da Praia, Santos, São Paulo, Brazil	MZSP 114920	–	–	–
Aplysia aff *brasiliana*	Camocim beach, Vitória, Espírito Santo, Brazil	MNRJ 10633	–	–	–
Aplysia aff *brasiliana*	Mar do Norte, Casimiro de Abreu, Rio de Janeiro, Brazil	MNRJ 4221	–	–	–
Aplysia aff *brasiliana*	Conchas beach, Cabo Frio, Rio de Janeiro, Brazil	MNRJ 18228	–	–	–
Aplysia aff *brasiliana*	Conchas beach, Cabo Frio, Rio de Janeiro, Brazil	MNRJ 18229	–	–	–
Aplysia aff *brasiliana*	Boa Viagem beach, Guanabara Bay, Niterói, Brazil	MZSP 89678	–	–	–
Aplysia aff *brasiliana*	Itacurussá Island, Sepetiba Bay, Rio de Janeiro, Brazil	MNRJ 10629	–	–	–
Aplysia aff *brasiliana*	Ilha Grande Island, Rio de Janeiro, Brazil	MNRJ 1902	–	–	–
Aplysia aff *brasiliana*	Angra dos Reis, Rio de Janeiro, Brazil	MZSP 50343	–	–	–
Aplysia aff *brasiliana*	Búzioz, Rio de Janeiro, Brazil	MZSP 89681	–	–	–
Aplysia aff *brasiliana*	Ingleses beach, Bombinhas, Santa Catarina, Brazil	MZSP 104507	–	–	–
Aplysia brasiliana	São Sebastiao, São Paulo, Brazil	MZSP 103217	–	KM272295	KM272300
Aplysia brasiliana	Ponta da Praia, Santos, São Paulo, Brazil	MZSP 109903	KM272284	–	KM272297
Aplysia brasiliana	Ponta da Praia, Santos, São Paulo, Brazil	MZSP 113776	KM272285	KM272293	–
Aplysia brasiliana	Ponta da Praia, Santos, São Paulo, Brazil	MZSP 113777	KM272289	–	–
Aplysia brasiliana	Ponta da Praia, Santos, São Paulo, Brazil	MZSP 104038	KM272288	KM272294	–
Aplysia brasiliana	Ponta da Praia, Santos, São Paulo, Brazil	MZSP 35128	–	–	–
Aplysia brasiliana	Ponta da Praia, Santos, São Paulo, Brazil	MZSP 104044	–	–	–
Aplysia brasiliana	Ponta da Praia, Santos, São Paulo, Brazil	MZSP 109975	KM272286	–	–
Aplysia brasiliana	Ponta da Praia, Santos, São Paulo, Brazil	MZSP 109976	–	–	–
Aplysia brasiliana	Ponta da Praia, Santos, São Paulo, Brazil	MZSP 109977	–	–	–
Aplysia brasiliana	Ponta da Praia, Santos, São Paulo, Brazil	MZSP 109978	–	–	–
Aplysia brasiliana	Ilha Bela, Búzios Island, São Paulo, Brazil	MZSP 103233	–	–	–
Aplysia brasiliana	Ilha Bela, Serraria Island, São Paulo, Brazil	MZSP 103251	–	–	–
Aplysia brasiliana	Biscayne Bay, Florida, United States	–	AF192296	AF343426	–
Aplysia parvula	Stocking Island, Exuma, Bahamas	CPIC 00039	JX560139	JX560149	JX560158
Aplysia fasciata	Mediterranean Sea, Israel	–	AF192298	AF343428	
Aplysia kurodai	Isozaki, Hitachinaka, Japan	CPIC 00548	JX560137	JX560147	JX560156
Aplysia kurodai	Isozaki, Hitachinaka, Japan	CPIC 00549	JX560138	JX560148	JX560157
Aplysia dactylomela	Stocking Island, Exuma, Bahamas	CPIC 00093	JX560134	JX560144	JX560153
Aplysia argus	Hekili Point, Maui, Hawaiian Islands	CPIC 00297	JX560133	JX560142	JX560151
Phyllaplysia taylori	Friday Harbor, Washington, United States	–	AF156139	AF156155	–

Partial sequences are indicated with an asterisk

PCR amplification and sequencing

The master mix was prepared using 34.75 mL H_2O, 5 mL PCR Buffer (500 mM KCl; 100 mM Tris, pH 8.3), 5 mL 50 mM $MgCl_2$, 1 mL 40 mM dNTPs, 1 mL 10 mM primer 1, 1 mL 10 mM primer 2, 0.25 mL 5 mg/mL Taq, and 2 mL extracted DNA. Reaction conditions for H3 and 16S rRNA were as follows: an initial denaturation for 2 min at 94 °C, 35 cycles for 30 s at 94 °C, annealing for 30 s at 50 °C, and elongation for 1 min at 72 °C, and a final elongation for 7 min at 72 °C. Reaction conditions for CO1 (universal) were as follows: lid heated to 105 °C and initial denaturation for 4 min at 95 °C, 40 cycles for 45 s at 94 °C, annealing for 45 s at 48 °C, and elongation for 90 s at 72 °C, followed by a final elongation step for 10 min at 72 °C.

Gel electrophoresis was used to determine whether PCR resulted in products with the band size of the approximate length for the targeted gene (approximately 375 base pairs [bp] for H3; 475 bp for 16S rRNA, 195 bp for 16S fragments, 700 bp for CO1), then were purified using the GeneJET PCR Purification Kit (Thermo Scientific). Cleaned PCR samples were quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). These samples were sequenced at the Eton Biosciences (San Diego, CA).

Sequence analyses

Sequences for each gene were assembled and edited using Geneious Pro 4.7.4 (Drummond et al. 2009). Geneious was also used to extract the consensus sequence and to construct the alignment for each gene using the default parameters and to concatenate the alignments. The sequences were not trimmed after alignment. The lengths of the gene fragments used in the phylogenetic analyses are 305–328 bp for H3, 440–454 bp for 16S, and 501–658 bp for COI. To assess whether the three genes have significantly conflicting signals, the incongruence length difference (ILD) test (Mickevich and Farris 1981; Farris et al. 1994), implemented in PAUP*4.0 as the partition homogeneity test (Swofford 2002), was calculated for all genes combined with 2,000 replicates. Phylogenetic analyses were conducted for all genes concatenated including those specimens for which at least two genes (including H3) were available. The best-fit models of evolution (GTR + I for CO1; HKY + I for 16S, and GTR + I+G for H3) were determined using the Akaike information criterion (Akaike 1974) implemented in MrModelTest (Nylander 2004). Bayesian analyses were conducted using MrBayes 3.2 (Ronquist et al. 2012), partitioned by gene (unlinked). The Markov chain Monte Carlo analysis was run with two runs of six chains for 20 million generations, with sampling every 100 generations. The default 25 % burn-in was applied before constructing the majority-rule consensus tree. Convergence was inspected using Tracer 1.5 (Rambaut and Drummond 2007). Maximum-likelihood analysis was conducted for the entire concatenated alignment with RAxML GUI 1.0 (Silvestro and Michalak 2012) using the bootstrap + consensus option (10,000 replicates) and the GTR + I model.

To visualize the genetic structure of *A. brasilana*, a haplotype network was constructed using all COI sequences of *A. brasiliana* using the program TCS 1.21 (Clement et al. 2000) with a 95 % connection limit. The same analyses were not conducted for 16S and H3 because all the sequences for *A. brasiliana* and *A.* aff *brasiliana* were identical.

Arlequin 3.5 (Excoffier and Lischer 2010) was also used to calculate F_{ST} values as a measure of pairwise differences between both morphotypes of *A. brasiliana* for both COI and 16S. The significance of the pairwise F_{ST} value was estimated by performing 10,000 permutations.

Results

Morphological study

The morphological examination of the specimens of *A. brasiliana* from Brazil revealed the presence of two distinct morphotypes. The main differences found between morphotypes include the external coloration and morphology. Specimens of morphotype 1 (the typical *Aplysia brasiliana*) posses a greenish and/or brownish background color, with few yellowish patches on the body and parapodia (Fig. 1a); the parapodia are relatively narrow, as wide as the rest of the body; there is a small pore (p) above the shell, which is covered by a papilla (Fig. 1b); the edge of the siphon does not completely cover the opening (s1), forming a semicircle (Fig. 1c); the oral tentacles are small (Fig. 1d). Morphotype 2 (*Aplysia* aff. *brasiliana*) has a black, gray or dark reddish background color, with conspicuous white spots scattered throughout the mantle and parapodia (Fig. 1e); the parapodia are broad, twice as wide as the rest of the body; the small pore above the shell is not covered by a papilla (Fig. 1f); the edge of the siphon covers the opening completely (s2) forming a circle (Fig. 1g); and the oral tentacles are large (Fig. 1h).

Examination of the radular morphology also revealed some differences between *A. brasiliana* and *A.* aff *brasiliana*. The rachidian teeth of *A. brasiliana* are broad with a denticulate central cusp and a secondary smaller cusp on each side; the bases of the teeth are convex (Fig. 2a). The mid-lateral teeth have an elongate cusp with several denticles on each side (Fig. 2b). The outer teeth are similar in shape (Fig. 2c). On the contrary, *A.* aff. *brasiliana* has much narrower rachidian teeth, with a shorter, more rounded central cusp, the teeth are triangular in shape, and the bases are less convex than in *A. brasiliana* (Fig. 2d). The lateral teeth have shorter cusps than in *A. brasiliana* (Fig. 2e). The outermost lateral teeth are very similar to those of *A.brasiliana* (Fig. 2f).

Molecular study

The ILD test showed no significant conflicting signals between the three genes combined 16S versus CO1 ($P = 0.918$), CO1 versus H3 ($P = 0.909$), and 16S versus H3 ($P = 1$). The maximum-likelihood bootstrap consensus and Bayesian consensus trees for all three genes combined

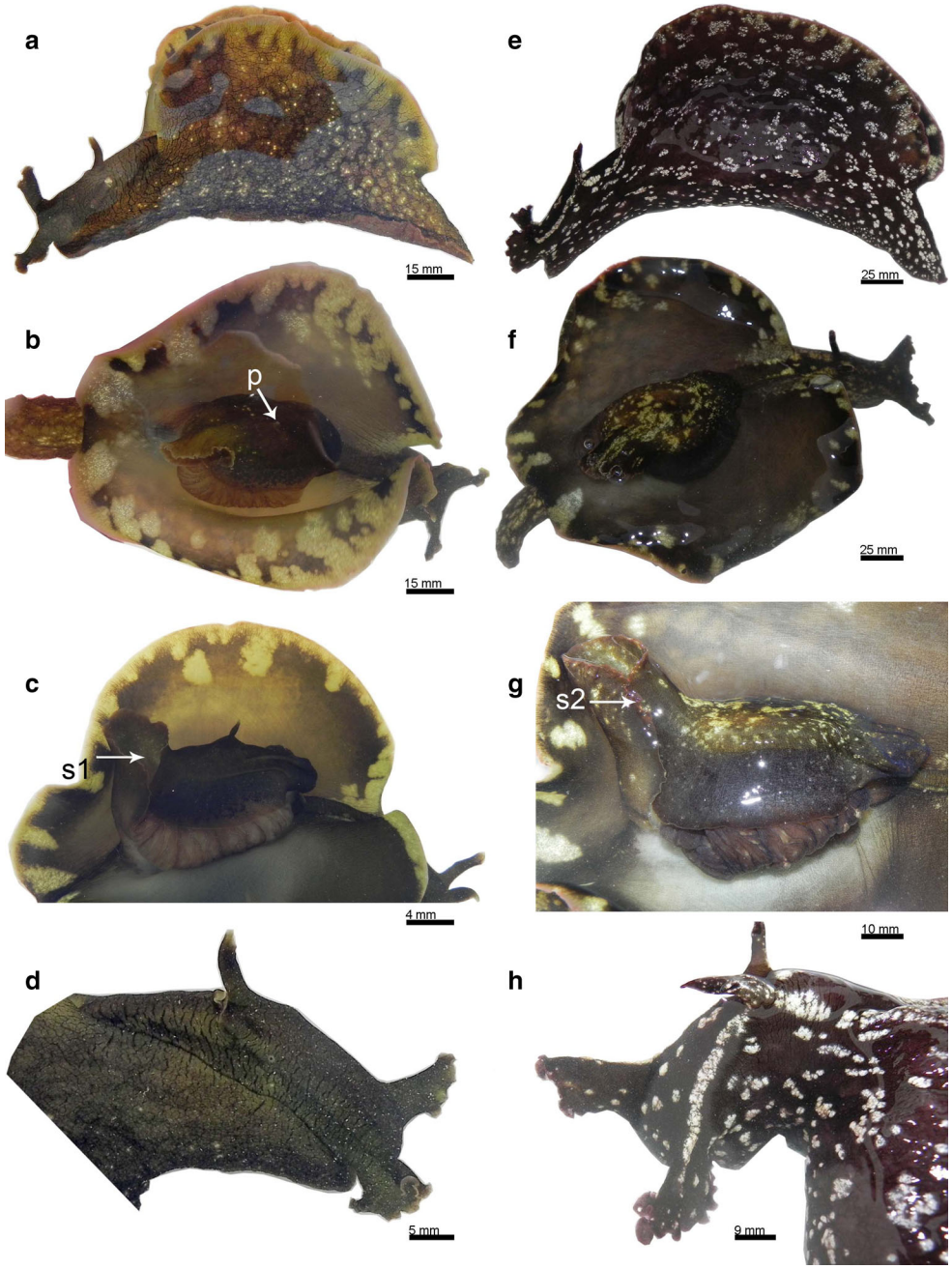

Fig. 1 **a–d** *Aplysia brasiliana* morphotype. **a** Background greenish and/or brownish with few yellowish patches around the body and parapodium. **b** Parapodium end a small pore (p) covered by a papilla. **c** The edge of the siphon (s1). **d** Oral tentacles. **e–h** *Aplysia* aff *brasiliana* morphotype. **e** Background black, gray or dark reddish with predominant white spots scattered throughout the mantle and parapodium. **f** Parapodia. **g** The edge of the siphon (s2). **h** Oral tentacles

recovered three clades (Fig. 3). One clade, including all specimens of *A. brasiliana* and *A.* aff *brasiliana* had a posterior probability (PP) of 0.88 and bootstrap value (BS) of 100. A second clade including specimens of *A. dactylomela* PP of 1 and BS of 98. A third clade including specimens of *A. kurodai* from Japan had a PP of 1 and a BS of 100. The specimens of *A. brasiliana* and *A.* aff *brasiliana* do not form distinct clades.

The CO1 haplotype network (Fig. 4) reveals the presence of 6 distinct haplotypes in *A. brasiliana* including the specimen from Florida. However, there is no clear structure in the haplotype network in relation to the morphology of

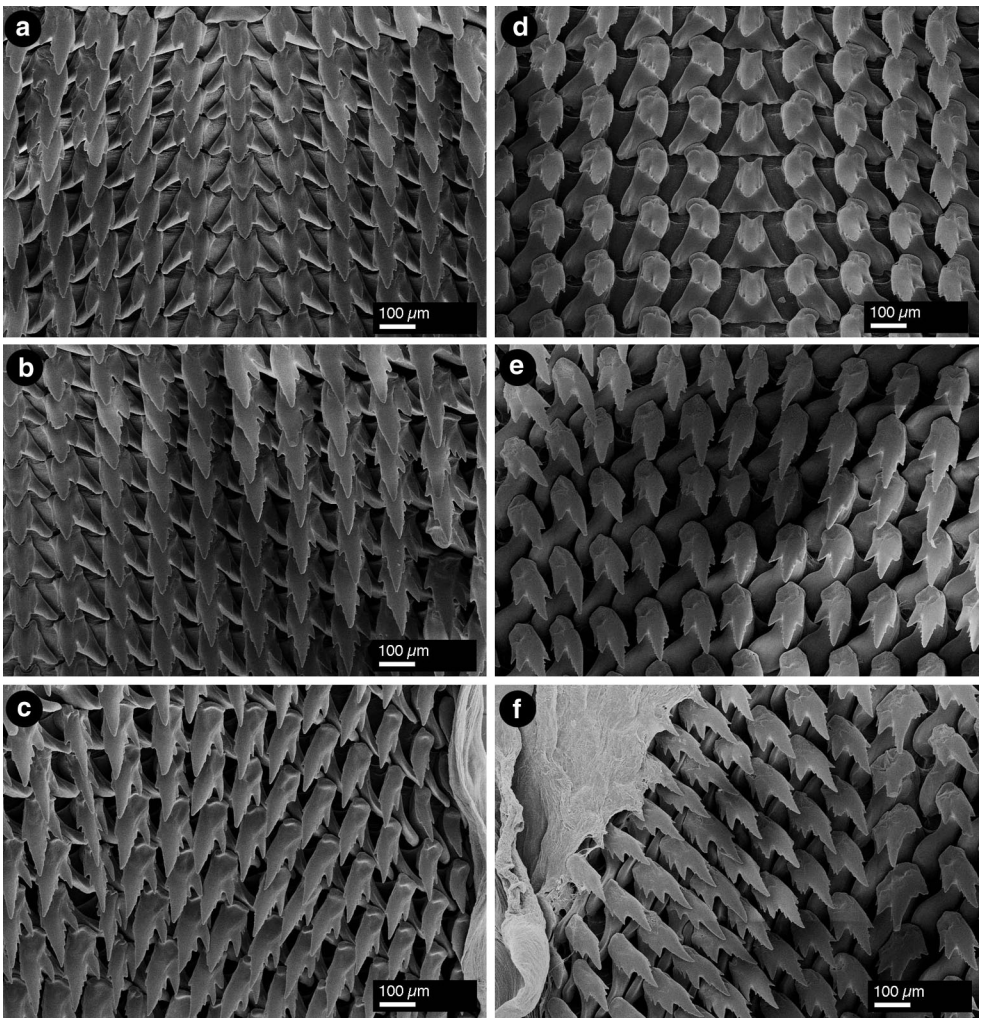

Fig. 2 **a–c** *Aplysia brasiliana* radula. **a** Rachidian tooth. **b** Lateral teeth. **c** Marginal teeth**. d–f** *A.* aff *brasiliana* radula. **d** Rachidian tooth. **e** Lateral teeth. **f** Marginal teeth

the animals, and one of the sequences of *A.* aff. *brasiliana* is identical to three sequences of *A. brasiliana*.

The result of the pairwise F_{ST} analysis between the two morphotypes was not significant for both COI and 16S (Table 2), confirming that there are no significant genetic differences between the two morphotypes.

Discussion

Although color pattern and morphological traits have been used traditionally to distinguish "opisthobranch" sea slug species, recent studies have challenged this view in light of newly available molecular data (Pola et al. 2006; Ornelas-Gatdula et al. 2011; Alexander and Valdés 2013; Valdés et al. 2013). The present study shows that specimens of *A. brasiliana* with distinctive morphologies and colorations are not genetically different and constitute morphotypes of the same species. As in some of the other examples mentioned above, the differences between the two morphotypes of *A. brasiliana* appear to be discrete and consistent, with no apparent intermediate specimens between the two morphotypes and detectable in a range of internal and external traits.

The radular differences are particularly conspicuous. Other studies on sea hares have shown that some species display substantial radular variability. For example, Martínez and Ortea (2002) showed that radular morphology of *Aplysia fasciata* can vary between large and small animals. Although this has not been tested for sea hares, it has been shown that diet can influence tooth morphology (size and number) in other "opisthobranch" sea slugs. For example, in sacoglossans, smooth teeth can be associated with filamentous food (Bleakney 1990; Jensen 1993).

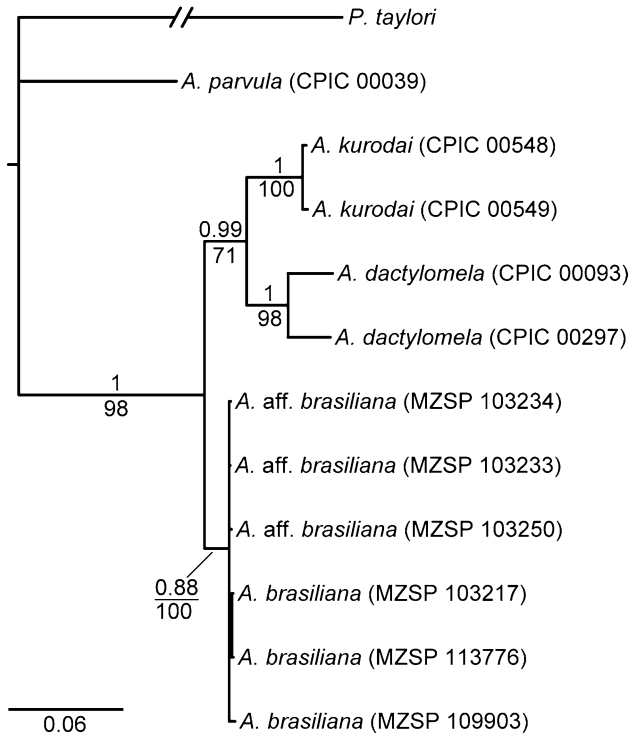

Fig. 3 Schematic representation of the Bayesian consensus phylogenetic tree of Aplysiidae based on three molecular markers. Statistical support percentages are shown on internal branches. Bayesian posterior probabilities precede maximum-likelihood bootstrap values

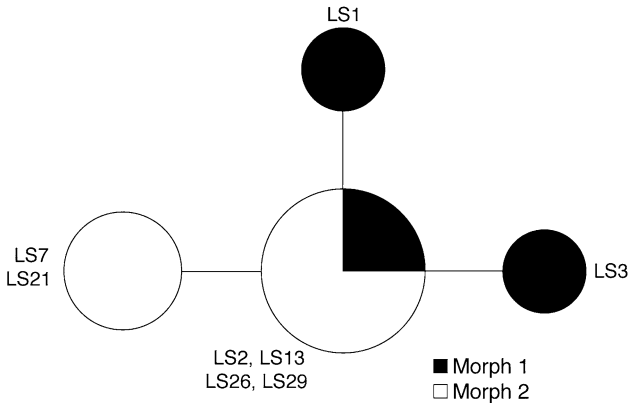

Fig. 4 Haplotype network (with sequence IDs) of CO1 haplotypes generated with TCS. *Pie charts* indicate the proportion of different haplotype clusters found in each locality. Haplotypes identified from the two morphotypes of *A. brasiliana* from Brazil and from other localities are color coded

Bleakney (1989) also noted some other morphological differences in the teeth of a species of "opisthobranchs" from different geographic regions.

The combination of morphological and molecular data in an integrative framework is the ideal approach to resolve difficult taxonomic problems. This is particularly important in "opisthobranchs", which appear to be morphologically plastic. Because of this and other studies, it is becoming more evident that many taxonomic decisions based on morphology will have to be re-evaluated in light of molecular evidence. Radular morphology and external coloration have been widely used to distinguish species of "opisthobranch", but these data may not be as useful as previously thought. This hypothesis will have to be tested in a wider variety of taxa.

Table 2 Population comparisons results for COI and 16S, with FST values and associated *p* values

Gene	F_{ST}	*p*	Error
COI	−0.16039	0.61657	±0.0044
16S	0.18644	0.32541	±0.0047

Significant values are $p < 0.005$

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