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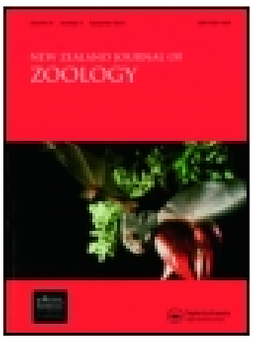

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RESEARCH ARTICLE

Genetic barcoding confirms the presence of the neurotoxic sea slug *Pleurobranchaea maculata* in southwestern Atlantic coast

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ABSTRACT

This work extends a previous report on the outbreak of an unknown neurotoxic sea slug along the southwestern Atlantic coast by providing identification to species level. In this study a c. 550 base pair (bp) region of the cytochrome c oxidase subunit 1 mitochondrial gene used for genetic barcoding was sequenced from three individual slugs sourced from three locations in Argentina. These data, in concert with previous description on the morphology and the presence of a neurotoxin of similar effect of that produced by pleurobranchs from New Zealand, confirm the identity of these sea slugs as *Pleurobranchaea maculata*. This is the first record of the species in the Atlantic Ocean, thus suggesting an exotic origin. Given the fast spread of reports in the southwestern Atlantic (c. 2000 km of coastline in 6 years) we recommend urgent studies to determine the origin, toxicity, population genetics and impacts on local ecosystems.

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Genetic barcoding; neurotoxic; sea slugs; southwestern Atlantic; specimen identification

Introduction

The ability to determine whether a newly found organism is a non-native species is critical to enable rapid management responses. Taxonomic uncertainty based solely on morphological descriptions is common among marine invertebrates, with accurate identification at species level often requiring great taxonomic expertise and the integration of evidence from diverse sources. In the last few years molecular tools have become commonplace in this integrative view of taxonomy. In particular, DNA barcoding has been demonstrated to be a powerful tool for specimen identification (Geller et al. 2010, 2013). DNA barcoding has been successfully used in studies dealing with the identification of marine molluscs, including gastropods (Hunt et al. 2010; Jennings et al. 2010; Jörger et al. 2012; Krug et al. 2012, 2013; Layton et al. 2014).

Recently, we reported on the sudden appearance and subsequent increase in abundance of an unknown neurotoxic sea slug of the genus *Pleurobranchaea* along the southwestern Atlantic coast (Farias et al. 2015). A few individuals were first observed in 2009 at the port

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of Mar del Plata (Farias 2009) and thereafter new records arose from several other locations in Argentina (Buenos Aires from Mar del Plata to Bahía Blanca, up to 60 m depth, and in the San Matias and Nuevo Gulfs, in the provinces of Rio Negro and Chubut respectively; NE Farias, Universidad Nacional de Mar del Plata, unpubl. data), spanning c. 2000 km of coastline.

Pleurobranchs are easily identifiable by the plumose gill located on the right side of the body. However, identification at species level is challenging, even with detailed analysis of the internal anatomy. Based solely on morphological evidence, Farias et al. (2015) showed that the newly observed sea slug was not *Pleurobranchaea inconspicua*, the only *Pleurobranchaea* previously known to occur in this region (Muniain et al. 2006), and suggested *Pleurobranchaea maculata* as the most likely candidate. Individuals of *P. maculata* are commonly found in shallow subtidal areas around New Zealand, and have also been recorded in southeastern Australia, China, Sri Lanka and Japan (Willan 1983). Further evidence to support this assertion came from mouse bioassay data indicating that the sea slug contained a neurotoxin with a similar mode of action to the tetrodotoxin identified in *P. maculata* from New Zealand (McNabb et al. 2010). Therefore, obtaining molecular data to identify the species became a priority to determine whether this represented the early stages of a biological invasion. Here, we use genetic barcodes (cytochrome c oxidase subunit 1 gene [CO1]) of three individuals sourced from distant locations along the southwestern Atlantic coast to enable identification of the specimens to a species level.

Materials and methods

Sampling, DNA extraction, PCR amplification and sequencing

Single individuals were collected from three distant locations: off Buenos Aires, Argentina (38°52′57″ S 59° 35′6″ W, 40 m depth, trawling onboard the R/V *Puerto Deseado-CONICET*); San Antonio Oeste, Rio Negro, Argentina (40°43′37.27″ S 64°56′47.63″ W, 0 m depth, by hand); and Puerto Madryn, Chubut, Argentina (42°44′15.22″ S 65°01′40.27″ W, 3–5 m depth, SCUBA diving). Specimens were photographed, cryo-anaesthetised and fixed directly in 96% ethanol following Geiger (2006). DNA was extracted from a small piece of mantle using the E.Z.N.A. Mollusc DNA Kit (Omega) according to the protocol supplied by the manufacturer. Polymerase chain reactions (PCRs) were performed in 50 µL volumes containing 25 µL of AmpliTaq Gold 360 master mix (Life Technologies), 5 µL bovine serum albumin (0.2 mg/mL; Life Technologies), 0.4 µM of each primer targeting the CO1 (dg LC01490 and dgHC02198; Geller et al. 2013) and template DNA (c. 20 ng). Cycling conditions were 94 °C 3 min, followed by 40 cycles of 94 °C 30 s, 50 °C 30 s, 72 °C 90 s and a final extension of 72 °C 10 min. PCR products were visualised with 1% agarose gel electrophoresis with Red Safe DNA Loading Dye and UV illumination and purified using an AxyPrep PCR cleanup kit (Axygen). Bi-directional sequencing was undertaken using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Data analysis

The sequences of Argentinian specimens were first compared with those of *P. maculata* from Wood et al. (2012) available on NCBI GenBank database (www.ncbi.nlm.nih.gov/GenBank)

using MegaBlast (Benson et al. 2013), and with sequences from other Pleurobranchomorpha available in BOLD (Barcode of Life Database, http://www.boldsystems.org/) using the BOLD Identification System (Ratnasingham & Hebert 2007). Additionally, we assembled a CO1 barcoding dataset with all the sequences available to date of representatives of the family Pleurobranchidae from the above public databases, including the three from the Argentine specimens presented here (hereafter we will refer to this dataset simply as Pleurobranchidae). The sequences were aligned manually with BioEdit (Hall 1999) and the extremes cut to fit the length of the fragments obtained in this work. Sequences shorter than those of our specimens were discarded.

To determine the existence of a barcode gap in Pleurobranchidae we calculated the pairwise distance matrix for intra- and inter-specific genetic distances under the maximum composite likelihood method (Tamura et al. 2004). Then, we conducted an evolutionary analysis including only the sequences of the three species of the genus *Pleurobranchaea*, our unidentified specimens, with the aim of testing whether our sequences were clustered with those of *P. maculata* within the resulting phylogenetic tree. As outgroup we used a CO1 sequence of *Pleurobranchus areolatus* from GenBank (JN675223). The evolutionary history was inferred using the neighbor-joining method (Saitou & Nei 1987). The analyses were conducted using MEGA6 (Tamura et al. 2013)

Finally, we applied Automatic Barcoding Gap Discovery (ABGD; Puillandre et al. 2012) to the whole Pleurobranchidae dataset. The analysis was performed directly from the web interface (http://www.abi.snv.jussieu.fr/public/abgd/, accessed 5 January 2016). This program uses an algorithm that automatically detects significant barcode gaps in the dataset under analysis and then sorts the sequences into different partitions (hypothetical species). Under the hypothesis that our specimens were *P. maculata* we expected to recover the sequences from Argentina in the same partition with those *P. maculata* from New Zealand included in Pleurobranchidae. ABGD analyses were initially performed with the default value of relative gap width ($X = 1.5$) and using all the distance metrics available in the system. The other parameter values were left as defaults.

The specimens and the sequences obtained in this study were deposited in the invertebrate collection of the University of Mar del Plata and uploaded to the NCBI GenBank database, respectively, under the following voucher ID/GenBank accession numbers: UNMDP-ZINV209/KU310926; UNMDP-ZINV219/KU310927; and UNMDP-ZINV225/ KU310928.

Results and discussion

The final sequences (564 bp) obtained for the three individuals from Argentina were identical. MegaBlast analysis showed high similarity (99%) with the four sequences available for *P. maculata* in GenBank, all from New Zealand (GenBank accession numbers JN675220 to JN675223). Sequence similarity with other pleurobranchs in the NCBI or BOLD databases was much lower: *P. meckeli* from northwest Africa (MegaBlast 83%; FJ917499 and AY345026) and *P. inconspicua* from Florida, USA (BOLD IDS 82%, FPMAR057-08).

After alignment and editing, the final CO1 barcoding dataset of Pleurobranchidae consisted of 82 CO1 sequences of 530 bp length, representing 22 nominal species distributed in five genera plus our three unidentified specimens. Figure 1 shows that the frequency

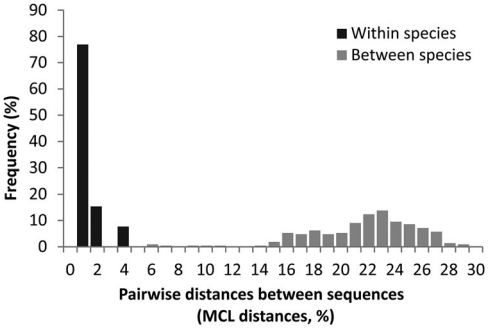

Figure 1. Frequency distribution of intra-specific and inter-specific pairwise genetic distances in Pleurobranchidae. The plot is based on 82 sequences of 530 bp obtained from BOLD system representing 22 nominal species distributed in five genera plus the three specimens from Argentina presented in this work. Pairwise genetic distances were calculated under the maximum composite likelihood (MCL) method.

distributions of the intra-specific and inter-specific pairwise genetic distances do not overlap, thus supporting the use of CO1 barcoding for identification of representatives of Pleurobranchidae at a species level.

The neighbor-joining analysis produced a tree with three groups in accordance to the species assigned a priori. The three specimens from Argentina were clustered with the specimens of *P. maculata* from New Zealand, as predicted (Figure 2).

During the ABGD analysis the relative width of barcoding gap (X-value) had to be lowered to 1 to get any partitions, the rest of the parameters were left as default. In full agreement with our a priori delimitation of species, the ABGD grouped the sequences into 22 partitions (hypothetical species) with a prior of intra-specific divergence up to 0.0129 irrespective of the model used to calculate genetic distances. The three specimens from Argentina were always recovered in the same group with the four sequences of *P. maculata* from New Zealand.

The congruence of the results from the molecular evidence analysed by independent methods, in concert with the morphological descriptions in Farias et al. (2015), confirms the identification of the recently observed sea slug along the southwestern Atlantic coast as *Pleurobranchaea maculata*. Given the presence of neurotoxins in Argentinian specimens (Farias et al. 2015), and the close CO1 sequence similarity, it is possible that New Zealand populations may be the original source for this incursion. Further genetic studies with more individuals from each locality and different populations from the putative native distribution are required to test this hypothesis.

Pleurobranchaea maculata has many of the traits described for successful invaders (Whitney & Gabler 2008; Sargent & Lodge 2014): fast generation times (Gibson 2003; Khor et al. 2014); high likelihood of reproduction at very low population densities since they are hermaphrodite (Puurtinen & Kaitala 2002); planktotrophic larvae with long development in the water column which favours wide dispersion (Gibson 2003); the ability to survive at extremely high densities (e.g. up to 0.8 individuals m^{-2}, Khor et al. 2014; Taylor et al. 2015); and a diet with a wide range of (new) food types (SA Wood, Cawthron Institute, unpubl. data; Ottaway 1977, under the synonym *P. novaezelandiae*; Willan 1984). Given these life history traits, the extensive geographical spread, the absence of

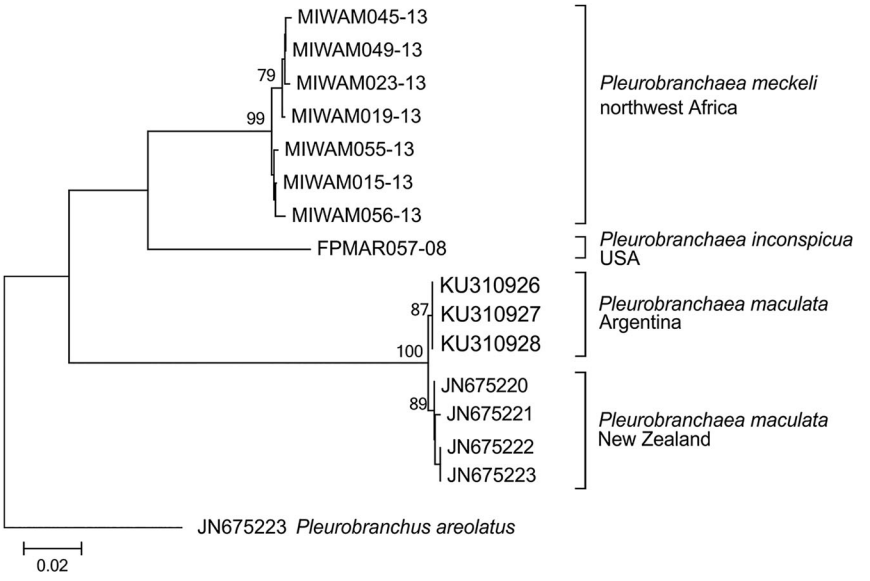

Figure 2. Evolutionary relationship of the genus *Pleurobranchaea*. Neighbor-joining phylogenetic tree performed with 15 CO1 sequences of 530 bp from representatives of the genus *Pleurobranchaea*. The optimal tree with the sum of branch length = 0.272 is shown. Numbers above nodes are bootstrap support values (1000 replicates).The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Terminal values are the identifiers for the individual CO1 sequences available for *Pleurobranchaea maculata* in GenBank and of *P. meckeli* and *P. inconspicua* in the Public Data Portal of the BOLD system.

records in previous studies within the region (Carcelles 1944; Carcelles & Williamson 1951; Bastida 1971; Valentinuzzi de Santos 1971; Scarabino 1977; Bremec 1989; Olivier et al. 1966a,b; Genzano et al. 2011) and the rapid increase in local abundances (NE Farias, S Obenat and E Schwindt, pers. obs.), it is possible that this represents a new incursion of an invasive species. Further studies on *P. maculata*'s toxicity, population genetics and impact on local ecosystems are urgently needed.

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Disclosure statement

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