DNA barcoding of marine polychaetes species of southern Patagonian fjords

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INTRODUCTION

Sustainable conservation of species requires, among other things, appropriate knowledge about the diversity of life at different hierarchical levels, including physiological, ecological, biogeographical, and systematic information (MacArthur 1984, Spicer & Gaston 1999, Avise 2000, Gaston & Blackburn 2000, Lomolino et al. 2006). However, a basic prerequisite for all these research programmes is the successful identification and delimitation of species (Gotelli 2004, Raven 2004). While concern has been voiced over the decline of taxonomy and the number of practicing...
taxonomists (Hopkins & Freckleton 2002, Gotelli 2004, Raven 2004), accurate species identification remains an imperative condition to investigate on biodiversity and conservation. To date, traditional taxonomy relies mostly on diagnostic morphological characters, requiring expert knowledge to identify specimens. In this regard, DNA barcoding has proved to be a useful alternative method for rapid global biodiversity assessment, providing an accurate identification system for living organisms (Hebert et al. 2003, Jia Min & Hickey 2007, Valentini et al. 2009, Tang et al. 2010). DNA barcoding translates expert taxonomic knowledge into a widely accessible format, DNA sequences, allowing a much broader range of scientists to identify specimens (Kerr et al. 2007). This method of species identification is based on detecting sequence diversity in a single standardized DNA fragment, namely, mitochondrial Cytochrome c Oxidase Subunit I (COI) (Hebert et al. 2003). Examination of nucleotide sequence diversity of this gene allows the grouping of unknown specimens with a priori-defined taxonomic species (Monaghan et al. 2005, Vogler & Monaghan 2006) based on the assumption that intraspecific genetic divergence is lower than the interspecific one (Hebert et al. 2003, Meyer & Paulay 2005, Waugh 2007). This method has provided a high degree of taxonomic resolution (> 94%) for most of the species examined across several animal groups (Hebert et al. 2003, Clare et al. 2007, Waugh 2007).

In marine organisms, such as invertebrates and macroalgae, species identification using standard taxonomic analyses can be notoriously difficult (Kelly et al. 2007). This is because these taxa often show morphologic convergence and phenotypic plasticity, resulting in taxonomic lumping or splitting of species. In particular, marine polychaetes constitute a large and diverse group of invertebrates highly diverse in terms of their morphologies and ecologies (Rouse & Pleijel 2001, Struck et al. 2007). However, the identification of species based on morphological traits is complex due to the high levels of homoplasy (Glasby & Alvarez 1999, Pleijel 1999, Eklöf 2010). In this regard, the use of complementary techniques such as DNA sequences may enhance taxonomic and systematic studies.

The phylogenetic relationships of polychaetes is controversial, and some studies supported the monophyly of this group (Rouse & Fauchald 1997, but see McHugh 2000, 2005). In this sense, while many studies have used the COI gene for evaluating explicit phylogenetic hypotheses, they have not examined the properties of DNA barcoding as a molecular tool for evaluating species identification (e.g., Nylander et al. 1999, Dahlgren et al. 2000, Struck et al. 2002, Nygren & Sundberg 2003, Jördens et al. 2004, Nygren et al. 2005, Wiklund et al. 2005, Halanych & Janosik 2006, Ruta et al. 2007). These studies encountered problems with the use of this gene, for example, a low phylogenetic signal for resolving closed relationship among polychaetes, and conclude that the COI region is not useful, when used alone, for inferring phylogenetics relationship among polychaetes. Nevertheless, in a molecular taxonomy perspective, Meyer & Paulay (2005) have indicated that DNA barcoding holds promise for identification in taxonomically well-understood and thoroughly sampled clades. Thus, DNA barcoding should be carried out only if based on solid taxonomic foundations. In this respect, the taxonomy of marine polychaetes of the Magellanic Patagonian fjords in the southeastern Pacific of the Chilean coast has recently received a particular attention (Rozbaczylo et al. 2005, 2006a, b, c). These studies represent an important improvement of species identification of a particularly rich and complex fauna. In line with Meyer & Paulay (2005), the intensive taxonomic analyses carried out on marine polychaetes, provide an excellent opportunity to test the efficacy of barcode-based species delimitation in a particularly challenging group. DNA barcoding might be a potentially valuable method for identifying polychaetes species, making it available to a much broader range of researchers and particularly to non-specialists. The development of DNA barcoding for polychaetes is still scarce, although some studies are currently being conducted in the Scandinavian waters, as well as in the Arctic and Antarctic Oceans (see Nygren et al. 2007, Carr & Hebert 2008, Grant & Linse 2009). Recently, the Chilean National Oceanographic Committee (CONA) has supported a barcode initiative to assess marine biodiversity along the Chilean coast through the improvement of molecular tools for taxonomic studies in marine polychaetes. Here, we assess intraspecific and interspecific genetic divergence among marine polychaetes, using COI. Our specific goal was to test the degree of accuracy of DNA barcoding to discriminate morphological described species of marine polychaetes at different taxonomical levels.

**Materials and methods**

**Sampling and identification**

Polychaetes species were collected in the sublittoral soft-bottoms from Boca del Guapo (43°45′S) to Estero Elefante (46°28′S) in the Magellanic Patagonian fjords of southern Chile. All the biological material was collected using an Agassiz Trawl ranging from 126 to 337 m depth during the
CIMAR-13 Fjords Chilean research cruise. Samplings were conducted between July and August of 2007 on board R/V AGOR Vidal Gormaz (Table 1). Individual specimens were assigned to polychaetes species based on traditional morphology, following the classification of Hartmann-Schröder (1962, 1965), Fauchald (1977) and Rozbaczylo et al. (2005, 2006a, b, c). These taxa were grouped in clades following Rouse & Fauchald (1997) and Rouse & Pleijel (2001). Voucher samples from this study were deposited in «Colección de Flora y Fauna Profesor Patricio Sánchez Reyes (SSUC)», Departamento de Ecología, Pontificia Universidad Católica de Chile, Santiago, Chile.

**DNA isolation, amplification and sequencing**

The 50 specimens belonging to 13 polychaetes species were preserved in 95% ethanol for subsequent analyses and whole DNAs were extracted from soft tissues using the Extraction Kit procedure ‘DNeasy Blood & Tissue’ (Qiagen). Partial fragments of the mitochondrial gene Cytochrome c Oxidase Subunit I were amplified using universal primers described by Folmer et al. (1994). Polymerase chain reaction (PCR) amplifications were made in a 25 μL reaction volume consisted of 2.5 μL 10X buffer (50 mM KCl, 10 mM Tris-HCl, pH 8), 1.0 μL 50 mM MgCl2, 1 μL (20 mM) dNTP’s, 0.8 μL 1 μg μL⁻¹ BSA, 0.5 μL of each primer, 1U Taq polymerase (Invitrogen), 15.4 μL of double-distilled water; and 30 ng of DNA. PCR cycling parameters included an initial denaturation phase at 97°C for 10 min, followed by 40 cycles at 94°C for 1 min, 48.5°C for 1 min, and 72°C for 2 min, and ended with a final extension at 72°C for 13 min. Amplified products were visualized in agarose gels (2%) stained with ethidium bromide and purified using the kit Wizard® SV gel and PCR Clean-Up system (Promega). Finally, all amplicons were automatically sequenced in both direction at Macrogen S.A. Korea (www.macrogen.com). All sequences obtained in this study have been deposited in GenBank under accession numbers JF731012-JF731024.

<table>
<thead>
<tr>
<th>Clade</th>
<th>Family</th>
<th>Species</th>
<th>N</th>
<th>r</th>
<th>Hap</th>
<th>Locality</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Depth (m)</th>
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<td>Sabellariidae</td>
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<td>4</td>
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EDITION, ALIGNMENT AND STATISTICAL ANALYSES

COI sequences were edited with ProSeq 2.91 (Filatov 2002) and multiple alignments were done using Clustal W (Thompson et al. 1994). Following alignments, COI sequences were translated to aminoacids to check for the presence of premature stop codons that indicate the presence of nuclear pseudogenes or sequencing errors. The probability of substitutional saturation for the COI was determined statistically using DAMBE 4.5.56 (Xia & Xie 2001).

Sequence divergence was estimated using the Kimura two-parameters (K2P) model of base substitution (Kimura 1980). Phenetic reconstruction was done using a distance based method, Neighbor-Joining (NJ), carried out in MEGA4 software (Tamura et al. 2007) with the K2P model of substitution. Support for nodes in NJ analyses was assessed using non-parametric bootstrapping with 20,000 full heuristic pseudo-replicates. We only considered clusters that were supported by at least 95% (Felsenstein 1985). For comparative purposes, we used the sequence of the sipunculid *Sipunculus nudus* Linné, 1767 (Genbank Accession Number: EF521189.1) to root the tree.

RESULTS

A total of 31 specimens, from 13 polychaetes species, were successfully sequenced and analyzed using a 555 bp fragment of the COI gene (Table 1). On average, sequences were adenine and thymine (A-T) rich (59.8%) compared to mean guanine and cytocine (C-G) content (41.2%). Polychaetes species exhibited high levels of variation among their sequences, 299 sites were variable (53.88%) and 280 of them (50.45%) were parsimonious informative. As expected for coding regions, no indels or stop codons were detected. Saturation tests did not detect significant levels of saturation at the third codon position. Mean pairwise sequence distances comparisons based on K2P within species ranged from 0.2 to 0.4%. In contrast, interspecific comparisons were much higher and ranged between 18 to 47% (Table 2), with the exception of the congeneric species *Asychis chilensis* (Hartmann-Schröder 1965) and *Asychis amphiglypta* (Ehlers 1897) that did not conform reciprocal monophyly (Fig. 1).

DISCUSSION

In the seminal report describing DNA barcoding, Hebert et al. (2003) suggested that DNA-based identification founded on the mitochondrial Cytochrome c Oxidase Subunit I would serve as the core of a global bioidentification system for animal life. This line of reasoning holds that when fully developed, COI identification system will provide a reliable, cost-effective and accessible solution to the current problem of species identification, generating also new insights into the diversification of life and the rules of molecular evolution.
Hebert et al. (2003) proposed two principal elements in the DNA barcoding initiative. First, the ability to assign an unknown sample to a known species, and second, the ability to detect previously unsampled species as distinct. Many researchers have criticised this approach, particularly when used in a molecular systematic context. These criticisms focus mainly on the limitations of using a single-locus system to identify species and quantify global biodiversity (Stoeckle 2003, Tautz et al. 2003, Moritz & Cicero 2004, Meyer & Paulay 2005, Valentini et al. 2009, but see Baker et al. 2009).

Also, it is well known that identical or similar mitochondrial DNA sequences can be in different related species due to introgression or incomplete lineage sorting since the speciation event (Meyer & Paulay 2005, Valentini et al. 2009). On the other hand, heteroplasmy, the presence of mixtures of more than one type of an organellar genome within a cell or individual can obscure the use of a single mitochondrial marker. It has also been argued that DNA barcoding may have limited phylogenetic resolution because it does not incorporate an adequate sampling of variation within species (Moritz & Cicero 2004, Meyer & Paulay 2005). However, barcoding used as identification tool does not suffer such criticisms. Assigning an unknown to a known element is promising especially for well-known, comprehensively sampled groups studied by genetic and morphological taxonomy, thus, DNA barcoding holds promise for identification in taxonomically well-understood groups (Meyer & Paulay 2005). Given that the taxonomy and systematic of the Class Polychaeta is complex and still being developed (McHugh, 2000, 2005, Rouse & Pleijel 2001, 2003, Bleidorn et al. 2003, Nygren & Sundberg 2003, Halanych & Janosik 2006, Rousset et al. 2007, Struck et al. 2007), the DNA barcoding provides undoubtedly a useful potential tool for polychaete species identification. In this regard, a series of recent studies have provided a solid understanding of the morphological taxonomy of marine polychaetes found along the south eastern Pacific, particularly in the Chilean Magellanic Patagonian fjords (Rozbaczylo et al. 2005, 2006a, b, c). These contributions represent an important improvement of available criteria for taxonomic species identification, allowing us to test the effectiveness of DNA barcoding method for marine polychaetes in this area.

Figure 1. Neighbor-joining tree analysis of the COI sequences (K2P) for 13 polychaetes species from the Patagonian Fjords of southern Chile. Only non-parametric bootstraps values ≥ 95% from 20,000 iterations are shown / Análisis de neighbor-joining (K2P) utilizando las secuencias de COI de 13 especies de poliquetos obtenidos en los fiordos Patagónicos del sur de Chile. Se muestran sólo los valores de bootstrap ≥ 95% obtenidos a partir de 20.000 iteraciones.
The results obtained in this study support the effectiveness of DNA barcoding for identification of intraspecific and interspecific genetic divergence among marine polychaetes, like in *Phragmatopoma* spp. (Drake *et al.* 2007), and agreement with the values reported for other animal taxa (see Waugh 2007). Thus, the DNA barcoding method effectively discriminates the two species within the genus *Ninoe* with 18% of mean pairwise sequence distances (see Table 2 and Fig. 1). However, it was not able to discriminate two congeneric species of the genus *Asychis*. In this respect, it is interesting to note that the two species studied within the genus *Asychis* (*A. chilensis* and *A. amphiglypta*) do not present strong morphological differences. The diagnostic character between these nominal species is that the cephalic plate in *A. chilensis* is bilobulate, while in *A. amphiglypta* is oblique (Hartmann-Schröder 1965). Thus, a deeper understanding of the morphological taxonomy with the development of the molecular taxonomy in this genus is required for future application of barcoding.

This study has showed the first data of DNA barcoding in marine polychaetes from Magellanic Patagonian Fjords, and we have established the effectiveness of DNA barcode for polychaetes species identification in the southern of Chile, making it available to a much broader range of scientists, with possibilities to extend to marine polychaetes in other biogeographic provinces of the southeastern Pacific Ocean (see Grant & Linse 2009 for Antarctica). The degree of taxonomic resolution in the studied species is comparable to the results in other marine and terrestrial taxa, such as nematodes (Bhadury *et al.* 2006), marine hydroids (Moura *et al.* 2008), bivalves (Mikkelsen *et al.* 2007), stomatopod larvae (Tang *et al.* 2010), echinodermes (Ward *et al.* 2008), marine and fresh-water fishes (Ward *et al.* 2005, Ardura *et al.* 2010), birds (Hebert *et al.* 2004) and bats (Clare *et al.* 2007). Despite its methodological shortcomings and limitations, DNA barcoding studies have reinvigorated the development of systematic studies and taxonomic inventories around the world. The increased development of molecular systematics and taxonomy provide exciting opportunities to enrich our understanding of ancient and widespread taxa such as marine polychaetes, and support the initiatives for conserving global biodiversity.

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**LITERATURE CITED**


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