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Article

DNA barcoding of marine polychaetes species of southern Patagonian fjords

Barcoding de poliquetos marinos de los fiordos patagónicos del sur de Chile

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Resumen.- La identificación de especies se establece como una de las principales etapas para cualquier estudio sobre biodiversidad, en especial frente a cambios globales y sus repercusiones. Es por esto que se requiere un amplio conocimiento taxonómico en los diferentes grupos de organismos. La herramienta de ADN barcoding ha sido descrita como una importante alternativa a los estudios morfológicos tradicionales, permitiendo complementar las técnicas de identificación en un amplio número de taxa. En este estudio, se analiza la divergencia genética intraespecífica e interespecífica entre poliquetos marinos de los fiordos patagónicos del sur de Chile, utilizando el gen Citocromo c Oxidasa Subunidad I (COI). Los resultados muestran que las 31 secuencias obtenidas de 13 especies analizadas exhiben altos niveles de variación interespecífica. La comparación intraespecífica de distancias genéticas basadas en K2P varió entre 0,2 a 0,4%. En contraste, las comparaciones interespecíficas fueron mucho mayores y variaron entre 18 a 47%, con excepción de las especies congenéricas *Asychis chilensis* y *Asychis amphiplypta,* las cuales no presentaron monofilia recíproca. Este trabajo representa el primer estudio que muestra resultados mediante la herramienta de barcoding en poliquetos de la zona sur de Chile. Además establece la efectividad de esta herramienta alternativa para la identificación de especies de poliquetos marinos en los fiordos Patagónicos, y así disponerlo a la comunidad científica para sus futuras aplicaciones.

Palabras clave: Taxonomía molecular, Polychaeta, Citocromo c Oxidasa Subunidad I, CIMAR-13 Fiordos, Chile

Abstract.- Accurate species identification remains a basic first step in any study of biodiversity, particularly for global changes and their consequences. Thus, there is a pressing need for taxonomic expertise in a broad range of taxa. DNA barcoding has proved to be a powerful alternative method to traditional morphological approaches, allowing to complement identification techniques for living organisms. In this study, we assess intraspecific and interspecific genetic divergence among marine polychaetes from Patagonian fjords of southern Chile, using mitochondrial Cytochrome c Oxidase Subunit I (COI) gene. Our results showed that a total of 13 polychaetes species identified in this study exhibited high levels of interspecific variation among 31 analyzed sequences. 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%, with the exception of the congeneric species *Asychis chilensis* and *Asychis amphiglypta* that showed high levels of genetic similarities and absence of reciprocal monophyly. This study presents the first information on DNA barcoding for polychaetes species in the southern Chile, and it establishes the effectiveness of DNA barcoding for identification of marine polychaetes species from Patagonian Fjords, thus making it available to a much broader range of scientists.

Key words: Molecular taxonomy, Polychaeta, Cytochrome c Oxydase Subunit I, CIMAR-13 Fjords, Chile

INTRODUCTION

Sustainable conservation of species requires, among other things, appropiate 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 southestern 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 Guafo (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 Gormáz (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 of reaction volume consisted of 2.5 μL 10X buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.0), 1.0 μL 50 mM $MgCl^2$, 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 1. Summary of taxonomic classification, number of individuals sequenced (N), number of replicates used (r), number of haplotypes registered for each species, sampling localities, georeferences and depth obtained for polychaetes species collected from the Patagonian fjords of southern Chile / Resumen de la clasificación taxonómica, número de individuos secuenciados (N), número de réplicas usadas por especie (r), número de haplotipos encontrados para cada especie, localidades de muestreo, georeferenciación y profundidad de las especies de poliquetos recolectadas en los fiordos Patagónicos del sur de Chile

Clade	Family	Species	N	r	Hap	Locality	Latitude	Longitude	Depth (m)
Sabellida	Sabellariidae	*Idanthyrsus macropalea* Schmarda, 1861	4	4	4	Canal Puyuguapi	44°49.40' S	72°55.40' W	270
Terebellida	Ampharetidae	*Melinna cristata australis* Hartmann-Schröder, 1965	1	4	1	Estero Quitralco	45°39.98' S	73°16.41' W	230
	Pectinariidae	*Cistenides ehlersi* (Hessle, 1917)	3	3	3	Boca Canal Puyuguapi	44°56.30' S	73°16.20' W	323
	Terebellidae	*Artacama valparaisiensis* Rozbaczylo & Méndez, 1996	2	2	1	Canal Puyuguapi	44°49.40' S	72°55.40' W	270
		Thelepus plagiostoma (Schmarda, 1861)	1	4	1	Canal Puyuguapi	44°53.00' S	73°02.10' W	170
	Trichobranchidae	*Terebellides stroemi kerguelensis* McIntosh, 1885	1	5	1	Canal Puyuguapi	44°49.40' S	72°55.40' W	270
	Sternaspidae	*Sternaspis scutata* Ranzani, 1817	3	4	1	Seno Aysén	45°22.10' S	73°04.20' W	224
Phyllodocida	Lumbrineridae	*Eranno chilensis* (Kinberg, 1865)	4	4	3	Boca Canal Puyuguapi	44°56.30' S	73°16.20' W	323
		Ninoe chilensis Kinberg, 1865	2	2	1	Seno Aysén	45°28.10' S	72°49.90' W	126
		Ninoe leptognatha Ehlers, 1900	3	5	2	Canal Puyuguapi	44°49.40' S	72°55.40' W	270
Eunicida	Onuphidae	*Onuphis pseudoiridescens* Averincev, 1972	1	4	1	Paso del Medio	45°22.90' S	73°31.90' W	337
Scolecida	Maldanidae	*Asychis chilensis* (Hartmann-Schröder, 1965)	3	3	2	Seno Aysén	45°19.60' S	73°19.70' W	218
		Asychis amphiglypta (Ehlers, 1897)	3	3	3	Canal Costa	45°30.80' S	73°31.70' W	297

Table 2. Mean pairwise sequence distances comparisons based on K2P (in percentage) between the analyzed taxa / Comparación de distancias genéticas basada en K2P (en porcentaje) entre los diferentes taxa analizados

Species	1	2	3	4	5	6	7	8	9	10	11	12	13
1 *Artacama valparaisiensis*	****												
2 *Cistenides ehlersi*	30	****											
3 *Idanthyrsus macropalea*	33	31	****										
4 *Ninoe chilensis*	32	30	32	****									
5 *Ninoe leptognatha*	30	31	29	18	****								
6 *Asychis chilensis*	35	36	30	29	31	****							
7 *Asychis amphiglypta*	35	36	30	29	31	N/A	****						
8 *Eranno chilensis*	37	31	29	23	22	32	32	****					
9 *Sternaspis scutata*	41	43	44	42	41	47	47	40	****				
10 *Melinna cristata australis*	27	26	30	27	28	30	30	30	40	****			
11 *Thelepus plagiostoma*	29	33	36	37	34	36	36	33	44	34	****		
12 *Terebellides stroemi kerguelensis*	28	25	29	32	32	32	32	31	39	20	32	****	
13 *Onuphis pseudoiridescens*	23	32	32	34	32	32	32	34	43	26	31	29	****

Edition, alignment and statistical analyses

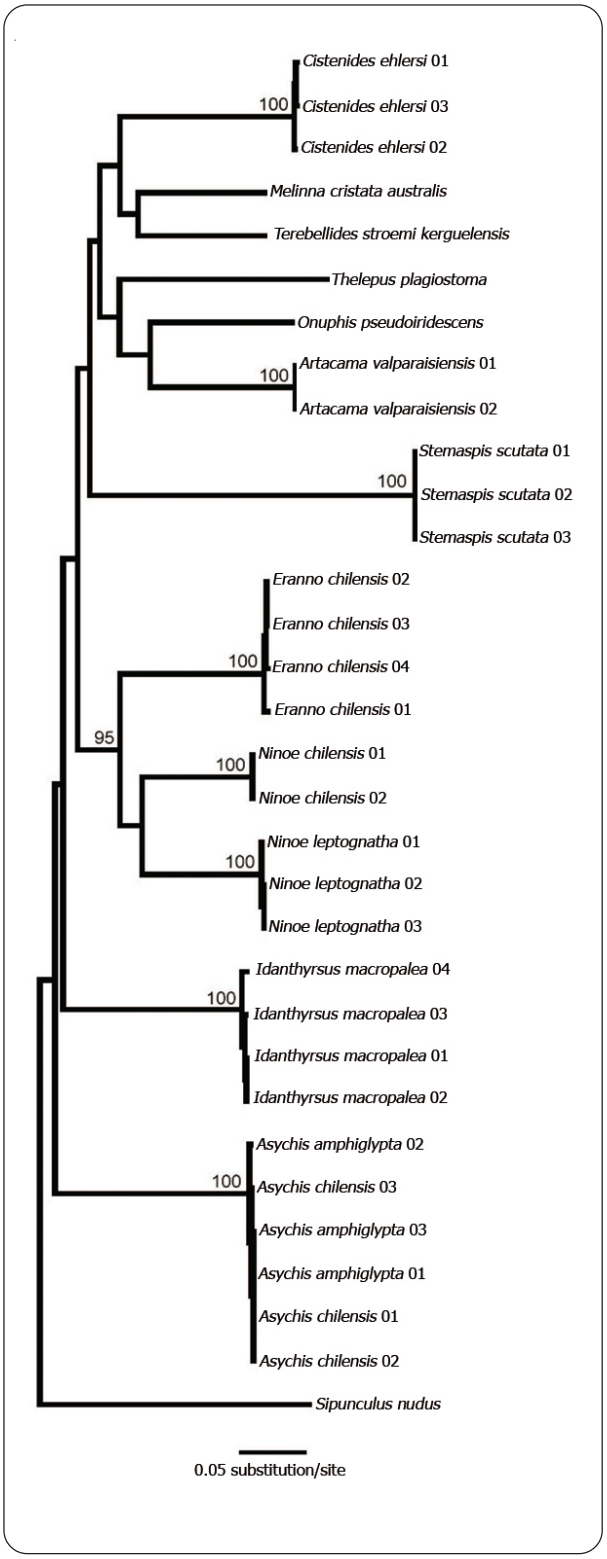
COI sequences were edited with ProSeq 2.91 (Filatov 2002) and multiple alignments were done using Clustal W (Thompson *et al.* 1994). Following alignments, COI sequences were translated to aminoacids to check for the presence of premature stop codons that indicate the presence of nuclear pseudogenes 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, comprehensivelly 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 extendend 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), echinoderms (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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