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Reliability of mitochondrial DNA in an acanthocephalan: The problem of pseudogenes☆

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Abstract

The utility of mitochondrial DNA as a molecular marker for evolutionary studies is well recognized. However, several problems can arise when using mitochondrial DNA, one of which is the presence of nuclear mitochondrial pseudogenes, or Numts. Pseudogenes of cytochrome oxidase I were preferentially amplified from *Acanthocephalus lucii* (Acanthocephala) using a universal PCR approach. To verify the presence and abundance of pseudogenes, length heterogeneity analysis of the PCR fragments was performed. PCR products obtained with universal primers often contained fragments of different sizes. Cloned sequences from universal PCR products nearly always contained sequence abnormalities such as indels and/or stop codons. Based on these sequences, new primers were developed to specifically target mitochondrial DNA. Sequences obtained with these specific primers lacked abnormalities. Phylogenetic analysis produced a single most parsimonious tree in which pseudogenes obtained with universal primers grouped together as did putative mitochondrial DNA sequences obtained with specific primers. The pattern of codon bias observed in the pseudogenes suggests a single nuclear integration event from the mitochondria. This is the first reported occurrence of pseudogenes in an acanthocephalan, and it demonstrates the potential dangers associated with the use of universal primers.

Keywords: Pseudogene; Numt; Cytochrome oxidase I; Mitochondrial DNA; Acanthocephalan; *Acanthocephalus*

1. Introduction

Mitochondrial DNA (mtDNA) has been extensively used in phylogenetic studies particularly at the population level. Several unique properties of mtDNA relative to nuclear DNA are responsible for its utility. These properties include high copy number, easy amplification (i.e. many primers available), high mutation rate, neutrality, little to no recombination and rapid lineage sorting due to haploidy and exclusively maternal inheritance (Avise et al., 1987; Moritz et al., 1987). On the other hand, a variety of problems such as heteroplasmy, paternal leakage, non-neutrality and variable rate of sequence evolution have been identified which may limit the usefulness of mtDNA (reviewed by Ballard and Whitlock, 2004). Additionally, the presence of mitochondrial-like sequences in the nuclear genome has been recognized as a potential confounder for evolutionary studies utilizing mtDNA (reviewed by Bensasson et al., 2001).

Nuclear mitochondrial pseudogenes (herein referred to as Numts after Lopez et al., 1994) are nuclear sequences exhibiting a high degree of similarity with mtDNA sequences. Numts have been identified from a wide range of taxa (Bensasson et al., 2001), and can occur in very high copy numbers (e.g. Lopez et al., 1994; Williams and Knowlton, 2001). The majority of Numts have been reported from vertebrates, particularly mammals and birds, but this is likely a consequence of the disproportionate attention these taxa receive relative to other groups. On the other hand, some heavily studied organisms, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, apparently have very few Numts (Bensasson et al., 2001). Thus, the abundance of Numts seems to be unevenly distributed across taxa. As more organisms, encompassing a wider taxonomic distribution, are studied genetically, the reported frequency and abundance of Numts is likely to increase.

☆Nucleotide sequence data presented in this paper are available in the EMBL database under the accession numbers AMO39832-AMO39884.

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The documentation of Numts, in addition to being of general scientific interest, is of practical importance. The evolutionary constraints experienced by mtDNA versus Numts are quite dissimilar given their different cellular locations and degrees of functionality, so if they are unknowingly used concurrently for phylogenetic analysis, erroneous relationships can be proposed (Sorenson and Quinn, 1998). Thus, identification and avoidance of Numts is necessary in any study utilizing mtDNA.

The phylum Acanthocephala has been little studied at the genetic level, and few investigations involving this group have used mtDNA (Giribet et al., 2004; O'Mahoney et al., 2004; Perrot-Minnot, 2004; Steinauer et al., 2005). While examining the geographic distribution of mitochondrial sequence variation in the acanthocephalan *Acanthocephalus lucii*, poor PCR amplification and problems with direct sequencing were common. After cloning and sequencing PCR products, it was evident that multiple sequences had been amplified, which suggested the presence of pseudogenes. Here, we present the first evidence suggesting the incidence of Numts in an acanthocephalan. The evolutionary significance of Numts as well as the possible dangers associated with the use of universal primers are discussed.

2. Materials and methods

2.1. Sample collection

Fish were collected from several localities in Finland, Europe, and North America. Fish were dissected and adult *Acanthocephalus* species were collected from the intestine. Collection localities and host species for the different *Acanthocephalus* samples are listed in Table 1.

2.2. DNA extractions, PCR amplification, and length heterogeneity analysis

Samples were stored in 70–100% ethanol. To extract total genomic DNA, samples were allowed to dry and then were incubated in 400 µl TE-buffer, 40 µl 10% sodium dodecyl sulphate (SDS), and 20 µl of 10 mg/ml Proteinase K overnight at 55 °C. DNA was extracted with chloroform-isoamyl alcohol (24:1) and then precipitated with isopropanol and 0.2 M NaCl. DNA pellets were washed with ice cold 70% ethanol, re-centrifuged and then allowed to dry. Pellets were then dissolved in 50 µl TE.

Sample DNA was amplified using universal primers HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′)

Table 1
Sample information and sequence characteristics for *Acanthocephalus* species

Species	Location code[a]	Host[b]	Method[c]	No. of sequences with indels and/or stop codons/total no. of sequences obtained	Uncorrected % divergence within an individual[d]	Uncorrected % divergence btw. sequences within a location[e]
A. lucii	KVS	PF	UC	1/1	–	0–6.1
			SD	0/4	–	–
	KYR	PF	SD	0/4	–	0.5–0.7
	NIE	PF	UC	6/6	0.4–3.7	0–6.9
			ND	2/2	–	–
			SD	0/3	–	–
	UKO	PF	UC	6/6	0–6.1	0–1.9
			ND	5/5	–	–
	POS	PF	UC	7/7	0.2–2.1	0–1.4
	SKN	PF	UC	1/1	–	–
	RAU	PF	UC	1/1	–	–
	KAL	PF	UC	2/2	–	1.5
	KOL	PF	UC	1/1	–	–
	KIE	PF	UC	1/1	–	–
	LEP	PF	UC	1/1	–	–
	LAT	PF	UC	1/1	–	–
	GER	GA	UC	1/1	–	–
	ENG	AA	UC	0/2	–	0
A. anguillae	ENG	AA	UC	0/1	–	–
	AUS	AA	UC	0/1	–	–
A. clavula	IRE	AA	UC	0/1	–	–
A. dirus	USA	–	UC	1/1	–	–
Total				37/53	0–6.1	0–6.9

[a] KVS, Kuivasjärvi, Finland; KYR, Kyrkösjärvi, Finland; NIE, Niemisjärvi, Finland; UKO, Ukonvesi, Finland; POS, Posiojärvi, Finland; SKN, Skåne, Sweden; RAU, Rautalampi, Finland; KAL, Kallavesi, Finland; KOL, Kolho, Finland; KIE, Kierikka, Finland; LEP, Leppävesi, Finland; LAT, Latvia; GER, Germany; ENG, England; AUS, Austria; IRE, Ireland; USA, U.S.A.

[b] PF, Perca fluviatalis; GA, Gasterosterus aculeatus; AA, Anguilla anguillae; host for the Acanthocephalus dirus sample is unknown.

[c] UC, PCR with universal primers, cloning; SD, PCR with specific primers, direct sequencing; ND, nested PCR, direct sequencing.

[d] Represents divergence between the six clones sequenced from each of three individuals, and is based on the maximum number of aligned bases (624–628 bp).

[e] Based on maximum number of aligned bases given the different methods of sequence acquisition (434 bp).