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Decapod Crustacean Phylogenetics

edited by

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Contents

Mitochondrial DNA and Decapod Phylogenies: The Importance of Pseudogenes and Primer Optimization

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ABSTRACT

Not much more than fifteen years ago, the first decapod phylogenies based on mitochondrial DNA (mtDNA) sequences revolutionized decapod phylogenetics. Initially, this method was accepted only reluctantly. However, a wider understanding of the methods, and the realization that credibility of specific branching patterns can be measured by statistic confidence values, allowed the recognition of molecular systematics as just another phylogenetic approach, in which homologous characters are compared and interpreted in terms of apomorphic or plesiomorphic status, and best possible trees are calculated based on distances, parsimony, or likelihoods. Similar to morphological characters, some of the shared molecular characters can result from convergence, but the large quantity of potential characters to be compared (15,000–17,000 in mtDNA) promises to reveal phylogenetic signal. For many years, preference was given to mitochondrial genes among the molecular markers, because of the relative ease with which they can be amplified (stable and numerous copies per cell) and interpreted (because they are only maternally inherited and lack introns and recombination), and because of higher mutation rates and thus greater variability than nuclear DNA. More recently, some of these apparent advantages were interpreted as shortcomings of mtDNA, and the discovery of selective sweeps, mitochondrial introgressions, and nuclear copies of mtDNA (numts) have questioned the credibility of phylogenies based exclusively on mtDNA. Here, I revisit the history and importance of mtDNA-based phylogenies of decapods, present two examples of how numts can produce erroneous phylogenies, and emphasize the need for primer optimization for better PCR results and avoidance of numts. Mitochondrial DNA has distinct advantages and disadvantages and, if used in combination with other phylogenetic markers, is still a very effective tool for phylogenetic inference. In most cases, and when used with the necessary care, phylogenies and phylogeographies based on mtDNA will render absolutely reliable results that can be tested and confirmed with other molecular and non-molecular approaches.

1 INTRODUCTION

Only a few years after the first publications announced the potential use of mitochondrial DNA for animal phylogenetics and population studies (e.g., Avise et al. 1987; Cann et al. 1987; Moritz et al. 1987) and the mitochondrial genome organization in *Artemia* was described (Batuecas et al. 1988), Cunningham et al. (1992) and Knowlton et al. (1993) published the first mtDNA-based phylogenies for Crustacea. It is noteworthy that these studies were based on sequences of the genes corresponding to the large ribosomal subunit 16S rRNA (16S; Cunningham et al. 1992) and the cytochrome oxidase subunit 1 (Cox1; Knowlton et al. 1993). Up to now, sequences of these genes continue to predominate in molecular phylogenetic studies of Crustacea, even though in many other animal taxa (including humans) other genes, like cytochrome b or the variable mitochondrial control region, have experienced at least a similarly wide use.

The proposal of Cunningham et al. (1992) that king and stone crabs (Anomura: Lithodidae) not only evolved from within the hermit crabs, but from within the genus *Pagurus*, cast a lot of doubt on the methodology and did not help to make the approach very popular among decapod crustacean systematists, causing a lot of skepticism concerning molecular phylogenies in general. For many years, it appeared that evolutionary biologists with molecular methods and taxonomists with morphological methods would continue their research separately. Consequently, there were only a few decapod molecular phylogenies published in the following years, most of them dealing with specific groups with special life history traits (Levinton et al. 1996; Patarnello et al. 1996; Sturmbauer et al. 1996; Tam et al. 1996; Kitaura et al. 1998; Schubart et al. 1998a; Tam & Kornfield 1998), rather than with phylogeny and taxonomy per se. Only in Crandall et al. (1995) and Crandall & Fitzpatrick (1996), and in subsequent papers on crayfish systematics and phylogeny (Ponniah & Hughes 1998; Lawler & Crandall 1998), was there an explicit goal to establish molecular systematics, which only Spears et al. (1992) had undertaken previously for decapods, by proposing phylogenetic relationships among brachyuran crabs using nuclear 18S.

This slowly changed as species descriptions became based on, or were accompanied by, mitochondrial DNA data (Daniels et al. 1998; Schubart et al. 1998b, 1999; Gusmão et al. 2000; Macpherson & Machordom 2001, Daniels et al. 2001; Guinot et al. 2002; Guinot & Hurtado 2003; Gillikin & Schubart 2004; Lin et al. 2004, and later papers), when species were synonymized based on mtDNA in the absence of morphological characters (Shih et al. 2004; Robles et al. 2007; Mantelatto et al. 2007), and especially when phylogenetic relationships within genera and families were reconstructed with mtDNA in order to establish new taxonomic classifications (Schubart et al. 2000a, 2002; Kitaura et al. 2002; Tudge & Cunningham 2002; Chu et al. 2003; Lavery et al. 2004; Klaus et al. 2006; Schubart et al. 2006). Only recently, mtDNA has been used as part of multi-locus studies to reconstruct phylogenies at higher levels within decapod Crustacea (Ahyong & O'Meally 2004; Porter et al. 2005; Daniels et al. 2006).

For this kind of higher-level taxonomy, the exclusive use of mitochondrial DNA as a molecular marker is inappropriate (see Schubart et al. 2000b). This is due to the fact that mtDNA is characterized by a relatively high mutation rate, which makes it very useful at low taxonomic levels (intraspecific to intrafamilial levels) but causes increasing saturation when older splits are analyzed. When that occurs, the ratio between "phylogenetic noise," mostly caused by molecular convergence (homoplasy), and phylogenetic signal becomes more and more unfavorable and restricts the use of mtDNA at these levels. Therefore, and because of other potential problems of mtDNA (see Discussion), today the combination of mtDNA with more conserved nuclear markers is essential when reconstructing higher order phylogenies.

mtDNA still has many advantages over nuclear DNA. First, its ring-shaped structure makes it a more stable molecule than the chromosomes in the nucleus. Furthermore, there are hundreds to thousands of mitochondrial genomes per cell (with up to 10 copies per mitochondrion, see Wiesner et al. 1992), whereas there is only one nuclear genome per cell. This makes mtDNA much easier to amplify than nuclear DNA (nDNA), and DNA quality becomes a less critical issue than it is for nDNA. As a result, it is now possible to sequence mtDNA from museum specimens that were preserved in ethanol 150 years ago (e.g., Schubart et al. 2005) or longer, something that would be much more difficult with nDNA. mtDNA is also characterized by the absence of introns, so that basically all DNA is informative. Nevertheless, mutation rates are much higher in mtDNA than in nDNA, allowing phylogenetic signal to accumulate at shorter time frames. The fact that mtDNA appears to not have recombination, and in most cases is only maternally inherited, makes its interpretation much easier and allows for extrapolation, as for example in the calibration of molecular clocks. More recently, the increasing number of multiple gene sequencing of mitochondrial genomes (many of them complete) and their comparison allows the detection of gene rearrangements that may be used to support phylogenetic conclusions (mitogenomics) (e.g., Hickerson & Cunningham 2000; Kitaura et al. 2002; Morrison et al. 2002).

After having listed these well-known and traditionally accepted advantages of mtDNA, below I will discuss potential disadvantages of mtDNA for the reconstruction of decapod crustacean phylogenies. This will be exemplified by the presentation of new data on pseudogenes and a subsequent discussion of their consequences and ways of avoiding them.

2 MATERIALS & METHODS

Samples of three species of the genus *Cardisoma* (Brachyura: Thoracotremata: Gecarcinidae) were collected or obtained between 1996 and 2005 from both tropical American coastlines and from western Africa (Table 1). The goal was to establish genetic differentiation between the western African species *C. armatum* Herklots, 1851, and both American species, *C. guanhumi* Latreille, 1828 (western Atlantic), and *C. crassum* Smith, 1870 (eastern Pacific). In a second study, we used single specimens of *Geryon trispinosus* (Herbst, 1803), *G. longipes* (A. Milne-Edwards, 1882), and *Chaceon granulatus* (Sakai, 1978) as part of a study investigating phylogenetic relationships within the Geryonidae and the superfamily Portunoidea (see Schubart & Reuschel this volume). Molecular studies were carried out at the University of Regensburg. DNA was extracted with the Gentra Systems buffer combination. After discovering multiple copies and strongly deviating products in some of our sequencing products, mtDNA enrichment techniques were applied during extractions, such as differential centrifugation in a saccharose gradient and a Triton X-100 treatment (see Burgener & Hübner 1998 and discussion below). This allowed us to work with two separate fractions from the same individual, one with potentially enriched mtDNA, the other with enriched nDNA. Selective amplification of an approximately 580-basepair region of the mitochondrial large ribosomal subunit 16S rRNA was carried out by PCR. Primers used were 16L29, 16L12, 1472, 16H10, 16H12 (see Tables 2, 3). In order to obtain clean sequences from otherwise mixed PCR products in *Cardisoma*, we designed specific primers for the presumed mtDNA (16L13J: 5'-TGTAGATATAAAGAG TTTAA-3') and the presumed nuclear derivate (16L13P: 5'-TGTAGATATAAAGAGTTTAG-3') for PCR and sequencing reactions. These primers differ only in the last nucleotide (3'-end) and should preferentially anneal to one of the two available products.

PCR amplifications were carried out with four minutes denaturation at 94°C, 40 cycles, with 45 s 94°C, 1 min 48°C, 1 min 72°C, and 10 min final denaturation at 72°C. PCR products were purified with Microcon 100 filters (Microcon) or Quick-Clean (Bioline) and then sequenced with the ABI BigDye terminator mix followed by electrophoresis in an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, USA). Forward and reverse strands were obtained for most products. New sequence data were submitted to the European molecular database EMBL (see Table 1 for accession numbers). In addition, the following sequences from the molecular database were included in our analyses: *Cardisoma guanhumi* (Z79653, from Levinton et al. 1996), *Cardisoma crassum* (AJ130805, from Schubart et al. 2000b), *Chaceon quinquedens* (Smith, 1879) and *C. fenneri* (Manning & Holthuis, 1984) (AY122641 to AY122646 from Weinberg et al. 2003) and *Chaceon affinis* (A. Milne-Edwards & Bouvier, 1894) (AF100914 to AF100916 from Weinberg et al. 2003 and previously unpublished by J. Bautista and Y. Alvarez).

Sequences were aligned and corrected manually with BioEdit (Hall 1999) or XESEE 3.2 (Cabot and Beckenbach 1989). The model of DNA substitution that best fit our data was determined using the software MODELTEST 3.6 (Posada and Crandall 1998). Reconstruction of phylogenetic trees with the corresponding models (TrN+I for *Cardisoma*; TVM+I+G for Geryonidae) in a Bayesian inference analysis (BI) with MrBayes v. 3.0b4 (Huelsenbeck and Ronquist 2001) and without models in a maximum parsimony analysis (MP) with PAUP* (Swofford 2001) revealed that the majority of genetic differences at the interindividual level were so small that the position of most operational taxonomic units was unresolved in major consensus clades. Therefore, a distance-based reconstruction with minimum evolution (ME) (Rzhetsky & Nei 1992) and Maximum Composite Likelihood as implemented in MEGA4 (Tamura et al. 2007) was carried out with 2000 bootstrap pseudoreplicates

Table 1. Crab specimens used for phylogenetic reconstruction of pseudogenes with locality of collection, museum catalogue number for vouchers, and genetic database accession numbers.

Species	Collection Locality	Coll. Date	voucher	mtDNA	numt
Cardisoma					
Cardisoma guanhumi R40	Jamaica (St. Ann): Priory	8 Oct. 2000	SMF 32773	n.a.	FM 208132
Cardisoma guanhumi CA1	Jamaica (Hanover): Negril	14 Oct. 2005	leg	FM 208123	FM 208133-35
Cardisoma guanhumi CA2	Jamaica (Hanover): Negril	14 Oct. 2005	SMF 32745	FM 208123	FM 208136-37
Cardisoma guanhumi CA3	Jamaica (St. James): Montego Bay	Oct. 2005	leg	FM 208124	FM 208132
Cardisoma guanhumi CA21	Jamaica (Trelawny): Glistening W.	22 March 2003	SMF 32772	FM 208124	n.a.
Cardisoma guanhumi CA27	Jamaica (Hanover): Negril	14 Oct. 2005	leg	FM 208123	n.a.
Cardisoma guanhumi	Cuba (Pinar de Río): El Rosario	21 Sept. 1999	SMF 25747	FM 208123	n.a.
Cardisoma guanhumi	Honduras (Islas de la Bahía): Utila	18 Aug. 2000	SMF 26006	FM 208123	n.a.
Cardisoma guanhumi	Panama (Caribbean): La Galeta	3 March 1996	ULLZ 3796	FM 208123	FM 208129-31
Cardisoma armatum tradeSG	West Africa (from aquarium trade)	1992	ZRC 1996.121	FM 208125	208127
Cardisoma armatum tradeD	West Africa (from aquarium trade)	2000	leg	FM 208126	208128
Cardisoma armatum R13	Ghana: Elmina	3 July 2001	SMF 27534	FM 208125	n.a.
Cardisoma crassum	Costa Rica: Rincón	18 March 1996	SMF 24543	AJ130805	n.a.
Geryonidae					
Geryon longipes	Spain (Ibiza): Sta. Eulalia fish market	28 March 2001	SMF 32747	FM 208120	FM 208119
Geryon trispinosus	North Sea: Flade Grounds	2000	SMF 32746	FM 208121	
Chaceon bicolor	Singapore fish market	2000	ZRC 2000.2830	FM 208122	
Chaceon granulatus	Japan		SMF 32762	FM 208775	

SMF: Senckenberg Museum, Frankfurt a.M.; ULLZ: University of Louisiana at Lafayette Zoological Collection, Lafayette.
ZRC: Zoological Reference Collection, Raffles Museum, National University of Singapore.

Table 2. Decapod-specific primers used for amplification of the 16S rRNA–$tRNA_{Leu}$–NDH1 complex and of the Cox1 gene.

16S towards NDH1:
16L2: 5′–TGCCTGTTTATCAAAAACAT–3′ (Schubart et al. 2002)
16L12: 5′–TGACCGTGCAAAGGTAGCATAA–3′ (Schubart et al. 1998)
16L12b: 5′–TGACYGTGCAAAGGTAGCATAA–3′ (new)
16L15: 5′–GACGATAAGACCCTATAAAGCTT–3′ (Schubart et al. 2000c)
16L29: 5′–YGCCTGTTTATCAAAAACAT–3′ (Schubart et al. 2001 as "16L2")
16L6: 5′–TTGCGACCTCGATGTTGAAT–3′ (new)
16L37: 5′–TTACATGATTTGAGTTCARACCGG–3′ (new)
16L11: 5′–AGCCAGGTYGGTTTCTATCT–3′ (new)
16LLeu: 5′–CTATTTTGKCAGATDATATG–3′ (new)

NDH1 towards 16S:
NDH4: 5′–CAAGCYAAATAYATYARCTT–3′ (new)
NDH2: 5′–GCTAAATATATWAGCTTATCATA–3′ (new)
NDH5: 5′–GCYAAYCTWACTTCATAWGAAAT–3′ (new)
NDH1: 5′–TCCCTTACGAATTTGAATATATCC–3′ (new)
16HLeu: 5′–CATATTATCTGCCAAAATAG–3′ (new)
16H10: 5′–AATCCTTTCGTACTAAA–3′ (new)
16H11: 5′–AGATAGAAACCRACCTGG–3′ (new)
16H37: 5′–CCGGTYTGAACTCAAATCATGT–3′ (Klaus et al. 2006)
16H6: 5′–TTAATTCAACATCGAGGTC–3′ (new)
16H12: 5′–CTGTTATCCCTAAAGTAACTT–3′ (new)

Cox1 forward (L) and reverse (H):
COL6: 5′-TYTCHACAAAYCATAAAGAYATYGG-3′ (new, substitute COL1490)
COL14: 5′-GCTTGAGCTGGCATAGTAGG-3′ (Roman & Palumbi 2004, unnamed)
COL19: 5′-ATAGTAGAAAGAGGRGTWGG-3′ (new)
COL7: 5′-GGTGTKGGMACMGGATGAACTGT-3′ (new)
COL8: 5′-GAYCAAATACCTTTATTTGT-3′ (new)
COL4: 5′-TAGCHGGDGCWATYACTAT-3′ (new)
COL12: 5′-GCHATTACTATACTTCTWACWGAYCG-3′ (new)
COL1b: 5′-CCWGCTGGDGGWGGDGAYCC-3′ (new, substitute for COIf)
COL3: 5′-ATRATTTAYGCTATRHTWGCMATTGG-3′ (Reuschel & Schubart 2006)
COH7: 5′-TGWARAGAAAAAATTCCTA-3′ (new)
COH14: 5′-GAATGAGGTGTTTAGATTTCG-3′ (Roman & Palumbi 2004, unnamed)
H7188: 5′-CATTTAGGCCTAAGAAGTGTTG-3′ (Knowlton et al. 1993)
COH6: 5′-TADACTTCDGGRTGDCCAAARAAYCA-3′ (Schubart & Huber, 2006, substitute HCO2198)
COI(10): 5′-TAAGCGTCTGGGTAGTCTGARTAKCG-3′ (Baldwin et al. 1998)
COH3: 5′-AATCARTGDGCAATWCCRSCRAAAAT-3′ (Reuschel & Schubart 2006)
COH8: 5′-TGAGGRAAAAAGGTTAAATTTAC-3′ (new)
COH4: 5′-GGYATACCRTTDARTCCTARRAA-3′ (Mathews et al. 2002)
COH12: 5′-GGYATACCRTTTARTCCTAARAA-3′ (new, substitute for COH4)
COH1b: 5′-TGTATARGCRTCTGGRTARTC-3′ (new, substitute for COIa)
COH18: 5′-CTA TGG AAG ATA CGA TGT TTC-3′ (Reuschel & Schubart 2007)
COH16: 5′-CATYWTTCTGCCATTTTAGA-3′ (new)

and was used for presentation of the phylogenetic relationships as a dichotomous tree (*Cardisoma*) or radiation tree (Geryonidae).

3 RESULTS

The aligned region of the 16S rDNA fragment of the three species of *Cardisoma* consisted of 594 basepairs (bp), of which 56 were variable and 39 parsimony-informative, whereas the length of the 16S sequence alignment from the species of *Geryon* and *Chaceon* consisted of 556 bp, of which 34 were variable and 18 parsimony-informative.

Phylogenetic analyses with three reconstruction methods (BI, MP, ME) revealed the evolutionary history of nuclear copies of the mitochondrial 16S rDNA by comparisons of the two products and with closely related species. The resulting topologies were most informative for the ME analysis, which was therefore selected for representation, even if most of the interior branches were not significantly supported. These topologies are not in conflict with the ones produced by BI and MP. In both examples, the successfully recognized numts do not represent the closest related sequence to the mtDNA of the corresponding species, and thus they would confound phylogenetic relationships if erroneously taken for, and treated as, the mitochondrial product.

The phylogenetic tree of the American and West African representatives of the genus *Cardisoma* shows a clear separation (MP bootstraps and BI posterior probabilities 100%) of the mitochondrial sequences, corresponding to three species from different nuclear products of two of the species, the Atlantic *C. guanhumi* and *C. armatum* (see Fig. 1). Clean sequences of numts were

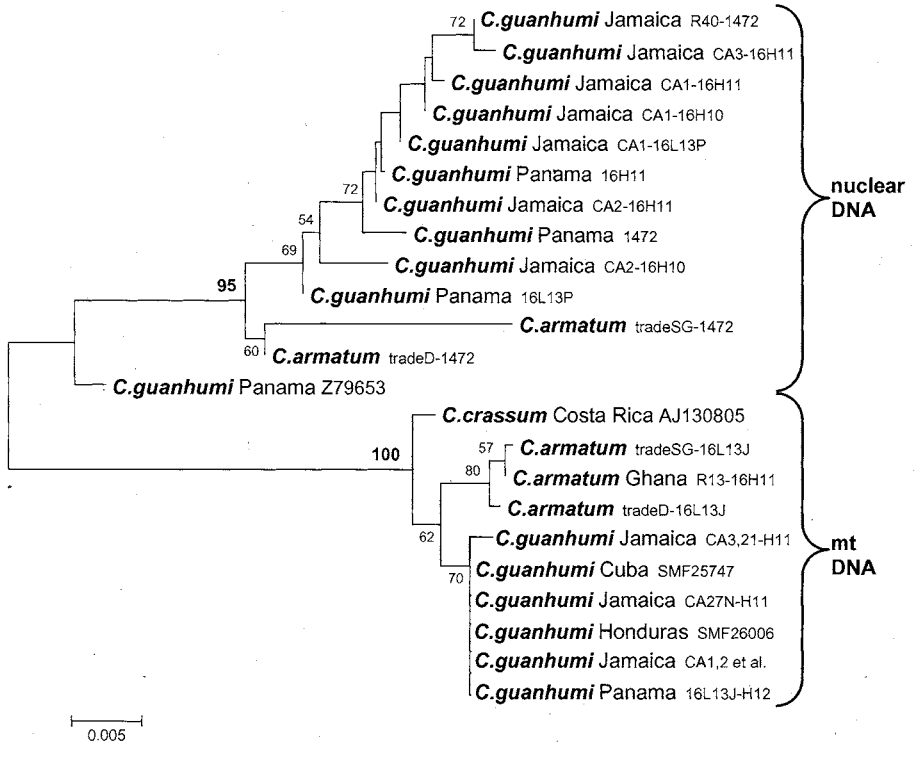

Figure 1. Phylogenetic tree of mitochondrial 16S rDNA sequences and nuclear copies obtained from the same individuals of crabs from the genus *Cardisoma* (Brachyura: Thoracotremata: Gecarcinidae). Topology of a Minimum Evolution analysis with confidence values (only $\geq$ 50) corresponding to confidence values after 2000 bootstrap pseudoreplicates.

obtained from four freshly preserved specimens of *C. guanhumi* from Jamaica and Panama, especially with the specifically designed primer 16L13P. Older museum specimens like those from Cuba and Honduras never showed signs of the presence of numts, another possible indication of the higher stability of mtDNA compared to nDNA. A pseudogene for the eastern Pacific species *C. crassum* was revealed by double products after PCR, but it has not yet been recovered as a clean sequence. Overall it appears that the evolution of the pseudogenes predates the separation of the mtDNA of the three species involved. Two sequences from GenBank were also included: *C. crassum* AJ130805 fits well within the mitochondrial clade, whereas there are clear indications that *C. guanhumi* Z79653 represents a pseudogene sequence, quite distinct from the other numts from this study, which most likely is the result of the use of different primer combinations (see below).

Phylogenetic reconstruction of all species of the genera *Geryon* and *Chaceon* for which 16S rDNA is available is presented as a radiation tree (unrooted) in Figure 2. This form of representation better demonstrates the phylogenetic position of the nuclear copy of the 16S rDNA from *Geryon longipes*, with respect to not only its mitochondrial counterpart but also to other 16S sequences of the genera *Geryon* and *Chaceon*. Also, the mitochondrial sequence of *G. longipes*

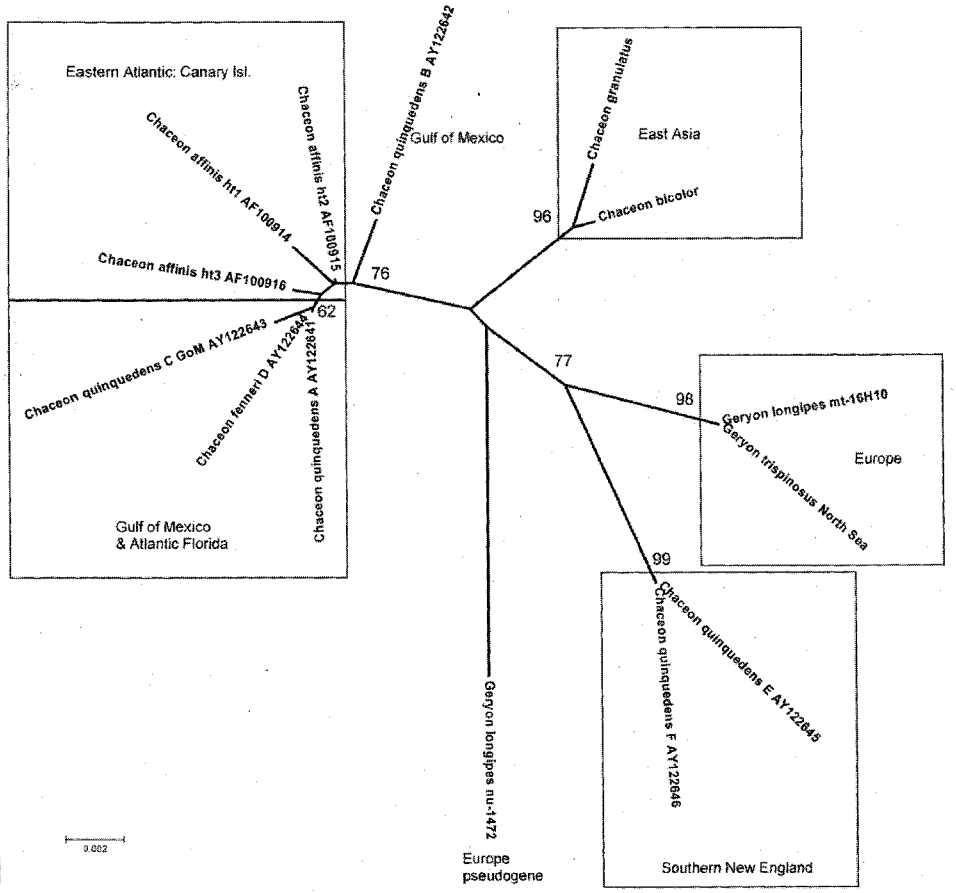

Figure 2. Radiation tree (Minimum Evolution, Maximum Composite Likelihood, 2000 bootstrap pseudoreplicates) of representatives from the crab genera *Geryon* and *Chaceon* (Brachyura: Heterotremata: Geryonidae) based on the mitochondrial 16S rDNA sequences and one nuclear copy of the species *G. longipes*.

is more closely related to other species, and even to representatives of another genus, than it is to its corresponding numt. However, available sequences in GenBank for three species of *Chaceon* demonstrate that the taxonomy of this genus is not settled. The American species *Chaceon quinquedens* is especially in need of revision; the North England representatives of this species seem to be more closely related to the genus *Geryon* (two species including the pseudogene) than to their "conspecifics" from the Gulf of Mexico (see also Weinberg et al. 2003). On the other hand, the population of *C. quinquedens* from the Gulf of Mexico is composed of several haplotypes, which do not cluster together but rather cluster with haplotypes of *C. affinis* from the Canary Islands and even share their most common haplotype with *C. fenneri* from Florida (see also Weinberg et al. 2003). If the morphological taxonomy and classification of these species are correct, this represents a case of incomplete lineage sorting, a typical phenomenon following recent speciation events, but a phenomenon that is not unique to mtDNA.

4 DISCUSSION

Phylogenies based entirely on mitochondrial DNA have recently and increasingly been criticized, especially because 1) only the maternal evolutionary lineage is considered, 2) there is possible introgression of mtDNA among species (e.g., Llopart et al. 2005), 3) early saturation due to homoplasy in the variable positions is possible (favored by an A&T-bias) (e.g., Chu et al. this volume), and 4) there is the potential for misinterpretation caused by the inclusion of pseudogenes (e.g., Williams & Knowlton 2001). Furthermore, all mitochondrial genes are located on the same molecule and thus cannot be used separately as independent sources of evidence (Moore 1995). I will not list again all the arguments in favor of using mtDNA for phylogenies (already highlighted in the Introduction),

Table 3. Large ribosomal subunit 16S rRNA universal primers 16Sbr (Palumbi et al. 1991) and 1472 (Crandall & Fitzpatrick 1996) in 5′-3′ direction and the corresponding sequence in selected decapod Crustacea.

16Sbr:	
CCGGTCTGAACTCAGATCACGT	16Sbr (Palumbi et al. 1991)
CCGGTCTGAACTCAGATCATGT	*Litopenaeus vannamei* NC 009626
CCGGTCTGAACTCAAATCATGT	*Penaeus monodon* NC 002184
CCGGTCTGAACTCAAATCATGT	*Halocaridina rubra* NC 008413
ATGGTTTGAACTCAAATCATGT	*Macrobrachium rosenbergii* NC 006880
CCGGTCTGAACTCAAATCATGT	*Panulirus japonicus* NC 004251
CCGGTCTGAACTCAAATCATGT	*Cherax destructor* NC 001243
CCGGTCTGAACTCAAATCATGT	*Pagurus longicarpus* NC 003058
CCGGTCTGAACTCAAATCATGT	*Pseudocarcinus gigas* NC 006891
CCGGTCTGAACTCAAATCATGT	*Callinectes sapidus* NC 006281
CCGGTCTGAACTCAAATCATGT	*Portunus trituberculatus* NC 005037
CCGGTTTGAACTCAAATCATGT	*Geothelphusa dehaani* NC 007379
CCGGTTTGAACTCAAATCATGT	*Eriocheir sinensis* NC 006992
CCGGTCTGAACTCAAATCATGT	16H7 (new)
CCGGTTTGAACTCAAATCATGT	16H3 (Reuschel & Schubart, 2006)
1472:	
AGATAGAAACCAACCTGG	1472 (Crandall & Fitzpatrick 1996)
AGATAGAAACCGACCTGG	*Litopenaeus vannamei* NC 009626
AGATAGAAACCGACCTGG	*Penaeus monodon* NC 002184
AGATAGAAACTAACCTGG	*Halocaridina rubra* NC 008413
AGATAGAAACCAACCTGG	*Macrobrachium rosenbergii* NC 006880
AGATAGAAACCGACCTGG	*Panulirus japonicus* NC 004251
AGATAGAAACCAACCTGG	*Cherax destructor* NC 001243
AGATAGAAACCAACCTGG	*Pagurus longicarpus* NC 003058
AGATAGAAACCAACCTGG	*Pseudocarcinus gigas* NC 006891

Table 3. (Continued)

AGATAGAAACCAACCTGG	*Callinectes sapidus* NC 006281
AGATAGAAACCGACCTGG	*Portunus trituberculatus* NC 005037
AGATAGAAACCGACCTGG	*Carcinus maenas* FM 208763
AGATAGAAACCGACCTGG	*Geryon trispinosus* FM 208776
AGATAGAAACCAACCTGG	*Geothelphusa dehaani* NC 007379
AGATAGAAACCAACCTGG	*Eriocheir sinensis* NC 006992
AGATAGAAACCGACCTGG	*Grapsus grapsus* (unpublished)
AGATAGAAACCRACCTGG	16H11 (new)

Table 4. Cytochrome oxidase subunit I primers LCO1490 and HCO2198 (Folmer et al. 1994) in 5'-3' direction, recommended to be used for barcoding studies and the corresponding sequence in selected decapod Crustacea.

Forward:	
GGTCAACAAATCATAAAGATATTGG	LCO1490
TTTCTACAAACCACAAAGACATTGG	*Litopenaeus vannamei* NC 009626
TTTCTACAAATCATAAAGACATCGG	*Penaeus monodon* NC 002184
TCTCAACAAACCATAAAGACATTGG	*Halocaridina rubra* NC 008413
TCTCCACCAACCATAAAGATATTGG	*Macrobrachium rosenbergii* NC 006880
TCTCTACTAATCATAAAGACATTGG	*Panulirus japonicus* NC 004251
TTTCAACAAATCATAAAGATATTGG	*Cherax destructor* NC 001243
TCTCTACTAACCACAAAGACATTGG	*Pagurus longicarpus* NC 003058
TTTCTACAAATCATAAAGACATTGG	*Pseudocarcinus gigas* NC 006891
TTTCTACAAATCATAAAGACATTGG	*Callinectes sapidus* NC 006281
TTTCTACAAATCATAAAGATATTGG	*Portunus trituberculatus* NC 005037
TTTCCACAAACCATAAAGATATCGG	*Geothelphusa dehaani* NC 007379
TTTCTACAAATCATAAAGATATTGG	*Eriocheir sinensis* NC 006992
TCWACAAATCATAAAGAYATTGG	COL6a (new)
ACAAATCATAAAGATATYGG	COL6b (Schubart & Huber 2006)
TYTCHACAAAYCATAAAGAYATYGG	COL6 (new)
Reverse:	
TAAACTTCAGGGTGACCAAAAAATCA	HCO2198
TATACTTCTGGGTGACCGAAGAATCA	*Litopenaeus vannamei* NC 009626
TATACTTCAGGATGACCGAAAAATCA	*Penaeus monodon* NC 002184
TAGACTTCTGGGTGGCCGAAAAATCA	*Halocaridina rubra* NC 008413
TATACTTCTGGGTGCCCAAAGAATCA	*Macrobrachium rosenbergii* NC 006880
TAAACTTCGGGATGACCGAAAAACCA	*Panulirus japonicus* NC 004251
TAGACCTCCGGGTGCCCAAAGAATCA	*Cherax destructor* NC 001243
TAAACCTCGGGGTGACCAAAAAACCA	*Austropotamobius torrentium* (unpublished)
TAAACTTCTGGGTGGCCGAAAAATCA	*Pagurus longicarpus* NC 003058
TACACTTCAGGGTGTCCAAAAAATCA	*Pseudocarcinus gigas* NC 006891
TAAACTTCAGGATGTCCGAAAAATCA	*Callinectes sapidus* NC 006281
TAGACTTCAGGATGACCAAAAAATCA	*Portunus trituberculatus* NC 005037
TATACTTCGGGATGACCAAAGAACCA	*Pachygrapsus transversus* (unpublished)
TAAACTTCTGGGTGACCAAAAAACCA	*Geothelphusa dehaani* NC 007379
TAAACTTCAGGGTGACCGAAAAATCA	*Eriocheir sinensis* NC 006992
TADACTTCDGGRTGDCCAAARAAYCA	COH6 (Schubart & Huber 2006)

because I think that there are and will be sufficient studies giving evidence of the suitability and credibility of mtDNA-based phylogenies at certain taxonomic levels (see, for example, Schubart & Reuschel this volume). I will also not discuss whether mtDNA or nDNA is the "better" option for reconstructing molecular phylogenies, because this will always depend on the evolutionary time scale to which the respective question refers, and because it is the combination of both that will give us most information (see also Klaus et al. this volume). It is similar to discussions of the potential uses of morphology and genetics when trying to understand evolution of natural lineages; the comparison of both will always increase information content, and it is to no one's advantage to ignore the other source of evidence.

Instead, I will use this discussion to respond to some of the criticisms that mtDNA phylogenies are receiving (e.g., Moore 1995; Zhang & Hewitt 2003; Mahon & Neigel 2008; Tsang et al. 2008; Chu et al. this volume). The topics of introgression and exclusive reconstruction of maternal lineages (criticisms 1 and 2) are important and must be considered in our understanding of the evolution of mtDNA. However, they are biological phenomena and not artifacts. There is nothing that can be done to avoid them, but we need to try to reconstruct and incorporate them in our models of evolution, aided by the independent insights we obtain from other sources of information (e.g., nDNA as, for example, in Shaw 2002). The early saturation of variable positions in mtDNA (criticism 3) may indeed be a problem, when, for example, DNA sequence data of Cox1 are used to reconstruct a phylogeny of the animal kingdom. In those instances, the obvious advantage at low taxonomic levels (i.e., availability of phylogenetic information even for younger differentiation events) becomes a potential problem at higher levels. However, there are ways to avoid this "phylogenetic noise" as a consequence of saturation. In coding genes, third positions can be omitted, as they are the ones most affected by silent mutations; transitions can be omitted, or the translated amino acid sequences used for phylogenetic inference. In their original proposal for implementation of DNA barcodes, Hebert et al. (2003a), for example, presented two independent phylogenetic trees of seven animal phyla and eight insect orders using the amino acid sequences corresponding to the Cox1 gene, while they switched to the DNA sequences (raw data) of the same gene when comparing 200 lepidopteran species. Thus, there are different levels of phylogenetic information that can be obtained from the same mitochondrial marker, depending on the question and on the amount of saturation that may blur the phylogenetic information. Similarly in mitochondrial rRNA genes, exclusion of hypervariable regions in higher-order phylogenies in response to alignment difficulties probably has a similar effect of reducing some of the noise caused by saturation (Schubart et al. 2000a). Nowadays, special software is available to perform these exclusions and avoid subjectivity during the process (Castresana 2000; Talavera & Castresana 2007).

The remaining problem of mtDNA, but also of nDNA, is the occurrence of paralogous copies, such that paralog and homolog DNA sequences may be confounded in comparative studies (criticism 4). The occurrence of non-functional pseudogenes as nuclear copies of mitochondrial genes (numts) is known from the literature and has been demonstrated with two examples in the Results section. Therefore, I would like to dedicate most of the Discussion to this phenomenon, the possibilities of avoiding amplification of paralogs, and the chances that arise when recognizing pseudogenes and possibly using them, together with the functional genes, for phylogenetic reconstruction.

4.1 *Pseudogenes*

The present examples of the occurrence of pseudogenes in the crab genera *Cardisoma* and *Geryon*, and their possible role in confusion of phylogenetic signal, highlight one of the possible problems of mtDNA. Schubart et al. (2000b: 826) noted that the discovery of pseudogenes in 16S rDNA and other mitochondrial genes "suggest[s] that the occurrence of pseudogenes is not an unusual phenomenon and is a potential source of artifacts." In *Menippe mercenaria* and *M. adina*, Schneider-Broussard & Neigel (1997) and Schneider-Broussard et al. (1998) were able to sequence and compare the mitochondrial 16S gene and its nuclear derivative. In this species complex,

separation of the two "species" was not possible with both of these sequencing products. In contrast, the South American sister species, *Menippe nodifrons*, represents an outgroup to both the mtDNA and the pseudogene of the *M. mercenaria* complex, when phylogenetically compared with other species (Schubart et al. 2000b), suggesting that the pseudogene evolved relatively recently and after separation of the North and South American forms.

This is not true for other occurrences of pseudogenes, including my examples here, where the nuclear copies must have evolved before the more recent separations within the genus *Cardisoma* (Fig. 1) and before the split of the genera *Geryon* and *Chaceon*, if they are confirmed as monophyletic taxa (Fig. 2). In the case of *Cardisoma*, we provide evidence that more than one nuclear copy of the 16S rDNA may be present in the same individual. Three presumed pseudogenes were obtained from one specimen of *C. guanhumi* from the Caribbean coast of Panama, in addition to the mitochondrial product, depending on the primer combination used for PCR (Fig. 1). Additionally, two specimens from Jamaica, CA1 and CA2, seem to have undergone more than one translocation event with three and two nuclear copies, respectively, detected in our analyses. The only 16S sequence of *C. guanhumi* that had been previously deposited in GenBank (Z79653, from Levinton et al. 1996) also seems to be a very derived pseudogene, not closely related to the pseudogenes obtained in this study (differing in a number of important indels), but also clearly not belonging to the mitochondrial complex of sequences. This can be explained by the fact that Levinton et al. (1996) used the Palumbi et al. (1991) primer combination 16Sar-br, which is suboptimal for most decapod Crustacea (see Table 3 and discussion below) and was not used in our analyses. Weinberg et al. (2003) also noticed "variability in PCR and sequencing results" when using the primers by Palumbi et al. (1991) and designed a new primer for *Chaceon*, thereby considerably shortening the resulting alignment. It is quite possible that this reported "variability" was due to the presence of pseudogenes, since we also detected the existence of such a nuclear copy in the closely related species *Geryon longipes* (Fig. 2). The position of the pseudogene of *G. longipes* in the phylogenetic tree demonstrates how inadvertent amplification of it, and alignment with otherwise mitochondrial products, could easily lead to wrong phylogenetic conclusions, based on the fact that non-homologous evolutionary products would be compared.

The existence of multiple nuclear copies of mitochondrial genes had previously been documented by Williams & Knowlton (2001), who cloned PCR products of the Cox1 gene corresponding to ten species of the snapping shrimp genus *Alpheus*, for which they previously had difficulties in obtaining "good sequences" for Cox1. They found up to seven nuclear copies of the mitochondrial genes per species (from fifteen clones), demonstrating that pseudogenes are a common phenomenon in decapod Crustacea and are often present in more than one copy. Differences among the sequences of pseudogenes from the same individual reached levels of up to 20%. Multiple nuclear Cox1 derivatives have also been found in the ghost crab *Ocypode quadrata* (author's unpublished data).

However, the phenomenon of multiple gene derivates is not unique to mtDNA; it is also a problem in nuclear DNA. By being diploid, there are already at least two copies (maternal and paternal) of all genes present in the nucleus of each individual, and these alleles may differ from each other, complicating the reading of sequences (especially when including length differences) and rendering subsequent analyses more difficult. In addition, many genes are known to be present in multiple copies on different loci throughout the genome. These multiple copies can be functional and on the same chromosome (as, for example, the 28S–5.8S–18S complex) to increase the amount of transcribed DNA, but they can also be nonfunctional and appear as pseudogenes on different chromosomes. This shows that the problem of multiple copies is not unique to mtDNA but is also prevalent in nDNA, where it may be even more difficult to recognize due to the underlying diploidy. Therefore, the challenge for all molecular phylogenetic studies is to recognize pseudogenes and make sure that they, as well as the functional product, are treated independently. Sequences representing pseudogenes do not have to be discarded, but recognized, labelled, and submitted as such. Phylogenies can be built based on functional products as well as on pseudogenes (independently or combined), as long as it is known which sequences are homologous.

There are different approaches to avoid amplification of pseudogenes. One of them would be to generate cDNA through reverse transcriptase out of mRNA (e.g., Palmero et al. 1988; Williams & Knowlton 2001). This would ensure that only DNA that is transcribed, i.e., the functional DNA, is amplified, and that nonfunctional DNA is avoided. However, fresh or frozen material is recommended, or special fixatives like DMSO solutions, to properly preserve the RNA and allow use of this method. It is difficult to apply this method to specimens preserved in ethanol.

Another way to reduce the effect of pseudogenes is enrichment of mtDNA during the extraction process. This can be achieved using mt-rich tissue, by miniprep DNA purification (Beckman et al. 1993) and/or differential centrifugation in a caesium chloride or saccharose gradient (Anderson et al. 1981). Burgener & Hübner (1998) provide a protocol in which the tissue is first exposed to a buffer including Triton-X-100. This commonly used non-ionic detergent makes the mitochondrial membrane soluble, allowing the mtDNA to dissolve in the supernatant, while nDNA stays within the nuclei that remain intact and can be spun down (see also Solignac 1991). However, these methods only allow the enrichment of mtDNA in relation to nDNA and not its isolation. In our study with *Cardisoma* (see above), it was not always possible to obtain clean mtDNA product, even after applying these enrichment methods.

4.2 *Primer optimization*

The best way to avoid pseudogenes is most likely the use of optimized primers. It can be assumed that pseudogenes exist for all mitochondrial genes and maybe for most, if not all, species. Nevertheless, since a normal cell has many more copies of the mitochondrial genome compared to the nuclear genome, the mitochondrial product should be favored in PCRs if both products do not differ in their primer affinities. If, however, the primers have a better fit to the nuclear pseudogene than to the mtDNA, they will preferentially anneal to the nDNA, despite the increased number of mtDNA copies. The result would be a mix of products or a clean sequence corresponding to the pseudogene. In my experience, the occurrence of pseudogenes strongly decreases when using taxon-specific primers. Also, the recorded pseudogenes by Williams and Knowlton (2001) were recovered only from those species "for which good sequences for Cox1 were difficult to obtain from gDNA." Tables 3 and 4 demonstrate how commonly used universal primers are suboptimal for a wide range of decapod Crustacea. The use of these universal primers, which initially were the only ones available, will therefore often result in sequences that have double products or do not represent the mitochondrial product. To help crustacean workers avoid some of the more problematic universal primers, I offer here a list of decapod-specific primers for 16S and Cox1 (Table 2) in addition to comparing the universal primers to "real" DNA sequences in the homologous region of decapod mtDNA (Tables 3, 4).

In 16S rRNA, the primer 16Sar by Palumbi et al. (1991) (formerly considered a forward primer, but according to newest GenBank entries actually the reverse) has a perfect fit to all sequences except for the relatively unimportant first position of the 5'-end, which in most cases is a T instead of C (see primers 16L2 and 16L29 in Table 2). However, the corresponding "reverse" primer 16Sbr (now the forward) has 2 or 3 positions in which it deviates from most decapod sequences. Most critical is a consistent difference at the third from last position, which in the primer is always a T instead of a C as recorded for all known decapod sequences. Since it is relatively close to the 3'-end, which is decisive for primer annealing, it could cause serious problems when amplifying decapod 16S rDNA. I use the primers 16H3, 16H7, or the consensus of the two 16H37 (Tables 2, 3) to avoid this problem when amplifying the corresponding fragment. Probably because of problems adherent to 16Sbr, an alternative forward primer is being frequently used: 1472 by Crandall & Fitzpatrick (1996). This primer normally works very well in combination with 1471 (Crandall & Fitzpatrick 1996), 16Sar (Palumbi et al. 1991), 16L2, or 16L29 (Table 2). However, in some cases it fails to amplify or results in pseudogenes (unpublished observations). After obtaining longer sequences and reading through that primer region, it turned out that in 1472 the seventh position

from the 3'-end often is a G instead of an A. This is confirmed with the alignment of a number of decapod sequences for which the entire mitochondrial DNA is known. Therefore, I propose the alternative primer 16H11, which allows easy amplification of sequences with G or A at that position (see Table 3).

For the other most popular mitochondrial marker, Cox1, two regions with a limited overlap have been used for phylogenetic studies: the "Palumbi region" with primers COIa and COIf (Palumbi et al. 1991) (e.g., Knowlton et al. 1993; Schubart et al. 1998a) and the "Folmer region" with primers LCO1490 and HCO 2198 (Folmer et al. 1994) (e.g., Harrison & Crespi, 1999; Trontelj et al. 2005). Subsequent to suggesting the "Folmer region" as a potential molecular barcode gene (Hebert et al. 2003a, b), the number of studies using that region has markedly increased, including the study by Costa et al. (2007) testing the suitability of this Cox1 region for barcoding studies in Crustacea. However, as can be seen in Table 4, the original primers by Folmer et al. (1994) are not optimized for decapod Crustacea, and their usefulness may be limited or could also result in the amplification of pseudogenes. LCO1490 starts with two Gs, which are not found in any of the decapod species with a known sequence of the entire gene. Probably more problematic is that the third position and especially the sixth from last position from the 3'-end (both third positions of the amino acid reading frame) show variability. In LCO1490 they are both Ts, but there are several occasions when they are found to be a C (see Table 4). In Schubart & Huber (2006), an alternative forward primer was suggested that does not include the double G at the beginning and accounts for the possible Cs at the third last position. Alternatively, COL6a can be used, in case the sixth from last position has mutated to C, which is often the case (Table 4). To consider both possible mutations, I propose the primer COL6, which has the same length as the original LCO1490 but accounts for almost all differences that have been observed in decapod crustaceans for which the entire mtDNA has been sequenced (Table 4). Likewise, the primer HCO2198 has some inherent potential problems. In this case, even more decapod species show mutations at the third from last position (C instead of T) and at the sixth from last position (G instead of A), these being again the third positions of the amino acid reading frames, which do not necessarily translate into new amino acids if modified. Also in this case, Schubart & Huber (2006) have proposed the new primer COH6 in their population study of the European crayfish *Austropotamobius torrentium*. This primer fits the sequences of most decapod species much better than the original HCO2198 and, due to its degenerate third and sixth from last positions, is less prone to fail when these mutate (Table 4).

I consider the variability of third positions in coding genes a big disadvantage for their use as universal barcoding genes. Unless taxon-specific primers are used, there is a greater risk of running into amplification problems or generating pseudogenes than in the conserved regions of ribosomal DNA (see Vences et al. 2005). Generation and use of taxon-specific primers should alleviate this problem and make the resulting sequences more trustworthy. In any case, mitochondrial genes will remain the target molecular markers for current and future animal barcoding approaches. They do have a number of advantages, but they must be treated properly. Once genetic barcoding proceeds, there will be a multitude of mitochondrial sequences that can and will be used for reconstructing phylogenies, even if this is not the explicit purpose of the Barcode of Life initiative. Therefore, mitochondrial sequences will continue to be used for molecular phylogenies, and it is easy to predict that there will always be more mitochondrial sequences available for comparisons at different phylogenetic levels than nuclear ones. Nevertheless, it will be important and advisable to complement phylogenies with independent evidence from the nuclear genome (and vice versa) to possibly recognize methodological problems and to distinguish the evolution of maternal lineages from the evolution of entire populations.

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