Available online at www.sciencedirect.com
SCIENCE DIRECT
Molecular Phylogenetics and Evolution 40 (2006) 50–60
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Phylogeny of sharks of the family Triakidae (Carcharhiniformes) and its implications for the evolution of carcharhiniform placental viviparity

J. Andrés López [a,*], Julie A. Ryburn [b], Olivier Fedrigo [c], Gavin J.P. Naylor [b]

[a] *Florida Museum of Natural History, University of Florida, Gainesville, FL 32611, USA*
[b] *School of Computational Science, Florida State University, FL 32306, USA*
[c] *Department of Biology, Duke University, NC 27708, USA*

Received 27 September 2005; revised 8 February 2006; accepted 10 February 2006
Available online 27 March 2006

Abstract

We present a study of inter- and intra-familial relationships of the carcharhiniform shark family Triakidae aimed at testing existing hypotheses of relationships for this group and at improving understanding of the evolution of reproductive traits in elasmobranchs. Our analyses and conclusions are based on evidence from DNA sequences of four protein-coding genes (three from the mitochondrial genome and a single copy nuclear gene) from eight of the nine genera and 20 of the 39 species currently assigned to the Triakidae. The sequence data offer strong support for the following previously proposed triakid clades: Galeorhinini (*Hypogaleus* + *Galeorhinus*); a subset of the Iagini (*Furgaleus* + *Hemitriakis* but not *Iago*); and part of the Triakinae (*Mustelus*, *Scylliogaleus* and part of *Triakis*). Interestingly, the molecular data provide considerable evidence of paraphyly of the genera *Triakis* and *Mustelus*. Our results suggest that the subgenera *Triakis* and *Cazon* of *Triakis* represent two distinct lineages that are only distantly related and that the genus *Mustelus* as currently defined does not constitute a monophyletic assemblage unless *S. quecketti* and some species of *Triakis* (subgenus *Cazon*) are included in *Mustelus*. Within our sample of species of *Mustelus* (including *Cazon* and *Scylliogaleus*), the sequence data support two well-defined clades that can be diagnosed by mode of reproduction (placental vs. aplacental species). The phylogenetic framework presented here is used to infer key events in the evolution and loss of placental viviparity among carcharhiniform sharks.

Keywords: *Mustelus*; *Triakis*; Galeomorphii; Placental viviparity; Chondrichthyan reproduction

1. Introduction

The evolutionary history of elasmobranchs has almost certainly featured the convergent evolution of adaptations related to their reproductive biology. For example, the occurrence of limited histotrophy (where developing embryos obtain some nourishment from absorption or ingestion of uterine secretions) among the distantly related squaliform and carcharhiniform lineages is best explained by independent acquisition of that trait (Fig. 1A; Musick and Ellis, 2005). Other instances where convergence or parallelism are suspected include the loss of placental viviparity in the carcharhinid *Galeocerdo cuvieri* and in some members of the family Triakidae (Fig. 1B). However, to refine our understanding of the mode and tempo of the evolution of reproductive strategies in sharks it is first necessary to critically examine existing hypothesis of phylogeny using modern methodology. In the present report, we focus on the family Triakidae because based on current understanding of carcharhiniform phylogeny and reproductive biology, the study of triakid lineages promises insights into the evolution of placental viviparity.

The family Triakidae (houndsharks, smooth-hounds, tope, and whiskery sharks) is one of eight families that compose one of the most species-rich orders of sharks, the Carcharhiniformes. Triakids are generally small- to

* Corresponding author. Fax: +1 352 846 0287.
E-mail address: andresl@ufl.edu (J.A. López).

1055-7903/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2006.02.011

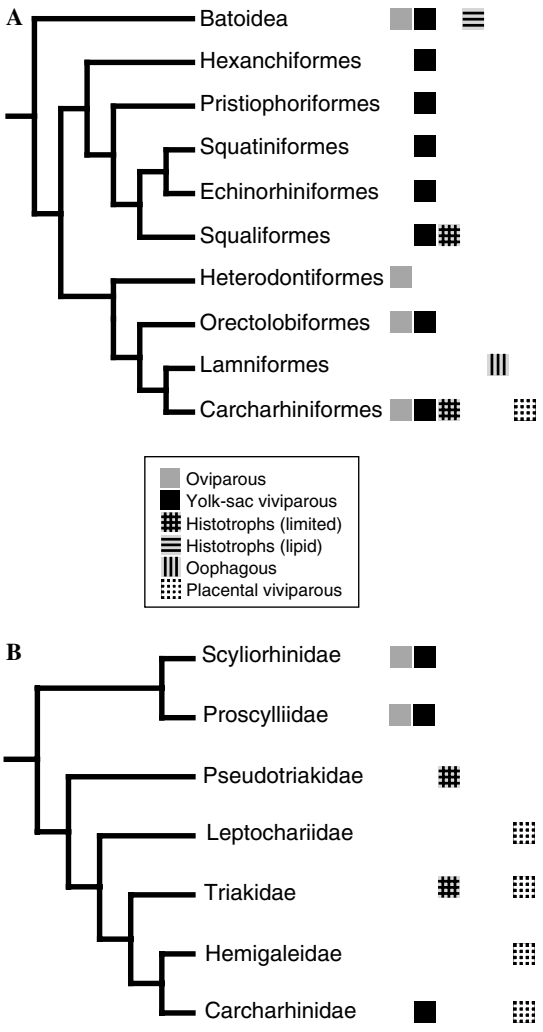

Fig. 1. Phylogenetic distribution of different modes of reproduction among shark orders (A) and families of the order Carcharhiniformes (B). Modified from Musick and Ellis, 2005.

medium-sized sharks that most frequently inhabit coastal regions in tropical and temperate seas throughout the world and feed primarily on benthic crustaceans, cephalopods and bony fish. Many triakid species (e.g. *Mustelus* spp., *Galeorhinus galeus*) are targets of commercial fisheries of local to regional significance (e.g., Conrath et al., 2002; Simpfendorfer et al., 2002). Triakids exhibit placental viviparity and limited-histotroph viviparity (aplacental viviparity), however the lack of a well-corroborated phylogenetic hypothesis for the family and for the order hinders inferences regarding the frequency, timing and consequences of changes between those two reproductive modes in the evolutionary history of these sharks.

As currently defined, the Triakidae is thought to include the living representatives of an "intermediate evolutionary phase" between the basal carcharhiniforms (e.g., Scyliorhinidae and Proscyllidae) and the so-called "higher carcharhinids" (e.g., Carcharhinidae and Sphyrnidae; Compagno, 1988). The patterns of variability in mode of reproduction seem to reflect that putative evolutionary trend. Oviparity and yolk-sac viviparity are the prevailing reproductive modes among basal carcharhiniforms; among triakids there are species with limited histotrophy and species with placental viviparity; and the "higher carcharhinids" are almost invariably placental viviparous (Fig. 1B; Musick and Ellis, 2005).

The language used by Compagno (1970, 1988) to describe carcharhiniform sub-groups (e.g., higher carcharhinoids, intermediate carcharhinids) is symptomatic of weaknesses in the methods he employed to develop hypotheses of relationships. Specifically, those hypotheses were based on a combination of phenetic, cladistic and evolutionary considerations without an explicit statement of the optimality criteria used to arrive at the phylogenetic conclusions (reviewed in McEachran, 1989). Thus, while those hypotheses may capture substantial information regarding the morphological variability of different shark groups, it is unclear how well they correspond to optimal phylogenetic hypotheses as currently understood; certainly, the analyses used to produce them cannot be replicated based on published information. Here we turn our attention to the development of a more readily testable phylogenetic framework for the Triakidae to provide an objective context for the study of elasmobranch reproductive biology and evolution.

1.1. Triakid systematics

The history of changes in the taxonomy of the Triakidae reflects the difficulty systematists have encountered when searching for characters to unambiguously diagnose the family and groups of species within the family. When first given family rank (White, 1936, 1937), the triakids included the carcharhinid genus *Triaenodon*. The placement of this genus among triakids later led to the provisional merging of triakids and carcharhinids in a single family (Compagno, 1970). Further study resulted in the recognition of the currently accepted assemblage of triakid genera: *Mustelus*, *Galeorhinus*, *Triakis*, *Scylliogaleus*, *Hemitriakis*, *Furgaleus*, *Hypogaleus*, *Iago*, *Gogolia* (Compagno, 1973). Nevertheless, the lack of clearly recognizable apomorphies shared by these genera puts into question the monophyly of this family (Compagno, 1988, p. 388). An effort to devise a classification of carcharhiniforms based partly on cladistic considerations yielded ambiguous results concerning triakid relationships, offering support for two alternative proposals of phylogeny (Compagno, 1988, p. 396). In summary, the relationships among species currently included in the Triakidae are poorly understood due in part to the small number of studies focusing on this question. Further, no compelling body evidence has been offered to date in support of the monophyly of the family or of that of the most species-rich genus within the family (i.e., *Mustelus*).

The current classification of triakid taxa divides the genera between the sub-families Triakinae and Galeorhininae. The former includes the species in the genera *Triakis*, *Mustelus*, and *Scylliogaleus*, and the latter, the species in the

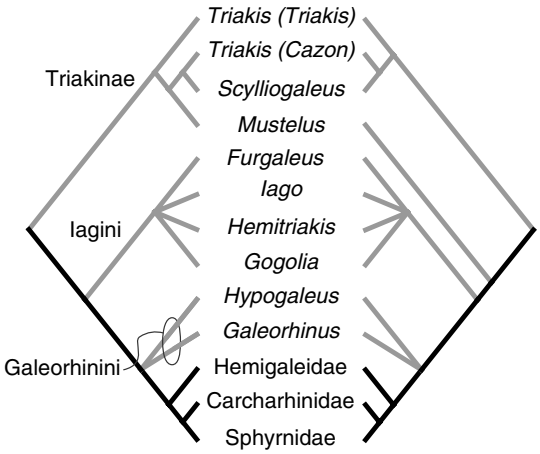

Fig. 2. Compagno's (1988) alternative arrangements of genera assigned to the family Triakidae. Branches in grey represent the lineages in the current definition of the group.

remaining six triakid genera. Galeorhinin species are further divided into iagins (*Iago*, *Furgaleus*, *Hemitriakis*, and *Gogolia*) and galeorhininins (*Galeorhinus* and *Hypogaleus*). The two alternative arrangements of triakid taxa offered by Compagno (1988, pp. 395–397) differ in the placement of the genus *Mustelus*. Fig. 2 shows the cladograms presented by Compagno (1988). Significantly, the proposed classifications imply the paraphyly of: Triakidae, Triakinae, Galeorhininae, and *Triakis*.

Due to the tenuous case for a monophyletic Triakidae, any efforts to improve our understanding of triakid evolutionary relationships and the evolution of placental viviparity in charcharhiniform sharks demands extensive sampling of the species currently assigned to the Triakidae. Here we report a phylogenetic investigation of triakid inter- and intra-family relationships based on analyses of DNA sequences from four protein-coding genes (three mitochondrial and one single copy nuclear) from representatives of all but one of the nine genera and 20 of the 39 nominal species currently assigned to the family. The only triakid genus not included in this study is *Gogolia*, which is only known from type material originally preserved in formaldehyde.

2. Materials and methods

2.1. Taxonomic sampling

Tissue samples were obtained from freshly caught animals and preserved in 95% ethanol or a solution of 20% DMSO, 0.25 M EDTA, and NaCl to saturation (Seutin et al., 1991). In all cases, specimens were identified in the field using Compagno's (1984) and Heemstra's (1973, 1997) identification keys by G. Naylor or by other investigators experienced in the identification of the shark faunas of specific regions. In all cases two or more individuals from each species were included in our sample. Because the monophyly of the Triakidae is in question, we included potential outgroups from basal and deeply nested carcharhiniform families. The triakid and outgroup taxa included in this study are listed in Table 1. We were unable to amplify DNA from the formalin-preserved specimens that constitute the only known record of the triakid genus *Gogolia*. In addition, our taxonomic sample included two taxa that could not be unambiguously identified in the field and which may represent undescribed or recently discovered triakids. The undetermined samples were: *Hemitriakis* sp., which corresponds to a newly described species from Japan (Takahashi and Nakaya, 2004) and *Iago* sp. from the Philippines.

2.2. DNA amplification and sequencing

We obtained complete coding sequences for the mitochondrial genes cytochrome *b* (Cyt *b*) and NADH dehydrogenase subunit 2 (ND2); and partial coding sequences for NADH dehydrogenase subunit 4 (ND4) and the single copy nuclear gene *recombination-activating gene* 1 (RAG1). In each case, we used amplification and sequencing primers specifically designed to target a wide diversity of species of galeomorph sharks. New primers were designed as needed when amplification or sequencing of specific genes and taxa proved problematic. Primers sequences are those of Naylor et al. (2005). PCR amplification conditions were optimized for each primer set and, in some cases for problematic taxa. In general, annealing temperatures ranged between 48 and 55 °C, and the number of cycles ranged between 32 and 40. Detailed amplification protocols for specific taxa and gene are available from the authors upon request.

We purified PCR products by centrifugation through size selective filters (Millipore, MA) according to manufacturer's recommendations. We then used the purified PCR products as templates in chain-termination reactions using fluorescently labeled chain terminators (PerkinElmer, MA). We purified the chain-termination reaction products by ethanol precipitation and submitted this purified products to the DNA Synthesis and Sequencing facility at Iowa State University for sequence determination by electrophoresis on automated sequencers (PerkinElmer, MA). Table 1 lists the species and gene sequences included in the analyses.

2.3. Phylogenetic analyses

We manually aligned the sequences using the program Se-Al (Rambaut, 1996). We excluded all missing or ambiguous characters from all subsequent analysis. Prior to performing phylogenetic analysis, we tested the hypothesis of base composition homogeneity for each gene using the χ^2 test implemented in PAUP* (Swofford, 1998) to determine if deviations from stationarity in this parameter had the potential to affect the results of phylogenetic analyses.

We conducted phylogenetic analyses on the mtDNA and nuclear sequences independently using maximum parsimony (MP), maximum likelihood (ML), and Bayesian criteria as implemented in the programs PAUP* (v4.0b10;

Table 1
Triakid and outgroup taxa sampled in this study and the genes from which DNA sequences were obtained

Subfamily	Tribe	Genus	Species	Cyt *b*	ND2	ND4	RAG1
Galeorhininae	Galeorhinini	*Galeorhinus*	*galeus*	X	X	X	X
		Hypogaleus	*hyugaensis*	X	X	X	X
	Iagini	*Furgaleus*	*macki*	X	X	X	X
		Hemitriakis	*japanica*	X	X	X	X
			sp. (Japan)			X	
		Iago	*omanensis*	X	X	X	X
			sp. (Philippines)	X	X	X	
Triakinae		*Mustelus*	*antarcticus*		X	X	X
			asterias	X	X	X	X
			californicus	X	X	X	X
			canis	X	X	X	X
			henlei	X	X	X	X
			manazo	X	X	X	X
			mosis	X	X	X	X
			mustelus	X	X	X	X
			norrisi	X	X	X	X
			schmitti	X	X	X	X
			sinusmexicanus	X		X	X
		Scylliogaleus	*quecketti*	X	X	X	X
		Triakis	*megalopterus*	X	X	X	X
			scyllium	X	X	X	
			semifasciata	X	X	X	X
Outgroup taxa		*Carcharhinus*	*acronotus*	X	X	X	X
		Prionace	glauca	X	X	X	X
		Eusphyra	*blochii*	X	X	X	X
		Sphyrna	*mokarran*	X	X	X	X
		Galeocerdo	*cuvier*	X	X	X	X
		Hemigaleus	*microstoma*	X	X	X	X
		Chaenogaleus	*macrostoma*	X	X	X	X
		Leptocharias	*smithii*	X	X	X	X
		Pseudotriakis	*microdon*	X	X	X	X
		Gollum	*attenuatus*	X	X	X	X
		Apristurus	*macrorhynchus*	X	X	X	X
		Poroderma	*pantherinum*	X	X	X	X

Swofford, 1998), PhyML (Guindon and Gascuel, 2003) and MrBayes (version 3.0b4 and 3.1; Huelsenbeck and Ronquist, 2001). In MP, we conducted heuristic searches with 1000 random addition sequence (RAS) replicates starting from random trees followed by tree-bisection–reconnection (TBR) branch swapping and determined indices of node support from the majority consensus tree from 1000 bootstrap pseudo-replicates of the data sets with 10 RAS and random starting tree per replicate. To determine if transitions have introduced significant levels of homoplasy into the sequences, we repeated the aforementioned MP analyses considering only transversions. In ML, we conducted tree searches through the PhyML server (http://atgc.lirmm. fr/phyml/) under the general time reversible model of DNA sequence evolution with among site rate variation and invariant sites (GTR + G + I) with estimation of all model parameters and 100 bootstrap pseudo-replications. We also conducted ML tree searches in PAUP* with model parameters selected through the likelihood ratio test implemented in Modeltest (Posada and Crandall, 1998). To gauge the extent of the incongruence between the optimal trees supported by the molecular data and currently accepted triakid clades, we examined the magnitude of the parsimony and likelihood penalties incurred by enforcing the monophyly of each of those clades using the Kishino–Hasegawa test to determine if the incongruence may be considered significant (Hasegawa and Kishino, 1989). Similarly, we carried out Bayesian runs with estimation of all parameters of the GTR + G + I model and with fixed parameters for models derived from likelihood ratio tests. Finally, we conducted MP, ML (GTR + G + I) and Bayesian (partitioned by gene and by codon) analyses on the combined data set to determine whether labile and weakly supported nodes found in the independent analyses were the result of insufficient information.

To derive a hypothesis of triakid relationships on which to map different reproductive modes and infer their underlying transformations, we examined the tree topologies resulting from all the different analysis in search of clades that were consistently recovered by all data sets and methods of phylogenetic reconstruction. We deemed clades that are recovered by all analyses (both, in optimal trees and in pseudoreplicate consensus trees) to be sufficiently well

defined to use as the framework for exploring the dynamics of evolution of placental viviparity in triakid lineages. On the other hand, we considered lineages whose placement varies widely between different analyses insufficiently stable to profitably explore those questions.

3. Results and discussion

3.1. Sequence composition and divergence

The base composition of the mtDNA sequences is largely homogeneous across all the taxa in this dataset. The sequences with the most distinct base composition are those from the outgroup species *Gollum attenuatus* and *Pseudotriakis microdon*. However, even with those two sequences considered, the hypothesis of stationarity of base composition is not rejected using the χ^2 statistic ($P = 0.115$), thus we do not expect the phylogenetic inference to be primarily shaped by homoplasy in base composition. But it is possible that localized codon usage profiles can skew specific aspects of the inference. The composition of the RAG1 sequences is homogeneous across all taxa in this study ($P = 1.0$) so it is unlikely to be an important determinant of the tree topology supported by those sequences.

The sequence diverge among the triakid taxa in our study ranges from 2.3 to 15.3% for mitochondrial DNA and from <0.5 to 3.7% for the RAG1 gene. In both cases, the sequences from *Iago omanensis* show the greatest levels of divergence when compared to other triakids. When triakid and outgroup taxa are considered, the extent of divergence reaches 22.5 and 16.1% among mitochondrial and RAG1 sequences, respectively. Generally, the highest levels of divergence are observed between the two scyliorhinid outgroups and the remaining taxa. This is in accord to expectations derived from the current understanding of carcharhiniform relationships, which places the scyliorhinid lineage as the sister group to all remaining carcharhiniforms. However, because the goal of the present study is to examine triakid relationships and not the relationships among carcharhiniform families, we rooted all trees at the branch that defines the scyliorhinid + pseudotriakid + proscyllid clade. To test the placement of the root of the carcharhiniform phylogeny it will be necessary to include non-carcharhiniform outgroups.

3.2. Carcharhiniform phylogeny—outgroups

The trees that result from all analyses of mtDNA and RAG1 sequences are congruent with a clade composed of *Pseudotriakis microdon* and *Gollum attenuatus* (Figs. 3–5). The evidence supporting this arrangement is strong, as indicated by bootstrap values and posterior probabilities. The level of divergence between the mitochondrial and RAG1 sequences from these two taxa (8.8 and 1.1%, respectively) is less than that observed between some pairs of species within the genus *Mustelus*. Compagno (1988) remarked on the extensive similarity between the then proscyllid *Gollum* and the monotypic Pseudotriakidae, but alluded to the

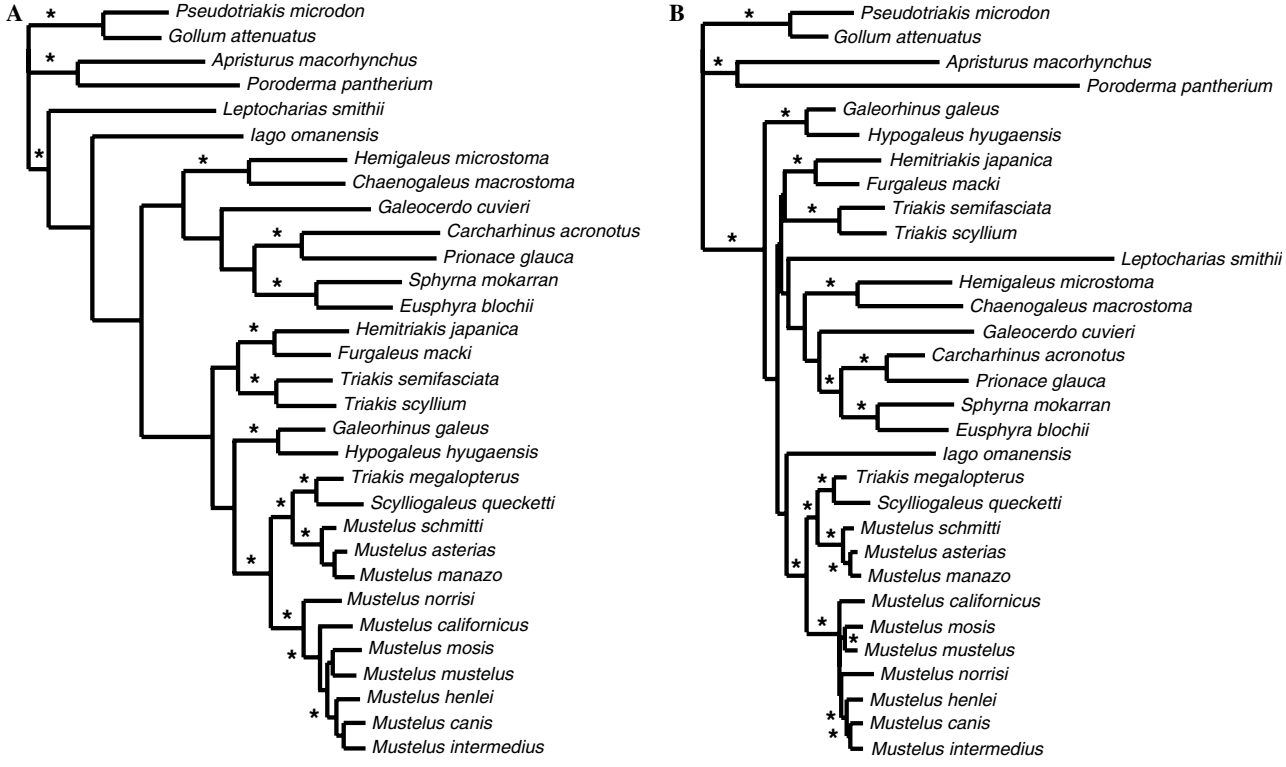

Fig. 3. mtDNA MP (A) and ML (B) bootstrap consensus topologies. Bootstrap analysis parameters are given in the text. Asterisks indicate clades that appear in >80% of the bootstrap pseudoreplicates.

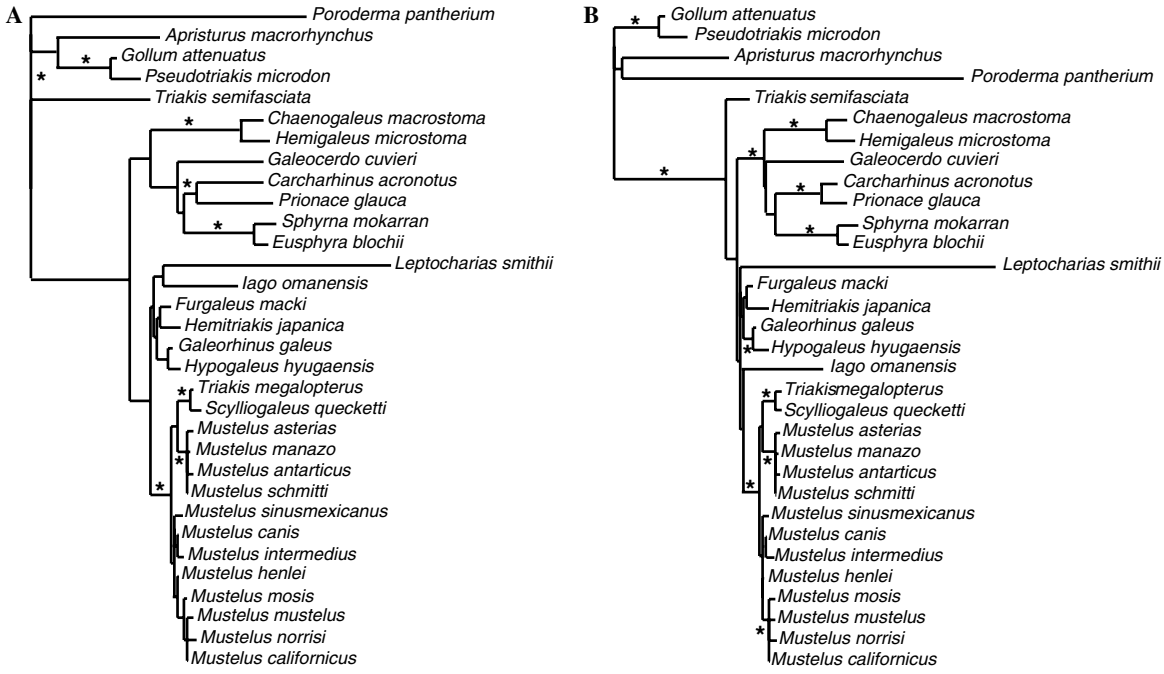

Fig. 4. RAG1 MP (A) and ML (B) bootstrap consensus topologies. Bootstrap analysis parameters are given in the text. Asterisks indicate clades that appear in >80% of the bootstrap pseudoreplicates.

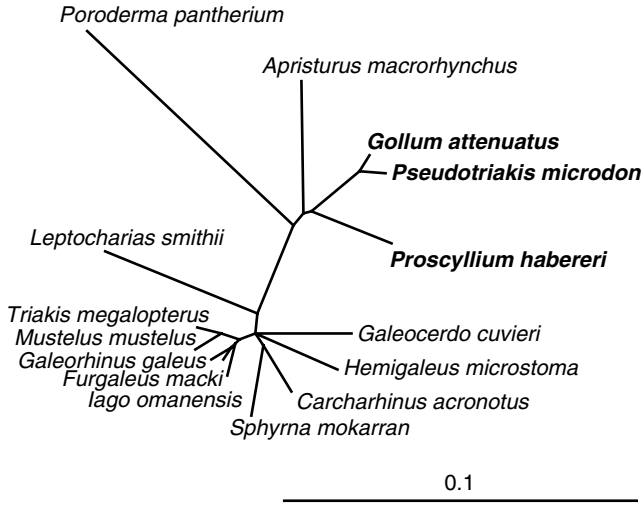

Fig. 5. NJ unrooted phylogram based on RAG1 sequences from the Genbank accession of *Proscyllium habereri* and a reduced set of taxa sequenced in this study. The pseudotriakids and the proscyllid are in bold.

abundance of autopomorphies in *Pseudotriakis microdon* as the justification to continue using the family designation. The DNA sequence data suggests that the unique traits in *Pseudotriakis* are of relatively recent origin and that the relatively high taxonomic rank this lineage received in the past was not justified. Further, it is important to note that although *Gollum* was deemed the most 'pseudotriakid-like' of the four proscyllid genera (Compagno, 1988), more recent treatments of shark systematics have reclassified this genus as a pseudotriakid (Compagno, 1999). The low divergence between *Gollum* and *Pseudotriakis* is congruent with the most recent classification of *Gollum*. Further, DNA sequence data reported by Iglésias et al. (2005) suggest that the proscyllid *Proscyllium habereri* and *Pseudotriakis microdon* do not have the close affinity that the latter shows with *Gollum* in the present study. We conducted a preliminary analysis by adding the sequence of RAG1 from *Proscyllium habereri* (GenBank AY462183) to our RAG1 dataset to determine the cause of the discrepancy. The results of this analysis strongly suggest that *Gollum* and *Pseudotriakis* form a clade that excludes *Proscyllium* (e.g., Fig. 5). This finding lends support to Compagno's (1999) rearrangement of the Pseudotriakidae. Further tests of the monophyly of the Proscyllidae will require a data set that includes representatives of all proscyllid and psudotriakid genera together with and an appropriate set of outgroup taxa (i.e., representatives of other carcharhiniform families).

All analyses consistently and strongly support the grouping of *Leptocharias smithii* with the remaining carcharhiniforms examined (i.e., Leptochariidae + Triakidae + Hemigaleidae + Carcharhinidae + Sphyrnidae). However, the placement of *L. smithii* within that group is labile depending on sequence and analysis types. Compagno (1988) had difficulty placing the monotypic Leptochariidae among carcharhiniform families because the anatomy of *L. smithi* is characterized by extensive autopomorpies. In this respect the DNA sequences from *Leptocharias* correspond well with the morphological observations. All the ML and Bayesian analyses reconstruct the branch leading to *Leptocharias* as significantly longer than other branches of similar implied age (i.e., diverging from the same node). In this context, the labile placement of *Leptocharias* is not unexpected because long branches are

known to be problematic in phylogenetic analyses (Bergsten, 2005; Felsenstein, 1978). In summary, neither morphological nor molecular data collected to date lend strong support for any particular placement of the Leptocharíidae among the triakid to carcharhinid group of carcarhiniform lineages. Given the taxonomic sampling used in this and Compagno's (1988) studies, it seems unlikely that the inclusion of more taxa will improve our understanding of *Leptocharias* relationships. The answer to that question may require types of evidence other than DNA sequences or gross morphology.

Finally, among the outgroup taxa included in this study all analyses are consistently congruent with a clade composed of the carcharhinids, sphyrnids and hemigaleids (Figs. 3 and 4). Within this clade, there is consistent support for a clade that comprises *Carcharhinus acronotus*, *Prionace glauca* and the two sphyrnids to the exclusion of *Galeocerdo cuvieri*. All analyses result in marginal but consistent support for a clade corresponding to carharhinids + sphyrnids to the exclusion of the Hemigaleidae. These results strengthen the argument for the paraphyly of the Carcharhinidae as currently defined and support a carcharhiniform group composed of the families Carcharhinidae, Sphyrnidae, and Hemigaleidae.

3.3. Carcharhiniform phylogeny—triakid monophyly

Our results, like Compagno's (1970, 1988) are equivocal regarding the monophyly of the Triakidae. None of the analyses suggests that the sequence data are most congruent with a monophyletic Triakidae. However, the specific aspects of the resulting topologies that are incongruent with triakid monophyly vary with analysis type and are always weakly supported by the data. For example, the trees that result from analyses of the mtDNA sequences place *Iago* (MP, Fig. 3A and ML with GTR + G + I estimation, not shown) or the *Galeorhinus* + *Hypogaleus* clade (ML with Modeltest derived parameters, Fig. 3B and Bayesian, not shown) as the sister group to a clade composed of the remaining triakids, carcharhinids, hemigaleids, and sphyrnids. On the other hand, the RAG1 analyses support the placement of *Triakis semifasciata* as the sister group of all remaining triakids, carcharhinids, hemigaleids, and sphyrnids (Fig. 3). Similarly, the combined and transversion-only analyses (not shown) do not recover the monophyly of the family. The MP and ML trees from the combined data set place *Iago* outside the triakids (Fig. 6, MP) while Bayesian analysis of those data place *Triakis* and *Iago* outside triakids (not shown). Given the widespread use of the triakid family concept it would be premature to propose a new classification based on the present evidence. However, future studies of so-called 'higher' carcharhiniform phylogeny would be best conducted under the conservative assumption implied by an unresolved polytomy of the deepest nodes that relate those lineages.

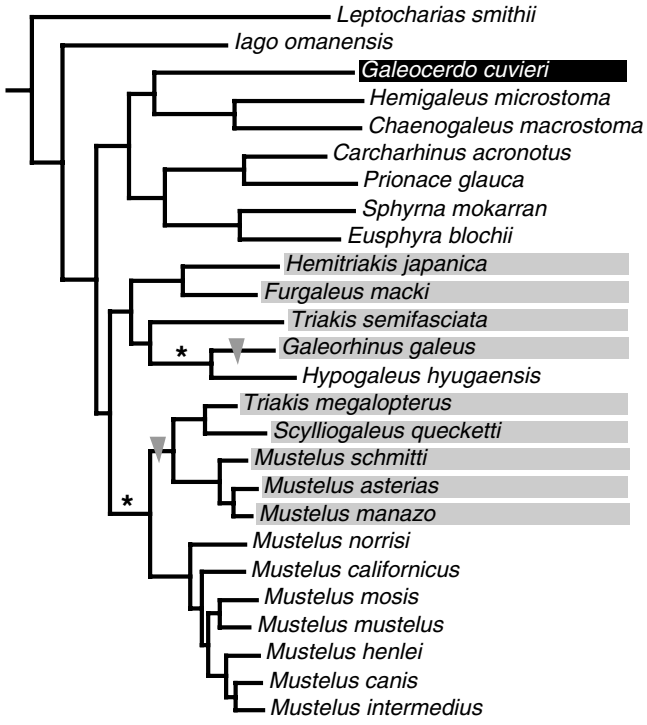

Fig. 6. Single MP tree from analysis of combined mtDNA and RAG1 sequences showing the distribution of placental viviparity and limited histotrophy among ingroup taxa. Grey, white, and black backgrounds denote limited histotrophy, placentatrophy, and yolk-sac viviparity, respectively. Asterisks highlight two clades with strong and consistent support in all the phylogenetic analyses. Triangles mark the two inferred changes of placentatrophy that are discussed in the text. Outgroup taxa are omitted.

3.4. Carcharhiniform phylogeny—triakid inter-relationships

The mtDNA and RAG1 data consistently support (Figs. 3, 4 and 6): (1) *Galeorhinus* and *Hypogaleus* as sister taxa; (2) *Furgaleus* and *Hemitriakis* as sister taxa; (3) *Triakis megalopterus* and *Scylliogaleus quecketti* as sister taxa; (4) a clade that comprises all the species of *Mustelus*, *T. megalopterus* and *S. quecketti*; (5) within this last clade, two reciprocally monophyletic groups that segregate the placental and aplacental species.

Although Compagno (1988) placed the two monotypic genera *Galeorhinus* and *Hypogaleus* in the Galeorhinini, no synapomorphies were offered in support of this group. Further, the two alternative cladograms of triakid relationships depict a trichotomy formed by the galeorhinin genera and a clade composed of the Hemigaleidae, Carcharhinidae, and Sphyrnidae (Fig. 3). The monophyly of the Galeorhinini receives unanimous and robust support from the DNA sequence data. The sequence divergence between the two galerorhininin taxa is lower than the highest levels observed between species of *Mustelus* (7.74% vs. 9.64% in mtDNA; 0.59% vs. 1.28% in RAG1). Thus, the evidence supports the continued grouping of these two genera in a clade, which is particularly significant because the variation in reproductive mode it contains. *Galeorhinus* is an aplacental viviparous genus and *Hypogaleus* is placental viviparous.

The analyses consistently support a sister group relationship between *Furgaleus* and *Hemitriakis*. This arrangement is more robustly supported by the mtDNA data, but it is also present, if weakly supported, in the topologies supported by the RAG1 sequences. These two genera together with *Iago* and *Gogolia* form the Iagini (Compagno, 1988). The position of *Iago* varies widely by analysis and dataset. Enforcing the monophyly of the Iagini results in MP trees that are 26 and 2 steps longer than the optimal unconstrained tree for the mtDNA and RAG1 datasets, respectively (Table 2). Neither penalty is statistically significant, thus although the Iagini does not appear in any of the topologies resulting from our analyses, the monophyly of the Iagini cannot be rejected.

Triakis megalopterus and *Scylliogaleus quecketti* are invariably and robustly placed as sister taxa in the results of all analyses. This arrangement is inconsistent with the monophyly of the genus *Triakis*. Enforcing the monophyly of *Triakis* results in MP trees that are 64 and 11 steps longer than the optimal unconstrained trees for the mtDNA and RAG1 datasets, respectively. These are significant differences, thus the genus seems to be in need of redefinition. Because not all species of *Triakis* are included in this study, it is premature to offer such a rearrangement here. But it should be noted that *Triakis megalopterus* is currently considered a member of the subgenus *Cazon* and that Compagno (1988) considered the subgenus a close relative of *Scylliogaleus*. Therefore, the paraphyly uncovered by the DNA sequence data may be an indication that the two subgenera of *Triakis* represent in fact two distantly related clades with more recent affinities to other carcharhiniform lineages.

Among triakid genera, *Mustelus* is not only the most species-rich but also the most problematic with respect to taxonomy and systematics. Scarce comparative material and paucity of informative morphological variation have hindered the delineation of species boundaries within the genus (Heemstra, 1973, 1997). Our study is aimed at improving our understanding of triakid inter-generic relationships so the systematics of the genus *Mustelus* fall outside of its scope. However, the sample of species of *Mustelus* that represent that genus in our study allows us to draw some preliminary conclusions on the taxonomy of *Mustelus*.

Table 2
Parsimony and likelihood penalties of enforcing topological constraints implied by different aspects of the currently accepted classification of triakids

Constraint	Parsimony (Diff. steps)		Likelihood (Diff. $-\ln L$)	
	mtDNA	RAG1	mtDNA	RAG1
Triakidae	14	5	7.64362	6.7710
Triakinae	6	3	0.92123	4.60326
Galeorhininae	22	2	10.17556	1.9319
Iagini	26	2	3.12007	4.61201
Mustelus	6	1	14.4604	3.77905
Triakis	64*	11*	104.41302*	42.88343*

Constraints correspond to the groups illustrated in Fig. 1A. Asterisks denote a statistical significant difference (Hasegawa and Kishino, 1989).

The DNA sequences we analyzed in this study offer strong support for the monophyly of an expanded *Mustelus* (with the inclusion of *T. megalopterus* and *S. quecketti*) and for the subdivision of the expanded genus into two groups. In our taxonomic sample, this categorization delineates a group of species with viviparous reproduction with a yolk-sac placenta (the 'mustelus' clade) and one with ovoviviparous reproduction (the 'asterias' clade). The sequence data gives strong support for these two clades but because our sampling of species of *Mustelus* is not exhaustive it remains possible that the pattern does not extend to all extant species in the group. However, if this division is supported by future studies that include a greater diversity of species of *Mustelus* then the significance of the different mode of reproduction between the two groups of *Mustelus* may justify dividing the genus in two. Under such a scenario, members of the 'mustelus' clade would conserve their current generic designation because the type species (*M. mustelus*) is a member of that clade and the members of the 'asterias' clade, including *T. megalopterus* and *S. quecketti* would be assigned to a different genus. From a nomenclatural perspective, a more conservative alternative would be to reassign *Scylliogaleus* and the species of *Cazon* (assuming this subgenus is monophyletic) to an expanded *Mustelus*.

Aside from the two well-defined clades, there are only a few consistently supported relationships among the species of *Mustelus* in our study. Overall, the magnitude of divergence between the sequences from species of *Mustelus* is relatively low (mtDNA: 2.36–9.64%; RAG1: <0.5–1.28%), which is in agreement with the relatively minor morphological variation observed in this genus. The low levels of divergence among species of *Mustelus* suggests that a better understanding of the taxonomy and systematics of the genus will come from improved understanding of the distribution of genetic variation among populations and species to justify the delineation of species boundaries (as suggested by Penny, 2005). Gardner and Ward (2002) used one such approach to better understand the genetic diversity within some of the species of *Mustelus* from Australia and the relationships between these nominal taxa, however the taxonomic scope of that study was limited geographically, which limits the generality of the conclusions reached. In addition, given the low levels of sequence divergence and the overlap in geographic distribution of many species of *Mustelus*, it is likely that hybridization and/or incomplete lineage sorting occurs in the genus (e.g., Tinti et al., 2003), thus increasing the complexity of the task of delineating species and devising methods for species identification in the field. Under these conditions, thorough taxonomic samples and large sample sizes for each putative taxonomic unit are imperative.

3.5. Evolution of placental viviparity

Among vertebrate radiations, mammals and chondrichthyans are unique in the frequency of viviparity within

those clades. Viviparity in chondrichtyans takes a variety of forms, which have been categorized based on the source of nutrition used by the developing embryo in utero. Assuming oviparity as a hypothetical simplest mode of reproduction, the categories of viviparity may be arranged in the following progression: lecithotrophy, histotrophy (of varying degrees) and placentatrophy. All these forms of reproduction are manifested among extant chondrichtyans. The unique adaptations associated with placentation (e.g., vascularization, nutrient exchange, endocrinology) have made it the focus of much anatomical and physiological study (reviewed by Hamlett et al., 2005).

The results of our phylogenetic analyses are ambiguous regarding the deeper nodes in the tree of triakid relationships (Figs. 3, 4 and 6). However, the topologies supported by the molecular data do shed light on some important aspects of the evolution of placental viviparity in carcharhiniforms. First, the distribution of placental viviparity among species in the most inclusive clade that finds strong support in this analysis (illustrated in Fig. 6) support the idea that placental viviparity in carcharhiniforms first evolved in the common ancestor of the (Leptochariidae + Triakidae + Hemigaleidae + Carcharhinidae). Under this scenario, aplacental viviparity within the group is the result of the loss of placentatrophy. Weak support for deep nodes in this clade make any statement about the total number of such losses purely speculative. However, two well-supported clades include placental and aplacental species: (a) the expanded *Mustelus*; and (b) *Hypogaleus* + *Galeorhinus*. These nodes imply placental viviparity has been independently lost a minimum of two times in triakid lineages (Fig. 6). Future refinements of a hypothesis of phylogeny for this group will help determine whether other aplacental triakid lineages independently arrived at that condition and, perhaps more interestingly, whether placentatrophy has been secondarily regained. On this point, we note that although the MP tree from combined analysis implies a secondary gain of placental viviparity in *Hypogaleus*, the nodes that support that scenario are weakly supported and sensitive to analysis conditions. Thus, assuming unresolved relationships for those nodes makes the loss of placentatrophy in *Galeorhinus* the simpler explanation at present.

The identification of two well-supported clades that contain placental and aplacental species (Fig. 6) provides us an opportunity to estimate the window of divergence during which transformations between the two modes of reproduction took place. In the expanded *Mustelus* clade the reproductive mode switch must have postdated the divergence between the two groups in the clade and it must have predated the earliest divergence within the group with the derived condition (e.g., loss of placentatrophy). The average divergence in mtDNA sequences between the two clades of *Mustelus* is 8.8%. Thus, these two lineages originated ~4.4% mtDNA 'divergence units ago.' The average divergence between terminals that span the deepest nodes in the aplacental clade is 7.4%, thus the species radiation in this clade began no later than 3.7% mtDNA 'divergence units ago.' These estimates place the loss of placental viviparity in *Mustelus* within a relatively narrow window of 0.7% divergence.

In the other well-supported clade with placental and aplacental species, the two galeorhinin species differ in 7.3% of their mtDNA sequences thus the divergence per lineage is approximately 3.6%. This divergence is the maximum limit on the timing of the putative loss of placentatrophy in the *Galeorhinus* lineage. Unfortunately, available fossil data are insufficient to derive well-founded estimates of the timing of these transformations. Although shark fossil teeth are abundant, their assignment to extant lineages is tenuous and they only provide minimum age constraints. As our ongoing studies of shark relationships continue (Naylor et al., 2005; present study) we expect to be able to develop sound calibrations of shark molecular clocks to estimate the timing of key events in their evolution.

Finally, the observed variation in placental morphologies in carcharhiniforms raises the possibility of multiple evolutionary origins for this trait. Detailed descriptions of carcharhiniform placental morphology have identified at least three distinct types: globular, entire and discoidal (Compagno, 1984, 1988; Heemstra, 1973). The last two are most similar and have been found in species of Carcharhinidae. The globular placenta is only present in *Leptocharias smithii*. Because our phylogenetic results point to repeated losses of placentatrophy among the triakids it is unnecessary to postulate an independent origin for the globular placenta of *Leptocharias*, thus the observed variation is suggestive of modifications from a common ancestral condition in the evolution of carcharhiniform placental viviparity. In this respect it is interesting to consider that *Leptocharias* is characterized by both a distinct placental morphology and a significantly higher rate of molecular substitution than other carcharhiniforms.

3.6. The unassigned specimens

Mitochondrial DNA sequences from the previously unidentified specimens of *Hemitriakis* collected around the Ryukyu Island of Japan in the same region inhabited by *H. japanica* lend weight to the argument that these specimens represent a distinct taxon not previously documented. These specimens of were collected and examined by Takahashi and Nakaya (2004), who determined that they did not fit the diagnostic characteristics of any known species of *Hemitriakis* (summarized in Compagno and Stevens, 1993). The specimens from Japan can be distinguished from the Australian species of *Hemitriakis* by differences in vertebral counts, morphometrics and color pattern in the young. The DNA sequences of these specimens differ from those of *H. japanica* by more than 4%. Also, it may be significant that the specimens from Japan and the Australian species of *Hemitriakis* are each parasitized by different species of copepods of the genus *Lernaeopoda* (M. Takahashi, pers. comm.). The molecular evidence suggests that these

specimens represent a genetically distinct unit. The taxonomic sample in the present study is insufficient to reach conclusions regarding relationships among the species of Hemitriakis.

The specimens of *Iago* from the Philippines also seem to belong to a previously unreported taxon that is closely related to *Iago omanensis*. Field identification of these specimens indicated that they do not represent individuals of *I. garricki*, which was described from the surroundings of Vanuatu and may extend to northwestern Australia (Compagno, 1984; Fourmanoir and Rivaton, 1979). The mitochondrial sequences of these two taxa show greater divergence (9.5%) than that observed between some triakid genera (e.g., 9.3% between *Furgaleus* and *Hemitriakis*) but they are invariably supported as sister taxa. Compagno (1984) noted that specimens of *Iago* from this region, although similar to *I. garricki* probably correspond to an undescribed species. Our species sample did not include specimens of *I. garricki* therefore we cannot determine with certainty the relationships between that species and the specimens from the Philippines, however together with the distinct morphological characteristics of these individuals, the sequence data supports Compagno's (1984) report of an undescribed species from the area.

3.7. Conclusions

The most significant findings of this study are: (1) the rejection of the hypotheses of monophyly of the triakid genera *Mustelus* (paraphyletic) and *Triakis* (polyphyletic) as they are currently defined; (2) the correspondence between the two clades formed by the species of *Mustelus*, *Cazon*, and *Scylliogaleus* and the reproductive mode of their member species; and (3) the inference of at least two independent losses of placental viviparity among triakid lineages. The combination of (a) the consistency and robustness of the results concerning *Mustelus*, *Triakis* and *Scylliogaleus*; (b) the correspondence of the 'mustelus' clades with reproductive traits; and (c) Compagno's (1984) statements on the affinities of the subgenera of *Triakis* and of *Scylliogaleus* point to the need for a revision of the taxonomy of these taxa. Lastly, the data we present fails to reject the monophyly of the Triakidae but at the same time it does not provide clear evidence to support it. None of the optimal or consensus trees from our analyses contain a monophyletic Triakidae.

The distribution of reproductive modes on the phylogeny depicted in Fig. 6 suggests that placental viviparity is the ancestral condition for the 'higher carcarhinoid' clade (e.g., Leptochariidae, Triakidae, Carcharhinidae, Hemigaleidae, and Sphyrnidae). The widely varying placement of several triakid lineages prevents a confident reconstruction of the events that led to the diversity of reproductive modes within this clade. However, consensus between different analyses implies at minimum two changes between placental viviparity and aplacental gestation among triakids (i.e., in the asterias clade of 'mustelus' and *Galeorhinus galeus*). Because these two changes are implied by parts of the tree topology that are robustly and consistently supported, we consider it unlikely that further tests of carcharhiniform phylogeny will support a single loss of placental reproduction among triakids. Therefore, the study of the evolution of this reproductive mode in the Triakidae has the potential to improve our understanding of the parallel loss of relatively complex biological traits. Future efforts should focus on better defining the placement of the other aplacental carcharhiniform lineages to test the hypothesis that placentatrophy in these sharks shares a common origin.

Acknowledgments

This work was made possible with support of NSF Grants DEB 0103640, DEB 0089533, and DEB 9707145. We are greatly indebted to a very long list of friends and colleagues who have and continue to provide samples. We also thank an anonymous reviewer for suggestions to improve this report.

References

Bergsten, J., 2005. A review of long-branch attraction. Cladistics 38, 163–193.

Compagno, L.J.V., 1970. Systematics of the genus Hemitriakis (Selachii: Carcharhinidae), and related genera. Proc. Cal. Acad. Sci. 38, 63–98.

Compagno, L.J.V., 1973. Gogolia filewoodi, a new genus and species of shark from New Guinea (Carcharhiniformes; Triakidae), with a redefinition of the family Triakidae and a key to the genera. Proc. Cal. Acad. Sci. 39, 383–410.

Compagno, L.J.V., 1984. FAO species catalogue. Vol. 4, Sharks of the world. An annotated and illustrated catalogue of shark species known to date. United Nations Development Program/Food and Agriculture Organization.

Compagno, L.J.V., 1988. Sharks of the Order Carcharhiniformes. Princeton University Press, Princeton, New Jersey.

Compagno, L.J.V., 1999. Systematics and body form. In: Hamlett, W.C. (Ed.), Sharks, Skates and Rays: The Biology of Elasmobranch Fish. Johns Hopkins University Press, Baltimore and London, pp. 1–42.

Compagno, L.J.V., Stevens, J.D., 1993. Hemitriakis falcata n. sp. and H. abdita n. sp., two new Houndsharks (Carcharhiniformes: Triakidae) from Australia. Records of the Australian Museum 45, 195–220.

Conrath, C.L., Gelsleichter, J., Musick, J.A., 2002. Age and growth of the smooth dogfish (Mustelus canis) in the northwest Atlantic Ocean. Fishery Bull. 100, 674–682.

Felsenstein, J., 1978. Cases in which parsimony and compatibility methods will be positively misleading. Syst. Zool. 27, 401–410.

Fourmanoir, P., Rivaton, I., 1979. Poissons de la pente recifale externe de Nouvelle—Caledonie et des Nouvelles—Hebrides. Cah. Indo-Pacif. 1, 405–443.

Gardner, M.G., Ward, R.D., 2002. Taxonomic affinities within Australian and New Zealand Mustelus sharks (Chondrichthyes: Triakidae) inferred from allozymes, mitochondrial DNA and precaudal vertebrae counts. Copeia, 356–363.

Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 696–704.

Hamlett, W.C., Jones, C.J.P., Paulesu, L.R., 2005. Placentatrophy in sharks. In: Hamlett, W.C., Jamieson, B.G.M. (Eds.), Reproductive Biology and Phylogeny, vol. 3. Science Publishers, Inc., Enfield, NH, pp. 463–502.

Hasegawa, M., Kishino, H., 1989. Confidence limits on the maximum-likelihood estimate of the hominoid tree from mitochondrial-DNA sequences. Evolution 43, 672–677.

Heemstra, P.C., 1973. A Revision of the Shark Genus Mustelus (Squaliformes: Carcharhinidae). University of Miami, Miami. p. 187.

Heemstra, P.C., 1997. A review of the smooth-hound sharks (Genus Mustelus, Family Triakidae) of the western Atlantic Ocean, with description of two new species and a new subspecies. Bull. Mar. Sci. 60, 894–928.

Huelsenbeck, J.P., Ronquist, F., 2001. MrBayes: Bayesian inference of phylogenetic trees. Biometrics 17, 754–755.

Iglésias, S.P., Lecointre, G., Sellos, D.Y., 2005. Extensive paraphylies within sharks of the order Carcharhiniformes inferred from nuclear mitochondrial genes. Mol. Phylogenet. Evol. 34, 569–583.

McEachran, J.D., 1989. Review—Sharks of the order Carcharhiniformes. Copeia 1989, 528–530.

Musick, J.A., Ellis, J.K., 2005. Reproductive evolution of chondrichthyans. In: Hamlett, W.C., Jamieson, B.G.M. (Eds.), Reproductive Biology and Phylogeny, vol. 3. Science Publishers, Inc., Enfield, NH, pp. 45–79.

Naylor, G.J.P., Ryburn, J.A., Fedrigo, O., López, J.A., 2005. Phylogenetic relationships among the major lineages of modern elasmobranches. In: Hamlett, W.C., Jamieson, B.G.M. (Eds.), Reproductive Biology and Phylogeny, vol. 3. Science Publishers, Inc., Enfield, NH, pp. 1–25.

Penny, D., 2005. Relativity for molecular clocks. Nature 436, 183–184.

Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Rambaut, A., 1996. Se-Al: Sequence Alignment Editor. Available at http://evolve.zoo.ox.ac.uk/.

Seutin, G., White, B.N., Boag, P.T., 1991. Preservation of avian blood and tissue samples for DNA analyses. Can. J. Zool. 69, 82–90.

Simpfendorfer, C.A., Kitchingman, A.M., Mcauley, R.B., 2002. Distribution, biology and fisheries importance of the pencil shark, Hypogaleus hyugaensis (Elasmobranchii: Triakidae), in the waters off south-western Australia. Mar. Freshwater Res. 53, 781–789.

Swofford, D.L., 1998. PAUP*. Phylogenetic analysis using parsimony (* and other methods). Sinauer Associates, Sunderland, Massachussetts.

Takahashi, M., Nakaya, K., 2004. *Hemitriakis complicofasciata*, a new whitefin topeshark (Carcharhiniformes: Triakidae) from Japan. Ichthyol. Res. 51, 248–255.

Tinti, F., Ungaro, N., Pasolini, P., De Panfilis, M., Garoia, F., Guarniero, I., Sabelli, B., Marano, G., Piccinetti, C., 2003. Development of molecular and morphological markers to improve species-specific monitoring and systematics of Northeast Atlantic and Mediterranean skates (Rajiformes). J. Exp. Marine Biol. 288, 149–165.

White, E.G., 1936. A classification and phylogeny of the elasmobranch fishes. Am. Mus. Novit. 837, 1–16.

White, E.G., 1937. Interrelationships of the elasmobranchs with a key to the order Galea. Bull. Am. Mus. Nat. Hist. 74, 25–138.