



Phylogeny of elasmobranchs based on LSU and SSU ribosomal RNA genes

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Abstract

The dominant view of the phylogeny of living elasmobranchs, based on morphological characters, is that batoids (skates and rays) are derived sharks, joined with saw sharks, and angel sharks in the clade Hypnosqualea [S. Shirai, *Squalean Phylogeny: A New Framework of 'Squaloid' Sharks and Related Taxa*, Hokkaido University Press, Sapporo, 1992]. By contrast, a recent molecular-phylogenetic study based on mitochondrial genes for 12S and 16S rRNA and tRNA valine [C.J. Douady et al., *Mol. Phylogenet. Evol.*, 26 (2003) 215–221] supported the older view that batoids and sharks are separate lineages. Here, we tested these two different views using combined, nuclear large-subunit and small-subunit rRNA gene sequences (~5.3 kb) from 22 elasmobranchs, two chimeras, and two bony fishes. We used maximum likelihood, maximum parsimony, minimum evolution, and Bayesian inference for tree reconstruction, and found the large-subunit rRNA gene to contain far more signal than the small-subunit gene for resolving this mostly Mesozoic radiation. Our findings matched those of Douady et al. (2003) in separating batoids from sharks and in statistically rejecting Hypnosqualea. The angel shark (*Squatina*) was the sister group to squaliforms (dogfish sharks), and our findings are consistent with the idea that “orbitostylic” sharks form a monophyletic group (squaliforms + the hexanchiform *Chlamydoselachus* + *Squatina* + *Pristiophorus*). In the galeomorph sharks, however, lamniforms grouped with orectolobiforms, opposing the widely accepted ‘lamniform + carcharhiniform’ grouping. A tree based on the mitochondrial gene for cytochrome *b* also supported a separation of sharks and batoids, in contrast to Hypnosqualea. Among elasmobranchs, variation in the evolutionary rates of the nuclear rRNA genes was higher than that of cytochrome *b* genes, mainly due to the relatively rapid evolution of rRNA in some carcharhiniforms. In conclusion, several different molecular studies now refute the Hypnosqualea hypothesis of elasmobranch interrelationships.

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1. Introduction

The interrelationships of the extant elasmobranchs (neoselachians), a group that evidently originated in the Early Triassic (Cuny and Benton, 1999; Thies, 1982), are subject to debate. Most older classifications separated elasmobranchs into two monophyletic groups: batoids and sharks (e.g., Holmgren, 1941; Regan, 1906; White, 1937), but Compagno (1973, 1977) reconsidered this

separation and produced a different classification (Fig. 1A). Using phenetics, Compagno recognized four separate superorders: (1) Squalomorphii (Orders Hexanchiformes, Pristiophoriformes, Squaliformes); (2) Batoidea (Orders Rajiformes, Rhinobatiformes, Myliobatiformes, Torpediniformes, Pristiformes); (3) Squatinomorphii (one genus, *Squatina*); and (4) Galeomorphii (Orders Heterodontiformes, Carcharhiniformes, Lamniformes, Orectolobiformes).

Next, Maisey (1980) identified a potential synapomorphy of Squalomorphs and Squatinomorphs—an orbital process that projects from the upper-jaw cartilage inside the eye socket—and united these two superorders as the “orbitostylic” sharks. This proposal was coupled with arguments for a return to the classical separation of

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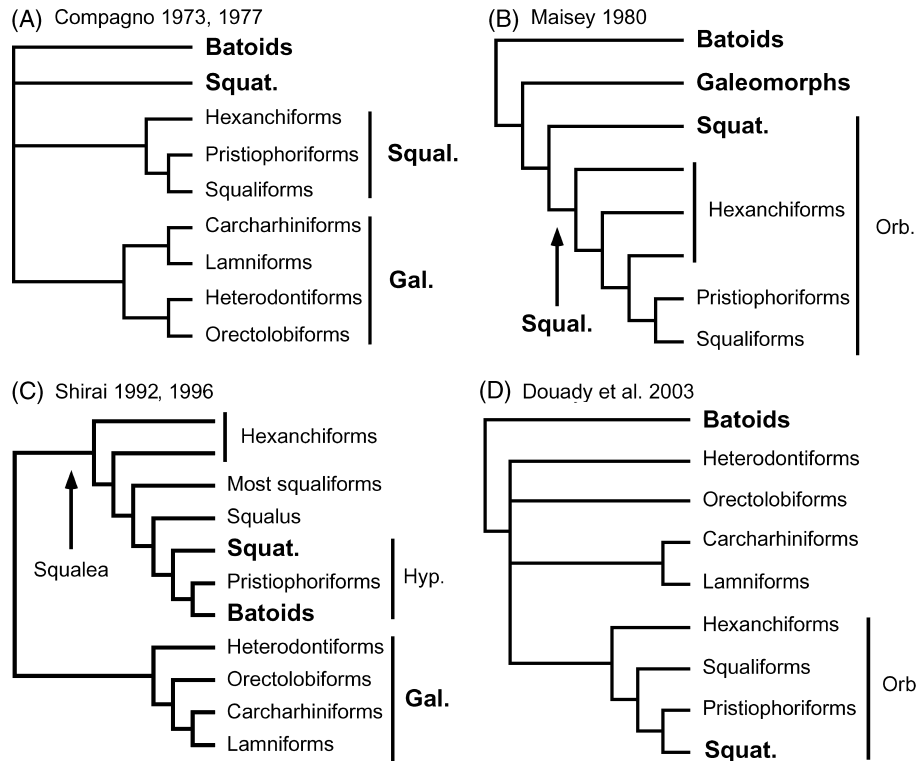


Fig. 1. Four previous hypotheses of elasmobranch relationships. Hypothesis C, which includes Hypnosqualea (Hyp.) and Squalea, is now the most widely accepted morphology-based hypothesis. Hypothesis D is based on the most recent molecular evidence. Compagno's (1973, 1977) superorders are in bold. Other abbreviations: Gal., galeomorphs; Orb., orbitostylic sharks; Squal., squalomorphs; Squat., squatinomorphs.

sharks and batoids, and for hexanchiform paraphyly (see Fig. 1B).

By comparing external, skeletal, and muscular characters in a cladistic analysis, Shirai (1992) revolutionized elasmobranch taxonomy. His optimal tree showed batoids as derived sharks, grouped with pristiophoriforms and *Squatina* (details are in Fig. 1C). Furthermore, these fishes (called hypnosqualeans) were in the squalomorphs, with which they formed supraorder "Squalea," the sister group of galeomorphs. Shirai based the Squalea clade on several putative synapomorphies, including an orbital articulation (i.e., Maisey's (1980) orbitostylic process—a trait Shirai felt was secondarily lost in batoids); a basal angle on the sub-orbital cranium; and widely separated nasal capsules. After Shirai's initial work, three subsequent studies obtained similar results (de Carvalho, 1996; de Carvalho and Maisey, 1996; Shirai, 1996), so the Hypnosqualea and Squalea clades became widely accepted (McEachran et al., 1996; Moyle and Cech, 2000; also see <http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html>). This morphology-based consensus recognizes only two main groups of elasmobranchs, Squalea and Galeomorphs, although the scarcity of good morphological synapomorphies for the latter group is widely acknowledged (Compagno, 1999; Maisey, 1984).

Despite the consensus, problems arise from using morphology to assess elasmobranch phylogeny (Fech-

helm and McEachran, 1984; Martin, 1995). First, the cartilage endoskeleton preserves poorly in fossil elasmobranchs, making it difficult to identify pleiomorphic character states. Second, the closest extant outgroup to living elasmobranchs, the chimeras, exhibit divergent features of the musculoskeletal system, making them inappropriate for polarizing many character transitions. Third, convergent evolution of anatomical traits may be widespread in elasmobranchs, as some groups have similar ecological roles (e.g., benthic predators, pelagic predators). Fourth, shark morphology is conserved in general, so recognizable synapomorphies are scarce for certain groups. Given these problems, data independent of morphological characters; e.g., molecular data, are needed to test phylogenetic hypotheses.

The first wave of molecular studies used low numbers of taxa (Arnason et al., 2001; Dunn and Morrissey, 1995; Kitamura et al., 1996), but in general did not support the Hypnosqualea/Squalea hypotheses. In the early study with the most taxa, Stock (1992) sequenced 18S ribosomal RNA (rRNA) genes from 14 elasmobranchs and found moderate support for orbitostylic sharks (squalomorphs + *Squatina*), a group that is inconsistent with the Hypnosqualea hypothesis because it separates batoids from *Squatina* and pristiophoriforms. However, these 18S rRNA data showed almost no support for galeomorph monophyly and little support

for batoid monophyly. Stock (1992) believed these biases were due to high variation in evolutionary rates of this gene across taxa, although another explanation is that 18S genes evolve too slowly to resolve Mesozoic divergences, such as those of neoselachians (Hillis and Dixon, 1991).

In the most extensive molecular-phylogenetic study to date, Douady et al. (2003) recently used mitochondrial 12S and 16S and tRNA valine genes (~2400 nucleotides) from over 20 elasmobranch and outgroup species. Results indicated that batoids are separate from sharks, that sharks are monophyletic with *Squatina* and pristiophoriforms being squalomorphs, and they statistically rejected the Hypnosqualea clade. This is a strong challenge to prevailing ideas.

The present study used an even larger, independent set of gene sequences to evaluate which of the two major hypotheses is supported: batoids as sharks (Shirai, 1992, 1996) or batoids as separate from sharks (Douady et al., 2003). We used nearly complete nuclear rRNA genes, consisting of large-subunit (LSU, or 28S and 5.8S) plus small-subunit (SSU, or 18S) sequences. These sequences were about 5300 nucleotides long, from 22 elasmobranchs, with two chimeras and two bony fishes as outgroups. Combined LSU + SSU data sets have been found to resolve deeper-level vertebrate and animal phylogeny (Giribet, 2002; Lockyer et al., 2003; Mallatt and Sullivan, 1998; Mallatt et al., 2001; Mallatt and Winchell, 2002; Mallatt et al., in press; Medina et al., 2001; Telford et al., 2003; Winchell et al., 2002), and here we show that these genes—especially LSU—are useful in the Class Chondrichthyes.

2. Materials and methods

2.1. Specimens and sequences

Table 1 lists all 26 taxa used. Multiple species from all four described elasmobranch superorders were sampled. We obtained 99%-complete 28S sequences (all but the last ~41 nucleotides from the 3' end) from *Callorhynchus*, *Alopias*, *Mitsukurina*, *Carcharias*, *Galeocerdo*, *Scyliorhinus*, *Squatina*, *Pristiophorus*, *Rhinobatos*, *Urobatis*, and *Potamotrygon*; a 98%-complete 28S sequence (missing the first 50 and last ~41 nucleotides) from *Hemiscyllium*; and 92%-complete sequences (all but the last ~300 nucleotides at the 3' end) from *Heterodontus*, *Orectolobus*, *Apristurus*, *Chlamydoselachus*, *Dalatis*, *Deania*, *Centroscymnus*, and *Centroscyllium*. We sequenced the 18S rRNA gene from all the above taxa (except *Heterodontus* and *Chlamydoselachus*, whose 18S sequences were from Stock (1992)), and sequenced the last 50 bases of the 5.8S gene from all taxa except *Hemiscyllium*. These sequences are available in GenBank under Accession No. AY049805-61. Other

sequences were taken from the literature, as documented in Table 1.

Genomic DNA was obtained by standard phenol–chloroform extraction, and in most cases we first digested the tissues in CTAB buffer (Winnepenninckx et al., 1993). All tissues had been preserved in 70–95% ethanol. We performed DNA amplification, purification, sequencing, fragment assembly, and alignment as described in Mallatt and Sullivan (1998) and Winchell et al. (2002).

This study used more information from the 28S gene than did our previous studies of higher-order phylogeny, because ~1300 bases from the variable *divergent domains* could be aligned and included in the analysis, in addition to the ~2100 bases from the conserved *core* of this 28S gene (see Hassouna et al., 1984, and Mallatt et al., 2001). Alignment was by eye, rigidly based on the LSU and SSU secondary-structure models of *Xenopus laevis* (Gutell, 1994; Schnare et al., 1996). The alignment is available upon request (winchell@ucla.edu). Overall, we used 1780, 50, and 3475 aligned sites in the 18S, 5.8S, and 28S genes, respectively; these represent 98%, 33%, and 93% of the entire lengths of the respective genes.

2.2. Phylogenetic analyses

Three data sets were analyzed: (1) LSU genes only, (2) SSU genes only, and (3) combined LSU + SSU genes. Two main model-based methods were used to infer phylogenetic relationships: maximum likelihood (ML) and Bayesian inference based on the likelihood function. For ML (Huelsenbeck, 1995; Swofford et al., 1996; Yang, 1994), the optimal tree was estimated with an iterative search strategy (Sullivan et al., 1997) in which the GTR + I + Γ model with four Γ rate categories fit our data best. Non-parametric bootstrapping with 100 replicates was used to assess support for nodes of the tree. To assess support under different objective criteria, we also bootstrapped the data (1000 replicates) under unweighted maximum parsimony (MP) and under minimum evolution (ME) in which pairwise distances were calculated under the GTR + I + Γ likelihood model. We conducted all these searches with PAUP* 4.0 beta 8 (Swofford, 2001).

Bayesian inference was implemented with MrBayes (Version 1.11, Huelsenbeck, 2000; Huelsenbeck et al., 2001). We specified a GTR + I + Γ model with no initial values assigned to these parameters, and with empirical nucleotide frequencies. We ran four separate Markov chains for 1 million generations and sampled them every 100 generations to create a posterior probability distribution of 10,000 trees. We discarded the first 1000 trees as burn-in before stabilization, then constructed a 50% majority-rule tree from the subsequent trees. Posterior probabilities $\geq 95\%$ were considered significant (Murphy et al., 2001; Wilcox et al., 2002).

Table 1
Taxa used for phylogenetic comparison in the current study

Classification ^a	Type of fish (common name)	GenBank Accession numbers and references
Class Actinopterygii	Ray-finned fishes	
<i>Acipenser brevirostrum</i>	Shortnose sturgeon	28S, 18S: U34340 Zardoya and Meyer (1996), Stock (1992)
Class Sarcopterygii	Lobe-finned fishes	
<i>Latimeria chalumnae</i>	Coelacanth	28S, 18S: U34336 Zardoya and Meyer (1996), L11288 Stock et al. (1991)
Class Chondrichthyes	Cartilaginous fishes	
Subclass Holocephalii	Chimeras	
<i>Callorhynchus milii</i> ^b	Southern elephantfish	5.8S, 28S, 18S: AY049811-13 (this study)
<i>Hydrolagus collii</i>	Ratfish	5.8S, 28S, 18S: AF061799 Mallatt and Sullivan (1998); Stock (1992)
Subclass Elasmobranchii	Sharks, skates, rays	
Superorder Batoidea	Skates and rays	
Order Rhinobatiformes	Guitarfishes	
<i>Rhinobatos productus</i> ^c	Shovelnose guitarfish	5.8S, 28S, 18S: AY049850-52 (this study)
Order Rajiformes	Skates	
<i>Raja schmidt</i> ^d	Browneye skate	5.8S, 18S, 28S: AF405506, AF278682-83 Winchell et al. (2002)
Order Myliobatiformes	Stingrays	
<i>Potamotrygon histrix</i> ^c	Porcupine river stingray	5.8S, 28S, 18S: AY049844-46 (this study)
<i>Urobatis jamaicensis</i> ^f	Caribbean yellow stingray	5.8S, 28S, 18S: AY049859-61 (this study)
Superorder Galeomorphii	Bullhead, carpet, mackerel, and ground sharks	
Order Heterodontiformes	Bullhead sharks	
<i>Heterodontus francisci</i> ^g	Horn shark	5.8S, 28S, 18S: AY049836-37 (this study), Stock (1992)
Order Orectolobiformes	Carpet sharks	
<i>Hemiscyllium ocellatum</i> ^d	Epaulette shark	28S, 18S: AY049834-35 (this study)
<i>Orectolobus ornatus</i> ^d	Ornate wobbegong shark	5.8S, 28S, 18S: AY049841-43 (this study)
Order Carcharhiniformes	Ground sharks	
<i>Apristurus profundorum</i> ^h	Deepwater cat shark	5.8S, 28S, 18S: AY049808-10 (this study)
<i>Galeocerdo cuvier</i> ⁱ	Tiger shark	5.8S, 28S, 18S: AY049831-33 (this study)
<i>Scyliorhinus torazame</i> ^j	Cloudy cat shark	5.8S, 28S, 18S: AY049853-55 (this study)
<i>Triakis semifasciata</i>	Leopard shark	18S, 5.8S, 28S: AF212180-82 Winchell et al. (2002)
Order Lamniformes		
<i>Alopias pelagicus</i> ^k	Pelagic thresher shark	5.8S, 18S, 28S: AY049805-07 (this study)
<i>Carcharias taurus</i> ⁱ	Sand tiger, or grey nurse shark	5.8S, 18S, 28S: AY049814-16 (this study)
<i>Mitsukurina owstoni</i> ^l	Goblin shark	5.8S, 18S, 28S: AY049838-40 (this study)
Superorder Squalomorphii	Squalomorph sharks	
Order Hexanchiformes	Cow and frilled sharks	
<i>Chlamydoselachus anguineus</i> ^j	Frilled shark	5.8S, 28S, 18S: AY049823-24 (this study), Stock (1992)
Order Pristiophoriformes	Saw sharks	
<i>Pristiophorus cirratus</i> ^l	Longnose saw shark	5.8S, 28S, 18S: AY049847-49 (this study)
Order Squaliformes	Dogfish sharks	
<i>Centroscyllium fabricii</i> ^h	Black dogfish	5.8S, 28S, 18S: AY049817-19 (this study)
<i>Centroscymnus owstoni</i> ^l	Roughskin dogfish	5.8S, 28S, 18S: AY049820-22 (this study)
<i>Deania sp.</i> ^j	Birdbeak dogfish	5.8S, 28S, 18S: AY049828-30 (this study)
<i>Dalatias licha</i> ^l	Kitefin shark	5.8S, 28S, 18S: AY049825-27 (this study)
<i>Squalus acanthias</i>	Piked dogfish shark	28S, 18S: AF061800 Mallatt and Sullivan (1998), M91179 Bernardi et al. (1992)
Superorder Squatinomorphii	Angel sharks	
<i>Squatina californica</i> ^m	Pacific angel shark	5.8S, 28S, 18S: AY049856-58 (this study)

^a The classification scheme presented here follows Compagno (1973, 1977, 1999). Although he considered some aspects of it to be provisional, it provides a good framework for comparing hypotheses of elasmobranch phylogeny.

^b Muscle tissue: collected in New Zealand by Malcolm Francis, National Institute of Water and Atmospheric Research; Kilbirnie, Wellington; New Zealand.

^c Muscle and gill tissue collected in Panama by A. Martin.

^d Liver tissue: collected in Mexico by Janine Caira, Dept. of Ecology and Evolutionary Biology, University of Connecticut; Storrs, CT, USA.

^e Muscle tissue: collected in South Carolina by Michael Janech, Marine Biomedicine and Environmental Sciences Program, Medical University of South Carolina; Charleston, SC, USA.

^f Fin clip: collected in Florida by Demian D. Chapman and Daniel P. Fahy, Nova Southeastern University Oceanographic Center; Dania Beach, FL, USA.

^g Muscle and gill tissue: collected in California by A. Martin.

^h Fin clip: collected in Newfoundland by Jason Treberg, Ocean Science Centre, Memorial University of Newfoundland; St. John's, NF, Canada.

ⁱ Muscle and gill tissue: collected in South Africa by Jeremy Cliff, Natal Sharks Board; KwaZulu-Natal, South Africa.

^j Muscle and gill tissue: collected in Japan by Masaki Miya, Natural History Museum and Institute, Dept. of Zoology, Chiba, Japan.

^k Muscle and gill tissue: collected in Taiwan by George Chen.

^l Muscle tissue: collected in Australia by Gordon Yearsley and Daniel Gledhill, CSIRO Marine Research; Hobart, Tasmania, Australia.

^m Muscle tissue: collected in Mexico by Manuel Grijalva Chon, Dpto. de Investigaciones Científicas y Tecnológicas, Universidad de Sonora; Hermosillo, Sonora, Mexico.

2.3. Statistical tests of alternate hypotheses

To test whether the rRNA data support anatomy-based hypotheses of elasmobranch interrelationships, we used parametric bootstrapping (Efron, 1985; Huelsenbeck et al., 1996) and the SH test (Shimodaira and Hasegawa, 1999). Each of these tests has strengths and weaknesses: Parametric bootstrapping is more statistically rigorous but it is heavily reliant on the accuracy of the evolutionary model it uses to simulate sequence data and thus is prone to Type 1 statistical errors, but the SH test makes so many simplifying assumptions that it is highly prone to Type 2 errors (Antezana, 2003; Goldman et al., 2000). Thus, we included both tests as guides in assessing the actual level of support for the alternate (i.e., anatomy-based) hypotheses.

In parametric bootstrapping, the null hypothesis is that stochastic variation accounts entirely for the difference between the optimal tree and a model tree that was constrained to match the alternate phylogenetic hypothesis. The test statistic δ is the difference between the ln likelihood scores of these two trees, both having been calculated from the original data. The bootstrap tests were performed, involving 100 simulations per hypothesis tested, as described elsewhere (Mallatt and Sullivan, 1998; Wilcox et al., 2002). Data were simulated with the Seq-Gen v1.2.3 program (Rambaut and Grassly, 1997).

Using the same optimal and model likelihood trees that had been generated for parametric bootstrapping, we implemented the SH test in PAUP*. We tested all the alternate hypotheses simultaneously (doing so automatically adjusts resulting p -values for multiple comparisons), and used RELL approximation of likelihood values (1000 replicates).

2.4. Evaluating nucleotide substitution rates

Here, we used not only the nuclear rRNA sequences, but also ~750 nucleotides of cytochrome *b* sequences obtained from GenBank (see the legend to Fig. 3 for references). We tested these gene data for clock-like evolution using a log-likelihood ratio test (Huelsenbeck and Rannala, 1997) and assessed absolute rates of nucleotide substitution by dividing branch lengths for specific lineages by their first-appearance times in the fossil record.

3. Results

Of the 5305 homologous sites in our aligned rRNA sequences, 862 were variable and 492 were phylogenetically informative under the criterion of maximum parsimony. Nucleotide frequencies for all the taxa were 0.224, 0.255, 0.315, and 0.206 for A, C, G, and T, respectively. The χ^2 test of homogeneity in PAUP* indi-

cated these frequencies did not differ across taxa ($\chi^2 = 9.97$; $p = 1.00$), even when only phylogenetically informative sites were tested ($\chi^2 = 88.20$; $p = 0.14$). This is important because all the tree-recovery algorithms used in this study (ML, Bayesian inference, MP, ME) require homogeneity of nucleotide frequencies for optimal performance (Omilian and Taylor, 2001).

Fig. 2 shows the ML trees calculated from combined LSU + SSU rRNA (Fig. 2A), LSU only (Fig. 2B), and SSU only (Fig. 2C). In examining the ML-bootstrap values on these trees (the top numbers), one sees that LSU is better than SSU at recovering (that is, shows higher bootstrap support for) established clades such as elasmobranchs, squaliforms, lamniforms, and orectolobiforms, and that the LSU + SSU tree is only slightly better resolved than the LSU tree—showing higher support for only squaliforms and carcharhiniforms. Supported nodes ($\geq 60\%$) in the combined-gene tree (Fig. 2A) include Chondrichthyes, elasmobranchs, batoids, Squaliformes, Carcharhiniformes, and Orectolobiformes. The orbitostylic sharks crownward of *Chlamydoselachus* were monophyletic (86%). Orectolobiformes were the sister group of Lamniformes (65%). Other nodes, which were present but not significantly supported by ML bootstrapping ($<60\%$), include: sharks, *Chlamydoselachus* in orbitostylic sharks, 'Heterodontus + orbitostylic sharks,' and 'carcharhiniforms with lamniforms + orectolobiforms.'

Bayesian analysis of the LSU + SSU data (see bottom numbers in Fig. 2A) gave essentially the same results as ML. All significantly supported clades ($\geq 95\%$) matched those in the ML tree (monophyly of batoids, of orbitostylic sharks beyond *Chlamydoselachus*, 'orectolobiforms + lamniforms,' etc.). Like ML, the Bayesian tree (not shown) failed to support shark monophyly, its placements of *Heterodontus*, *Chlamydoselachus*, and batoids were uncertain; and its nodal support for 'carcharhiniforms + lamniforms + orectolobiforms' was not significant.

A notable feature of the rRNA trees is a high variation in branch lengths (Fig. 2A). The test of clock-like evolution was soundly rejected ($A = 633$; $df = 22$; $p < 0.0001$). As shown in Table 2, the rRNA genes of some old lineages have evolved extremely slowly (*Squatina*, *Pristiophorus*) and those of some young lineages relatively quickly (*Urobatis*, *Potamotrygon*), but by far the fastest rRNA evolution has occurred in some carcharhiniforms (e.g., *Galeocerdo*). The variation in these evolutionary rates is huge, the fastest rate being 65 times greater than the slowest (rates range from 0.6 to 39×10^{-5} substitutions per site per million years). By comparison, the mitochondrial gene for cytochrome *b* evolved faster but with less variation across lineages, its fastest and slowest rates differing by a factor of only 6.5 (Table 2). It must be noted that the huge range of rRNA rates is mostly due to the rapidly evolving

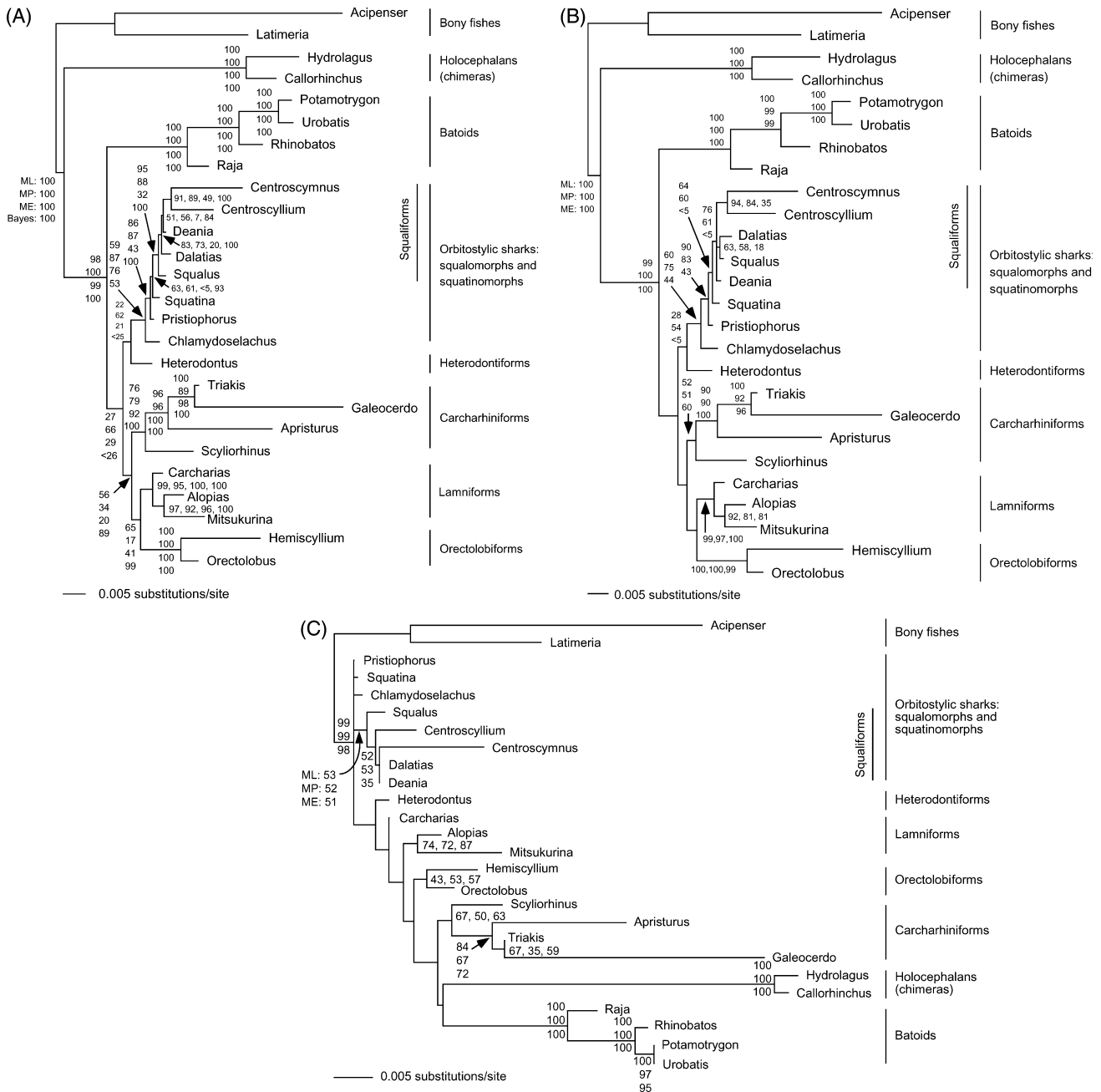


Fig. 2. Maximum likelihood trees. (A) From combined LSU + SSU rRNA genes (ln likelihood = 16858.091); (B) From LSU gene only (ln likelihood = 11799.965); (C) From SSU gene only (ln likelihood = 4937.198). Of the numbers shown next to the nodes, the first, second, and third are the maximum likelihood (ML), maximum parsimony (MP), and minimum evolution (ME) bootstrap percentages, respectively. The fourth numbers, in part A, are Bayesian posterior probabilities. Nodes with <50% support by all methods are unnumbered.

carcharhiniform sequences, because when *Galeocerdo* is omitted, the range is only about 14 times, closer to that of cytochrome *b*.

When it became clear that our LSU + SSU tree disagreed with many of the morphology-based hypotheses from the literature, we tested these hypotheses statistically against our data with parametric bootstrapping and the SH test (Table 3). The five hypotheses tested were the topologies of (A) Compagno (1973) (Fig. 1A);

(B) Maisey (1980) (Fig. 1B); (C) Shirai (1992) (Fig. 1C); plus topologies that were constrained to show (D) Hypnosqualea, or (E) Galeomorph monophyly, but were otherwise optimized to fit our rRNA data. As seen in Table 3, both hypotheses that propose Hypnosqualea, hypotheses C and D, were rejected by both tests. In addition, Compagno's (1973) hypothesis was strongly rejected by parametric bootstrapping ($p < 0.01$) and was nearly rejected by the conservative SH test at a 0.05 level

Table 2
Rates of nuclear rRNA and cytochrome *b* evolution

Terminal lineage	Age (million years = MY); Cappetta (1987)	rRNA branch length: Substitutions/site	rRNA rate: Substitutions/ site/ MY, $\times 10^{-5}$	Cyt. <i>b</i> branch length: Substitutions/site (see Fig. 3)	Cyt. <i>b</i> rate: Substitutions/ site/ MY, $\times 10^{-5}$
<i>Squalus</i>	152	0.0027	1.8	0.1506	99
<i>Squatina</i>	157	0.0016	1	0.262	167
<i>Pristiophorus</i>	157	0.0009	0.6	0.322	205
<i>Chlamydoselachus</i>	200	0.0033	1.7	0.39	195
<i>Mitsukurina</i>	145	0.0103	7.1	0.171	118
<i>Alopias</i>	121	0.00598	4.9	0.116	96
<i>Carcharias</i>	121	0.00251	2.1	0.143	118
<i>Galeocerdo</i>	112	0.0435	39	0.188	168
<i>Scyliorhinus</i>	112	0.0087	7.8	0.253	226
<i>Heterodontus</i>	194	0.00454	2.3	0.188	97
<i>Urobatis</i>	35	0.00304	8.7	0.222	634
<i>Potamotrygon</i>	35	0.00281	8	0.195	557
<i>Rhinobatos</i>	85	0.00492	5.8	0.288	339
<i>Raja</i>	85	0.00405	4.8	0.358	421
			Range: 0.6–39 = 65 \times		Range: 97–634 = 6.5 \times

Table 3

Results of hypothesis testing by parametric bootstrapping and the Shimodaira–Hasegawa (SH) test, from LSU + SSU rRNA sequences of the taxa in Fig. 2A

Hypothesis	$\delta_{\text{OBS}}^{\text{a}}$	$d_{95\%}^{\text{b}}$	P_{PB}^{c}	P_{SH}^{d}
A. Compagno (1973) (Fig. 1A)	35.19	0–4.58	<0.01	0.051
B. Maisey (1980) (Fig. 1B)	18.03	0–1.99	<0.01	0.270
C. Shirai (1992, 1996) (Fig. 1C)	63.8	0–3.10	<0.01	0.000
D. Hypnosqualea	48.77	0–2.43	<0.01	0.033
E. Galeomorph monophyly (<i>Heterodontus</i> + lamniforms + carcharhiniforms + orectolobiforms)	3.09	0–1.23	0.01	0.859

^a Observed test statistic for parametric bootstrapping = ln likelihood score of the model tree – ln likelihood score of the optimal ML tree.

^b Range for 95% of δ 's expected distribution in parametric bootstrapping; these are one-tailed tests, so upon rejection (see *p*-values), it can be concluded that the optimal ML tree is statistically better than the model tree.

^c Parametric bootstrapping *p*-values; the Bonferroni correction for multiple comparisons is applied, so the critical value for rejection of a hypothesis is 0.05/5 = 0.01.

^d SH-test *p*-values; these have already been adjusted for multiple comparisons.

($p = 0.051$), so we conclude that this topology is also inconsistent with our data. Maisey's (1980) hypothesis, with its orbitostylic sharks, should not be rejected, due to the high SH *p*-value (0.270). Galeomorph monophyly (E) was not rejected by either test (after correcting the parametric bootstrapping *p*-values for multiple comparisons); therefore, Galeomorphii is consistent with our data.

4. Discussion

4.1. Main conclusion

Our results from nuclear LSU and SSU rRNA are remarkably similar to those of Douady et al. (2003) from three mitochondrial genes. The key point of agreement is that both studies place *Squatina* and *Pris-*

tiophorus with squaliform sharks rather than with batoids, and statistically reject the Hypnosqualea hypothesis (de Carvalho, 1996; de Carvalho and Maisey, 1996; Shirai, 1992, 1996). The hypnosqualean subgroups were said to be united by many anatomical characters including aspects of the skull, shoulder girdle, and the dorsal and caudal fin musculature (Shirai, 1992, 1996). Given the new molecular evidence that batoids and sharks are independent lineages, these shared "hypnosqualean" characters can be interpreted as homoplasious (Douady et al., 2003), perhaps convergent adaptations to benthic living.

4.2. LSU versus SSU genes

Past studies of higher-order animal phylogeny that compared results of LSU-only, SSU-only, and combined LSU + SSU data sets (Mallatt and Sullivan, 1998;

Mallatt and Winchell, 2002; Medina et al., 2001; Winchell et al., 2002) found that LSU and SSU genes produced broadly similar trees, and that the combined-gene results were more robust. In general, SSU sequences proved better than LSU at resolving higher-order relations (between phyla and subphyla). However, the opposite holds true for the present study of lower-order relations: LSU genes are better than the SSU genes, and the combined data set is not much better than LSU alone (Fig. 2). Why SSU gave so little resolution here is not entirely clear. The branches on the SSU tree (Fig. 2C) are about the same average length as those on the LSU tree (Fig. 2B), so differences in evolutionary rates of the two genes probably cannot explain the differences between their respective trees. Perhaps our LSU sequences did better simply because they have twice as many nucleotides as the SSU sequences, and thus contain more information.

4.3. Other higher-elasmobranch relationships

Although most taxonomists unite lamniforms and carcharhiniforms within galeomorphs (Fig. 1), our results support a lamniform + orectolobiform group (Fig. 2A). White (1936) advocated such a group, naming it *Isurida*, but Compagno (1973) rejected it because he considered its defining characters—similarities in vertebral calcification, ring intestinal valves, and no nictitating membrane—to be homoplasious since some carcharhiniforms also have them. However, the lamniform + carcharhiniform clade is also plagued by homoplasy: Of the six or more characters supporting this clade, only one (tripodal rostrum) is not homoplasious, i.e., has a consistency index of 1.0 (de Carvalho, 1996, p. 44). Perhaps the characters used to unite lamniforms and carcharhiniforms are convergent adaptations for pelagic carnivory. Incidentally, the mitochondrial data of Douady et al. (2003) cannot answer these questions because they included only one orectolobiform sequence, and this was highly divergent.

The taxonomic positions of *Heterodontus* and *Chlamydoselachus* are controversial, although the dominant, morphology-based view (Fig. 1A–C) is that *Heterodontus* is a galeomorph, and *Chlamydoselachus*, plus other hexanchiforms, are squalomorphs (Compagno, 1973; Holmgren, 1940; Holmgren, 1941; Maisey, 1983). Our rRNA-based study did not strongly place either genus with any other clade (Fig. 2A), so it does not resolve the controversies. The positions were so unstable that we cannot disprove the idea that *Heterodontus* is in a monophyletic Galeomorphii (Table 3) nor the older ideas that *Heterodontus* and *Chlamydoselachus* are basal neoselachians or even “primitive,” non-neoselachian sharks (Allis, 1923; Goodrich, 1909; Gudger and Smith, 1933; Regan, 1906; White, 1937). The findings of Douady et al. (2003) were almost the

same as ours: They found an apparently basal but unresolved position of *Heterodontus* within sharks and a weak-to-good association of hexanchiforms with squalomorphs.

4.4. Within elasmobranch subgroups

Incomplete taxon sampling limits our ability to discuss relationships within smaller elasmobranch groups, but our rRNA results are mostly consistent with morphological studies. For example, within squaliforms, de Carvalho (1996) and Compagno (1973) placed *Squalus* (and related genera) farthest basally; and this fits our findings (Fig. 2A). However, the relations among our other squaliform genera do not match other published trees.

In Carcharhiniformes, it is thought that scyliorhinids represent the basal lineage, that triakids branched off second, and that carcharhinids and sphyrnids (hammerheads) are the most derived (Compagno, 1973, 1988; White, 1937). Also, Maisey (1984) found evidence of extensive paraphyly among the carcharhiniform families. Our trees fit these conclusions in showing paraphyletic scyliorhinids (*Scyliorhinus*, *Apristurus*) situated basal to *Triakis* + *Galeocerdo*.

Within the lamniforms, both molecular and anatomical data placed *Mitsukurina* basal to all other lamniforms (Compagno, 1999; Martin, 1999; Martin and Burg, 2002; Martin et al., 2002; Naylor et al., 1997;

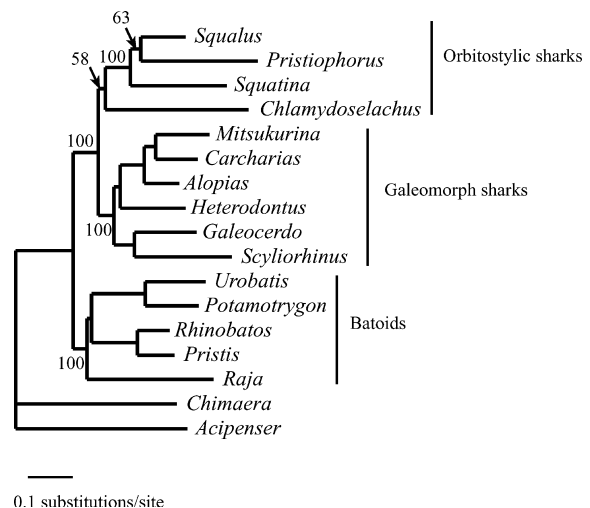


Fig. 3. Cytochrome *b* tree, Bayesian topology with the branch lengths estimated using ML, and posterior probabilities (>50%) indicated above the nodes. The cytochrome *b* sequences of the four orbitostylic sharks, plus *Rhinobatos*, and *Pristis* are from Kitamura et al. (1996), of the three lamniforms from Naylor et al. (1997), *Heterodontus* from Martin and Palumbi (1993), *Galeocerdo* from Heist and Gold (1999), *Scyliorhinus* from DeLarbre et al. (1998), *Urobatis* from Martin (1995), *Potamotrygon* from Martin et al. (1998), *Raja* from Rasmussen and Arnason (1999), *Chimaera* from Arnason et al. (2001), and *Acipenser* from Ludwig et al. (2000).

Shirai, 1996). Our trees seem to disagree because they place *Carcharias* basal to a 'Mituskurina + *Alopias*' clade (Fig. 2A). However, an MP analysis using more-complete rRNA sequences and closer outgroups (i.e., using more of the 28S gene to yield 5706 sites, and limiting the taxa to just lamniforms plus one carcharhiniform and one orectolobiform) did place *Mitsukurina* basal to the other lamniforms; that is, there was 77% bootstrap support for a '*Carcharias* + *Alopias*' clade.

Because we did not sample Torpediniformes (electric rays) or Pristiformes (saw fishes), we can say little about batoid interrelationships. However, because relationships among the rajid skates, some guitarfish genera, and stingrays are uncertain (McEachran et al., 1996), our trees placing *Raja* basally and *Rhinobatos* closer to the myliobatiforms (*Potamotrygon*, *Urobatis*) could be informative.

4.5. Cytochrome *b*-based phylogeny

Elasmobranch phylogeny was also calculated from ~750 nucleotides of the cytochrome *b* gene, a mitochondrial gene that was not used by Douady et al. (2003). The resulting tree (Fig. 3) resembles our rRNA tree and that of Douady et al. (2003) in separating batoids from sharks and in showing a clade of orbitostylic sharks. However, it shows more support for galeomorph monophyly. Like the other mitochondrial genes (Douady et al., 2003), cytochrome *b* shows stronger support for shark monophyly than do the nuclear rRNA genes.

4.6. Summary

Several molecular-phylogenetic studies, based on both nuclear (LSU and SSU rRNA) and mitochondrial genes (12S and 16S rRNA, tRNA valine, and cytochrome *b*) now refute the dominant, morphology-based Hypnoqualea hypothesis, in favor of batoids and sharks as separate lineages. The classical hypothesis most consistent with these new molecular findings is Maisey's (1980) concept of monophyletic orbitostylic sharks (Fig. 1B). Outstanding problems to be addressed by future gene-based studies are: (1) relations of *Heterodontus* and hexanchiforms to other sharks; (2) relations among lamniforms, carcharhiniforms, and orectolobiforms; and (3) batoid interrelationships.

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