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Venom Evolution Widespread in Fishes: A Phylogenetic Road Map for the Bioprospecting of Piscine Venoms

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Abstract

Knowledge of evolutionary relationships or phylogeny allows for effective predictions about the unstudied characteristics of species. These include the presence and biological activity of an organism's venoms. To date, most venom bioprospecting has focused on snakes, resulting in six stroke and cancer treatment drugs that are nearing U.S. Food and Drug Administration review. Fishes, however, with thousands of venoms, represent an untapped resource of natural products. The first step involved in the efficient bioprospecting of these compounds is a phylogeny of venomous fishes. Here, we show the results of such an analysis and provide the first explicit suborder-level phylogeny for spiny-rayed fishes. The results, based on ~1.1 million aligned base pairs, suggest that, in contrast to previous estimates of 200 venomous fishes, >1,200 fishes in 12 clades should be presumed venomous. This assertion was corroborated by a detailed anatomical study examining potentially venomous structures in >100 species. The results of these studies not only alter our view of the diversity of venomous fishes, now representing >50% of venomous vertebrates, but also provide the predictive phylogeny or "road map" for the efficient search for potential pharmacological agents or physiological tools from the unexplored fish venoms.

> Until such fundamentals as the anatomical distribution of fish venoms have been determined, the pharmacological and chemical characterization of these compounds will continue to be unstudied.
>
> —Halstead (1988, p. XXI) in the introduction to his treatise on venomous marine organisms.

Venomous organisms produce and use venom, a toxin injected using a specialized apparatus, for interactions with predators, prey, and competitors (Bulaj et al. 2003; Halstead 1970, 1988). Venoms and their associated delivery systems have evolved in animal groups ranging from the simple box jellyfish (*Carybdea*) to the black widow spider (*Latrodectus*), lionfish (*Pterois*), and cobra (*Naja*). To date, snakes, scorpions, and spiders have been the principal focus of research into potential pharmacological agents or physiological tools found in the toxins of venomous animals (Ault 2004; Tan et al. 2003). This research has already led to the development of six pharmaceuticals derived from snake venoms with functions ranging from anticoagulation to antiangiogenesis; these drugs are currently in various stages of U.S. Food and Drug Administration regulatory review (Ault 2004; Tan et al. 2003). In comparison to these terrestrial groups, venomous fishes have been largely ignored as a source of potential pharmaceuticals (Church and Hodgson 2002; Halstead 1988; Tan et al. 2003). In fact, recent studies (Church and Hodgson 2002; Sosa-Rosales et al. 2005) indicate that roughly one dozen toxins have been identified and/or characterized from venomous fishes. This deficiency is due, in part, to the lack of reliable estimates of the number and diversity of venomous fish species. The number of venomous fishes has typically been reported as ~200 species (Church and Hodgson 2002; Haddad et al. 2003; Halstead 1970, 1988), but this value clearly underestimates the number of venomous fishes implied by the phylogenetic distribution of venom among ray-finned fishes. Given the potential pharmaceutical benefits offered by and the health threats posed by venomous fishes, it is surprising that no studies have ever examined the relationships of venomous fishes to infer the identity and number of venomous fish species. Phylogenies are crucial for predicting the distribution of such characteristics because they provide maximally efficient descriptions of

organismic attributes, which allow for effective predictions about organismal characteristics that have not yet been studied (Raven et al. 1994; Systematics Agenda 2000 1994). For this reason, a complete understanding of the phylogeny of venomous fishes would also be extremely valuable for the efficient bioprospecting of piscine venoms. However, generating this phylogenetic road map for exploring the biological activity and distribution of piscine venoms requires sufficient resolution of the >18,000 species "acanthomorph problem," which remains the major task facing systematic ichthyology (Johnson 1993; Miya et al. 2003; Nelson 1989; Stiassny et al. 2004).

Venomous ray-finned fishes are diverse with representatives spread across four orders (Church and Hodgson 2002; Haddad et al. 2003; Halstead 1988; Smith-Vaniz et al. 2001; Vetrano et al. 2002) and habitats ranging from mountain streams to coral reefs and oceanic midwaters (Nelson 1994). Their envenomations cause at least 50,000 reported injuries per year with symptoms ranging from blisters to intense pain, fever, and death (Haddad et al. 2003; Halstead 1970, 1988; Vetrano et al. 2002). The known venomous fishes are currently distributed among the catfishes (Siluriformes) and six groups of "acanthomorphs" or spiny-rayed fishes (Church and Hodgson 2002; Halstead 1970, 1988; Nelson 1994; Smith-Vaniz et al. 2001): toadfishes (Batrachoidiformes); scorpionfishes (Scorpaeniformes: Scorpaenoidei); surgeonfishes, scats, and rabbitfishes (Perciformes: Acanthuroidei); saber-toothed blennies (Perciformes: Blennioidei); jacks (Perciformes: Percoidei); and stargazers and weeverfishes (Perciformes: Trachinoidei). The distantly related catfishes (Nelson 1994; Stiassny et al. 2004) are not included in this analysis, but it is clear that envenomation structures have been gained and/or lost multiple times within the order (Friel J, personal communication; Smith WL, unpublished data). The remaining venomous fish groups are nested within the acanthomorph problem or Nelson's (1989) "bush at the top of the tree," which refers to our poor, and often contradictory, understanding of the relationships of the ~18,000 species of spiny-rayed fishes that represent the crown group of bony fish evolution (Johnson 1993; Miya et al. 2003; Nelson 1994; Stiassny et al. 2004). Our knowledge of the relationships of the venomous fish groups within the context of the acanthomorph problem is also poor, with recent studies (Chen et al. 2003; Miya et al. 2003; Smith and Wheeler 2004; Tang et al. 1999) challenging the integrity of many of these venomous assemblages. The incongruence between these recent studies and the traditional classification (Nelson 1994) only highlight the need for a large-scale phylogenetic analysis examining the relationships among spiny-rayed fishes as a whole to delimit venomous fish clades.

To resolve relationships among venomous spiny-rayed fishes, we analyzed ~4,700 bp in 233 species (approximately 1.1 Mb), including representatives of all suborders and venomous groups within the Acanthomorpha. The results of this study will allow us to (1) hypothesize the number and identity of venomous species, (2) hypothesize the number of times that venom apparatuses have originated by delimiting all venomous clades, and (3) make effective predictions about venomous fishes and venom evolution by providing a predictive phylogenetic framework or road map.

Materials and Methods

Taxon Sampling

The 233 analyzed taxa include 228 representatives from all suborders and orders of spiny-rayed fishes (Acanthomorpha) and two aulopiform and three myctophiform outgroups, with the Atlantic sabretooth (*Coccorella atlantica*) as the root. Representatives of all venomous acanthomorph groups (i.e., *Meiacanthus* blennies, thalassophrynine toadfishes, scatophagids, scomberoidine carangids, uranoscopids, siganids, acanthurids, trachinids, and scorpaenoids) and their putative allies were included to rigorously examine the evolution of venomous fishes. Species considered venomous in our molecular analysis have had the presence of venom confirmed, or it has been confirmed in one of their congeners (Church and Hodgson 2002; Halstead 1970, 1988; Smith-Vaniz et al. 2001; Sosa-Rosales et al. 2005).

Acquisition of Nucleotide Sequences

Fish tissues were preserved in 70%–95% ethanol prior to extraction of DNA. Total DNA was extracted from muscle or fin clips using a Qiagen (Valencia, CA) DNeasy Tissue Extraction Kit following the manufacturer's protocol. Polymerase chain reaction (PCR) was used to amplify four segments, representing five genes, from the mitochondrial and nuclear genomes. Double-stranded amplifications were performed in a 25-μl volume containing one Ready-To-Go PCR bead (Amersham Biosciences, Piscataway, NJ), 1.25 μl of each primer, and 2–5 μl of undiluted extracted DNA. To amplify and sequence these five gene regions, the following primer pairs were used (primer sequences can be found in Smith and Wheeler 2004): 12S, $tRNA^{Val}$, 16S fragment—primers 12SL13-L and TitusI-H, remaining 16S fragment—primers 16S ar-L and 16S br-H, 28S fragment—primers 28SV and 28SJJ, and histone H3 fragment—primers H3a-L and H3b-H. The analysis resulted in an alignment of 4,721 aligned nucleotides (based on the implied alignment, Wheeler 2003a). Amplifications for all fragments were carried out in 30–40 cycles with the following temperature profile: initial denaturation for 6 min at 94°C, denaturation for 45–60 s at 94°C, annealing for 45–60 s at 46°C–49°C, and extension for 1–2 min at 72°C, with an additional terminal extension at 72°C for 6 min. The double-stranded amplification products were desalted and concentrated using an ArrayIt PCR Product Purification Kit (TeleChem International, Sunnyvale, CA) on a Beckman BIOMEK 2000 laboratory automated pipetting workstation with minor modifications to the manufacturer's protocol. Both strands of the purified PCR fragments were used as templates and directly cycle sequenced using the original amplification primers and an ABI Prism Dye Terminator Reaction Kit V1.1. The sequencing reactions were cleaned with standard isopropyl-ethanol precipitation and resuspended in 10 μl formamide. The nucleotides were sequenced on an ABI 3700 or ABI 3730xl automated DNA sequencer. Contigs were built in SEQUENCHER 3.1 (Gene Codes, Ann Arbor, MI)

Table 1. Spiny-rayed fishes examined in this study for the presence or absence of a venom apparatus with a conspicuous venom gland. The abbreviation "ag" refers to an anterolateral glandular groove in a venomous dorsal- or anal-fin spine (see Figures 1 and 3)

Taxon	Venom apparatus condition	Museum voucher[a]
Acanthuridae—surgeonfishes		
Acanthurus pyroferus	ag without conspicuous venom gland[b]	AMNH 51847
Paracanthurus hepatus	ag with venom gland	AMNH 50752
Prionurus scalprum	ag with venom gland	AMNH 26891
Zebrasoma flavescens	ag without conspicuous venom gland[b]	AMNH 50762
Ambassidae—glassfishes		
Ambassis sp.	Neither ag nor venom gland	AMNH 231644
Apistidae-waspfishes		
Apistus carinatus	ag with venom gland	CAS 15975
Aploactinidae—velvetfishes		
Aploactis aspera	Neither ag nor venom gland	CAS 15611
Erisphex pottii	Neither ag nor venom gland	CAS 30316
Ptarmus jubatus	ag with venom gland	AMNH 19599
Batrachoididae—toadfishes		
Daector reticulata	Both opercular and dorsal glands	AMNH 7549
Opsanus beta	Neither opercular nor dorsal glands	AMNH 15482
Porichthys margaritatus	Neither opercular nor dorsal glands	AMNH 233797
Thalassophryne amazonica	Both opercular and dorsal glands	AMNH uncat.
Bembridae—deepwater flatheads		
Bembras japonica	Neither ag nor venom gland	AMNH 89899
Blenniidae—blennies		
Meiacanthus anema	Both fang and venom gland	AMNH 48593
Meiacanthus grammistes	Both fang and venom gland	AMNH 213840
Salarias fasciatus	Neither fang nor venom gland	AMNH 48746
Callionymidae—dragonets		
Callionymus lyra	Neither opercular spine gland nor ag with venom gland	AMNH 36841
Foetorepus agassizii	Neither opercular spine gland nor ag with venom gland	AMNH 85527
Caracanthidae—coral crouchers		
Caracanthus unipinna	ag with venom gland (at least in dorsal-fin spines)	AMNH 49681
Carangidae—jacks		
Oligoplites saurus	Not able to be confirmed in specimen[c]	AMNH 47846
Scomberoides lysan	Not able to be confirmed in specimen[c]	AMNH 1576
Congiopodidae—pigfishes		
Congiopodus leucopaecilus	Neither ag nor venom gland	AMNH 13481
Cottidae—sculpins		
Cottus bairdi	Neither ag nor venom gland	AMNH 68716
Icelinus quadriseriatus	Neither ag nor venom gland	AMNH uncat.
Creediidae—sandburrowers		
Limnichthys fasciatus	Neither cleitheral spine gland nor ag with gland	AMNH 57282
Dactylopteridae—helmet gunards		
Dactylopterus volitans	Neither ag nor venom gland	AMNH 64427
Emmelichthyidae—rovers		
Erythrocles schlegelii	Neither ag nor venom gland	AMNH 13066
Enoplosidae—old wives		
Enoplosus armatus	Neither ag nor venom gland	AMNH 31418, 222920
Gasterosteidae—sticklebacks		
Apeltes quadratus	Neither ag nor venom gland	AMNH 21932
Gnathanacanthidae—red velvetfishes		
Gnathanacanthus goetzeei	ag with venom gland	AMNH 223040
Hexagrammidae—greenlings		
Hexagrammos stelleri	Neither ag nor venom gland	AMNH 58940
Hoplichthyidae—ghost flatheads		
Hoplichthys citrinus	Neither ag nor venom gland	AMNH 89898
Kuhliidae—aholeholes		
Kuhlia rupestris	Neither ag nor venom gland	AMNH 215492
Lutjanidae—snappers		
Lutjanus fulviflamma	Neither ag nor venom gland	AMNH 213080
Monodactylidae—monos		
Monodactylus sebae	Neither ag nor venom gland	AMNH 226594
Moronidae—temperate basses		
Morone americana	Neither ag nor venom gland	AMNH 229546

Table 1. Continued

Taxon	Venom apparatus condition	Museum voucher[a]
Neosebastidae—gurnard perches		
Neosebastes entaxis	ag with venom gland	AMNH 4024
Neosebastes scorpaenoides	ag with venom gland	AMNH 91776
Neosebastes thetidis	ag (skeletal prep., so presence of gland not determined)	AMNH 99340SD
Pataecidae—prowfishes		
Aetapcus maculatus	Neither ag nor venom gland	NMV A 11847
Pataecus fronto	No ag (skeletal prep., so presence of gland not determined)	SU 67408
Percidae—perches and darters		
Perca flavescens	Neither ag nor venom gland	AMNH 228835
Sander vitreum	Neither ag nor venom gland	AMNH 29723
Peristediidae—armored sea robins		
Peristedion gracile	Neither ag nor venom gland	AMNH 220804
Platycephalidae—flatheads		
Platycephalus conatus	No ag (skeletal prep., so presence of gland not determined)	AMNH 88538SD
Platycephalus endrachtensis	Neither ag nor venom gland	AMNH 37843
Scathophagidae—scats		
Scatophagus tetracanthus	ag with venom gland	AMNH 232414
Selenotoca multifasciata	ag with venom gland (in specimens <50 mm standard length)	AMNH 48771
Scorpaenidae—scorpionfishes		
Iracundus signifer	ag with venom gland	AMNH uncat.
Neomerinthe beanorum	Glandular tissue on posterior margin of fin spines[d]	AMNH 74150
Neomerinthe hemingwayi	Glandular tissue on posterior margin of fin spines[d]	AMNH 83911
Parascorpaena mossambica	ag with venom gland	AMNH 213580
Pontinus furcirhinus	Glandular tissue on posterior margin of fin spines[d]	AMNH 224219
Pontinus longispinis	Glandular tissue on posterior margin of fin spines[d]	AMNH 83416
Pontinus rathbuni	Glandular tissue on posterior margin of fin spines[d]	AMNH 73608
Pterois volitans	ag with venom gland	AMNH 16883
Scorpaena calcarata	ag with venom gland	AMNH 82980
Scorpaena maderensis	ag with venom gland	AMNH 230444
Scorpaena plumieri	ag with venom gland	AMNH 30379
Scorpaenodes guamensis	ag with venom gland	AMNH 213867
Scorpaenodes kelloggi	ag with venom gland	AMNH 19154
Scorpaenodes xyris	ag with venom gland	SIO 70-167
Scorpaenopsis macrochir	ag with venom gland	AMNH uncat.
Sebastapistes galactacma	ag with venom gland	AMNH 72855
Taenionotus triacanthus	ag with venom gland	AMNH 49801
Sebastidae—rockfishes		
Helicolenus dactylopterus	ag with venom gland	AMNH 84711
Sebastes crameri	ag with venom gland	AMNH 97475
Sebastes saxicola	ag with venom gland	AMNH 38172
Sebastiscus marmoratus	ag with venom gland	AMNH 17446
Sebastolobus alascanus	Glandular tissue on posterior margin of fin spines[d]	AMNH 38179
Trachyscorpia cristulata	ag with venom gland	AMNH 84331
Serranidae—sea basses and groupers		
Acanthistius serratus	Neither opercular spine nor ag with venom gland	AMNH 219096
Centroprisis striata	Neither opercular spine nor ag with venom gland	AMNH 65236
Diplectrum formosum	Neither opercular spine nor ag with venom gland	AMNH 81346
Epinephelus merra	Neither opercular spine nor ag with venom gland	AMNH 72229
Niphon spinosus	Neither opercular spine nor ag with venom gland	AMNH 4008
Setarchidae—deepwater scorpionfish		
Ectreposebastes imus	ag with venom gland (at least in anal-fin spines)	AMNH 29775
Setarches guentheri	ag with venom gland	AMNH 84334
Siganidae—rabbitfishes		
Siganus doliatus	ag with venom gland	AMNH 213426
Siganus margaritiferus	ag with venom gland	AMNH 17023
Siganus stellatus	ag with venom gland	AMNH 232551
Synanceiidae—stonefishes		
Erosa erosa	ag with venom gland	AMNH 34900
Inimicus sinensis	ag with venom gland	AMNH uncat.
Minous monodactylus	ag with venom gland	AMNH 13121
Synanceia verrucosa	ag with large venom gland	AMNH uncat.

Table 1. Continued

Taxon	Venom apparatus condition	Museum voucher[a]
Tetrarogidae—waspfishes		
Gymnapistes marmoratus	ag with venom gland	AMNH 31009
Neocentropogon japonicus	ag with venom gland	AMNH 89901
Terapontidae—grunters		
Leiopotherapon unicolor	Neither ag nor venom gland	AMNH 35567
Trachinidae—weeverfishes		
Echiichthys vipera	ag and opercular spine with venom gland	AMNH 49662
Trachinus araneus	ag and opercular spine with venom gland	AMNH 9168
Trachinus draco	ag and opercular spine with venom gland	AMNH 57476
Triglidae—sea robins		
Bellator militaris	Neither ag nor venom gland	AMNH 84578
Chelidonichthys kumu	Neither ag nor venom gland	AMNH 219121
Lepidotrigla sp.	Neither ag nor venom gland	AMNH 89820
Uranoscopidae—stargazers		
Astroscopus guttatus	Cleithral spine with venom gland	AMNH 73984
Astroscopus y-graecum	Cleithral spine with venom gland	AMNH 75699
Kathetostoma albigutta	Cleithral spine with venom gland	AMNH 83576
Kathetostoma cubana	Cleithral spine (skeletal preparation, so presence of gland not determined)	AMNH 49656
Uranoscopus japonicus	Cleithral spine with venom gland	AMNH 13245
Zanclidae—moorish idols		
Zanclus cornuta	Neither ag nor venom gland	AMNH 32454
Zaniolepididae—combfishes		
Oxylebius pictus	Neither ag nor venom gland	SIO 67-139

[a] Museum catalog numbers are for specimens examined by gross dissection for the current study. The condition of the venom apparatus and gland is listed for all specimens examined whether or not they were previously listed as venomous.

[b] The presence of a venom gland could not be determined despite the presence of distinct anterolateral grooves; this may be due to the loss of venom glands in adults (Halstead 1988; Randall et al. 1997).

[c] Although specimens of *Scomberoides* and *Oligoplites* were examined in the current study, venom glands were not visible. This confirms Halstead's (1988, p. 936) statement that, for jacks, "the venom glands are not grossly visible."

[d] Although these taxa lack a venom gland associated with the anterolateral grooves on their dorsal- and anal-fin spines, they have a thick, glandular tissue on the caudal margin of each of the fin spines, which we believe is probably venomous (see Figure 3F).

using DNA sequences from the complementary heavy and light strands. Sequences were edited in SEQUENCHER and BIOEDIT (Hall 1999). All novel sequences were submitted to GenBank and assigned accession numbers (DQ532831-DQ533482) or were taken from our previous studies (Smith and Wheeler 2004; Sparks and Smith 2004a,b; see supplementary information). Histone H3 was unable to be amplified in the brotulid (*Brosmophycis*) and the 12S/tRNA^{Val} genes were unable to be sequenced in toadfishes (Batrachoidifomes except *Perulibatrachus*) and the soldierfish (*Gymnapistes*), so the unavailable data were treated as missing.

Phylogenetic Analyses

The parsimony analysis was run using direct optimization (Wheeler 1996), fixed states optimization (Wheeler 1999), and iterative pass (Wheeler 2003b) as implemented in the program POY 3.0 (Wheeler WC, Gladstein DS, DeLaet J, unpublished; http://research.amnh.org/scicomp/projects/poy.php) using default values unless listed otherwise below and run on the American Museum of Natural History (AMNH) parallel computing cluster.

The analysis began by generating 500 random addition sequences each under fixed states optimization and direct optimization for a total of 1,000 starting points for the analysis. These random addition sequences were improved with tree fusing (Goloboff 1999; specifying: fuselimit 5000, fitchtrees, and fusemingroup 3), tree bisection and reconnection (TBR) branch swapping, and 400 parsimony ratchet replicates (Nixon 1999; specifying: ratchettbr; ratchetseverity 3; ratchetpercent 40). This initial searching took 35 days on the AMNH parallel computing cluster and resulted in eight most parsimonious trees with a length of 27,625 steps. These optimal trees were submitted to POY for further TBR branch swapping, tree fusing (specifying: fuselimit 5000, fitchtrees, and fusemingroup 3), and 100 rounds of parsimony ratcheting (specifying: ratchettbr; ratchetseverity 3; ratchetpercent 40) using iterative pass and exact, which provide more thorough and less heuristic searches. This final round of the analysis took an additional 32 days.

The length of the resulting implied alignment was verified in NONA 3.0 (Goloboff PA, unpublished; http://www.cladistics.com). To estimate the robustness of the phylogenetic hypotheses recovered, Bremer supports and jackknife resampling percentages were calculated. Jackknife resampling analyses were performed using NONA (1,000 replications, five random addition sequences per replication), and Bremer supports were calculated with default values using

Table 2. Number and taxonomic distribution of the 2,000+ venomous vertebrates

Cartilaginous fishes—Chondrichthyes[a]	~200 venomous species
Chimaeras—Chimaeriformes	38
Hornsharks—Heterodontidae	8
Dogfishes—Squalidae	11
Stingrays—Dasyatidae	70
Butterfly rays—Gymnuridae	14
Eagle rays—Myliobatidae	41
Deepwater stingrays—Plesiobatidae	1
River stingrays—Potamotrygonidae	19
Lobe-finned fishes and tetrapods—Sarcopterygii[b]	~460 venomous species
Snakes—Colubroidea	450
Gila monsters—Helodermatidae	2
Lorises—Loridae	2
Platypus—Ornithorhynchidae	1
Solenodons—Solenodontidae	2
Shrews—Soricidae	2
Ray-finned fishes—Actinopterygii[c]	~1335–1650+ venomous species
Catfishes—Siluriformes	~750–1000+ venomous species
Spiny-rayed fishes—Acanthomorpha	~585–650 venomous species
Toadfishes—Thalassophryninae	11
Stargazers—Uranoscopidae	49
Weeverfishes—Trachinidae	9
Blennies—*Meiacanthus*	25
Jacks—Scomeroidinae	11
Rabbitfishes—Siganidae	27
Surgeonfishes—Acanthuridae	45-80
Scats—Scatophagidae	4
Gurnard perches—*Neosebastes*	12
Scorpionfishes	
Caracanthidae	4
Scorpaenidae	185–200
Sebastidae	115–130
Setarchidae	5
Stonefishes	
Aploactinidae[d]	3
Apistidae	3
Gnathanacanthidae	1
Synanceiidae	36
Tetrarogidae	42

[a] Froese and Pauly (2004) and Halstead (1970, 1988).

[b] Alterman (1995), de Plater et al. (1995), Jackson (2003), Mebs (1999), and Russell and Boger (1981).

[c] Cameron and Endean (1972), Church and Hodgson (2002), de Pinna (1993), Fishelson (1974), Froese and Pauly (2004), Halstead (1970, 1988), Hardman (2002), Imamura (2004), Ishida (1994), Karmakar et al. (2004), Nelson (1994), Poss (1982), Randall et al. (1997), Rifkin and Williamson (1996), Smith and Wheeler (2004), Smith-Vaniz et al. (2001), Sosa-Rosales et al. (2005), and Southcott (1975).

[d] Unpublished data suggest that the aploactinid genus *Ptarmus*, which was found to have a venom gland, may be more appropriately classified in the Gnathanacanthidae, but we retain this genus in the Aploactinidae following Froese and Pauly (2004).

TREEROT 2b (Sorenson MD, unpublished; http://people.bu.edu/msoren/TreeRot.html) in conjunction with PAUP* 4.0b10 (Swofford DL, unpublished; http://paup.csit.fsu.edu/). Character evolution on the recovered topologies was examined using NONA and WINCLADA 0.99 (Nixon KC, unpublished; http://www.cladistics.com).

Morphological Examination

After completing the molecular phylogeny, we examined preserved museum specimens for the presence of both a venom delivery structure (e.g., spine, teeth) and a conspicuous venom gland to test the effectiveness of our phylogeny for predicting the distribution of venomous spiny-rayed fishes. Testing using these anatomical features has been the dominant method for establishing whether a fish is venomous because venomous fishes must have both a toxin, housed in a discrete gland, and a specialized delivery system (Halstead 1970, 1988). Previous studies have shown that both the gland and the delivery system are visible by dissection in all groups except venomous jacks (Fishelson 1974; Halstead 1988).

The presence or absence of a venom apparatus was examined in 102 museum specimens spread across 42 families (Table 1). Our sampling focused on species that are predicted to be venomous (e.g., additional scorpionfishes, surgeonfishes, rabbitfishes), species listed as possibly venomous in previous studies (e.g., Halstead 1970), or species that were closely allied to the venomous clades recovered in our phylogeny.

The estimates for venomous cartilaginous and lobe-finned fishes were taken from the literature (as given in Table 2). Because the distribution and number of venomous ray-finned fishes have not been studied as extensively, these numbers were estimated using the previously reported distribution of venom in spiny-rayed fishes (Supplementary Table 1), the results of our examination of 102 museum specimens (Table 1), previously published phylogenies and taxonomies (Nelson 1994 and references listed in Tables 1 and 2), our current phylogeny, and the current number of described species in each clade (Froese and Pauly 2004). If the distribution of venom within a small clade that lacks diagnosed subgroups (e.g., *Acanthurus* surgeonfishes) was unclear because both venomous and nonvenomous forms have been noted, a range is given. Because the phylogeny and distribution of venom in catfishes is less well known and not examined in the current study, the estimated number of venomous catfishes was never extended beyond the level of the family. Therefore, the listed values for catfishes are clearly underestimates because there are countless anecdotal reports (Froese and Pauly 2004; Halstead 1970, 1988) of venom in other catfish families and the presence or absence of venom in most catfish families has not been reported.

Results

Molecular Phylogeny

The phylogenetic analysis resulted in a single most parsimonious hypothesis with 27,395 steps (Figures 1 and 2). This cladogram had a consistency index of 0.37 and a retention index of 0.57 when parsimony-uninformative characters

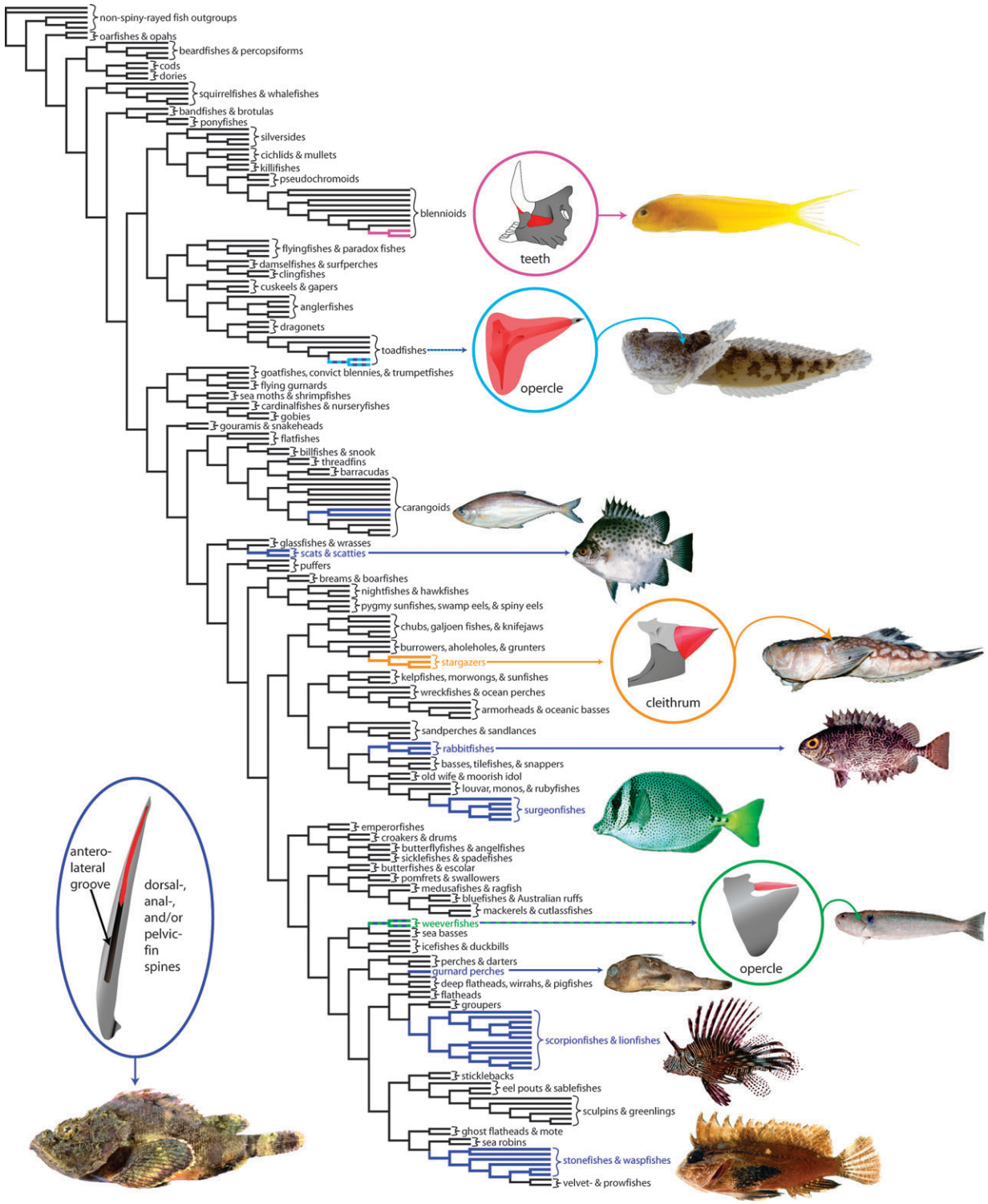

Figure 1. Phylogeny of venomous spiny-rayed fishes. A representative of each of the resulting venomous clades is figured adjacent to the group on the phylogeny. Venomous fish species have colored (nonblack) branches with their venom apparatus morphology optimized on the cladogram. All species with venomous dorsal-, anal-, and/or pelvic-fin spines have blue or blue-hatched branches (illustrated in the bottom-left corner of the figure). Species with other venom apparatuses are color coded and have the apparatus illustrated between the clade and the representative species figure. Venom glands are illustrated in red for each venom apparatus. See Figure 2 for scientific names of included taxa. Many of the schematic illustrations are modified from Halstead (1988) or Smith-Vaniz et al. (2001).

were retained (based on the implied alignment). Our results are largely congruent with the traditional classification of spiny-rayed fishes (Nelson 1994). Approximately two-thirds of the orders, suborders, and families that were represented by multiple species were recovered as monophyletic groups. The only order containing venomous species resolved as monophyletic in our phylogeny was the toadfishes (Batrachoidiformes). The remaining spiny-rayed fish orders with venomous species, Scorpaeniformes (scorpionfishes) and Perciformes (perchlike fishes), were resolved as polyphyletic, corroborating the results of recent studies (Chen et al. 2003; Johnson 1993; Miya et al. 2003; Smith and Wheeler 2004; Tang et al. 1999). Based on our phylogeny, venom apparatuses have originated 11 independent times in spiny-rayed fishes (Figures 1 and 2), nearly doubling previous estimates (Halstead 1988). Furthermore, our results, in combination with studies on the phylogeny of catfishes (de Pinna 1993; Hardman 2002), suggest that >1,200 fish species should be presumed venomous (Table 2), and it is likely that 1,500–2,000 ray-finned fishes may be venomous when catfishes are examined in detail. Clearly, this greatly increases the previous estimate of ~200 venomous fish species (Church and Hodgson 2002; Haddad et al. 2003; Halstead 1970, 1988; Vetrano et al. 2002; Supplementary Table 1). Our results also indicate that the most common venom apparatus (Halstead 1970, 1988), venom glands associated with fin spines, have convergently evolved in nine of the 11 venomous spiny-rayed fish clades (blue or hatched blue branches in Figure 1). The four remaining envenomation structures (i.e., teeth, dorsal opercular spines, central opercular spines, and cleithral spines; Halstead 1970, 1988; Smith-Vaniz et al. 2001) are unreversed and uniquely derived.

Morphological Examination

The results of our morphological examination (Table 1) provide evidence that our molecular phylogeny is highly effective at predicting the presence or absence of venom glands in spiny-rayed fishes. Of the 43 families examined in the morphological study, we were unable to find a conspicuous venom gland or any indications of a venom apparatus in 24 families (Table 1), despite previous suggestions that some species in these groups were venomous (e.g., *Chelidonichthys*, *Zanclus*).

Because of the diverse phylogenetic distribution of venomous fin spines, it is not surprising that there is variation in the morphology of these structures (Figures 1 and 3). The venomous toadfishes (Figure 3A) have a distinct venom gland surrounding their dorsal spines; this is in contrast to nonvenomous dorsal spines in other toadfish species that lack a venom gland (Figure 3B). All fishes with venomous spines (except the toadfishes and jacks) have distinct anterolateral grooves on the lateral surfaces of the fin spines (Figure 3C), where the venom gland is situated. Interestingly, the only clade in which we predict a reversal from the presence to the absence of venomous fin-spine glands (the velvetfish and prowfish clade; Figure 1) shows a reversion in the fin spines to the primitive condition where the anterolateral grooves are absent (Figure 3D). During the course of this morphological investigation, three scorpionfish genera (*Neomerinthe*, *Pontinus*, and *Sebastolobus*) were found to have a modified venom apparatus. Our examination of six species in these three genera indicates that anterolateral grooves are present in all six species, but conspicuous venom glands associated with these grooves are lacking. However, the caudal margin of their fin spines have conspicuous glandular tissue (Figure 3F) that differs significantly from the typical muscle tissue found on the posterior margin of the spines in most nonvenomous spiny-rayed fishes (e.g., toadfishes, Figure 3B). We tentatively identify this structure as a venom gland, pending further study. Interestingly, these three genera, although classified into two families, form a clade in recent molecular analyses (Smith and Wheeler 2004, unpublished data).

To illustrate the remarkable similarity of venomous fin spines across the diversity of venomous spiny-rayed fishes, the dorsal-fin spine of a rabbitfish is shown (Figure 3G) for comparison to the distantly related scorpaenoid *Ptarmus* (Figure 3E). Finally, the highly modified venomous spines in *Synanceia* stonefishes show their distinct venom glands and venom duct (Halstead 1988; Figure 3H).

The other venom apparatuses examined in this study were remarkably similar to previous descriptions (e.g., Halstead 1988). Briefly, the opercular spines of venomous (Figure 3I) and nonvenomous (Figure 3J) toadfishes are shown to illustrate the presence of an opercular venom gland surrounding an opercular spine. A similar morphology is seen in the weeverfishes where the opercular venom gland surrounds the distal margin of the dorsal opercular spine (Figure 3K). Finally, a venomous fang from a saber-toothed blenny is shown, highlighting the grooved tooth that delivers the venom (Figure 3L).

The results of our survey of museum specimens clearly indicate that our phylogeny is effective at predicting the presence or absence of venom in spiny-rayed fishes. Because of this high level of predictability, our estimates of the number of fishes predicted to be venomous were unaltered by the results of our museum survey, with one exception. Because the fin-spine morphology of the scorpionfish genera *Neomerinthe*, *Pontinus*, and *Sebastolobus* has not been previously described and the morphology of the "probable" venom gland has not been examined histologically, we have used a range for the estimated number of venomous scorpionfishes that either includes or excludes the species classified in these three genera, until additional study confirms the presence of venom in these fishes. The results of this museum survey provide strong corroborative evidence for our hypothesis that the presence of venom in fishes has been drastically underestimated in previous reviews.

Road Map for the Bioprospecting of Piscine Venoms

In addition to its use for understanding the evolution and diversity of venomous fishes, our phylogeny can be used to make predictions about the biological activity of the venoms themselves. An example (Systematics Agenda 2000 1994) of the predictive power of phylogenies for bioprospecting comes from the plant kingdom. The drug paclitaxel

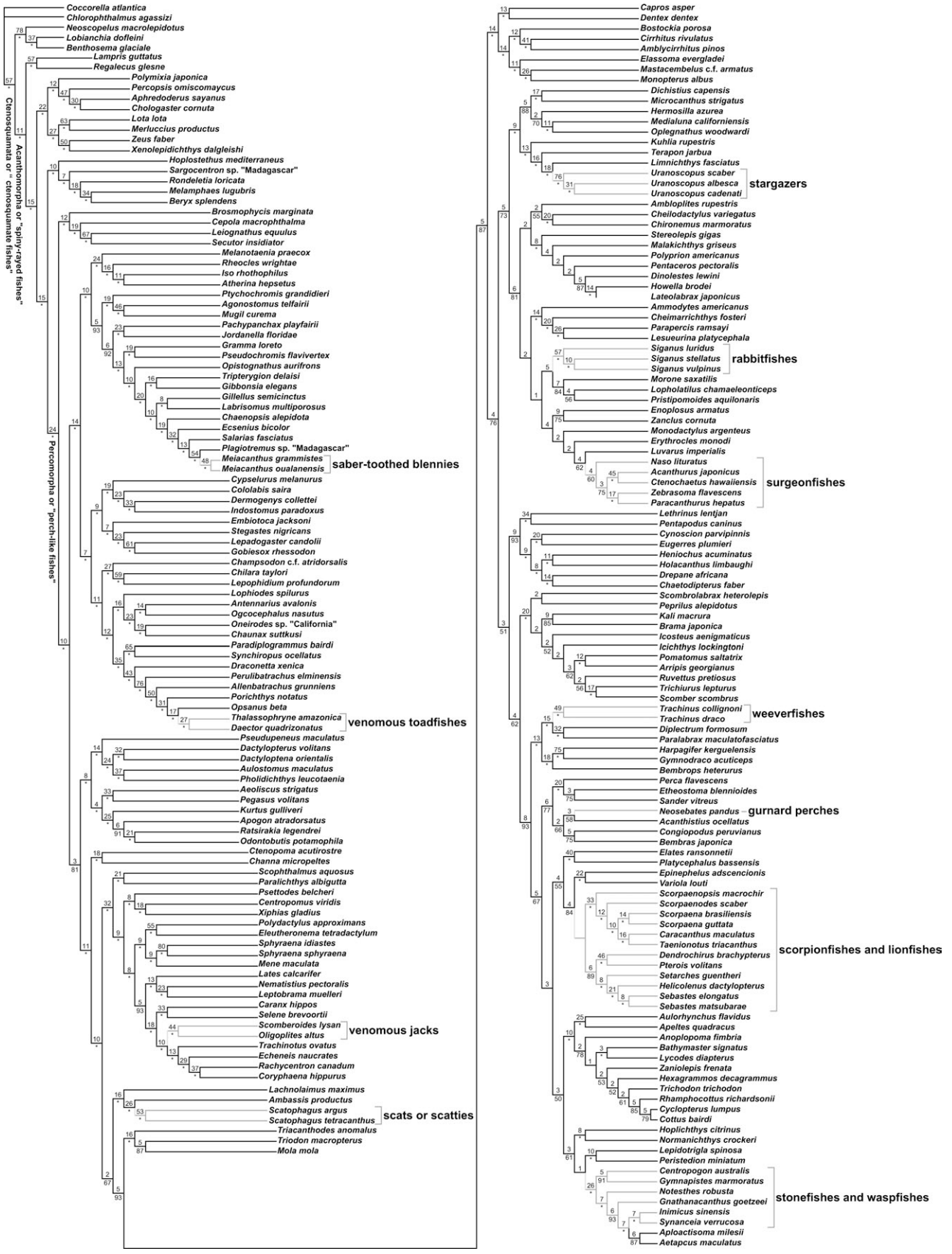
Coccorella atlantica
Chlorophthalmus agassizi
Neoscopelus macrolepidotus
Lobianchia dofleini
Benthosema glaciale
Lampris guttatus
Regalecus glesne
Polymixia japonica
Percopsis omiscomaycus
Aphredoderus sayanus
Chologaster cornuta
Lota lota
Merluccius productus
Zeus faber
Xenolepidichthys dalgleishi
Hoplostethus mediterraneus
Sargocentron sp. "Madagascar"
Rondeletia loricata
Melamphaes lugubris
Beryx splendens
Brosmophycis marginata
Cepola macrophthalma
Lelognathus equulus
Secutor insidiator
Melanotaenia praecox
Rheocles wrightae
Iso rhothophilus
Atherina hepsetus
Ptychochromis grandidieri
Agonostomus telfairii
Mugil curema
Pachypanchax playfairii
Jordanella floridae
Gramma loreto
Pseudochromis flavivertex
Opistognathus aurifrons
Tripterygion delaisi
Gibbonsia elegans
Gillellus semicinctus
Labrisomus multiporosus
Chaenopsis alepidota
Ecsenius bicolor
Salarias fasciatus
Plagiotremus sp. "Madagascar"
Melacanthus grammistes
Melacanthus oualanensis
saber-toothed blennies
Cypselurus melanurus
Cololabis saira
Dermogenys collettei
Indostomus paradoxus
Embiotoca jacksoni
Stegastes nigricans
Lepadogaster candolii
Gobiesox rhessodon
Champsodon c.f. atridorsalis
Chilara taylori
Lepophidium profundorum
Lophiodes spilurus
Antennarius avalonis
Ogcocephalus nasutus
Oneirodes sp. "California"
Chaunax suttkusi
Paradiplogrammus bairdi
Synchiropus ocellatus
Draconetta xenica
Perulibatrachus elminensis
Allenbatrachus grunniens
Porichthys notatus
Opsanus beta
Thalassophryne amazonica
Daector quadrizonatus
venomous toadfishes
Pseudupeneus maculatus
Dactylopterus volitans
Dactyloptena orientalis
Aulostomus maculatus
Pholidichthys leucotaenia
Aeoliscus strigatus
Pegasus volitans
Kurtus gulliveri
Apogon atradorsatus
Ratsirakia legendrei
Odontobutis potamophila
Ctenopoma acutirostre
Channa micropeltes
Scophthalmus aquosus
Paralichthys albigutta
Psettodes belcheri
Centropomus viridis
Xiphias gladius
Polydactylus approximans
Eleutheronema tetradactylum
Sphyraena idiastes
Sphyraena sphyraena
Mene maculata
Lates calcarifer
Nematistius pectoralis
Leptobrama muelleri
Caranx hippos
Selene brevoortii
Scomberoides lysan
Oligoplites altus
venomous jacks
Trachinotus ovatus
Echeneis naucrates
Rachycentron canadum
Coryphaena hippurus
Lachnolaimus maximus
Ambassis productus
Scatophagus argus
Scatophagus tetracanthus
scats or scatties
Triacanthodes anomalus
Triodon macropterus
Mola mola
Ctenosquamata or "ctenosquamate fishes"
Acanthomorpha or "spiny-rayed fishes"
Percomorpha or "perch-like fishes"
Capros asper
Dentex dentex
Bostockia porosa
Cirrhitus rivulatus
Amblycirrhitus pinos
Elassoma evergladei
Mastacembelus c.f. armatus
Monopterus albus
Dichistius capensis
Microcanthus strigatus
Hermosilla azurea
Medialuna californiensis
Oplegnathus woodwardi
Kuhlia rupestris
Terapon jarbua
Limnichthys fasciatus
Uranoscopus scaber
Uranoscopus albesca
Uranoscopus cadenati
stargazers
Ambloplites rupestris
Cheilodactylus variegatus
Chironemus marmoratus
Stereolepis gigas
Malakichthys griseus
Polyprion americanus
Pentaceros pectoralis
Dinolestes lewini
Howella brodei
Lateolabrax japonicus
Ammodytes americanus
Cheimarrichthys fosteri
Parapercis ramsayi
Lesueurina platycephala
Siganus luridus
Siganus stellatus
Siganus vulpinus
rabbitfishes
Morone saxatilis
Lopholatilus chamaeleonticeps
Pristipomoides aquilonaris
Enoplosus armatus
Zanclus cornuta
Monodactylus argenteus
Erythrocles monodi
Luvarus imperialis
Naso lituratus
Acanthurus japonicus
Ctenochaetus hawaiiensis
Zebrasoma flavescens
Paracanthurus hepatus
surgeonfishes
Lethrinus lentjan
Pentapodus caninus
Cynoscion parvipinnis
Eugerres plumieri
Heniochus acuminatus
Holacanthus limbaughi
Drepane africana
Chaetodipterus faber
Scombrolabrax heterolepis
Peprilus alepidotus
Kali macrura
Brama japonica
Icosteus aenigmaticus
Icichthys lockingtoni
Pomatomus saltatrix
Arripis georgianus
Ruvettus pretiosus
Trichiurus lepturus
Scomber scombrus
Trachinus collignoni
Trachinus draco
weeverfishes
Diplectrum formosum
Paralabrax maculatofasciatus
Harpagifer kerguelensis
Gymnodraco acuticeps
Bembrops heterurus
Perca flavescens
Etheostoma blennioides
Sander vitreus
Neosebates pandus
gurnard perches
Acanthistius ocellatus
Congiopodus peruvianus
Bembras japonica
Elates ransonnetii
Platycephalus bassensis
Epinephelus adscencionis
Variola louti
Scorpaenopsis macrochir
Scorpaenodes scaber
Scorpaena brasiliensis
Scorpaena guttata
Caracanthus maculatus
Taenionotus triacanthus
Dendrochirus brachypterus
Pterois volitans
Setarches guentheri
Helicolenus dactylopterus
Sebastes elongatus
Sebastes matsubarae
scorpionfishes and lionfishes
Aulorhynchus flavidus
Apeltes quadracus
Anoplopoma fimbria
Bathymaster signatus
Lycodes diapterus
Zaniolepis frenata
Hexagrammos decagrammus
Trichodon trichodon
Rhamphocottus richardsonii
Cyclopterus lumpus
Cottus bairdi
Hoplichthys citrinus
Normanichthys crockeri
Lepidotrigla spinosa
Peristedion miniatum
Centropogon australis
Gymnapistes marmoratus
Notesthes robusta
Gnathanacanthus goetzeei
Inimicus sinensis
Synancela verrucosa
Aploactisoma milesii
Aetapcus maculatus
stonefishes and waspfishes

Figure 3. Venom apparatus morphology. (**A**) Venomous dorsal spines of toadfish *Thalassophryne amazonica*, AMNH uncat. (**B**) Nonvenomous dorsal spines of toadfish *Opsanus beta*, AMNH 15482. (**C**) Anterolateral groove on dorsal spine of venomous gurnard perch *Neosebastes thetidis*, AMNH 99340SD. (**D**) Alizarin-stained (red) dorsal spine lacking anterolateral groove in prowfish *Pataecus fronto*, SU 67408. (**E**) Venomous dorsal spine of velvetfish *Ptarmus jubatus*, AMNH 19599. (**F**) Dorsal spine of scorpionfish *Neomerinthe hemingwayi*, AMNH 83911, showing a possible venom gland on the caudal margin of the spine. (**G**) Venomous dorsal spine of rabbitfish *Siganus stellatus*, AMNH 232551. (**H**) Venomous dorsal spine with enlarged venom glands in the stonefish *Synanceia verrucosa*, AMNH uncat. (**I**) Venomous opercular spine of a toadfish *Thalassophryne amazonica*, AMNH uncat. (**J**) Nonvenomous opercular spines of a toadfish *O. beta*, AMNH 15482. (**K**) Venomous opercular spine of a weeverfish *Trachinus araneus*, AMNH 9168. (**L**) Venomous fang from the lower jaw of saber-toothed blenny *Meiacanthus grammistes*, AMNH 213840. Abbreviations: ag, anterodorsal groove; m, muscle; os, opercular spine; and vg, venom gland.

(Taxol™), which is used to treat ovarian and breast cancer, was originally extracted from the bark of the threatened Pacific Yew (*Taxus brevifolia*), but each dose required the destruction of several trees. Fortunately, our understanding of the relationships among yews led researchers to examine its close relatives for similar natural products, resulting in the

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Figure 2. Phylogeny of venomous spiny-rayed fishes. The topology is identical to Figure 1. The scientific names for all the species are listed on the terminals, and the support values (Bremer above and jackknife below) are listed on the nodes. Jackknife support values <50% are omitted from the cladogram, and nodes with ≥95% jackknife support are marked with an "*." Venomous fishes are indicated by the use of gray branches.

discovery that the leaves of the European Yew (*Taxus baccata*) had 10-deacetylbaccatin III, which could be used as a precursor to synthesize paclitaxel without harm to yew populations. Our results provide biochemists with a similar predictive road map for the efficient testing and tracing of venoms for beneficial compounds. Without this road map, it would be prohibitively expensive and time consuming to isolate and characterize the thousands of venoms that are found in spiny-rayed fishes.

The generation of a piscine venom road map is particularly helpful for expanding our current limited understanding of fish venom biological activity. Recent reviews (Church and Hodgson 2002; Halstead 1988; Sosa-Rosales et al. 2005) indicate that fewer than one dozen fish venoms have been characterized and identified (Supplementary Table 1); therefore, this sampling only allows for a few preliminary trends to be noted. First, fish venoms are unusual in that only one to a few toxins per species possess all of the lethal or hemolytic activity (Church and Hodgson 2002). This is in stark contrast to cone snails or terrestrial animals, which often have hundreds of venoms per species (e.g., Bulaj et al. 2003). This small number of venoms per species will allow natural-product chemists to more easily trace the evolution of these individual toxins without the confounding homology problems inherent in studies tracing venoms in species with hundreds of toxins (e.g., gene identification, gene loss, gene duplication). Despite limited venom diversity within species, fish venoms exhibit a surprisingly wide range of pharmacological effects, including neuromuscular, cytolytic, hemolytic, and particularly cardiovascular activity (reviewed in Church and Hodgson 2002). Clearly, additional study may overturn these preliminary observations, but our current knowledge provides the starting point for future bioprospecting of fish venoms as potential pharmacological agents and physiological tools.

Ultimately, our study provides the first explicit suborder-level phylogeny of spiny-rayed fishes, which we used to delimit venomous clades. Using the predictive capabilities of phylogeny, prior knowledge of the distribution of venomous fishes, and an extensive survey for the presence or absence of conspicuous venom glands in spiny-rayed fishes, we have estimated the number and identity of venomous ray-finned fishes. Our results suggest at least a sixfold increase in the number of venomous species and a nearly twofold increase in the number of origins of envenomation structures. Additionally, our phylogeny provides a framework for studying the biological activity of piscine venoms in a predictive, evolutionary context. The next step in their bioprospecting is additional isolation and characterization of toxins from several species within each of the 11 venomous spiny-rayed fish clades. These preliminary assays can then be used, in conjunction with our phylogeny and further fine-scaled systematic studies (i.e., species-level phylogenies), to target species with venoms that provide novel structures or have desirable qualities for use as research tools or lead compounds for drugs. In an era when traditional bioprospecting and high-throughput screening of mass-produced combinatorial libraries have failed to meet expectations for the development of novel pharmaceutical compounds (Newman et al. 2003), a fresh approach is clearly needed. The phylogenetic approach favored by Halstead (1988, see quote above) and highlighted here serves as a model for the efficient discovery and exploitation of untapped natural products, which continue to play the dominant role in the discovery of leads for novel pharmaceuticals (Newman et al. 2003).

Supplementary Material

Supplementary Table 1 and other supplementary information are available online at http://jhered.oxfordjournals.org/.

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