(*13*), the *scid* fibroblasts had impaired coding join formation but, unlike xrs-6 and XR-1 cells, had a relatively normal level and fidelity (80% precise) of RS joins (Table 1). This finding suggested that the *scid* mutation affects a different gene than the xrs-6 and XR-1 mutations. To verify this conclusion, we made multiple cell hybrids between these lines and found a complete complementation of radiation sensitivity. Furthermore, V(D)J recombination activity was restored to normal in the one hybrid (xrs-6/*scid*) tested (Table 1). We conclude that xrs-6, XR-1, and *scid* represent three different genes involved in V(D)J recombination.

The xrs-6 and XR-1 mutations affect both RS and coding join formation (Fig. 1E); however, recognition and introduction of double-strand breaks appear to occur normally in these mutants because some RS or coding joins have at least one end at or near the RS or coding junction, and recovered RS and coding joins linked sequences from the expected side of the involved elements. Therefore, it is likely that these mutations either result in hyperexonucleolytic activity or impair the normal joining process such that only illegitimate recombination events are recovered. The presence of extra nucleotides resembling normal P elements [(*14*) and Fig. 3] in the XR-1 mutant suggests that the defect in this mutant affects a V(D)J recombination step downstream of that involved in the generation of these elements and the activity affected by the *scid* defect (*13*, *15*).

V(D)J recombination has some mechanistic similarities to certain transposition events. For example, P element transposition in *Drosophila* occurs by a cut-and-paste process usually followed by double-strand gap repair to restore the donor P element (*16*). A gene product (*mei-41*) necessary for recovery of P-bearing chromosomes undergoing transposition has also has been implicated in DSBR (*17*). Furthermore, protection of ends in DSB intermediates by a stable protein complex occurs in Tn7 and Tn10 transposition in *Escherichia coli* (*18*). In the radiosensitive Rad52 epistatis group of yeast, mutants show defects in meiotic and mitotic recombination (*19*, *20*). Furthermore, yeast exhibit extensive exonucleolytic processing of broken DNA ends during meiosis and mating-type switching (*21*). In this context, the xrs-6 or XR-1 mutations might lead to a failure to protect free ends from extensive degradation by either the V(D)J complex or by normal cellular exonucleases.

REFERENCES AND NOTES

1. F. W. Alt *et al.*, *Immunol. Today* **13**, 306 (1992).
2. D. G. Schatz, M. A. Oettinger, D. Baltimore, *Cell* **59**, 1035 (1989); M. A. Oettinger, D. G. Schatz, C. Gorka, D. Baltimore, *Science* **248**, 1517 (1990).
3. M. J. Bosma and A. M. Carroll, *Annu. Rev. Immunol.* **9**, 323 (1991).
4. B. A. Malynn *et al.*, *Cell* **54**, 453 (1988); M. R. Lieber *et al.*, *ibid.* **55**, 7 (1988); T. K. Blackwell *et al.*, *EMBO J.* **8**, 735 (1989).
5. G. M. Fulop and R. A. Phillips, *Nature* **347**, 479 (1990); K. A. Biedermann *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1394 (1991); E. A. Hendrickson *et al.*, *ibid.*, p. 4061.
6. J. E. Hesse, M. R. Lieber, M. Gellert, K. Mizuuchi, *Cell* **49**, 775 (1987).
7. One microgram of the corresponding recombination substrates pJH200 or pJH290 (*6*) was cotransfected with 3 μg of each of the genomic expression vectors RAG-1 and RAG-2 under the control of the RSV-LTR promoter (*1*) with the use of standard $CaPO_4$ method. Mock transfections were performed in the corresponding wild-type cell line transfected only with the recombination substrate. This transient assay procedure to test for ability to rearrange introduced V(D)J recombination substrates has been described (*6*).
8. J. H. J. Hoeijmakers and D. Bootsma, *Cancer Cells* **2**, 311 (1990).
9. P. A. Jeggo, *Mutat. Res.* **239**, 1 (1990).
10. C. N. Robson, A. L. Harris, I. D. Hickson, *ibid.* **217**, 93 (1989).
11. P. A. Jeggo *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6423 (1992).
12. A. J. Giaccia *et al.*, *Am. J. Hum. Genet.* **747**, 459 (1990).
13. M. A. Lieber, *FASEB J.* **5**, 2934 (1991).
14. J. J. Lafaille *et al.*, *Cell* **59**, 859 (1989).
15. D. B. Roth *et al.*, *ibid.* **70**, 1 (1992).
16. W. R. Engels, D. M. Johnson-Schlitz, W. B. Eggleston, J. Sved, *ibid.* **62**, 515 (1990).
17. S. S. Banga, A. Velazquez, J. B. Boyd, *Mutat. Res.* **255**, 79 (1991).
18. R. Bainton, P. Games, N. L. Craig, *Cell* **65**, 805 (1991); D. B. Haniford, H. W. Benjamin, N. Kleckner, *ibid.* **64**, 171 (1991).
19. A. Shinohara, H. Ogawa, T. Ogawa, *ibid.* **69**, 457 (1992).
20. E. C. Friedberg, *Microbiol. Rev.* **52**, 70 (1988).
21. L. Cao, E. Alani, N. Kleckner, *Cell* **61**, 1089 (1990).
22. The RS joins from recovered plasmids were amplified by PCR with the primers 5′-CTTTCATTGCCATACG-3′ and 5′-AGCGGATAACAATTTCACACAGGA-3′. Twenty-five cycles of amplification were performed in 50 μl of 10 mM tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, and 50 ng of each primer plus 1.0 U of Taq polymerase (Perkin-Elmer). Denaturation was at 94°C for 45 s, annealing was at 52°C for 1 min, and elongation was at 72°C for 3 min. The PCR product was digested with Hind III and size-separated by electrophoresis on a 2.5% agarose gel. Precise V(D)J recombination generates a 150-bp band that contains the RS without the 200-bp OOP sequence. Alkaline-lysed minipreps were prepared and sequenced with a Sequenase kit (United States Biochemical Corp.) according to the manufacturer's instructions.
23. W. Schuler, N. R. Ruetsch, M. Amsler, M. J. Bosma, *Eur. J. Immunol.* **21**, 589 (1991); L. Kienker, W. A. Kuziel, P. W. Tucker, *J. Exp. Med.* **174**, 769 (1991).
24. The xrs-6Rev and xrs-6/Ch2(D5) are xrs-6 cells that have been reactivated after treatment with azacytidine (*9*) or complemented by the presence of human chromosome 2 (*11*), respectively. XR-1:Ch5 is the corresponding hybrid containing human chromosome 5 that fully complements the DSBR defect (*12*). The *scid*/xrs-6 and XR-1/xrs-6 are the two different kind of hybrids generated by cell fusion as described elsewhere (*9*). The results presented for the xrs-6 and XR-1 mutants plus the complemented derivatives of these lines were reproduced in at least three independent transfection experiments.
25. The UV-sensitive cell lines (UV and EM9) were provided by L. Thompson. The *scid* fibroblast cell line SCGR11 was provided by D. Weaver. The bleomycin (BLM-1 and BLM-2)- and adryamicin (ADR-3)-resistant CHO cell lines were provided by I. Hickson and C. Robson. The substrates pJH200 and pJH290 were provided by J. Hesse and M. Gellert. Supported by NIH grant A.I. 20047 (to F.W.A.); the Howard Hughes Medical Institute, EC contract B17-0026 (to P.A.J.); NIH grant CA45277 (to T.S.); and postdoctoral fellowships from the Irvington Institute (to G.E.T.) and the Cancer Research Institute (to E.O.).

6 November 1992; accepted 11 January 1993

Evolution of Endothermy in Fish: Mapping Physiological Traits on a Molecular Phylogeny

Barbara A. Block,* John R. Finnerty, Alexandre F. R. Stewart, Jessica Kidd

Mackerels, tunas, and billfishes (suborder Scombroidei and Teleostei) provide an ideal taxonomic context in which to examine the evolution of endothermy. Multiple origins and diverse strategies for endothermy exist among these fish. Here a molecular phylogeny of the Scombroidei has been determined by direct sequencing of a portion of the mitochondrial *cytochrome b* gene. The distribution of endothermic species within this proposed genealogy indicates that the ability to warm the brain and retina arose independently in three lineages, each time in association with a movement into colder water. This suggests that the evolution of cranial endothermy in fish was selected in order to permit thermal niche expansion and not selected for increased aerobic capacity.

The majority of the 30,000 species of teleost fishes are ectotherms with body temperatures within 1° to 2°C of ambient water temperature. Endothermy, the ability to maintain elevated body temperature by metabolic means, has been documented only within one major assemblage of large oceanic teleosts, the Scombroidei (*1–3*). Sharks of the family Lamnidae and Alopiidae have convergently evolved endothermy (*3*, *4*). The transition from ectothermy to endothermy requires the elevation of aerobic capacity (and hence heat production) and the reduction of the rate of

Department of Organismal Biology and Anatomy, University of Chicago, Chicago, IL 60637.

*To whom correspondence should be addressed.

heat loss. An inability to limit heat loss while respiring through gills prevents most fish from being warmer than the water in which they swim. Except for the difference in the mode of respiration and the resultant heat loss, the physiological requirements for the evolution of endothermy in fish should parallel those in terrestrial amniotes.

The anatomical and physiological requirements for endothermy and its selective advantage have received intensive study, particularly in avian and mammalian lineages (*5*). One major unresolved issue is whether endothermy is the result of selection for a high and stable body temperature, which permits the exploitation of a broad thermal niche, or selection for increased aerobic activity and improved locomotor performance (*5*). Within the Scombroidei, endothermy is multiply derived and phenotypically variable among extant species. Therefore, a comparison of an endothermic scombroid taxon with its ectothermic sister group is possible, which makes the Scombroidei particularly amenable to a study of the evolution of endothermy.

Two distinct strategies for elevating tissue temperatures have evolved in the Scombroidei. Tunas (Scombridae: *Thunnus*, *Katsuwonus*, *Euthynnus*, and *Auxis*) achieve the endothermic condition in a manner similar to birds and mammals. They have exceptionally high metabolic rates and have reduced whole body thermal conductance (*1*, *6*). In contrast, billfishes (Xiphiidae: *Xiphias*, and Istiophoridae: *Istiophorus*, *Makaira* and *Tetrapturus*) use cranial endothermy and warm only the brain and eyes (*2*, *3*). The billfishes accomplish this more limited form of endothermy with the use of a thermogenic organ composed of highly modified extraocular muscle fibers (*7*). In billfishes, expression of the heater phenotype (thermogenic muscle cells that produce heat without force generation) is associated with the superior rectus eye muscle. A countercurrent heat exchanger retains the heat beneath the brain, and a distinct arterial supply directs warm blood to the retina (*2*, *7*). One other scombroid, the butterfly mackerel, *Gasterochisma melampus*, also uses cranial endothermy, but the thermogenic tissue in this species is derived from the lateral rectus eye muscle (*2*). The lateral and superior rectus muscles derive from different somites during ontogeny implying separate evolutionary origins of the thermogenic cell type in *Gasterochisma* and billfishes. However, heater cells of *Gasterochisma* and billfishes are structurally identical and have similar biochemical properties (*8*), suggesting they are homologous. A close phylogenetic relationship between these taxa would support this latter possibility, particularly if billfishes and *Gasterochisma* share a common ancestor to the exclusion of other species lacking the thermogenic tissue type.

Tunas possess no tissue specialization for heat production and instead retain metabolic heat by way of vascular countercurrent heat exchangers located in the brain, muscle, and viscera (*1*). Red aerobic muscle, which powers sustained swimming, contributes a major fraction of this metabolically derived heat. In most teleosts, the red muscle is located laterally, just beneath the skin. In tunas, this muscle occupies an atypi-

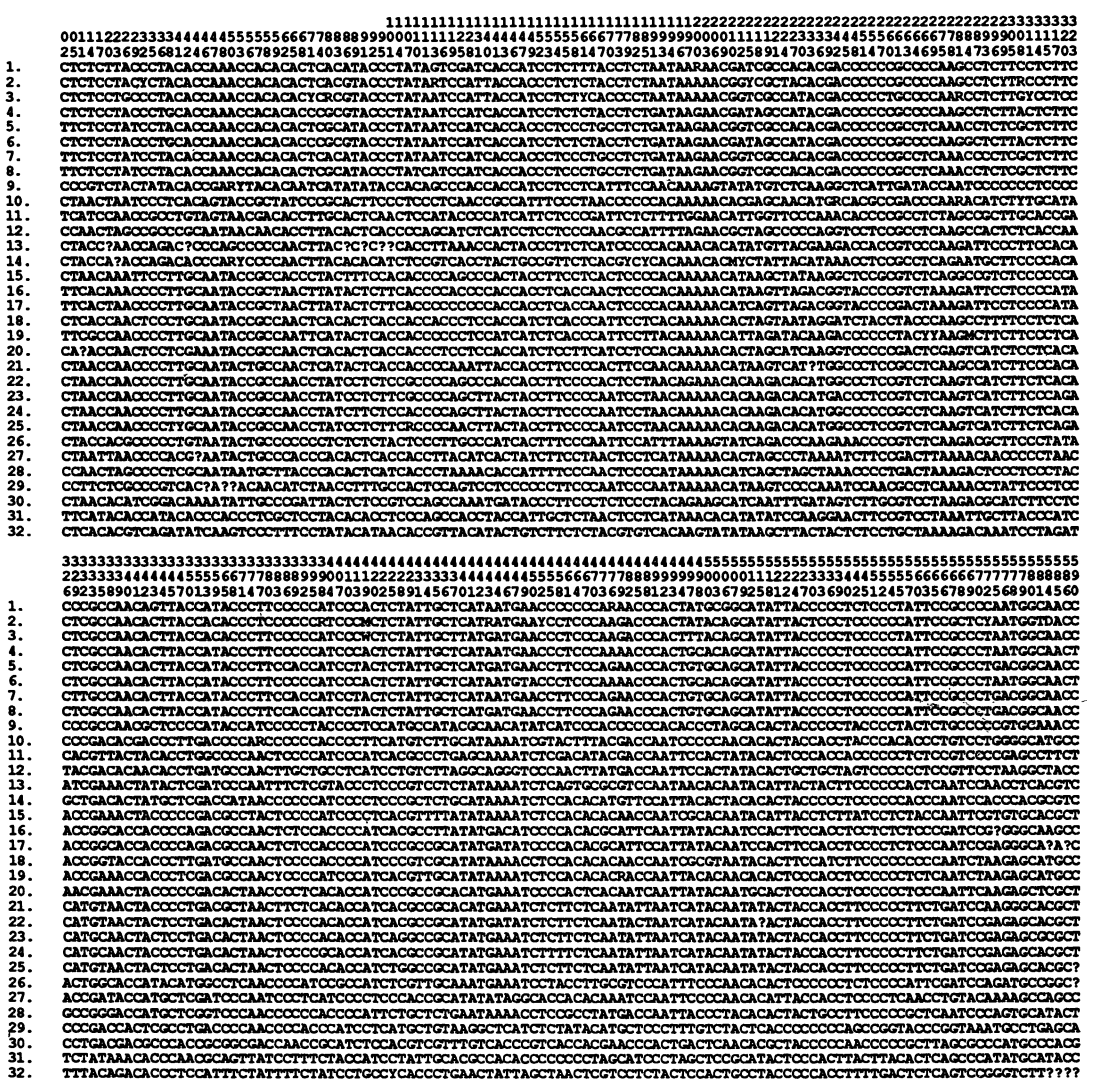

Fig. 1. Two hundred and eighteen phylogenetically informative characters derived from a 600–base pair fragment of the *cytochrome b* gene. Character numbers above the sequences refer to positions within this fragment. Position 1 corresponds to position 14,880 of the human mitochondrial genome. Numbers to the side of the sequence data refers to the following species: 1, *Makaira indica*; 2, *Makaira nigricans*; 3, *Istiophorus platypterus*; 4, *Tetrapturus albidus*; 5, *Tetrapturus angustirostris*; 6, *Tetrapturus audax*; 7, *Tetrapturus belone*; 8, *Tetrapturus pfluegeri*; 9, *Xiphias gladius*; 10, *Gasterochisma melampus*; 11, *Scomber japonicus*; 12, *Scomber scombrus*; 13, *Scomberomorus cavalla*; 14, *Scomberomorus maculata*; 15, *Sarda chiliensis*; 16, *Sarda sarda*; 17, *Auxis thazard*; 18, *Euthynnus affinis*; 19, *Euthynnus alletteratus*; 20, *Katsuwonus pelamis*; 21, *Thunnus alalunga*; 22, *Thunnus albacares*; 23, *Thunnus maccoyii*; 24, *Thunnus obesus*; 25, *Thunnus thynnus*; 26, *Gempylus serpens*; 27, *Lepidocybium flavobrunneum*; 28, *Ruvettus pretiosus*; 29, *Trichiurus lepturus*; 30, *Sphyraena sphyraena*; 31, *Serranidae*; 32, *Coryphaena equiselis*.

cal centralized location, and is closely apposed to the axial column (*1*, 9). The central positioning of red muscle in tunas has received much attention as a result of its role in endothermy; however, it may also reflect the unique locomotor style of the tunas, thunniform swimming (*10*). These fish generate thrust exclusively via the tail and restrict their propulsive movements to the caudal region (*11*). Evolution of the locomotor apparatus and endothermy may be tightly coupled in this lineage; thunniform swimming arises concomitantly with a movement of the aerobic red muscle internally, and this central aerobic muscle mass is the major source of metabolic heat in tunas (*12*).

Hypotheses concerning the evolution of endothermy in the Scombroidei require testing in a phylogenetic context (*13*). We address three specific points: (i) the number of times endothermy has originated in the Scombroidei; (ii) the selective pressures that favored the evolution of endothermy in this suborder; and (iii) the reason for the evolution of two distinct endothermic strategies. Recent studies based on morphological characters address systematic relationships within the suborder Scombroidei (*14*, *15*). These studies disagree over the taxonomic affinities of Xiphiidae, Istiophoridae, and *Gasterochisma* taxa that are key to the study of the evolution of endothermy within the suborder. In an attempt to resolve these relationships, we have produced a hypothesis of scombroid phylogeny based on molecular data. By mapping characters that are putatively associated with the evolution of endothermy on an independently derived phylogeny for the scombroid fishes, we provide a hypothesis for the evolution of diverse forms of endothermy in fish.

With the polymerase chain reaction (*16*) and dideoxy sequencing (*17*), we amplified two overlapping sections from the *cytochrome b* gene and sequenced 600 base pairs from 75 individuals including 29 scombroid species and 3 additional percoid taxa. Parsimony analysis (*18*) based on 218 phylogenetically informative characters (Fig. 1) identified a single minimum length tree (Fig. 2). Data presented here are an important addition to our understanding of scombroid systematics because the *cytochrome b* sequence is informative about the affinities of key taxa whose relations based on morphology are in dispute, in particular, the billfishes and *Gasterochisma melampus*. In contrast to recent morphological results (*14*, *15*), the molecular data support a monophyletic billfish clade (Istiophoridae plus Xiphiidae) with no close affinities to other scombroids (Scombridae, Trichiuridae, and Gempylidae). Both molecular and morphological data (*14*, *15*) support the monophyly of endothermic tunas (*Auxis*, *Euthynnus*, *Katsuwonus*, and *Thunnus*) and place ectothermic bonitos (*Sarda*) as their closest relatives. *Gasterochisma melampus* has proven difficult to place taxonomically by morphological criteria because it possesses a suite of primitive and uniquely derived characteristics (*2*, *7*, *19*, *20*). According to the *cytochrome b* sequence analysis, *G. melampus* falls within a radiation which includes primitive scombrids (*Scomber*) and gempylids (*Ruvettus*). However, the nodes within this region of the tree are not strongly supported because terminal branches are relatively long when compared to the internal branches. This placement of *Gasterochisma* is congruent with a recent osteological study (*19*). Both morphological and molecular data suggest this species evolved independently for a long period.

Mapping the distribution of the en-

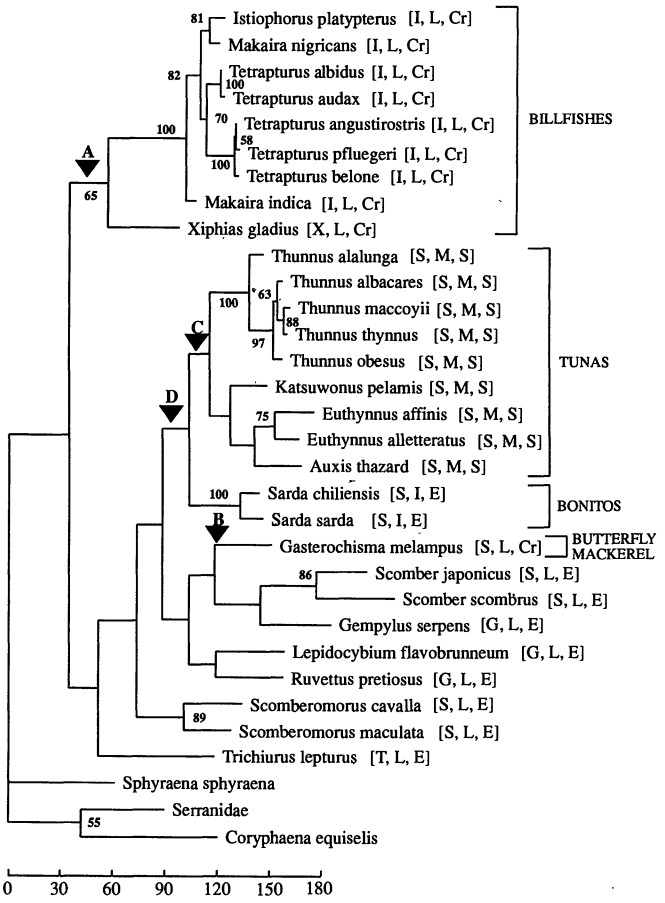

Fig. 2. Phylogeny of the Scombroidei. The single most parsimonious tree was generated by PAUP on 218 phylogenetically informative characters from a 600–base pair region of the *cytochrome b* gene of 29 scombroids and 3 outgroup taxa (*18*). The exact species identification of one of the outgroups (Serranidae) is unknown. Fifty replications of a heuristic search procedure were performed with random stepwise addition to generate starting trees. Tree length is 1292 steps (retention index, 0.589). Branch lengths depicted are proportional to the number of substitutions which occurred along the branch (scale at lower left). Numbers indicate the percentage of trials that support a given node in 300 replications of the bootstrap. Only nodes supported at >50% are indicated. Arrows indicate inferred evolutionary events: A, modification of the superior rectus muscle into a thermogenic organ (countercurrent heat exchanger formed from the carotid artery); B, modification of the lateral rectus muscle into a thermogenic organ (heat exchanger derived from the lateral dorsal aorta); C, systemic endothermy using vascular countercurrent heat exchangers in the muscle, viscera, and brain (heat exchanger in the brain is formed from the carotid artery); and D, some internalization of red muscle along the horizontal septum. Ten additional steps are required to produce a tree topology which indicates less than three independent origins of endothermy. Letters in brackets indicate family (I, Istiophoridae; X, Xiphiidae; S, Scombridae; G, Gempylidae; and T, Trichiuridae), red muscle positioning (L, lateral: red muscle is restricted to a lateral position; M, medial: internalization of red muscle along the horizontal septum occurs; I, intermediate: more red muscle is internalized than lateral condition), and metabolic strategy (Cr, cranial endothermy; S, systemic endothermy; and E, ectothermy). The outgroups (*Sphyraena*, Serranidae, and *Coryphaena*) are ectotherms with laterally placed red muscle.

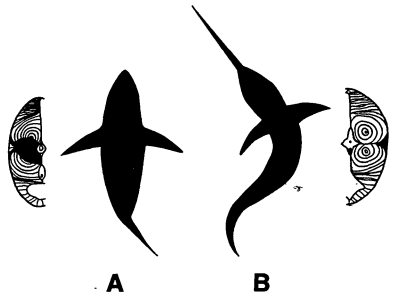

Fig. 3. Diagrammatic representation of dorsal view of swimming and body undulation in tunas versus billfishes. (**A**) In tunas, the body remains stiff whereas the caudal peduncle and tail are the point of flexion. A view of one-half of a cross section reveals central positioning of red fibers (dark area). In billfishes (**B**), most of the postcranial body is involved in propagation of the propulsive wave associated with swimming and a cross section reveals lateral placement of red fibers within the epaxial musculature.

dothermic strategies on the phylogeny indicates that endothermy evolved at least three times within the Scombroidei (Fig. 2). Cranial endothermy, which involves a thermogenic organ, evolved twice. Xiphiidae (*Xiphias*) and Istiophoridae (*Istiophorus*, *Makaira*, and *Tetrapturus*) share a common ancestor to the exclusion of other scombroids, indicating that a thermogenic organ associated with the superior rectus is a synapomorphy of these taxa (*21*). However, *Gasterochisma* is nested within a separate clade, which refutes the possibility that the thermogenic cell type of *Gasterochisma* is homologous to that of the billfishes. Endothermic tunas comprise a derived clade within the Scombridae, separate from *Gasterochisma*, which supports a single unique origin for the systemic endothermy characteristic of this group.

The repeated evolution of endothermy in the Scombroidei suggests that there is strong selection for this energetically costly metabolic strategy. All three endothermic scombroid lineages have expanded their ranges (*22*) into cool temperate waters (50°N and S); this supports the primacy of niche expansion over increased aerobic capacity as a selective force. The adaptive value of warming the brain and eyes, a characteristic of all three endothermic lineages, seems clear. Whereas brain and eye warming cannot improve locomotor performance or increase aerobic scope, the ability to warm the central nervous system permits these active, visually oriented predators to forage over a wide temperature range (*2*, *3*). For example, in swordfish, *Xiphias gladius*, brain temperature remains constant during deep dives when ambient water temperature changes as much as 19°C (*2*). Warming the brain permits the swordfish to feed on vertically migrating squid both at the surface and at depth. Other large predators (cetaceans, tunas) that exploit this vertically migrating community in the same intensive fashion also minimize variations in tissue temperature. Swordfish, as well as certain tunas and sharks, use behavioral and physiological thermoregulation to reduce conductive and convective heat loss and increase foraging time in cold waters (*2*, *23*). Thus, in many of the largest oceanic fishes, daily and seasonal movement away from oligotrophic tropical waters into colder, more nutrient rich seas, has been accompanied by the evolution of some form of endothermy.

In contrast to fishes with thermogenic organs, endothermy in tunas is not restricted to warming the central nervous system, but includes warming of the muscle and viscera (*1*). The ability to warm brain, muscle, and viscera in tunas represents at least three independent evolutionary events: the acquisition of countercurrent heat exchangers in three separate anatomical locations. Therefore, different selection pressures may have favored the evolution of brain and muscle warming mechanisms. Regardless of the selective advantage of warm muscles, their presence in tunas and absence in other scombroids may be a result of historical constraints. The phylogeny (Fig. 2) suggests a link between the tunas' ability to conserve metabolic heat from red muscle and their unique stiff-bodied mode of locomotion, thunniform swimming (*9*, *10*). Internalization of red muscle along the main horizontal septum is seen in the endothermic tunas which utilize the derived, stiff-bodied form of swimming (Fig. 3). Most scombroids utilize the ancestral, more undulatory carangiform swimming mode and have laterally placed red muscle. Carangiform swimming has been reported for the billfishes (Fig. 3) (*24*), *Scomber* and *Scomberomorus*, and most likely applies to *Gasteroschisma*, which has laterally positioned red muscle (*25*). The bonitos (*Sarda*) are the ectothermic sister group to the endothermic tunas (Fig. 2) and have in some species a degree of red muscle internalization intermediate between tunas and other scombroids (*26*). This suggests that internalization of red muscle (Fig. 2) preceded the evolution of vascular countercurrent heat exchangers and predisposed this clade for conserving metabolic heat from muscle. The swimming mode of the bonitos has not been extensively characterized. If the bonitos swim in a more stiff-bodied fashion than carangiforms, it would argue that internalization of red muscle was initially driven by mechanical demands of a newly evolved swimming form and later contributed to the endothermic condition in tunas. Laterally placed red muscle is a constraint against developing endothermy like that of tunas because of the inability to conserve heat sufficiently when the aerobic red muscle is located close to the body surface (*1*, *27*). Despite this historical limitation on their ability to maintain elevated muscle temperatures, billfishes and *Gasterochisma* appear to have evolved the minimum required endothermic capacity for expanding their thermal niches, heating the central nervous system.

REFERENCES AND NOTES

1. F. G. Carey and J. M. Teal, *Proc. Natl. Acad. Sci. U.S.A.* **56**, 1464 (1966); E. D. Stevens and F. E. J. Fry, *Comp. Biochem. Physiol.* **38A**, 203 (1971); I. Barett and F. J. Hester, *Nature* **203**, 96 (1964); J. B. Graham, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1964 (1973); F. G. Carey, *Sci. Am.* **238**, 36 (February 1973).
2. F. G. Carey, *Science* **216**, 1327 (1982).
3. B. A. Block, *Biochemistry and Molecular Biology of Fishes*, P. Hochachka and T. Mommsen, Eds. (Elsevier, New York, 1991), pp. 269–311.
4. F. G. Carey and J. M. Teal, *Comp. Biochem. Physiol.* **28**, 199 (1969); F. G. Carey, in *A Companion to Animal Physiology*, C. R. Taylor, K. Johansen, L. Bolis, Eds. (Cambridge Univ. Press, New York, 1982), pp. 216–234.
5. A. W. Crompton, C. R. Taylor, J. A. Jagger, *Nature* **272**, 333 (1978); A. F. Bennett and J. A. Ruben, *Science* **206**, 649 (1979); A. F. Bennett, *J. Exp. Biol.* **160**, 1 (1991); J. A. Ruben, *Evolution* **45**, 1 (1991).
6. R. M. Gooding, W. H. Neill, A. E. Dizon, *Fishery Bull.* **79**, 31 (1981); J. B. Graham and R. M. Laurs, *Mar. Biol.* **72**, 1 (1982); R. W. Brill, *Fishery Bull.* **85**, 25 (1987).
7. B. A. Block, *J. Morphol.* **190**, 169 (1986); B. A. Block and C. Franzini-Armstrong, *J. Cell Biol.* **107**, 1099 (1988); B. A. Block, *Am. Zool.* **31**, 726 (1991).
8. A. Tullis *et al.*, *J. Exp. Biol.* **161**, 383 (1991).
9. J. B. Graham, F. J. Koehrn, K. A. Dickson, *Can. J. Zoology.* **61**, 2087 (1983).
10. C. C. Lindsey, in *Fish Physiology*, W. S. Hoar and D. J. Randall, Eds. (Academic Press, New York, 1978), pp. 1–100.
11. H. L. Fierstine and V. Walters, *Mem. Soc. South. Calif. Acad. Sci.* **6**, 1 (1968).
12. J. B. Graham, *Fish. Bull. U.S.A.* **73**, 219 (1975).
13. D. R. Brooks and D. A. McLeannan, *Phylogeny, Ecology, and Behavior. A Research Program in Comparative Biology* (Univ. of Chicago Press, Chicago, 1991); P. H. Harvey and M. D. Pagel, Eds., *The Comparative Method in Evolutionary Biology* (Oxford Univ. Press, Oxford, 1991).
14. B. B. Collette, in *The Physiological Ecology of Tunas*, G. D. Sharp and A. E. Dizon, Eds. (Academic Press, New York, 1978), pp. 1–39; ———, T. Potthoff, W. J. Richards, S. Ueyanagi, J. L. Russo, Y. Nishikawa, in *Ontogeny and Systematics of Fishes*, H. G. Moser *et al.*, Eds. (Spec. Publi. no. 1, Supplement to *Copeia*, American Society of Ichthyologists and Herpetologists, Lawrence, KS, 1984), pp. 591–620.
15. G. D. Johnson, *Bull. Mar. Sci.* **39**, 1 (1986).
16. R. K. Saiki *et al.*, *Science* **239**, 487 (1988); U. B. Gyllensten and H. A. Erlich, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7652 (1988). DNA extraction and PCR were performed with the conditions and oligonucleotide primers from J. R. Finnerty and B. A. Block [*Mol. Mar. Biol. Biotech.* **1**, 206 (1992)], including primers L14841 and H15149 from T. D. Kocher *et al.* [*Proc. Natl. Acad. Sci. U.S.A.* **86**, 6196 (1989)].
17. F. Sanger, S. Nicklein, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977). Sequences for 24 species were obtained as in J. R. Finnerty and B. A. Block (in *16*). The remaining sequences were obtained by direct sequencing of PCR-generated double-stranded DNA according to J. L. Casanova, C. Pannetier, C. Jaulin, P. Kourilsky, *Nucleic Acids Res.* **18**, 4028 (1991).
18. Sequence data were analyzed by Phylogenetic Analysis Using Parsimony (PAUP), D. L. Swofford, PAUP 3.0s (Illinois Natural History Survey, Champaign, 1989). The sequences have been submitted to GenBank (accession numbers L11532–L11562).
19. H. Kohno, *Jpn. J. Ichthyol.* **31**, 268 (1984).
20. J. R. Finnerty and B. A. Block, unpublished data.
21. Allozyme and RFLP data (J. Graves, unpublished data) support relationships of billfishes as presented here, including the splitting of *Makaira* and placement of *Istiophorus* as sister-group to *M. nigricans*.
22. D. A. Shubnikov, *J. Ichthyology* **14** (5) 633 (1974); J. S. Beckett, U.S. Department of Commerce NOAA Technical Report NMFS SRF-675 (Washington, DC, 1974), pp. 103–107.
23. W. H. Neill and E. D. Stevens, *Science* **184**, 1008 (1974); F. G. Carey, in *Planning the Future of Billfishes*, R. H. Stroud, Eds. (National Coalition Marine Conservation, Savannah, GA, 1990), pp. 103–122; ——— and J. V. Scharold, *Mar. Biol.* **106**, 329 (1990); K. N. Holland, R. W. Brill, R. K. C. Chang, J. R. Sibert, D. A. Fournier, *Nature* **358**, 410 (1992).
24. J. H. Hebrank, M. R. Hebrank, J. H. Long, B. A. Block, S. A. Wainwright, *J. Exp. Biol.* **148**, 449 (1990).

25. M. W. Westneat, W. Hoese, C. A. Pell, S. A. Wainwright, unpublished results; B. A. Block and J. R. Finnerty, unpublished results.
26. G. D. Sharp and G. W. Pirages, in *The Physiological Ecology of Tunas*, G. D. Sharp and A. E. Dizon, Eds. (Academic Press, New York, 1978), pp. 41–78; K. Kishinouye, *J. Coll. Tokyo Agric. Imper. Univ.* **8**, 293 (1923); T. Kafuku, *Jap. J. Ichthyol.* **1**, 89 (1950).
27. E. D. Stevens and F. E. J. Fry, *Can. J. Zool.* **48**, 221 (1970); J. Dean, *Trans. Am. Fish. Soc.* No. 6, 709 (1976).

28. Supported by NSF PYI grant DCB-8958225 and NIH AR40246 to B.A.B. We thank A. Borisy, S. Eng, S. Malik, and D. Wang for technical assistance. We are indebted to C. Proctor, G. DeMetrio, P. Davie, J. Pepperell, K. Dickson, B. Collette, F. Carey, and P. Grewe for tissue samples. F. Carey, B. Collette, D. Johnson, A. Meyer, S. Norton, E. D. Stevens, and M. Westneat provided valuable assistance on the project and constructive comments on the manuscript.

30 September 1992; accepted 19 January 1993

Taxol and Taxane Production by *Taxomyces andreanae*, an Endophytic Fungus of Pacific Yew

Andrea Stierle, Gary Strobel, Donald Stierle

Taxomyces andreanae, a fungal endophyte, was isolated from the phloem (inner bark) of the Pacific yew, *Taxus brevifolia*. The fungus is hyphomyceteous and, when grown in a semi-synthetic liquid medium, produced taxol and related compounds. Taxol was identified by mass spectrometry, chromatography, and reactivity with monoclonal antibodies specific for taxol. Both [1-^{14}C]acetic acid and L-[U-^{14}C]phenylalanine served as precursors of [^{14}C]taxol in fungal cultures. No taxol was detected in zero-time cultures or in the small agar plugs used to inoculate the culture flasks.

Taxol, a highly derivatized diterpenoid (*1*), has shown promise as an anti-tumor agent in breast and ovarian cancers (Fig. 1) (*2*, *3*). The primary source of taxol has been the harvested and dried inner bark (phloem-cambial tissues) of *Taxus brevifolia* (Pacific yew) (*4*, *5*). Pacific yew is a slow-growing tree found in moist soils near streams and lakes in certain regions of the Pacific Northwest (6). Only 0.01 to 0.03% of the dry phloem weight is taxol, yet as much as 2 g of purified taxol is required for a full regimen of anti-tumor treatment (*5*). The supply issue is further complicated by the scarcity of the yew tree. Although all 11 species of *Taxus* make taxol, natural stands of these trees are often small and located in remote areas.

Here we report the production of taxol by *Taxomyces andreanae*, an endophytic fungus associated with *Taxus brevifolia* (*7*). We confirmed the presence of taxol and related taxanes in 3-week-old cultures of the fungus by mass spectrometry, immunochemistry, chromatographic methods, and radiochemical techniques.

The search for yew-associated microbes that produce taxol is justified by previous examples of plant-associated microbes producing "plant" compounds, such as gibberellins (*8*). The pathways of gibberellin biosynthesis in the fungus and the higher plant are identical up to GA_{12} (*9*). We examined microorganisms isolated from more than 25 *T. brevifolia* trees from 20 locations. Of the 200 microbes screened to date, only *T. andreanae* has demonstrated the ability to produce taxol. This fungus was isolated from the surface-disinfected (80% ethanol) inner bark of one tree in an old-growth cedar forest in northern Montana.

Taxomyces andreanae was cultured by transferring hyphal tips from water agar, on which bark pieces had been cultured, onto a modified-mycological agar (*10*). The mycelium was then successively transferred to eliminate traces of taxol or other taxanes carried over from the original tree source. Transfers of small agar plugs to broth cultures were made from mycelia that had grown 3 to 7 days. After this period, mycelia appeared to go into a quiescent state. *Taxomyces andreanae* was stored in water at 4°C and grown on a semi-defined culture medium.

The conidia of *T. andreanae* do not germinate, therefore we transferred pieces (0.5 by 0.5 cm) of agar block containing the mycelial mats to sterilized S-7 medium (*10*). Optimum conditions were as a still culture, at 25°C, with a surface-to-volume ratio of 1.3 (cm^2:ml).

After 21 days of incubation, the culture was filtered through cheesecloth. The residue was ground in a Sorvall Omnimixer and filtered again. The combined fluids were extracted with an equal volume of dichloromethane or chloroform-methanol (10:1 v/v). Solvent was removed from the organic phase by rotary evaporation at 30°C, yielding the organic extract. Thin-layer chromatography (TLC) of the organic extract demonstrated the presence of a compound that mimicked taxol in three solvent systems (*11*).

The organic extract was dissolved in 2 ml of chloroform and placed on a silica gel column. The column was rinsed with chloroform and eluted with 20 ml of acetoni-

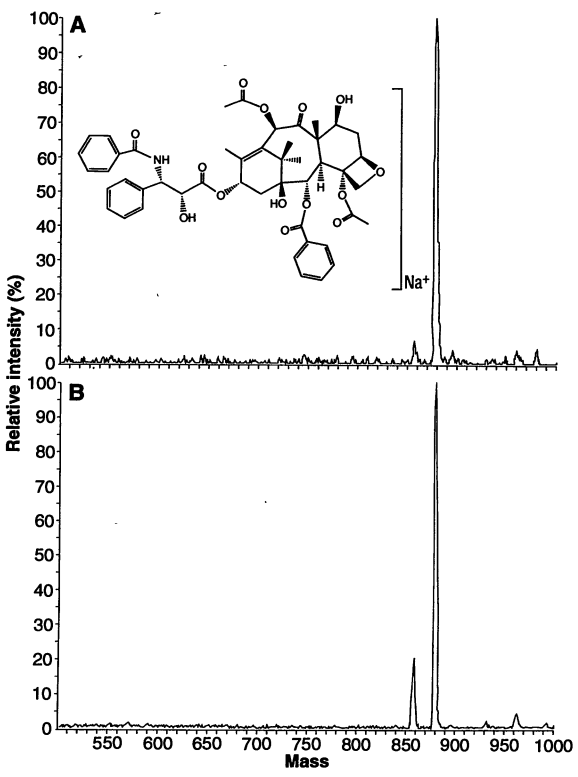

Fig. 1. Electrospray mass spectrum of fungal taxol and structure of yew taxol (**A**). The solvent was methanol–H_2O–acetic acid 50:50:1 v/v/v with a flow of 2 μl/min under a voltage of 2.2 kV. The sample was loop injected. Electrospray mass spectrum of sodiated yew taxol (**B**).

A. Stierle and G. Strobel, Department of Plant Pathology, Montana State University, Bozeman, MT 59717.
D. Stierle, Department of Chemistry and Geochemistry, Montana College of Mineral Science and Technology, Butte, MT 59701.