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Cranial Neural-Crest Migration and Chondrogenic Fate in the Oriental Fire-Bellied Toad *Bombina orientalis:* Defining the Ancestral Pattern of Head Development in Anuran Amphibians

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ABSTRACT We assess cranial neural-crest cell migration and contributions to the larval chondrocranium in the phylogenetically basal and morphologically generalized anuran *Bombina orientalis* (Bombinatoridae). Methods used include microdissection, scanning electron microscopy, and vital dye labeling, in conjunction with confocal and fluorescence microscopy. Cranial neural-crest cells begin migrating before neural-fold closure and soon form three primary streams. These streams contribute to all cranial cartilages except two medial components of the hyobranchial skeleton (basihyal and basibranchial cartilages), the posterior portion of the trabecular plate, and the otic capsule, the embryonic origin of which is unknown. Chondrogenic fate is regionalized within the cranial neural folds, with the anterior regions contributing to anterior cartilages and the posterior regions to posterior cartilages. A neural-crest contribution also was consistently observed in several cranial nerves and the connective tissue component of many cranial muscles. Notwithstanding minor differences among species in the initial configuration of migratory streams, cranial neural-crest migration and chondrogenic potential in metamorphosing anurans seem to be highly stereotyped and evolutionarily conservative. This includes a primary role for the neural crest in the evolutionary origin of the paired suprarostral and infrarostral cartilages, two prominent caenogenetic features of the rostral skull unique to anuran larvae. Our results provide a model of the ancestral pattern of embryonic head development in anuran amphibians. This model can serve as a basis for examining the ontogenetic mechanisms that underlie the diversity of cranial morphology and development displayed by living frogs, as well as the evolutionary consequences of this diversity.

Evolutionary developmental biology is emerging as a distinct field of research, asking such questions as "How do developmental processes and mechanisms evolve?" and "To what extent does development bias or constrain phenotypic evolution?" Although the intellectual roots of the field are ancient, it has experienced a renaissance in the last 15–20 years (Gould, '77; Bonner, '82, '88; Goodwin et al., '83; Thomson, '88; Müller, '91; Hall, '92). Accompanying this rebirth and maturation is the widespread recognition that, as in any field of comparative biology, analyses of evolutionary pattern and process are conducted most effectively in a rigorous phylogenetic context, including explicit hypotheses of phylogenetic relationship and the identification of primitive vs. derived character states (e.g., Northcutt, '90; Collazo and Marks, '94; Patel, '94; Wake and Hanken, '96). Typically, such analyses require the consideration of "non-model" organisms, because the model systems of developmental biology are a biased sample of organisms generally not chosen with evolutionary questions in mind (Hanken, '93; Bolker, '95).

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The primary focus of our research is the development and evolution of the head in anuran amphibians. We are particularly interested in the unique problems regarding mechanisms of cranial pattern formation posed by the group's ancestral, biphasic ontogeny (which involves, for instance, morphologically distinct skulls in larvae and adults), as well as the effects of the evolution of derived life histories, such as direct development, on cranial patterning (Hanken, '92, '93; Hanken et al., '92; Hanken and Thorogood, '93). Examination of these topics has been hampered by the lack of comprehensive baseline data regarding the presumed ancestral pattern of head development in frogs. For example, while there is considerable, detailed knowledge about cranial development in the pipid frog *Xenopus laevis* (Table 1), this species is highly derived phylogenetically (Cannatella and de Sá, '93) and has a very specialized skull morphology in both the larva and the adult (Trueb and Hanken, '92); it is an inappropriate baseline for evolutionary comparisons among anurans or between frogs and other vertebrates (Cannatella and Trueb, '88; Cannatella and de Sá, '93). And while important developmental parameters such as cranial neural-crest biology have been studied in many other species of anurans (Table 1), nearly all of these studies predate the application of modern, improved tools to study cell migration and lineage, such as scanning electron microscopy (SEM) and fluorescent cell labels (e.g., Collazo et al., '93).

In this paper, we 1) document the pattern and timing of cranial neural-crest emergence and migration during embryogenesis, and

TABLE 1. Published studies of cranial neural-crest biology in anuran amphibians[1]

Species	Analytical method(s)	Reference
Bombinatoridae		
Bombina orientalis	Histology of normal development, SEM, vital labeling	This study
B. pachypus	Homospecific and heterospecific grafts	Andres, '46, '49; Wagner, '48, '49, '55, '59; Baltzer, '50, '52; Chen and Baltzer, '54; Henzen, '57
B. variegata	Organ culture of tissue explants	Petricioni, '64
Discoglossidae		
Discoglossus pictus	Homospecific grafts	Fagone, '59; Cusimano et al., '62; Cusimano-Carollo, '63, '69, '72
Alytes obstetricans	Histology of normal development	Reisinger, '33
Pipidae		
Xenopus laevis	Histology of normal development	Nieuwkoop and Faber, '56
	Ablation, homospecific grafts	Seufert and Hall, '90
	SEM, heterospecific grafts	Sadaghiani and Thiébaud, '87
	Heterospecific grafts	Wagner, '49
	In situ hybridization	Bradley et al., '92; Dirksen et al., '93; Essex et al., '93; Papalopulu and Kintner, '93; Ho et al., '94; Winning and Sargent, '94; Brändli and Kirschner, '95
	In situ hybridization, immunocytochemistry	Song and Slack, '94
	Lectin and thiodigalactoside treatment	Varma et al., '94
Bufonidae		
Bufo viridis	Homospecific grafts	Raunich, '57
Leptodactylidae		
Eleutherodactylus coqui	SEM	Moury and Hanken, '95
Ranidae		
Rana sp.	Histology of normal development	Corning, 1899
R. esculenta	Homospecific grafts, organ culture of tissue explants	Raunich, '57, '58
R. fusca	Heterospecific grafts	Raven, '33
R. japonica	Histology of normal development, ablation, organ culture of tissue explants, homospecific and heterospecific grafts	Ichikawa, '35, '37; Okada and Ichikawa, '56; Okada, '57
R. palustris	Histology of normal development, ablation, vital labeling, homospecific grafts	Stone, '22, '27, '29, '32
R. pipiens	Histology of normal development	Knouff, '27
R. temporaria	Histology of normal development, ablation, homospecific grafts	Lundborg, 1899; Reisinger, '33

[1]Only those studies are listed that provide data on patterns of crest emergence, migration, or gene expression, or on cranial skeletal derivatives and differentiation. Species are listed according to family (Ford and Cannatella, '93).

2) assess the contribution of neural crest to the larval chondrocranium in the Oriental fire-bellied toad *Bombina orientalis*. This is a phylogenetically basal and morphologically generalized species that is among the most appropriate living taxa for inferring ancestral features of anurans (Cannatella and de Sá, '93). Previous studies of this, and closely related, species have documented cranial morphology in larvae and adults, as well as details of cranial embryogenesis and metamorphosis (Stadtmüller, '31; Ramaswami, '42; Slabbert, '45; Sokol, '81; Hanken and Hall, '84, '88; Hanken and Summers, '88; Hanken et al., '89; Smirnov, '89; Viertel, '91). Here we focus on the neural crest because of its prominent role in head development in vertebrates generally, including amphibians (reviewed in Hall and Hörstadius, '88), and because of its likely role in mediating many aspects of the evolution of cranial patterning (Hunt et al., '91; Hanken and Thorogood, '93; Langille and Hall, '93). Our analysis is based on a variety of techniques, including microdissection, SEM, and vital labeling. While *Bombina* has figured prominently in many classical studies of amphibian neural-crest biology (Table 1), neither patterns of cranial crest migration nor crest contributions to the entire larval skull have been reported for any species. Our primary aim is to derive a model of the ancestral pattern of head development in anuran amphibians. Such a model can serve as a basis for examining the ontogenetic mechanisms that underlie the pronounced diversity of cranial morphology and development displayed by living taxa, as well as the evolutionary consequences of this diversity.

MATERIALS AND METHODS

Embryos

Eggs were obtained from laboratory matings among wild-caught adults (Charles D. Sullivan Co., Inc., Nashville, TN) that are maintained as a breeding colony at the University of Colorado. Methods for breeding and husbandry followed established procedures (Carlson and Ellinger, '80; Frost, '82). Adults were injected subcutaneously with human chorionic gonadotropin (Sigma Chemical Co., St. Louis, MO) and allowed to spawn overnight in the dark at room temperature. Fertilized eggs were reared in 10% Holtfreter's solution at 10–25°C. Embryos and tadpoles were staged from external morphology according to the scheme of Gosner ('60), which defines a total of 46 stages from fertilization to metamorphosed froglet. In *B. orientalis,* larvae hatch at around stage 22 (Hanken, personal observations).

SEM

At least eight embryos each of stages 14 (neural plate) to 19 (beginning of heart beat) were prepared for SEM. Embryos were dejellied either chemically (0.63 g cysteine HCl, 0.12 g NaCl, 24 ml H_2O, buffered to pH 8.0 with 5 N NaOH) or manually with watchmaker's forceps; they were decapsulated manually and fixed at 4°C in modified Karnovsky fixative (1.5% glutaraldehyde/1.5% paraformaldehyde in 0.1 M cacodylate buffer; Karnovsky, '65) overnight or longer. The embryos were transferred to ice-cold 0.1 M phosphate buffer (PB), where the cranial epidermis was carefully removed with tungsten needles. Because neural-crest cells are dark and stand out against the lighter background of adjacent tissues in such preparations, camera-lucida drawings and photomicrographs were made at this stage as an aid to identifying neural crest later using SEM. Specimens then were postfixed in 1% OsO_4 in cacodylate buffer for 1 h. After thorough rinsing in PB, specimens were dehydrated in a graded series of ethanol and transferred to liquid CO_2 in a critical-point dryer. Dried specimens were mounted, sputtercoated with gold/palladium, and examined in a Philips CM 10 scanning electron microscope at 10 kV.

Vital labeling

Eighty-two live embryos were labeled with vital dye at stage 14 (neural plate; Fig. 1), immediately before the onset of cranial neural-crest migration. After being dejellied and decapsulated, embryos were immobilized in shallow trenches cut into 2% agar gelled in the bottom of Petri dishes. A 0.5% stock solution of the lipophilic dye DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine, perchlorate; Molecular Probes D-282, Eugene, OR) was prepared in 100% ethanol and stored at 4°C. Immediately before injection, it was diluted in 0.3 M sucrose to working concentrations of 0.1 and 0.05%. Micropipets pulled from thin-walled, 1.2 mm diameter glass microfilaments were filled with dye and attached to a Picospritzer II (General Valve Corp., Fairfield, NJ). Micropipet tips were broken to a diameter of about 20 μm. DiI was injected into one of six different sites in the left neural fold (Fig. 1) by inserting a micropipet and expelling a small amount

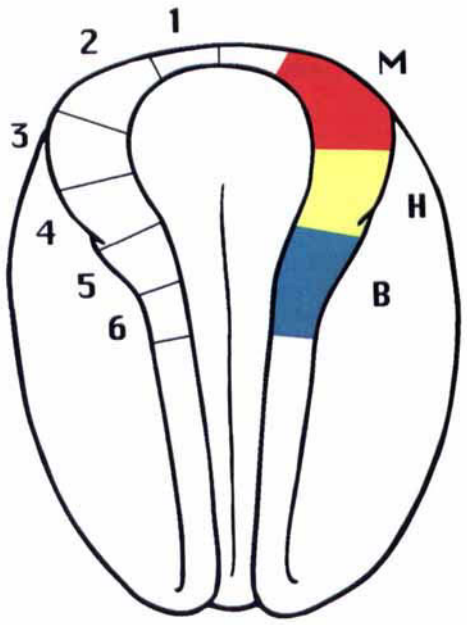

Fig. 1. Stage 14 (neural plate) embryo of *Bombina orientalis*. The left side depicts the six regions of the cranial neural fold that were injected with DiI. The right side depicts the approximate origins of the neural-crest cells that contribute to the three cranial migratory streams—mandibular (M), hyoid (H), and branchial (B). Dorsal view, anterior at the top.

of dye solution; the right side was not injected and served as a control. Injections were made by hand or with a micromanipulator. The total of six injection sites represents the maximum number of discrete regions of the cranial neural fold that could be injected with both a high degree of repeatability among embryos and a minimum of overlap with adjacent sites. All neural-crest and presumptive neural-tube cells within each site appeared to be labeled following injection.

Fluorescence microscopy of whole embryos and cryostat sections

Injected embryos were reared in 10% Holtfreter's solution containing 50 mg/l gentamicin sulfate (Sigma G-1264). They were maintained individually in 24-well tissue culture plates at 25°C. For fluorescence imaging, living embryos were temporarily mounted in rectangles cut into agar gelled at the bottom of custom-made brass slides with coverslip floors (provided by M. Klymkovsky, University of Colorado) and observed with a Leitz Dialux 20 epifluorescence microscope with a rhodamine (N2) filter block. They were photographed using a Wild MPS55 photoautomat and Kodak T-MAX P3200 or Ektachrome P1600 film, or digital images were recorded with a Panasonic WV-CL350 video camera and processed using Adobe Photoshop 3.0 on an Apple Macintosh 7100 computer. Photographic prints were produced with a Polaroid Palette on Kodak Ektachrome Elite 100 film.

Fifty-nine labeled tadpoles (stage 26—hindlimb bud appears) were prepared as cryostat sections. The specimens were killed in 30% aqueous chloretone (1,1,1-trichloro-2-methyl-2-propanol; Sigma T-5138) and fixed overnight or longer in 4% paraformaldehyde/0.25% glutaraldehyde in PB at 4°C. They were then washed thoroughly in PB, soaked in 15% sucrose solution overnight, transferred through two changes of a 15% sucrose/7.5% gelatin solution at 37°C for a total of at least 5 h, and embedded in fresh sucrose/gelatin solution at 4°C. Once set, the specimens were rapidly frozen in liquid nitrogen and stored at −40°C. Approximately 20 μm sections were cut using an International Harris cryostat (International Equipment Co., Needham Heights, MA). Sections were viewed unmounted using fluorescence microscopy and documented as described above.

Confocal microscopy

Twenty-three living, labeled embryos (stages 15–20) were mounted in agar on brass slides and scanned with an inverted, laser-scanning confocal microscope (Molecular Dynamics, Sunnyvale, CA). Images were recorded and processed with a 3000 Silicon Graphics Iris workstation using ImageSpace software (Molecular Dynamics). They were stored on a Sony Magneto-optical disk and photographed with Screenstar (Presentation Technologies, Sunnyvale, CA) on Kodak Ektachrome Elite 100 film.

RESULTS

Cranial neural-crest cell migration

Cranial neural-crest cell emergence and migration begin early in stage 15, before the neural folds fuse; most cells have begun migrating away from the neural tube by the end of that stage. At least initially, the cells migrate in dense streams. Two streams form first (Fig. 2A,B). The mandibular (or rostral) stream originates at the level of the prospective mesencephalon and migrates anteriorly

toward the optic vesicle. A second, posterior stream emerges soon after at the level of the prospective rhombencephalon; it is separated from the mandibular stream by a narrow, crest-free zone (Fig. 2B).

At stage 16, the mandibular stream is migrating rostrally around the optic vesicle. Most cells migrate along a ventral pathway, but some follow a dorsal route (Figs. 3A,B, 4A,B). The second stream divides into an anterior hyoid (rostral otic) stream and a posterior branchial (caudal otic) stream (Fig. 2C). Later in stage 16, the branchial stream further subdivides into two parallel cell masses (Figs. 2D, 3C,D).

At stage 17, the mandibular stream completely surrounds the optic vesicle. The hyoid stream migrates ventrally faster than either cell mass of the branchial stream and overtakes them (cf. Figs. 2D,E, 3E, 4C). Even at this relatively late stage, some branchial stream cells are present within the zone that separates its two cell masses (Fig. 3F).

By stage 18, the mandibular stream surrounds and extends rostral to the optic cup. The hyoid stream has migrated farther ventrally and nearly reaches the anlagen of the cement glands (Figs. 2E, 4C). After further subdivision, the branchial stream comprises four cell masses, each within a different branchial arch (Fig. 2F). After this stage, when cranial neural-crest cells begin to disperse from within their respective streams into the adjacent mesoderm, their migration and fates no longer can be followed by dissection and SEM. With confocal microscopy, labeled neural-crest cells are seen to migrate into the transient epidermal (external) gills that extend from branchial arches 1 and 2 (Fig. 4D).

Relative contributions of the six different injection sites (Fig. 1) to each cranial migratory stream were determined from DiI-labeled cells in living embryos examined using confocal and conventional fluorescence microscopy (Table 2). The mandibular stream was derived from cells originating within injection sites 2 and 3. The hyoid stream was derived almost exclusively from site 4; additional, minor contributions from sites 3 and 5 were observed in a few embryos. The branchial stream was derived from sites 5 and 6; site 5 contributed to the most anterior cell mass and site 6 to the posterior masses. No labeled cells from injection site 1 contributed to any migratory stream.

Neural-crest contribution to the larval skull

Contributions of DiI-injected cranial neural-crest cells to the cartilaginous larval skull were determined from cryosectioned tadpoles at stage 26. Only a small portion of the skull was labeled in any given embryo; results from all embryos were combined to give a composite image of the extent of crest contribution to each individual cartilage (Table 2, Fig. 5).

Cells from all six injection sites except site 1 (transverse neural folds) contributed to cranial cartilages. Cells derived from the mandibular stream (sites 2 and 3) were found in the upper and lower jaws (suprarostral, infrarostral, and Meckel's cartilages; Fig. 6A–C), the jaw suspensorium (palatoquadrate cartilage; Fig. 6E), and the rostral portion of the neurocranium (trabecular horns; Fig. 6D). The hyoid stream (sites 3–5) contributed to the ceratohyal cartilage, a prominent, anteroventral component of the hyobranchial (gill) skeleton, and to the anterior portion of the trabecular plate, which constitutes the floor of the neurocranium ventral to the brain (Fig. 6F,G). The branchial stream contributed to nearly all of the remainder of the hyobranchial skeleton, with cells from the anterior cell mass (site 5) contributing to the first ceratobranchial cartilage (CB I) and those from the posterior cell masses (site 6) contributing to CB II–IV (Figs. 6H, 7). DiI-labeled neural crest from posterior portions of site 6 did not contribute to any cranial cartilages.

There is no evidence of a cranial neural-crest contribution to either the basihyal or the basibranchial (two median, ventral cartilages in the hyobranchial skeleton), to the posterior portion of the trabecular plate, or to any portion of the cartilaginous otic capsule. The boundary between crest and non-crest-derived portions of the trabecular plate lies approximately at the level of the ascending process of the palatoquadrate (Fig. 5).

Other neural-crest derivatives

The presence of DiI-labeled cells was noted in several cranial tissues in addition to cartilage, although these additional tissues were not surveyed comprehensively (data not shown). Labeling was consistently observed in cranial nerves V, VII, IX, and X; in the connective tissue (non-contractile) elements of cranial muscles (especially the complex jaw abductor and adductors, e.g., orbitohyoideus); in specific portions of the eye (cornea,

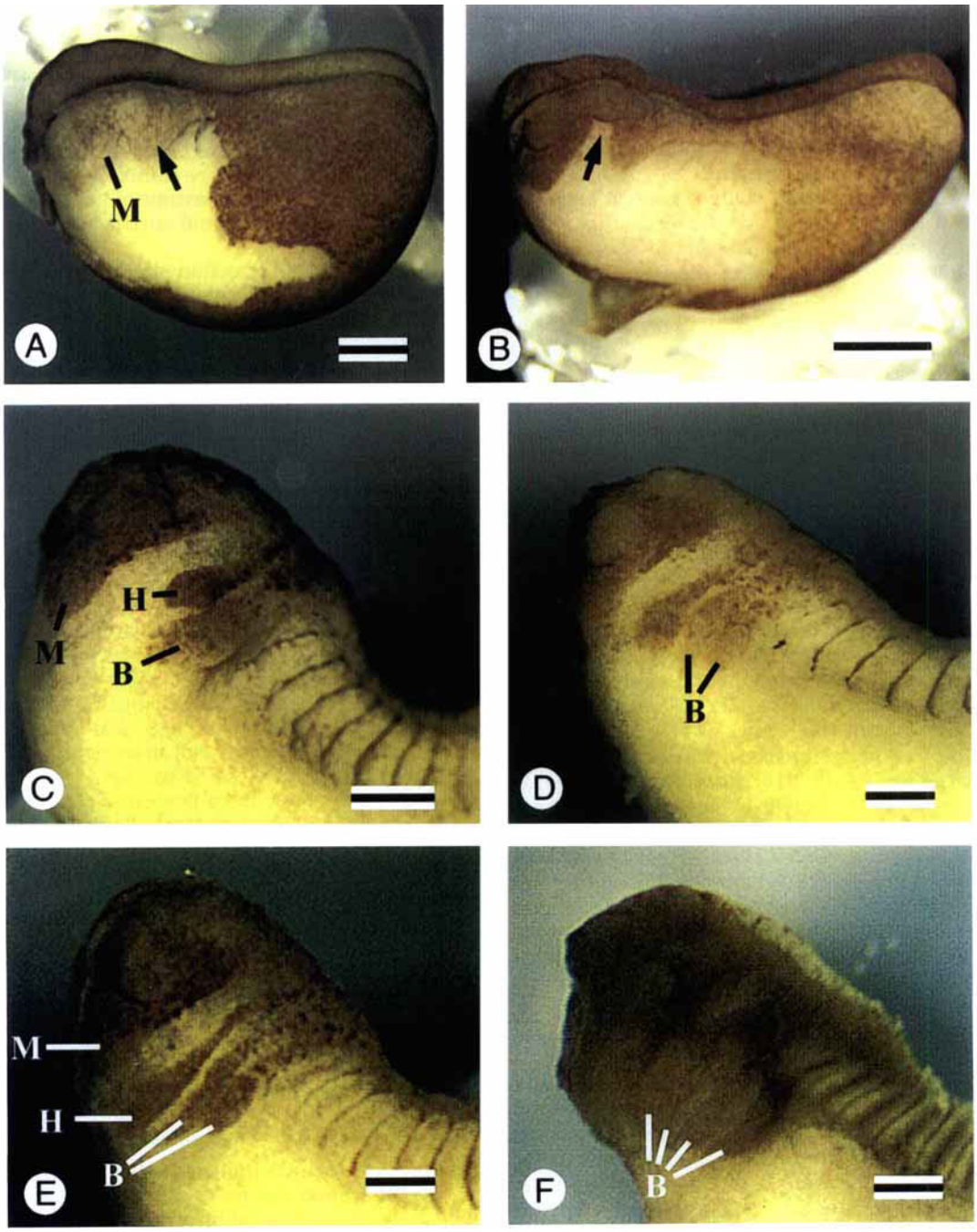

Fig. 2. Cranial neural-crest migration in *Bombina orientalis*. Preserved embryos are photographed in lateral view, anterior to the left, with the cranial epidermis removed to reveal the underlying migratory streams. **A:** Early in stage 15, crest cells migrate within anterior (mandibular, M) and posterior (arrow) streams. Scale = 0.2 mm. **B:** Later in stage 15, these two streams are separated by a crest-free zone (arrow). Scale = 0.5 mm. **C:** Stage 16. The posterior stream has divided into anterior (hyoid, H) and posterior (branchial, B) streams. Scale = 0.25 mm. **D:** Early stage 17. The branchial stream is further subdivided into two distinct cell masses. Scale = 0.25 mm. **E:** Late stage 17. All three streams have migrated further ventrally. Scale = 0.25 mm. **F:** Stage 18. The branchial stream comprises four parallel cell masses. Boundaries of all cranial streams are less distinct as neural-crest cells begin to disperse within the head. Scale = 0.25 mm.

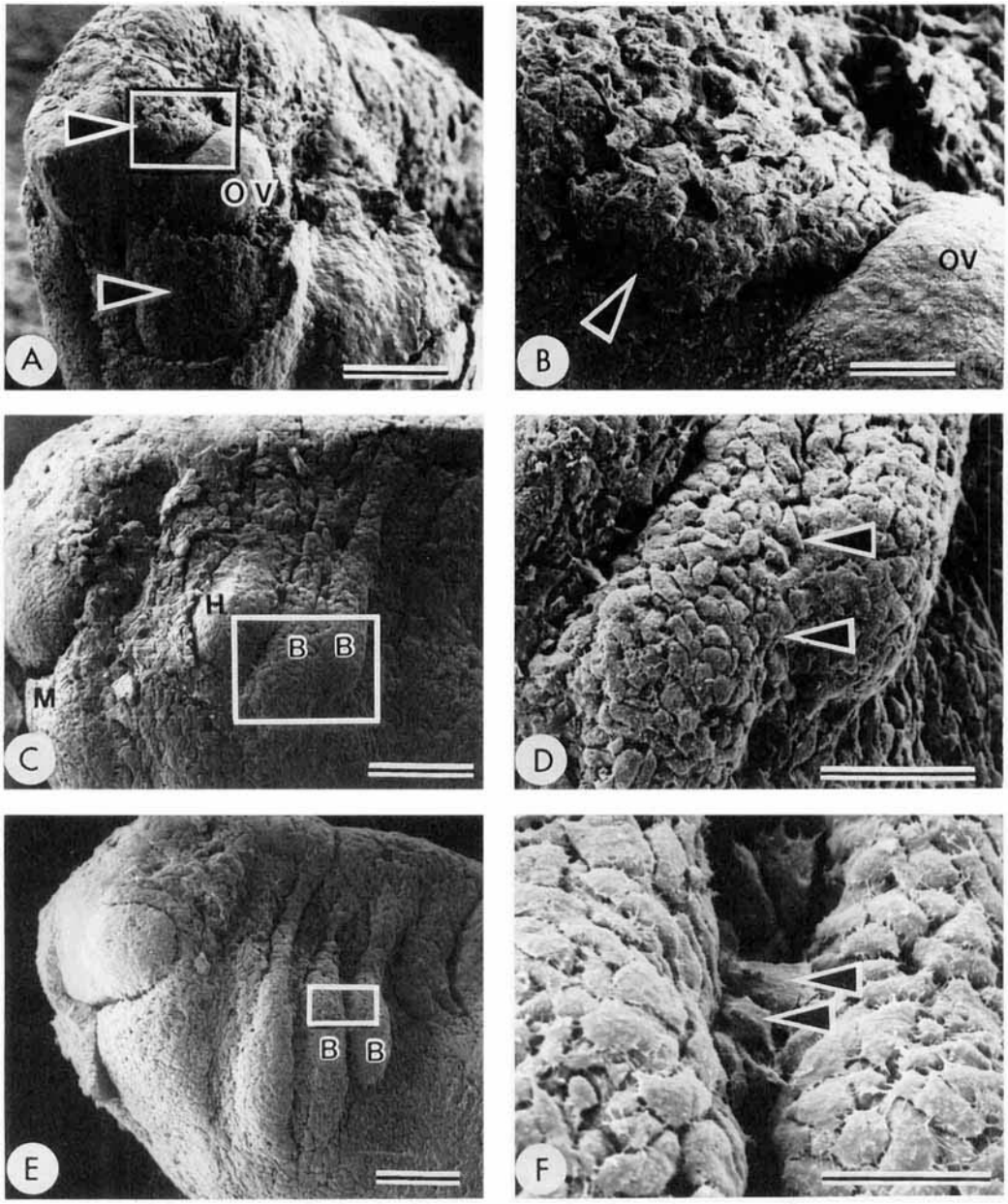

Fig. 3. Scanning electron micrographs of embryonic *Bombina orientalis* depicting migratory streams of cranial neural crest. All are lateral views (anterior to the left) unless indicated otherwise; overlying epidermis has been removed. **A:** Anterolateral view of the head at stage 16. Crest cells within the mandibular stream migrate rostrally around the optic vesicle (OV) via either dorsal or ventral pathways (arrowheads). Scale = 0.2 mm. **B:** Close-up of inset in A showing crest cells migrating around optic vesicle via dorsal pathway (arrowhead). Scale = 0.05 mm. **C:** Stage 16. Mandibular (M), hyoid (H), and branchial streams (B) are all prominent. Scale = 0.2 mm. **D:** Close-up of inset in C. The branchial stream is subdividing to form anterior and posterior cell masses; crest cells in the separation zone (arrowheads) are moving to either side. Scale = 0.1 mm. **E:** Stage 17. Mandibular stream, hyoid stream, and two cell masses of the branchial stream (B) are distinct. Scale = 0.2 mm. **F:** Close-up of inset in E. A few crest cells remaining in the separation zone within the branchial stream (arrowheads) are moving horizontally to join either cell mass. Scale = 0.05 mm.

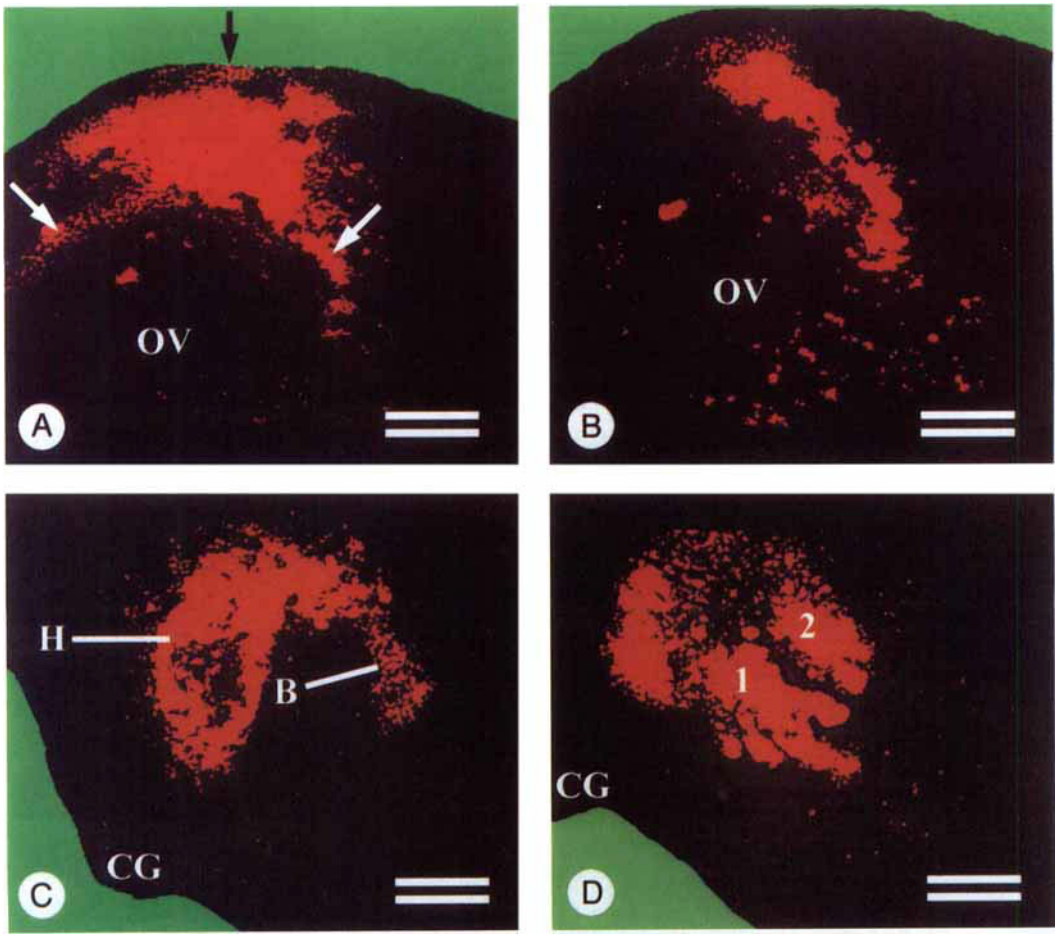

Fig. 4. Confocal microscopic images of heads of living embryos of *Bombina orientalis*, showing migration of cranial neural-crest cells labeled with DiI at stage 14. Lateral views, anterior to the left. **A:** Stage 16 embryo injected at site 2 (black arrow). Cells in the mandibular stream that are close to the epidermis migrate dorsally (left arrow) and ventrally (right arrow) around the optic vesicle (OV). **B:** In the same embryo at the same time, only cells in the thicker, ventral pathway migrate at a deeper level. **C:** Stage 17 embryo injected at site 4. Most labeled crest cells are within the hyoid stream (H); a few are within the anterior portion of the branchial stream (B). CG, cement gland. **D:** Stage 19 embryo injected at site 6. Labeled crest cells derived from the anterior two cell masses of the branchial stream are populating the transient epidermal (external) gills (Viertel, '91) on branchial arches 1 and 2. Scales = 0.05 mm.

choroid, and sclera); and in undifferentiated head mesenchyme. These results will be presented in a future paper.

DISCUSSION

Defining the ancestral pattern of head development in anurans

Basic features of cranial neural-crest cell emergence and migration in *Bombina orientalis* may be summarized as follows. Crest cell emergence begins early in stage 15, before neural-fold closure, and most crest cells have begun to migrate away from the developing neural tube by the end of this stage. The cells initially migrate in two streams (Fig. 2A,B), although the posterior stream soon divides in two (stage 16; Fig. 2C). The resulting three streams are the mandibular (or rostral), which supplies the first visceral arch; the hyoid (rostral otic), which supplies the second arch; and the branchial (caudal otic), which comprises a series of parallel cell masses that supply arches 3–6. These three streams or their component cell masses can

TABLE 2. Derivation of cranial neural-crest streams and their contribution to larval cranial cartilages in B. orientalis[1]

Neural-crest stream	Injection site	Cartilage
—	1	—
Mandibular	2, 3 (part)	Suprarostral, infrarostral, Meckel's, palatoquadrate, trabecular horns
Hyoid	3 (part), 4, 5 (part)	Anterior trabecular plate, ceratohyal
Branchial		
Anterior mass	5 (part)	Ceratobranchial I
Posterior masses	6	Ceratobranchials II–IV

[1]Locations of injection sites 1 (anterior) to 6 (posterior) within the cranial neural folds are depicted in Figure 1.

be recognized as discrete entities as late as stage 18, when the individual cells begin to disperse throughout the head.

Cranial neural crest is the predominant embryonic source of progenitor cells for the larval chondrocranium, which is first visible at stage 20. Indeed, the only larval cranial cartilages *not* derived from neural crest are two medial components of the hyobranchial skeleton (basihyal and basibranchial cartilages), the posterior portion of the trabecular plate, and the otic capsule (Fig. 5). All portions of the cranial neural folds are chondrogenic except for the most anterior (transverse) region (Fig. 1, site 1). The posterior boundary between cranial (chondrogenic) and trunk (non-chondrogenic) neural crest lies within the caudal portion of site 6. Skeletogenic fate is at least broadly regionalized within the neural folds, with the anterior regions (i.e., mandibular stream) contributing to anterior cartilages and the posterior regions (branchial stream) to posterior cartilages (Fig. 1, Table 2). Because we labeled premigratory neural-crest cells as groups rather than individually, we cannot assess segment restrictions as finely as has been done for some other vertebrates. In the zebrafish, for example, segment restrictions within premigratory branchial crest are, at least initially, much less precise than those of more anterior crest; individual crest-cell progenitors of branchial segments can contribute to as many as three adjacent arches (Schilling and Kimmel, '94). Our data, however, are at least consistent with this observation; thus, the same phenomenon may apply to anurans. Our data also do not allow us to assess neural-crest contributions to the osteocranium, which is first visible at stage 31 but not fully formed until after metamorphosis (stage 46; Hanken and Hall, '88), well after

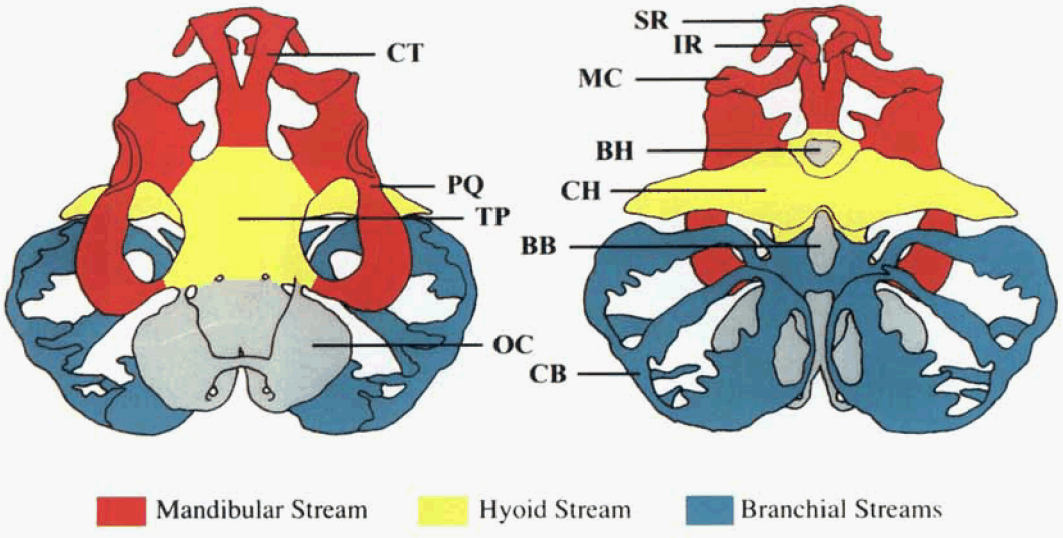

Fig. 5. Neural-crest contribution to the larval skull in *Bombina orientalis*. Crest-derived components are shaded according to migratory stream (see also Figs. 1, 7); non-crest-derived components are gray. Cartilage abbreviations: BB, basibranchial; BH, basihyal; CB, ceratobranchials I–IV; CH, ceratohyal; CT, cornua trabecula (trabecular horn); IR, infrarostral; MC, Meckel's cartilage; OC, otic capsule; PQ, palatoquadrate; SR, suprarostral; TP, trabecular plate. Skulls (stage 36) are redrawn from Hanken and Summers ('88) and depicted in dorsal (left) and ventral views.

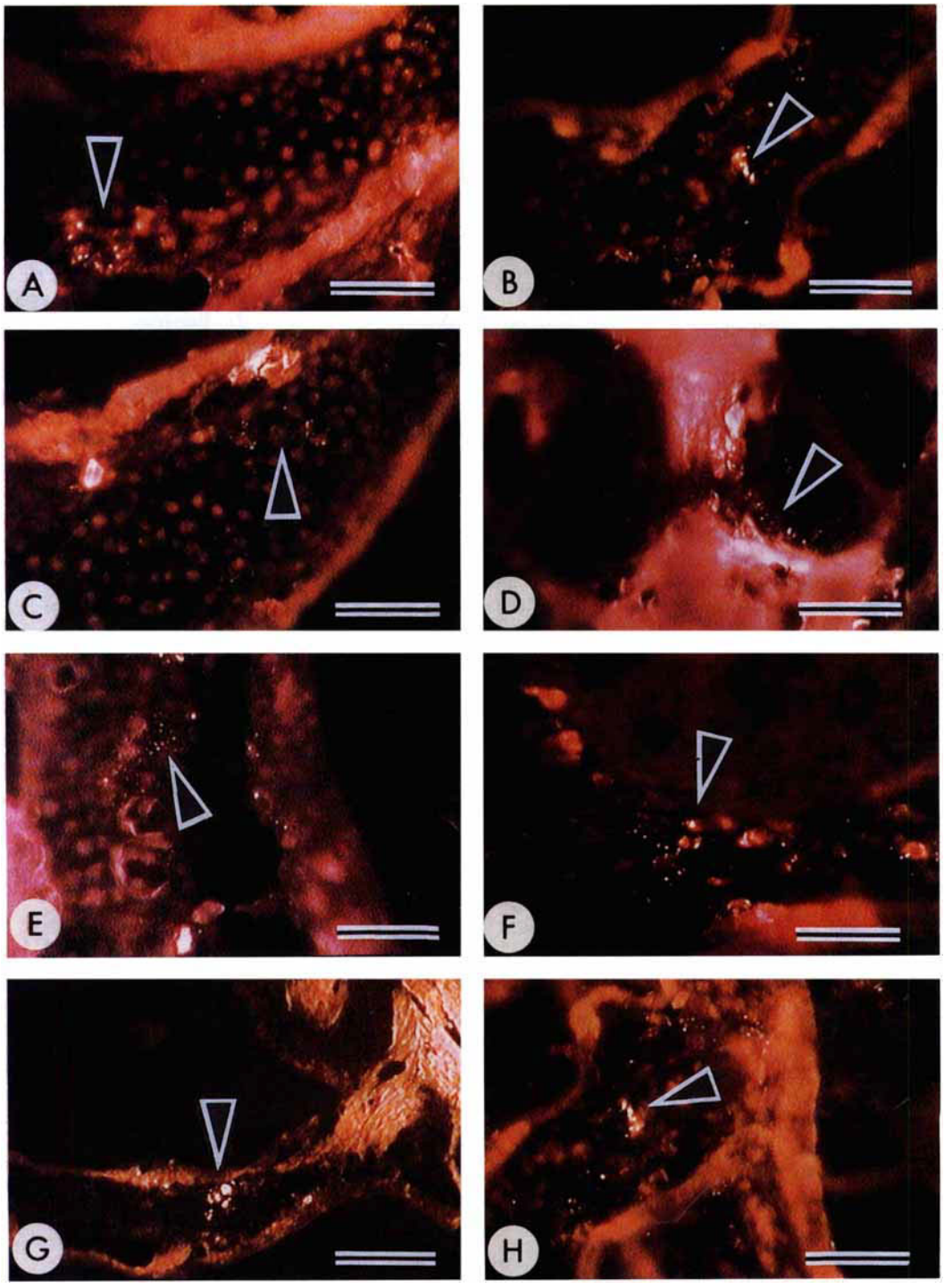

Fig. 6. Fluorescence microscopic images of cryosections showing DiI-labeled cranial cartilages in *Bombina orientalis*. Transverse sections, dorsal is up; all but D and F depict the left side, in which lateral is to the right. Arrowheads denote regions of labeled cells, which are seen as small bright dots of crystallized dye. Unlabeled chondrocytes appear as red circles; extracellular matrix is black. Areas of solid red or pink result from autofluorescence within adjacent connective tissues (e.g., muscles, perichondrium). See Figure 5 for the location of individual cartilages. **A:** Infrarostral (lower jaw) cartilage, labeled along its ventral margin. **B:** Suprarostral cartilage (upper jaw). **C:** Meckel's cartilage (lower jaw). **D:** Median section depicting paired cornu trabeculae (trabecular horns). Only the left side is labeled. **E:** Palatoquadrate cartilage. **F:** Median section depicting the trabecular plate, which underlies the brain (faint red tissue dorsally). **G:** Ceratohyal cartilage. **H:** First ceratobranchial cartilage (CB I). Scales = 0.05 mm, except in G (0.2 mm).

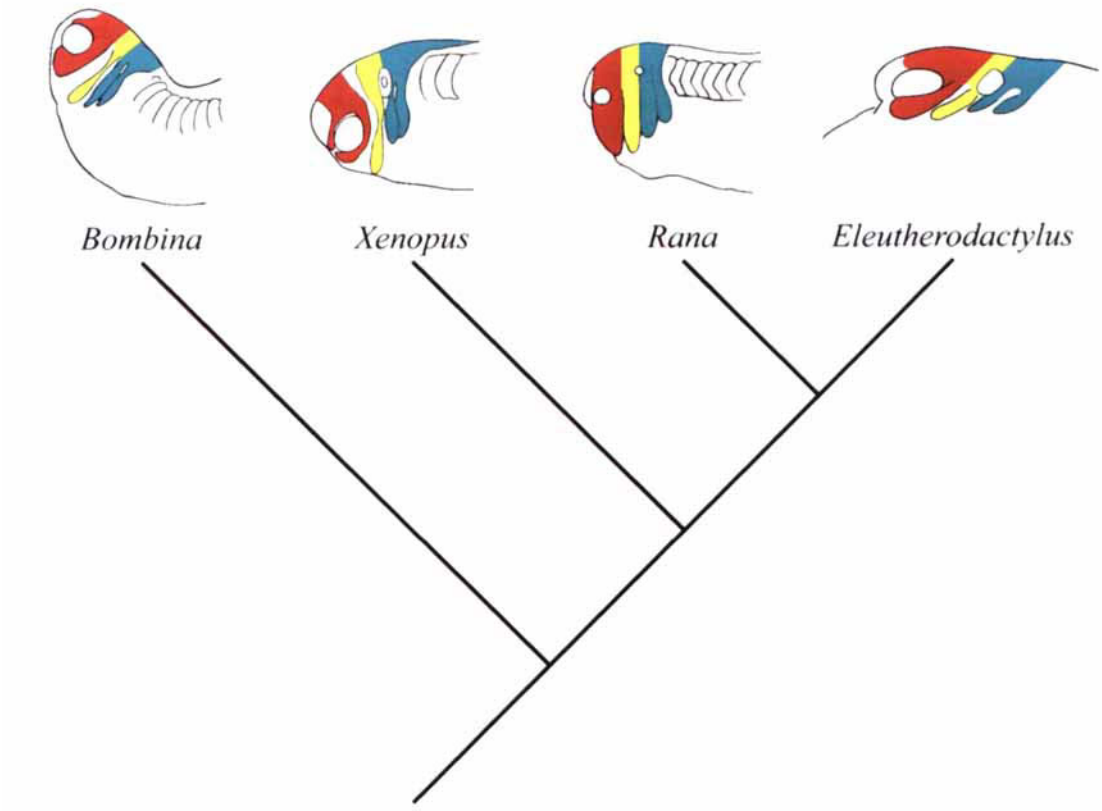

Fig. 7. Evolutionary conservation of gross patterns of cranial neural-crest migration in extant frogs that have been studied. The number and configuration of cranial neural-crest streams are remarkably constant among some distantly related anurans, including some with direct development (e.g., *Eleutherodactylus*). Hypothetical phylogenetic relationships are represented according to Ford and Cannatella ('93). Depictions of cranial neural-crest migration are based on the following sources: *Bombina*—this study; *Xenopus*—Sadaghiani and Thiébaud, '87; *Rana*—Stone, '29; *Eleutherodactylus*—Moury and Hanken, '95.

our labeled larvae were preserved. Resolution of DiI-labeled cells is technically much more difficult in a calcified tissue (such as bone) than in cartilage. This analysis, however, currently is underway in our laboratories.

Several of the above features of cranial neural-crest cell biology in *B. orientalis* are shared by all other anurans (Table 1); we regard these features as plesiomorphic among all living taxa. These features include the final number and configuration of principal migratory streams (Fig. 7), non-chondrogenic fate of the transverse neural fold, and the extensive contribution to the embryonic chondrocranium (e.g., Stone, '29; Sadaghiani and Thiébaud, '87; Seufert and Hall, '90). All three features are characteristic of vertebrates generally (Hall and Hörstadius, '88); presumably they were retained by the earliest anurans from their immediate ancestors.

Precocious onset of neural-crest emergence and migration relative to neural-fold closure may represent a fourth primitive character for anurans, but variation both among frogs and among other vertebrates makes this determination much more difficult. For example, whereas precocious crest emergence and migration are also characteristic of mammals (Tan and Morriss-Kay, '85, '86; Nichols, '86, '87; Chan and Tam, '88), neither feature occurs in urodeles, birds, or turtles. In the latter three taxa, crest emergence and migration commence much later, after neural-fold closure (Tosney, '78; Le Douarin, '82; Jacobson and Meier, '84; Hou and Takeuchi, '94). And while precocious crest emergence and migration long have been regarded as characteristic features of all living frogs, recent evidence suggests that, at least in *Xenopus*, cranial neural-crest emergence does

not begin until after neural-fold closure (Collazo et al., '94). Lack of sufficient comparative data precludes determination of the likely evolutionary transitions in this character among these taxa, or even its evolutionary polarity. Thus, it is not known whether precocious emergence and migration represent an evolutionary innovation that occurred one or more times within the Anura (independent of mammals), or if they were inherited from an ancestral taxon and lost at least once in living frogs (i.e., *Xenopus*).

Apparent variation in three additional characters suggests minor evolutionary trends affecting neural-crest migration among living anurans (Fig. 7). First, unlike *Bombina,* in which two initial cranial migratory streams subdivide to form three streams (Fig. 2A), *X. laevis* (Pipidae), four species of *Rana* (*R. japonica, R. palustris, R. pipiens,* and *R. temporaria;* Ranidae), and *Eleutherodactylus coqui* (Leptodactylidae) display three streams virtually from the inception of migration (Knouff, '27; Stone, '29; Reisinger, '33; Ichikawa, '37; Sadaghiani and Thiébaud, '87; Moury and Hanken, '95; Table 1). If *Bombina* displays the primitive character state, then the occurrence of a derived state in representative pipid, ranid, and leptodactylid taxa may evidence a heterochronic shift (predisplacement) that resulted in a much earlier subdivision of hyoid and branchial streams. Second, the large gap, or crest-free zone, that separates mandibular and hyoid streams in *Bombina* (Fig. 2B) is much smaller in *Xenopus* and *Eleutherodactylus* and absent, or nearly so, in *Rana* (Fig. 7). Finally, whereas the mandibular stream is substantially more massive than the other two cranial-crest streams in *Bombina,* in *Rana,* and in at least one bufonid (*Bufo bufo;* Olsson et al., unpublished data), all cranial streams are approximately equal in size in *Xenopus.* There is no evidence that variation in these features correlates with interspecific differences in neural-crest differentiation or fate. Nevertheless, it is both interesting and somewhat disconcerting that *X. laevis,* the anuran species most widely used to study neural-crest biology in these vertebrates, has an apparently derived pattern of variation in several characters and is arguably the most atypical species of frog considered so far.

These results confirm a prominent role for the neural crest in the evolutionary origin of the paired suprarostral and infrarostral cartilages, two prominent caenogenetic features of the rostral skull unique to larval anurans. The suprarostrals, which constitute the skeleton of the larval upper jaw, likely evolved via elaboration of the neural crest-derived anterior trabeculae typical of the neurocranium of all vertebrates and from which the suprarostrals extend anteriorly (Fig. 5). The infrarostrals, together with the laterally adjacent Meckel's cartilages, represent a doubling of the number of independent cartilages in the crest-derived mandibular (lower jaw) skeleton. Developmental mechanisms underlying these changes in skeletal patterning are unknown. While neural-crest derivation of both cartilages has been claimed in earlier studies based on crest ablation (Stone, '29; Seufert and Hall, '90), on transplantations between anurans and urodeles (Wagner, '49, '59), or on tissue explants (Raunich, '57; Cusimano et al., '62), reliability of these claims has been difficult to assess because of the technical problems or artifacts frequently associated with such methods. Sadaghiani and Thiébaud ('87) used a more refined technique for assessing embryonic derivation in *Xenopus* (interspecific grafts involving a permanent cell marker). These authors, however, provided only limited evidence of a neural-crest contribution to the anterior portion of the "ethmoid-trabecular cartilage" (= suprarostral plate; Trueb and Hanken, '92), a likely homologue of the suprarostrals, and did not evaluate the derivation of the infrarostrals. We find no support for Wagner's ('49, '59) suggestion that the suprarostrals form from crest cells that migrate dorsally from the stomodeum distinct from those that form the anterior trabeculae. Similarly, we do not corroborate the claims based on tissue-explant experiments in *Discoglossus* that supra- and infrarostral cartilages are derived from crest cells originating within the transverse neural fold (Fagone, '59; Cusimano et al., '62; Cusimano-Carollo, '63, '69, '72). Our results are fully consistent with the interpretation that, at least in anurans, neural-crest cells within the transverse neural fold have chondrogenic potential that is not expressed during normal development (Seufert and Hall, '90; Graveson, '93). The prominent role of supra- and infrarostral cartilages in larval mouth formation is well documented (Fagone, '59; Cusimano et al., '62; Cusimano-Carollo, '63, '69, '72; reviewed in Hall and Hörstadius, '88).

Conversely, our results are consistent with earlier claims regarding the lack of neural-

crest contribution to the basihyal and basibranchial cartilages, to the posterior trabecular cartilages, and to the otic capsule (e.g., Stone, '29). Thus, the resulting composite embryological origin of the chondrocranium is firmly established in several species of anurans and by the use of several alternate experimental methods, ranging from ablation (Stone, '29), to heterospecific chimeras (Sadaghiani and Thiébaud, '87), to vital labeling (this study); it is a characteristic feature of the skull in all frogs examined to date. The particular complement of non-crest-derived cartilages in anurans differs somewhat from that in other vertebrates. In birds, for example, neural crest contributes to a greater proportion of the neurocranium, including at least part of the otic capsule, and to the entire hyobranchial skeleton (Noden, '83a,b, '86b; Hall and Hörstadius, '88; Couly et al., '92, '93). The embryonic origin of the non-crest-derived components in anurans is unknown. Two likely and obvious candidates are paraxial cephalic mesoderm and somitic mesoderm, which are the source of non-crest-derived portions of the skull in chick-quail chimeras (Noden, '83b, '86b, '88; Couly et al., '92, '93), but the relative contribution of either cell population in anurans remains to be assessed. Sadaghiani and Thiébaud ('87; Table 1) indicated a contribution of "mesoderm" to the ethmoid-trabecular, ceratohyal, basihyal, and posterior branchial cartilages in *Xenopus,* but mesoderm was neither labeled nor ablated in their experiments, and no direct evidence for this assertion was provided.

Evolutionary conservatism of neural-crest pathways and fates

Notwithstanding the above minor differences in the initial configuration of migratory streams, cranial neural-crest development in metamorphosing anurans seems to be highly stereotyped and evolutionarily conservative; both migratory pathways and chondrogenic derivatives are nearly invariant among the 14 species studied (Table 1). For example, except for slight differences in the origin of the trabecular plate, the pattern of neural-crest derivation of the larval chondrocranium that we have produced for *Bombina* is virtually identical to that for *Rana* produced more than 65 years ago (Stone, '29; Fig. 5). These taxa represent clades that are among the most phylogenetically distant of all living frogs (Duellman and Trueb, '86: fig. 17-3); they likely diverged from a common ancestor no later than the Jurassic period, at least 144 million years ago. Indeed, the species *B. orientalis* may have differentiated as early as the Miocene (Maxson and Szymura, '79).

Interestingly, one situation in which this otherwise highly conserved pattern of neural-crest development has been perturbed is in the evolution of the alternate reproductive mode, direct development, as seen in the leptodactylid frog *E. coqui.* While many basic, ancestral features of cranial neural-crest cell emergence and migration have been retained (Moury and Hanken, '95), the apparently stereotyped pattern of crest derivation of the chondrocranium seen in metamorphosing taxa has been altered considerably; many larval-specific cranial cartilages do not form during embryogenesis, and the initial patterning of others is highly modified (Hanken et al., '92). Detailed aspects of this evolutionary loss of chondrogenic fate and repatterning currently are under investigation (Olsson and Hanken, in preparation).

Implications for cranial pattern formation

The composite embryonic origin of the chondrocranium as seen in anurans is a characteristic feature of skull development in vertebrates (Noden, '83b, '86b, '88; Hall and Hörstadius, '88; Couly et al., '92, '93). Indeed, this seemingly anomalous, but nevertheless consistent, mode of development has formed the basis for radical claims regarding the evolutionary origin of different parts of the skull, such as the origin of portions of the crest-derived anterior trabeculae from one or more ancestral anterior visceral arches (Bjering, '77). Regardless of its implications for head evolution, a composite origin also poses numerous intriguing problems regarding the mechanisms that specify cranial pattern (Thorogood, '93). There is, for example, overwhelming evidence of pattern specificity within premigratory neural crest that contributes to the visceral skeleton (Noden, '83a, '86a,b; Prince and Lumsden, '94). Is the patterning of skeletal elements derived from other sources also determined intrinsically, or do they instead receive their patterning cues extrinsically? One potential extrinsic cue is epithelially derived macromolecules such as type II collagen, which has been proposed to form a cranial prepattern that mediates skeletogenic cell migration or differentiation (Thorogood, '88, '93). While epithelially derived type II collagen is present in the head of embryonic *Xenopus,* it is expressed too late to

affect the gross patterning of neural crest (Seufert et al., '94). The role, if any, of this or other macromolecules in patterning non-crest-derived cartilages in anurans is yet to be evaluated. These challenges to models of cranial pattern formation are magnified in cases of composite origin that involve integrated functional systems that are also phylogenetically diverse (e.g., the hyobranchial skeleton), or even individual elements such as the trabecular plate. How is patterning coordinated between crest-derived and non-crest-derived portions of such components, both during ontogeny and during evolution? We hope to address these and similar questions in future studies.

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