Expression of *Hoxa-11* and *Hoxa-13* in the pectoral fin of a basal ray-finned fish, *Polyodon spathula*: implications for the origin of tetrapod limbs

Brian D. Metscher, a,b,* Kazuhiko Takahashi, b Karen Crow, b Chris Amemiya, c Daisuke F. Nonaka, b and Günter P. Wagner b

aDepartment of Biology, University of Southern Indiana, Evansville, IN 47712, USA
bDepartment of Ecology & Evolutionary Biology, Yale University, New Haven, CT 06520-8106, USA
cBenaroya Research Institute, Virginia Mason, Seattle, WA 98101, USA

*Author for correspondence (email: bmetsche@usi.edu)

SUMMARY Paleontological and anatomical evidence suggests that the autopodium (hand or foot) is a novel feature that distinguishes limbs from fins, while the upper and lower limb (stylopod and zeugopod) are homologous to parts of the sarcopterygian paired fins. In tetrapod limb development *Hoxa-11* plays a key role in differentiating the lower limb and *Hoxa-13* plays a key role in differentiating the autopodium. It is thus important to determine the ancestral functions of these genes in order to understand the developmental genetic changes that led to the origin of the tetrapod autopodium. In particular it is important to understand which features of gene expression are derived in tetrapods and which are ancestral in bony fishes. To address these questions we cloned and sequenced the *Hoxa-11* and *Hoxa-13* genes from the North American paddlefish, *Polyodon spathula*, a basal ray-finned fish that has a pectoral fin morphology resembling that of primitive bony fishes ancestral to the tetrapod lineage. Sequence analysis of these genes shows that they are not orthologous to the duplicated zebrafish and fugu genes. This implies that the paddlefish has not duplicated its *HoxA* cluster, unlike zebrafish and fugu. The expression of *Hoxa-11* and *Hoxa-13* in the pectoral fins shows two main phases: an early phase in which *Hoxa-11* is expressed proximally and *Hoxa-13* is expressed distally, and a later phase in which *Hoxa-11* and *Hoxa-13* broadly overlap in the distal mesenchyme of the fin bud but are absent in the proximal fin bud. Hence the distal polarity of *Hoxa-13* expression seen in tetrapods is likely to be an ancestral feature of paired appendage development. The main difference in *HoxA* gene expression between fin and limb development is that in tetrapods (with the exception of newts) *Hoxa-11* expression is suppressed by *Hoxa-13* in the distal limb bud mesenchyme. There is, however, a short period of limb bud development where *Hoxa-11* and *Hoxa-13* overlap similarly to the late expression seen in zebrafish and paddlefish. We conclude that the early expression pattern in tetrapods is similar to that seen in late fin development and that the local exclusion by *Hoxa-13* of *Hoxa-11* from the distal limb bud is a derived feature of limb developmental regulation.

INTRODUCTION

A major transition in vertebrate evolution was the origin of fully terrestrial animals, the tetrapods. One of the innovations that facilitated this transition from water to land was the tetrapod limb. Recent paleontological evidence suggests that the tetrapod limb originated about 360 million years ago (Mya) in aquatic animals living in shallow coastal waters (Clack 2002). At the morphological level the tetrapod limb arose from the paired sarcopterygian fins by the addition of a distal segment, the autopod, that is, the hand or foot (Coates 1994, 1996). Hence a main objective for understanding the developmental evolution of tetrapods is to determine the changes in paired appendage development instrumental in the origin of the autopod (Sordino and Duboule 1996; Wagner and Chiu 2001).

In tetrapod model organisms, that is, mouse, chick, and *Xenopus*, the development of the autopodium depends on the activity of *sonic hedgehog* (*Shh*) in the zone of polarizing activity (Riddle et al. 1993) and the expression of *AbdB*-like Hox genes from the *HoxA* and *HoxD* clusters (Dollé et al. 1989; Nelson et al. 1996). In the absence of *Shh* in the mouse, typical autodial structures fail to develop (Chiang et al. 2001; Litingtung et al. 2002). Lack of *Shh* activity in mouse also leads to a suppression of *Hoxa-13* because of the presence of the repressor form of Gli3-protein (Welscher et al. 2002). This regulatory link between *Shh* and *Hoxa-13* expression appears to be ancestral. In the zebrafish *Sonic-you*...
mutant, which knocks out the Shh gene, expression of Hoxa-13 is absent in the pectoral fin bud (Neumann et al. 1999). Furthermore the deletion of the 5′ HoxA and HoxD genes leads to the loss of autopodal structures, suggesting that Hoxa-13 acts upstream of the HoxD genes (Zákány et al. 1997).

The expression of Hoxa-11 and Hoxa-13 also suggests a role of Hoxa-13 in autopodal identity. Hoxa-13 is expressed in the prospective autopod while Hoxa-11 is only expressed proximal to the autopodial field (Haack and Gruss 1993; Nelson et al. 1996). This pattern of expression is likely to be homologous among the major Recent tetrapod taxa because it is found in chick and mouse, which share the most recent common ancestor of all amniotes, as well as in Xenopus (Blanco et al. 1998; Endo et al. 2000; Lombardo and Slack 2001), which shares with the amniotes the most recent common ancestor of all tetrapods. The only exceptions are urodeles, which are probably derived in their limb development (Wagner et al. 1999). Misexpression of Hoxa-13 in the proximal wing bud of chick leads to a homeotic transformation of the zeugopodial long bones into nodular bones, supporting the hypothesis that Hoxa-13 is involved in determining the autopodial identity (Yokouchi et al. 1995). In zebrafish the expression patterns of Hoxa-11 and Hoxa-13 are broadly overlapping (Sordino et al. 1995, 1996; Sordino and Duboule 1996). The interpretation of the zebrafish results, however, is complicated by the fact that the zebrafish genome is known to have a duplicated HoxA cluster (Amores et al. 1998) and has maintained both Hoxa-11 and Hoxa-13 a/b paralogs. Also, the development of the zebrafish and tetrapod endoskeletal elements is fundamentally different (Mabee 2000; Davis et al. 2004; Mabbe and Noordsy 2004). Hence it is not clear whether the expression patterns in zebrafish pectoral fin bud are indicative of the ancestral condition for the tetrapod lineage or whether it is instead derived and thus not informative for evolutionary inference. To address this problem we examined the Hox gene expression in an actinopterygian fish, the paddlefish Polyodon spathula, belonging to a basal lineage within the clade of ray-finned fishes.

Together with sturgeons the paddlefish belongs to the well supported clade of acipenseriform fishes, which split more recently from the teleost lineage than the bichir lineage (Polypterus) (Acanthias) and sarcopterygian lineages (Metscher and Ahlberg 1999). In this cladogram, Amia and the gars are shown in their traditional position as the sister lineage to the teleosts. Recent mitochondrial genome sequences, however, place the sturgeons and Amia into one clade (Inoue et al. 2003). To the right of the taxon names are shown sketches of the pectoral fin and forelimb skeleton, with anterior at the top. The metapterygium of the paddlefish is indicated by a gray oval. This skeletal system is homologous to the paired fin/limb skeleton of sarcopterygian fishes. Chondrichthyan (Acanthias) and bichir (Polypterus) from Sewertzoff (1926); lungfish (Neoceratodus) after Goodrich (1930); coelacanth (Latimeria) after Forey (1998); Amia after Grande and Bemis (1998).

Fig. 1. Phylogenetic position of paddlefish and variation in pectoral fin skeletons. Paddlefish and sturgeons (the Acipenseriformes) are the most basal living clade of actinopterygian (ray-finned) fishes aside from the bichirs (Polypterus; Grande and Bemis 1996; Janvier 1996). Considering availability of material and phylogenetic position, paddlefish are a practical choice for a representative of osteichthyan traits ancestral to both actinopterygian and sarcopterygian lineages (Metscher and Ahlberg 1999). In this cladogram, Amia and the gars are shown in their traditional position as the sister lineage to the teleosts. Recent mitochondrial genome sequences, however, place the sturgeons and Amia into one clade (Inoue et al. 2003). To the right of the taxon names are shown sketches of the pectoral fin and forelimb skeleton, with anterior at the top. The metapterygium of the paddlefish is indicated by a gray oval. This skeletal system is homologous to the paired fin/limb skeleton of sarcopterygian fishes. Chondrichthyan (Acanthias) and bichir (Polypterus) from Sewertzoff (1926); lungfish (Neoceratodus) after Goodrich (1930); coelacanth (Latimeria) after Forey (1998); Amia after Grande and Bemis (1998).
MATERIALS AND METHODS

Specimens
North American paddlefish (P. spathula) were sampled from spawns at the Kentucky State University Aquaculture Center (Frankfort, KY, USA). Hatching fish were fixed in 10% neutral-buffered formalin at daily intervals following hatching (approximately 7 days postfertilization). Hatchlings were fed fine pellet feed and maintained at seasonable water temperature, about 18°C, in large flow-through tanks. All animal procedures were performed under the guidance and approval of the University of Southern Indiana IACUC (protocol # BM-02).

Cloning and sequencing of Hoxa-11 and A-13 genes
Total genomic DNA was extracted from a frozen specimen of paddlefish (P. spathula). The coding region of Hoxa-11 and Hoxa-13 genes were amplified by polymerase chain reaction (PCR) using the primers A11FOR2 (ATGATTTGCCAAGTTGYAC) and DFNA11R2 (RTACTGCAGAGTTTCTCCTRTTMA) for Hoxa-11 and KTA13F1 (CTYTATCCGGCCTGATGTYA) and KTA13R1 (CCITTACGKYCTGTYGRAAC) for Hoxa-13. The reactions were carried out in 50 μl of a mixture of 100 ng of the extracted genomic DNA, 0.5 pmol of each primer, 0.25 mM of each dNTP, 0.5 ml of Advantage-GC2 Polymerase (Clontech, Palo Alto, CA), and buffers recommended by the manufacturer. Thirty cycles of PCR were carried out, with each cycle consisting of denaturation at 94°C for 30 sec, annealing at 50–60°C for 30 sec, and extension at 72°C for 2–3 min. Each sample was analyzed by electrophoresis in a gel that contained 1.0% (w/v) agarose in 1×TAE (Tris-acetate/EDTA) buffer. Bands of DNA were stained with ethidium bromide and visualized under UV irradiation. Products of PCR were ligated into the pGEM-T® Vector (Promega, Madison, WI). Cloned fragments were sequenced by dideoxy chain termination method. We amplified and sequenced a total of four to five clones for each gene independently to make single consensus sequences, to eliminate errors caused during PCR.

Sequence analysis
In addition to the sequences generated in this study we downloaded Hoxa-11 and Hoxa-13 sequences of the following taxa: shark (Heterodontis francisci), bichir (Polypterus senegalus), zebrafish (Danio rerio), and pufferfish (Takitus rubripes). The coelacanth (Latimeria chalumnae) sequences were obtained from the full HoxA cluster sequence. Sequences were aligned using the computer program Clustal V (Higgins et al. 1992), implemented by Sequence Navigator (Applied Biosystems, Foster City, CA). Gene genealogies were assessed by maximum parsimony (MP), neighbor joining (NJ), and Bayesian inference (Bayes) methods implemented by the software packages PAUP (Phylogenetic Analyses Using Parsimony version 4.0, Swofford 1998) and MrBayes (version 2.1, Huelsenbeck and Ronquist 2001). The most parsimonious trees were obtained using a heuristic search. Statistical confidence in nodes was evaluated using 1000 bootstrap replicates (Felsenstein 1985; Hedges 1992; Hillis and Bull 1993). The GTR model (unequal base frequencies and six substitution rates) was adopted for the likelihood analysis in MrBayes, and statistical confidence in nodes was evaluated by Bayesian posterior probabilities. Stationarity of tree likelihood, sampled every 100 cycles, was consistently achieved after 2500 generations and all sampled trees preceding stationarity were discarded.

Expression analysis
From the cloned PCR fragments parts of the exon-1 were sub-cloned into PGEM-T (Promega) for probe preparation. The subclones were amplified with the same forward primers as for the genomic DNA amplification (see above) and the following reverse primers:

- DFNA11RX: GCTGCTAGTTTCTCTCCATTGTG
- KTA13R8: TGTTGATTCTCCAGAGATGAGTCCG

The resulting clones correspond to nucleotide positions 1–528 for Hoxa-11 and 1–573 Hoxa-13, respectively, of the sequence deposited in Genbank (excluding primer sequences). The corresponding amino acid sequences of these probes are shown in Fig. 2 and exclude both the conserved homeodomains as well as the introns. A comparison between these two sequences did not show any similarities (less than 60% sequence identity in any 30 nucleotide window). These fragments were then BLAST-ed against the GenBank nucleotide sequence database to determine the likelihood of cross hybridization with other genes. In each case only either Hoxa-11 or Hoxa-13 sequences aligned. We thus conclude that these probe sequences likely are specific.

In situ hybridization was carried out using a protocol modified from Wilkinson (1992) and D. Stock (personal communication), with additional steps adapted from Breitschopf et al. (1992) and DeBlock and Debrouwer (1993). Digoxygenin-labeled (DIG) antisense RNA probes were prepared according the protocol from Roche Biochemical (Indianapolis, IN).

Paddlefish were anaesthetized in MS222 and fixed in phosphate-buffered 10% formalin for a week or more at 4°C. Specimens were then changed to 50% methanol/water, then to 100% methanol, and stored in a second change of 100% methanol at –20°C. Specimens were dissected in methanol, cleared in chloroform, and embedded in paraplast. Sections were cut with a standard microtome at 7–8 μm in a plane normal to the A-P axis of the fins (near a cross section for the body).

Slides were dewaxed in Anatech ProPar, rehydrated through an ethanol series to phosphate-buffered saline with Tween-20 (PBST), refixed in 10% formalin in PBST for 20 min and washed in PBST. Sections were then digested in protease K (10 μg/ml in PBST) at room temperature for 15 min, washed in PBST, then dehydrated in a methanol series and allowed to air dry. DIG-labeled anti-sense RNA probes were added to each slide, 75 μl per slide, at a concentration of approximately 0.2–1.0 μg/ml in the buffer described by Breitschopf et al. (1992). Slides were covered with Hybrislips (PGC Scientifics, Frederick, MD), and placed on Whatman 3MM chromatography paper wetted with 50% formamide in a flat plastic box with a fitted lid, which was then floated in a 65°C water bath overnight.

After hybridization, slides were washed 15 min in 50% formamide/1× SSC to remove coverslips, then twice for 30 min in the same solution, all at 65°C in plastic Coplin jars with screw-caps. Slides were then transferred to staining racks and washed twice in maleic acid buffer (MABT: 100 mM maleic acid, 150 mM NaCl,
0.1% Tween-20, pH 7.5) at room temperature in a staining jar with a small magnetic stir bar. Tissues were prepared for immunodetection by blocking in MABT containing 10% heat-inactivated sheep or calf serum and 0.5% Roche blocking reagent for 90 min or longer. Slides were then incubated overnight in an alkaline phosphatase-conjugated DIG antibody diluted 1:8000 in the same blocking solution.

The antibody solution was washed off with five or more changes of MABT at room temperature with stirring extended over 4 hours or more. Sections were prepared for staining with two washes (10 min each) in NTMT (100 mM NaCl, 50 mM MgCl2, 100 mM Tris, 0.1% Tween-20, pH 9.5). The alkaline phosphatase substrate chromogen NBT/BCIP (Roche) was applied to each slide in a solution of NTMT and 10% polyvinyl alcohol (30–70 kDa; Sigma, St. Louis, MO), 500 μl per slide. Slides were incubated at room temperature in a humidified flat plastic box from a few hours to overnight. The chromogen solution was washed off with distilled water, sections were dehydrated through an ethanol series, cleared twice in ProPar and once in xylene, then mounted with Cytoseal. A detailed protocol is available from the authors.

RESULTS AND DISCUSSION

The Polyodon Hoxa-11 and Hoxa-13 genes

A 1452-bp fragment of the paddlefish Hoxa-11 genomic locus was sequenced including the intron and the majority of the exon 1 and exon 2 (accession # AY661748, protein ID AAT75330). The intron is 709-bp long, comparable in size to other fish Hox-a-11 introns but only half as long as the human intron which has 1412 bp. The GC content of the intron is low, 33.8%, which matches the composition of the bichir and zebrafish Hoxa-11 introns, but is much lower than the human Hoxa-11 intron (55.3% GC). The high GC content in humans is likely to be a derived feature of mammals as it is also found in opossum (51.6% GC), but not in the turtle (38.7% GC). The paddlefish intron also contains a conserved 35-bp phylogenetic footprint shared among all Hox-a-11
introns examined to date (Chiu et al. 2000; Santini et al.
2003):

5′-TTATATGCTGGTTTTATAAGCATATAAGGGTT-
TAT-3′.

This sequence has been shown to bind nuclear proteins (Chiu
et al. 2000) and is thus likely to be a cis-regulatory
element.

The predicted amino acid sequence of the paddlefish
Hoxa-11 corresponds to amino acids 21–277 of the 282 amino
acid sequence of bichir Hoxa-11 (Fig. 2A; Chiu et al. 2004).
The alignment with the bichir sequence is 90.7% identical and
without gaps. The alignment with the shark sequence is only
84.2% identical and suggests an indel of two amino acid res-
ides that the shark is missing. The sequences of the dupli-
cated teleost genes are more divergent from each other as well
as from paddlefish, probably because of increased evolution
after gene duplication (Chiu et al. 2000).

The homeodomain sequence is identical with the canonical
Hoxa-11 homeodomain except for the first N-terminal amino
acids. In shark, bichir, and human Hoxa-11 and human Hoxc-
11 homeodomains this amino acid is a Threonine (T), but in
paddlefish it is predicted to be an Isoleucine (I). This amino
acid position is also variable within the teleost Hoxa-11 pro-
teins. In zebrafish Hoxa-11a it is a Phenylalanine (F) and in
fugu Hoxa-11b it is an Alanine (A).

Hoxa-13
A genomic fragment of 1172 bp was sequenced of the pad-
dlefish Hoxa-13 gene including the intron and most of exons 1
and 2 (accession # AY661749, protein ID AAT75331). The
intron is 384-bp long and has a very low GC content of 31%
GC. In comparison the human Hoxa-13 intron is almost
twice as long, 716 bp, and has 67% GC. Hence the length and
compositional differences between paddlefish and human in-
trons are similar to those described for Hoxa-11 above. The
amino acid sequence of the paddlefish Hoxa-13 coding region
aligns to that of the bichir Hoxa-13 without gaps (Chiu et al.
2004). The paddlefish fragment starts with amino acid 14 of
the predicted bichir amino acid sequence and continues up to
amino acid 279 (Fig. 2B). At the amino acid level the pad-
dlefish fragment is 95.5% identical with the corresponding
bichir sequence. The paddlefish sequence contains 48 amino
acids of the homeodomain that are identical to the shark and
bichir homeodomain sequences except for a Glutamine (Q) on
homeodomain position 38 instead of a Threonine (T) as in
the human and bichir Hoxa-13 homeodomain. The shark has a
Serine (S) in this position.

Gene trees
Gene trees were constructed from sequence alignments with
introns removed, with 467 and 732 bp for Hoxa-11 and
Hoxa-13 respectively. For Hoxa-11 a single most-parsimoni-
ous tree, identical to the neighbor-joining tree and to the
maximum-likelihood tree, was obtained (Fig. 3; tree
length = 546 steps). These data indicate that duplicated Hoxa-
11a paralogs in zebrafish and pufferfish form a gene lineage
clade, as are the Hoxa-11b paralogs. Together these dupli-
cated paralog groups were associated in a monophyletic clade.

Fig. 3. Cladograms produced from Hoxa-11 and Hoxa-13 se-
quences, based on 462 and 732 nucleotides, respectively. The sym-
 bols a and b refer to duplicated p a ralog s. Arrows indicate the stem
lineage where the HoxA cluster duplication event occurred. Sup-
port values for maximum parsimony/neighbor joining/Bayesian
posterior probabilities are shown at the nodes (based on 2000 boot-
strap replicates for MP and NJ and 1,000,000 generations sampled
every 100 cycles for Bayesian analysis. Blank spaces indicate boot-
strap values <50%. Note that the gene lineage of the paddlefish Hox
genes branches before the Hox gene duplication shared among ze-
brafish and fugu.
with 54% (NJ) bootstrap replicate support and a posterior probability of 97%. The Hoxa-11 genes for paddlefish and bichir were associated in monophyletic clade with support values of 62/76/99 for MP/NJ/Bayes.

For Hoxa-13 a single most-parsimonious tree, identical to the maximum-likelihood tree and similar to the neighbor-joining tree, was obtained (tree length = 732 steps; Fig. 3). The only difference was that the MP and likelihood trees did not recover a clade containing paddlefish and bichir for this locus; however, the NJ tree did and this topology was identical to the topology inferred from Hoxa-11. These data also indicate a monophyletic clade containing both duplicated Hoxa-13a and Hoxa-13b paralogs for zebrafish and pufferfish, with >93% bootstrap replicate support and a posterior probability of 100%.

The gene tree analysis for Hoxa-11 and Hoxa-13 implies that the most recent common ancestor of paddlefish and teleosts lived prior to the cluster duplication event that created the two HoxA clusters found in zebrafish and fugu. Hence the paddlefish and bichir HoxA clusters (Chiu et al. 2004) do not share the HoxA cluster duplication with zebrafish and pufferfish. This conclusion is consistent with the low degree of sequence divergence between shark, bichir and paddlefish exons compared with the strong divergence of the duplicated Hox genes (e.g. Chiu et al. 2000; Santini et al. 2003).
This result constrains the possible time frame for the HoxA cluster duplication to a time after the most recent common ancestor of teleosts and paddlefish. Paddlefish is a member of the well established clade of Acipenseriformes (Grande and Bemis, 1996). A recent study of the time of the teleost genome duplication based on the Fugu paranome came up with a molecular clock estimate of 320 Myr (Vanderpoele et al. 2004). This estimate is consistent with our result and the age of the acipenseriform lineage. Fossil evidence suggests that the most recent common ancestor of teleosts and sturgeons has to be older than 270 Myr (Gardiner, 1993). This suggests that the duplication might have happened shortly after the teleosts—sturgeon node to be consistent with the divergence time of 320 Myr estimated by Vanderpoele et al. (2004).

**Gene expression in the pectoral fin**

Section in situ hybridization using species-specific RNA probes was used to demonstrate the expression patterns of Hoxa-11 and Hoxa-13 during the early stages of Polyodon...
pectoral fin development, from 3 days posthatching (dph) to 7 dph (Figs. 4 and 5).

At 3 dph, hatchlings were 11–12 mm total length (TL). **Hoxa-11** is expressed in a concentrated region proximal to the body wall immediately subjacent to the pectoral fin bud. Expression is restricted to the mesenchyme. **Hoxa-13** is expressed within the fin bud mesenchyme in a domain distal to that of **Hoxa-11**. The two expression domains do not overlap, and both are restricted to the posterior and central areas of the fin bud.

At 4 dph, hatchlings were 12–13-mm TL. No chondrogenic condensation is visible yet. **Hoxa-11** expression is still restricted to the proximal fin mesenchyme, but is now distal to the body wall and slightly more diffuse. Expression appears faintly in a distal domain overlapping with **Hoxa-13** expression. **Hoxa-13** expression fills most of the mesenchyme within the fin bud distal to and overlapping with the proximal domain of **Hoxa-11**. Both domains are still restricted to the posterior-central portion of the fin bud.

At 5 dph, hatchlings were 13–14 mm TL, **Hoxa-11** expression is divided into dorsal and ventral mesenchymal domains by the central condensing (chondrogenic) mesenchyme. Expression of **Hoxa-11** appears to have shifted into a more extensive and distal domain overlapping with **Hoxa-13**. The expression of **Hoxa-13** is weaker than at 4 dph and more distal. Both transcripts are still only detected in mesenchyme, and both are restricted to a more posterior region than at earlier stages.

By 7 dph hatchlings were ~15 mm TL, **Hoxa-11** expression is much weaker and more diffuse, appearing in presumptive chondrocytes as well as presumptive myogenic mesenchyme (Neyt et al. 2000). **Hoxa-13** is still more distally restricted and is also seen in chondrocytes. The two expression domains still overlap, but **Hoxa-11** expression has diminished more than **Hoxa-13**. Neither transcript is detected in ectoderm.

Of the two genes investigated here **Hoxa-11** has the more dynamic expression pattern in paddlefish. It starts out in a proximally restricted pattern (early phase), then extends to the distal tip of the fin bud mesenchyme and finally gets distally restricted, largely coincidental with **Hoxa-13** expression (late phase). Qualitatively these phases of **Hoxa-11** expression are also seen in zebrafish. Neumann and collaborators (Neumann et al. 1999) show a 32 h pectoral fin bud with an expression pattern that does not extend to the distal mesenchyme, corresponding to the paddlefish early expression at 3 dph. Slightly later expression (37 h, Neumann et al. 1999; 38 h, Sordino et al. 1995, Sordino and Duboule 1996) extends all the way from the proximal to the distal tip of the fin bud mesenchyme, corresponding to the paddlefish expression at 4–5 dph. By 48 h, zebrafish **Hoxa-11** expression becomes distally restricted, similar to **Hoxa-13** expression with perhaps slightly less **Hoxa-11** distally than **Hoxa-13** (Sordino and Duboule 1996), corresponding to the 7 dph paddlefish. The differences between zebrafish and paddlefish at the later stages are minor, consisting of a slightly more posterior expression in paddlefish than in zebrafish. **Hoxa-13** is always distally expressed in both zebrafish and paddlefish.

This broad correspondence between the expression patterns of zebrafish and paddlefish imply that this pattern is likely to be ancestral for sarcopterygian fish, because it is found in both a derived (zebrafish) as well as a basal lineage (paddlefish) of its closest outgroup taxon, the ray-finned fishes (Fig. 1). This is unexpected, because the development of pectoral fin skeleton in paddlefish and zebrafish differ quite dramatically (Mabee 2000; Davis et al. 2004; Mabee and Noordsy 2004). In particular the paddlefish contains a so-called metapterygium (Fig. 1), which is homologous to the tetrapod limb skeleton, but this structure is missing in the zebrafish. The mode of development of the metapterygium is also different from that of the zebrafish radials. The zebrafish pectoral fin development is thus derived and so could be the gene expression pattern. The paddlefish data, however, show that the differences in gene expression between zebrafish and tetrapods is not because of the loss of metapterygial structures in the zebrafish, but probably ancestral, as hypothesized by Sordino et al. (1995).

In the tetrapod limb bud **Hoxa-11** expression differs most significantly from that in ray-finned fishes during late expression, starting in *Xenopus* at St 50.1 (Blanco et al. 1998), in mouse at E11.5 (Haack and Gruss 1993), and in chick at HH25 (Nelson et al. 1996). In these and later stages of limb bud development **Hoxa-11** is absent from the distal part of the limb bud mesenchyme but expressed in more proximal regions, locally exclusive to the **Hoxa-13** expression. This pat-
tern is never seen in zebrafish and paddlefish. The earlier stages of *Hoxa-11* expression are more similar with the fish expression patterns but are not as extensively documented. A proximal expression domain has been described in the mouse E9.75 limb bud (Fig. 5D in Haack and Gruss 1993), and in the HH19 wing bud (Fig. 5C in Nelson et al. 1996), but not in *Xenopus* (Blanco et al. 1998; Lombardo and Slack 2001). This lack of proximal expression pattern in *Xenopus* may be because of a reporting bias, since the earliest stage reported in both studies is stage 50, where the limb bud is already more advanced than the mouse E9.75 limb bud or the chick HH 19 wing bud. The proximal expression pattern in mouse has been associated with migrating myoblast cells (Haack and Gruss 1993). Whether the proximal expression of *Hoxa-11* in the paddlefish in the 3 dph pectoral fin bud is also related to myoblasts remains to be determined.

In all tetrapods examined, mouse, chick, *Xenopus*, and newt, there is a phase of *Hoxa-11* expression in which this gene is expressed at the very distal tip of the limb bud: newt (Wagner et al. 1999 and unpublished observations) *Xenopus* stage 50–51 (Blanco et al. 1998; Lombardo and Slack 2001), chick HH19-23 (Nelson et al. 1996), mouse E10.5-11.0 (Haack and Gruss 1993). This stage is similar to the zebrafish expression between 47 and 60 hpf (Sordino E10.5-11.0 (Haack and Gruss 1993). Whether the proximal expression of *Hoxa-11* in the paddlefish in the 3 dph pectoral fin bud is also related to myoblasts remains to be determined.

*Hoxa-13*, in contrast, seems to have a less variable expression pattern than *Hoxa-11*. In both the zebrafish as well as the paddlefish *Hoxa-13* is initially expressed with a clear distal polarity in the fin bud mesenchyme. There are smaller differences in the exact extent of the expression domains but no qualitative differences between zebrafish and paddlefish. Similarly in tetrapods *Hoxa-13* also always starts out to be expressed distally and remains expressed there throughout limb development (Haack and Gruss 1993; Nelson et al. 1996; Endo et al. 2000; Lombardo and Slack 2001). Hence the most parsimonious interpretation is that the distal part of the fin bud is characterized by the differential expression of *Hoxa-13* as is the case in the tetrapod limb bud, even though there does not seem to be a structure in the fin corresponding to the autopodium in limbs. The only distal elements that develop differently from the radials but similar to that of autopodial elements are the distal radials (Grandel and Schulte-Merker 1998; Davis et al. 2004; Mabee and Noordsy 2004). To our knowledge, however, there is no evidence that distal radials would be related to the skeleton of the autopod.

There is a short period in tetrapod limb development in which the expression patterns of *Hoxa-11* and *Hoxa-13* seem to recapitulate the late expression seen in zebrafish and paddlefish (see above). In tetrapods this apparently plesiomorphic (ancestral) pattern is quickly replaced with the tetrapod-typical pattern in which *Hoxa-11* and *Hoxa-13* expression domains do not overlap. The exclusion of *Hoxa-11* expression from the distal mesenchyme is not coincidental but depends on *Hoxa-13*: in mouse *Hoxa-13 —/— and Hd/Hd mutants *Hoxa-11* is expressed in the distal limb bud, showing that *Hoxa-13* is necessary to exclude *Hoxa-11* expression from the distal limb bud (Post and Innis 1999). We conclude that the negative regulation of *Hoxa-11* by *Hoxa-13* is a derived feature of limb development because it is neither found in a derived nor in a basal lineage of ray-finned fishes, the closest outgroup to sarcopterygian fishes. This conclusion supports the hypothesis that the autopodial field is a developmental novelty of the tetrapod lineage (Sordino and Duboule 1996; Zákány et al. 1997; Wagner and Chiu 2001).

**CONCLUSIONS**

The data presented in this study support three conclusions:

1. The most recent common ancestor of paddlefish and zebrafish did not share the duplicated HoxA clusters found in zebrafish and fugu. Hence duplicated HoxA clusters are characteristic of more derived actinopterygians. This raises the possibility that Hox cluster duplications could be associated with the teleost radiation.

2. The plesiomorphic (ancestral) expression pattern of *Hoxa-11* and *Hoxa-13* is (a) broadly overlapping and (b) restricted to the distal fin bud mesenchyme.

3. The nonoverlapping expression patterns of *Hoxa-11* and *Hoxa-13* in the tetrapod limb is derived, most likely by the acquisition of a negative regulatory influence on *Hoxa-11* from *Hoxa-13*.

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