

Evolution of digit identity in the three-toed Italian skink *Chalcides chalcides*: a new case of digit identity frame shift

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SUMMARY Digit identity in the avian wing is a classical example of conflicting anatomical and embryological evidence regarding digit homology. Anatomical in conjunction with phylogenetic evidence supports the hypothesis that the three remaining digits in the bird wing are digits 1, 2, and 3. At the same time, various lines of embryological evidence support the notion that these digits develop in positions that normally produce digits 2, 3, and 4. In recent years, gene expression as well as experimental evidence was published that supports the hypothesis that this discrepancy arose from a digit identity shift in the evolution of the bird wing. A similar but less well-known controversy has been ongoing since the late 19th century regarding the identity of the digits of the three-toed Italian skink,

Chalcides chalcides. Comparative anatomy identifies these digits as 1, 2, and 3, while embryological evidence suggests their derivation from embryological positions 2, 3, and 4. Here we re-examine this evidence and add gene expression data to determine the identity of the three digits of *C. chalcides*. The data confirm that the adult and the embryological evidence for digit identity are in conflict, and the expression of *Hoxd11* suggests that digits 1, 2, and 3 develop in positions 2, 3, and 4. We conclude that in *C. chalcides*, and likely in its close relatives, a digit identity frame shift has occurred, similar to the one in avian evolution. This result suggests that changes in of digit identity might be a more frequent consequence of digit reduction than previously assumed.

INTRODUCTION

As the basis of comparative biology, correctly assigning character homology is critical to the study of evolution. Although homologous characters, by definition, share common ancestry and descent, they can be highly divergent in both form and function. Moreover, the development of homologous characters (e.g., embryonic origins or developmental pathways) can likewise diverge (Hall 1994; Wilkins 2002; Wagner 2007), making identification of some homologies quite difficult.

Among the most debated and problematic cases are those concerning the identity of digits in taxa that have undergone partial digit reductions (Hinchliffe and Hecht 1984; Shubin 1994a; Wagner and Gauthier 1999; Shapiro 2002; Galis et al. 2003; Wagner 2005). The most controversial case is that of the avian wing, where anatomical and phylogenetic evidence is in conflict with embryological evidence regarding digit identity. To resolve this conflict a homeotic transformation of character identity—or the development of a particular character identity in a different embryological position (e.g., see Filler

2007; Vargas and Wagner 2009)—has been hypothesized to result in the development of digits 1, 2, and 3 in embryological positions that typically develop into digits 2, 3, and 4 in other amniotes (Fig. 1; Wagner and Gauthier 1999; Wagner 2005). Another less well known but similar case is that of the fore- and hindlimb of the Italian three-toed skink (*Chalcides chalcides*) (Fürbringer 1870; Sewertzoff 1931; Steiner and Anders 1946; Renous-Lecuru 1973; Raynaud et al. 1986; and see below for a brief review). In the avian forelimb, extensive empirical and experimental evidence provides strong support for the “Frame Shift Hypothesis (FSH)” (Wagner and Gauthier 1999; Larsson and Wagner 2002; Vargas and Fallon 2005a; Wagner 2005; Vargas et al. 2008; Vargas and Wagner 2009); however, no such investigation of the Italian three-toed skink has been done. Here, we review the historical controversy and re-examine the anatomical and embryological evidence of digit identity in *C. chalcides*, and suggest that, as in the avian forelimb, the FSH provides a resolution to this conflicting evidence. We provide detailed developmental and molecular evidence indicating a frame shift of digit identity in this group.

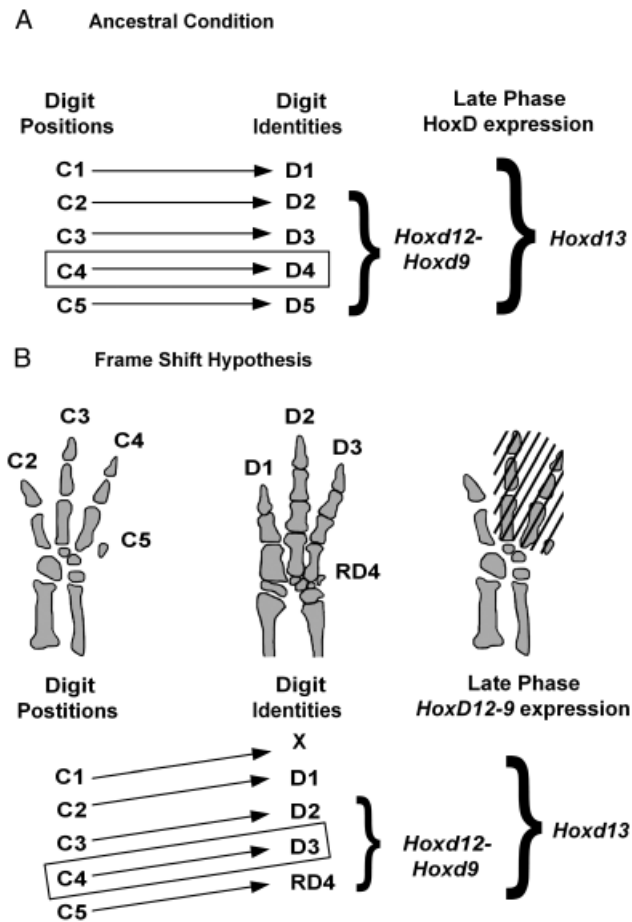


Fig. 1. Digit position, identity and gene expression patterns under ancestral conditions (A) and predicted by the frame shift hypothesis (FSH) (B). The boxes indicate the known (A) or predicted (B) position of the primary axis. (A) In the ancestral condition (e.g., *Chalcides ocellatus*) cell condensations in positions 1–5 (C1–C5) develop into digit identities 1–5 (D1–D5). During the late phase expression of the posterior HoxD genes, *Hoxd13* is expressed in all five developing digits while expression of *Hoxd12* through *Hoxd9* is restricted to posterior digits. (B) For taxa that have undergone a digit identity frame shift, there is a mismatch between digit position and digit identity. In *C. chalcides*, the FSH predicts that cell condensations in positions 2–5 (C2–C5) will result in the development of digits 1–3 (D1–D3) and rudimentary digit 4 (RD4). Associated with the shift in digit identity relative to position, a shift in expression of the posterior HoxD genes is predicted. In this case, *Hoxd13* is predicted to be expressed in C2–C5 and *Hoxd12* through *Hoxd9* are thought to be restricted to C3–C5. Hatched area indicates predicted expression pattern of *Hoxd12* through *Hoxd9* in *C. chalcides*.

BACKGROUND

Limb evolution in *chalcides*

Digit and limb reduction are common features among many extant and extinct tetrapod groups (e.g., Lande 1978; Caldwell 2003; Shapiro et al. 2007; Wiens et al. 2006; Brandley

et al. 2008). Among squamates, limb reduction is especially frequent, occurring independently 62 times in 53 lineages (Greer 1991; Wiens et al. 2006; Brandley et al. 2008), and has shaped major lineages most notably the snakes. Scincid lizards comprise a highly diverse group of squamates in which limb reduction has been a major feature, having occurred 31 times in 25 lineages (Greer 1991). One of the most important of these, in terms of morphological diversity, is the genus *Chalcides*, distributed over southwest Asia, southern Europe, northern Africa including the Canary Islands, and central western Africa (Pasteur 1981). This is a group of small to medium-sized terrestrial lizards with cryptic habits, including about 28 viviparous species that differ primarily in degree of body elongation and limb reduction (Caputo et al. 1995; Greer et al. 1998; Caputo et al. 2000; Caputo 2004). Members of *Chalcides* can be placed along a broad, graduated morphocline that goes from lacertiform lizards with pentadactyl fore- and hindlimbs to serpentiform ones with very small limbs bearing reduced or missing fingers and phalanges. These latter species tend to use lateral body undulation rather than limb movement for locomotion.

Chalcides species live in the topmost loose layer of soil, under stones, in leaf litter or in dense bushy or grass vegetation. Probably, as a consequence of the locomotory problems in such habitats, many tetrapod and pentadactyl species have relatively elongate bodies with 34–45 presacral vertebrae, compared with an apparent primitive number of 26 in skinks and other lizards (Table 1). The limb morphology of these species shows a typical phalange formula, except for the digit 4 in the manus and the digit 5 in the pes, having one phalange less than the number considered to be the ancestral complement for skinks (Caputo et al. 1995; Greer et al. 1998), with the exception of *Chalcides thierryi* that has lost a phalange in the fourth toe of the hindlimb as well (Table 1). In contrast to this relatively primitive morphology, some *Chalcides* have a much more elongate and slender body with 46–65 presacral vertebrae and very small limb showing digital reduction. *Chalcides mionecton* includes both pentadactyl and tetradactyl individuals, the latter being definitely predominant (Caputo et al. 1995). The tetradactyl ones lost the phalanges of the fifth digit on the fore and hindlimbs; metacarpal 5 is reduced, whereas metatarsal 5 is normally developed. The absence of the fifth digit in *C. mionecton* and other African skinks (see Raynaud et al. 1989) is a rather unusual mode of reduction as, in most tetradactyl scincomorphs, it is the first digit that is missing (e.g., Steiner and Anders 1946; Shapiro 2002). This mode of digit loss is similar to the one found in the theropod forelimbs, which leads to the retention of the three anterior digits (Wagner and Gauthier 1999). Another trait in which *Chalcides* appears to differ from other limb reduced lizards, in which the bone loss occurs concurrently with body elongation, is the retention of phalanges until a critical lengthening level is reached, after which the rate of loss is nearly linear

Table 1. Phalangeal formulas and range of presacral vertebrae in the genus *Chalcides* (with the exception of the species formerly attributed to the genus *Sphenops*) (elaborated from Caputo et al. 1995)

Species group (sensu Pasteur 1981)	Species	Phalanges		Presacral vertebrae
		Manus	Pes	
	Ancestral complement for skinks (Greer 1991)	2.3.4.5.3	2.3.4.5.4	26
<i>ocellatus</i>	14 species	2.3.4.4.3	2.3.4.5.4	34–45
<i>thierryi</i>	<i>Chalcides pulchellus</i>	2.3.4.4.3	2.3.4.5.4	40–41
	<i>Chalcides thierryi</i>	2.3.4.4.3	2.3.4.4.3	43–44
<i>mionecton</i>	<i>Chalcides mionecton</i> (pentadactyl individuals)	2.3.4.4.3(2)	2.3.4.5.3	46–49
	<i>C. mionecton</i> (tetradactyl individuals)	2.3.4.4.0	2.3.4.5.0	46–49
	<i>Chalcides armitagei</i>	0.2.3.3.0	0.2.3.4.0	50–51
<i>mauritanicus</i>	<i>Chalcides mauritanicus</i>	0.0.2(1).3.0	0.2.3.4.0	50–51
<i>chalcides</i>	Five species	0.2.3(2).3(2).0	0.2.3(2).3(2).0	56–65
	<i>Chalcides guentheri</i>	0.0.0.0.0	0.0.0.0.0	60–65

The alternative phalange conditions are given in parentheses.

(Greer et al. 1998) (Table 1). Indeed, limb reduction is most marked in the very elongate (over 50 presacral vertebrae) sand-swimmers *Chalcides armitagei* (tridactyl) and *Chalcides mauritanicus* (didactyl anteriorly, tridactyl posteriorly) and in the grass-swimmers (tridactyl or adactyl species in the *C. chalcides* group, sensu Pasteur 1981) (Table 1). Interestingly, although the phalanges of the fifth toe are the first to disappear, the corresponding metapodial element persists, even though in a rudimentary form, in the tri- and didactyl species. Its persistence presumably has functional value, linked to the fact that the fifth metapodial is an insertion point for muscles still involved in postural movements and digging even after the limbs lost a primary role in locomotion (see Rewcastle 1983). Concerning a possible evolutionary scenario of limb reduction and body elongation, parsimony reconstruction based on DNA phylogeny indicates that a degree of reduction in digit number has evolved independently at least three times in the genus *Chalcides* (Carranza et al. 2008; Fig. 2).

Digit reduction and identity in *C. chalcides*

Like other members of its clade, *C. chalcides* is a grass-swimming species that typically moves through vegetation without the use of limbs; however, the limbs do function to stabilize the animal while resting and are also reportedly used during slower movement to push away vegetation (Bruno and Mauergeri 1976; Orsini and Cheylan 1981; see also fig. 5 in Wagner 2005). As a result, while reduced in size and digit number, the limbs of *C. chalcides* are well-formed and functional. The reduced fore- and hindlimb contain three digits, originally identified as digits 1, 2, and 3 based on anatomical evidence (*C. chalcides* referred to as *Seps tridactylus*, Fürbringer 1870), with an additional rudimentary metapodial element positioned posterior to the other metacarpal and metatarsals (Fig. 3, A and B). The three digits have been shown to share characteristics of digits 1, 2, and 3 in other four- and five-toed

Chalcides species supporting their original identification (Steiner and Anders 1946; Renous-Lecuru 1973). However, studies tracking digit formation through development found that the digits develop in embryological positions that form digits 2, 3, and 4 in five-toed lizard species (Sewertzoff 1931; Raynaud et al. 1986), and showed that the rudimentary metapodial element develops in the typical embryological position of the fifth metapodial element (Raynaud et al. 1987). As a result, contrary to anatomical digit identities, the embryological studies identify the three *C. chalcides* digits at digits 2, 3, and 4.

Resolution of the conflict—the FSH

In *C. chalcides* anatomical and embryological evidence results in conflicting identification of the digits (reviewed in Wagner 2005 and above). A remarkably similar conflict of digit identity and position occurs in the avian hand. In this case, Wagner and Gauthier (1999) proposed the FSH as a way to reconcile the different sets of evidence for digit identity (i.e., anatomical/paleontological and embryological). The FSH suggests that over evolutionary time the developmental genetic determinants of digit identities 1, 2, and 3 shifted to positions 2, 3, and 4 (Fig. 1).

Since its original proposal, support for the FSH in the avian forelimb has come from several lines of evidence. First, central to the FSH is the location of the primary axis. The primary axis is the first digit anlage to develop. It develops in line with the ulna and ulnare in the forelimb and the fibula and calcaneum in the hindlimb, and invariantly develops in embryological position 4 (Shubin and Alberch 1986; Müller and Alberch 1990; Burke and Feduccia 1997). One requirement of the FSH is that the primary axis must develop in embryological position 4 and ultimately generate digit 3 in the bird hand. In fact, several developmental studies that visualize all five digit anlagen of the avian hand by staining the initial

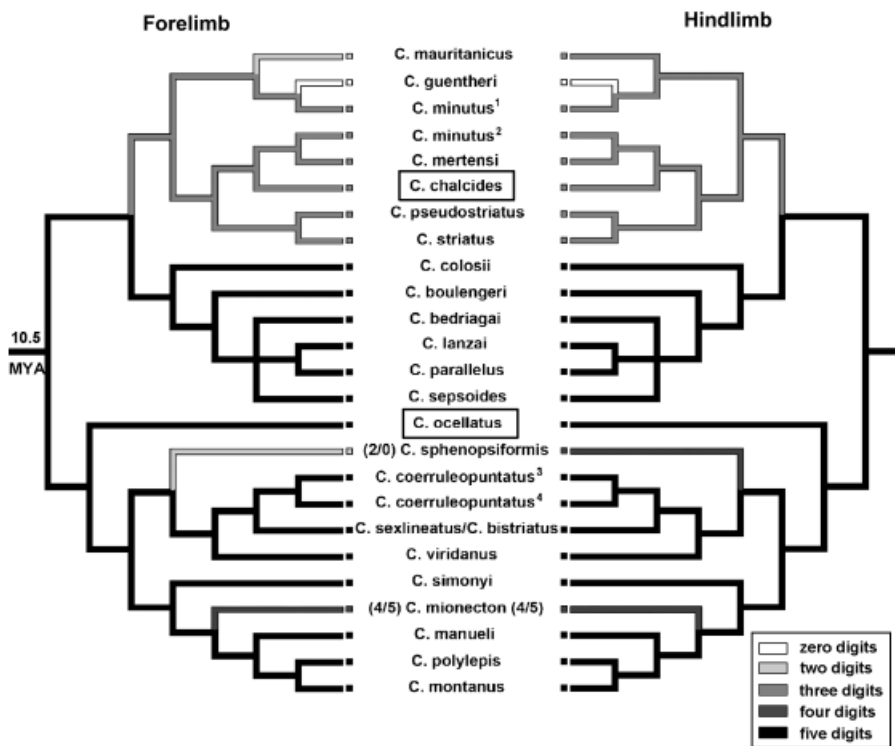


Fig. 2. Phylogeny of the Chalchids skinks redrawn from Carranza et al. (2008) including the taxa utilized for this study highlighted in the open boxes: the Italian three-toed skink *Chalcides chalcides* and the ocellated skink *Chalcides ocellatus*. Taxa are color coded by number of digits in the forelimb (left) and hindlimb (right), black indicates five digits, dark gray indicates 4 digits, gray indicates 3 digits, light gray indicates 2 digits and open indicates no digits. Variable digit numbers are indicated in the parentheses next to species name. Two taxa *Chalcides minutus* and *Chalcides coeruleopunctatus* are listed twice on the phylogeny due to high sequence divergence between geographically distinct populations (*C. minutus*: ¹Debdou, Morocco and ²Middle Atlas region, Morocco; *C. coeruleopunctatus*: ³La Gomera, Canary Island; ⁴El Hierro, Canary Islands; Carranza et al. 2008)

condensations of pre-chondrogenic cells found one pre-chondrogenic digit condensation posterior to the primary axis (i.e., the first developing digit anlage) and three digit condensations anterior to the primary axis (Feduccia and Nowicki 2002; Kundrat et al. 2002; Larsson and Wagner 2002; Welten et al. 2005). These results indicate maintenance of the primary axis in position 4, and subsequently support the hypothesis that digits 1, 2, and 3 of in the avian hand develop from embryological positions 2, 3, and 4.

Second, the FSH explicitly hypothesizes that a shift in the developmental genetic determinants of digit identity underlies the proposed homeotic frame shift (Wagner and Gauthier 1999). One critical determinant of digit identity is the expression patterns of the posterior HoxD genes (e.g., *Hoxd13* to *Hoxd9*) in the developing digits. Specifically, while *Hoxd13–9* are expressed during development of the posterior digits (i.e., digit identities 2–5), only *Hoxd13* is expressed in developing digit 1 (reviewed in Vargas and Fallon 2005b; see also Montavon et al. 2008). This pattern of HoxD expression is evolutionarily conserved across numerous vertebrate taxa (Morgan et al. 1992; Nelson et al. 1996; Zákány et al. 1997; Chiang et al. 2001; Vargas and Fallon 2005a; Vargas et al. 2008). In the bird forelimb, examinations of posterior HoxD expression patterns reveal that a shift in HoxD expression is associated with the digit identity shift of digit 1 to position 2. Digit 1 only expresses *Hoxd13*, whereas digits 2 and 3 express all the posterior HoxD genes (Nelson et al. 1996; Vargas and Fallon 2005a) indicating that underlying developmental ge-

netic features associated with digit identity have also undergone a frame shift.

Finally, in a recent experiment, Vargas and Wagner (2009) induced an additional homeotic frame shift of digit identity in the chicken forelimb that mimics the hypothesized transformation that occurred in the lineage of modern birds. The developing embryos were treated with the sonic hedgehog inhibitor cyclopamine (see Vargas and Wagner 2009 for further discussion). The treated embryos developed only two digits, digits 1 and 2. Instead of developing in embryological positions 2 and 3, as in untreated chicken embryos, these two digits develop in embryological positions 3 and 4. Beyond the shift in digit identity relative to position, Vargas and Wagner (2009) showed that a shift in expression of the posterior HoxD genes also occurred as are result of cyclopamine treatment. These findings provide evidence that a simultaneous homeotic frame shift of digits is developmentally plausible and suggest that it can occur through a relatively simple yet unknown mechanism.

While extensive studies in birds provide strong support for the FSH, the commonality of this mechanism in other cases of digit reduction remains unknown. Here, we ask whether a similar frame shift in digit identity has occurred in *C. chalcides*. To test the FSH, first, we verify the embryological positions of the digits by examining development of the *C. chalcides* autopod (i.e., hands and feet). Second, we assess characteristics of the developing and fully formed autopod to assign anatomical identity to the digits. Finally, we examine

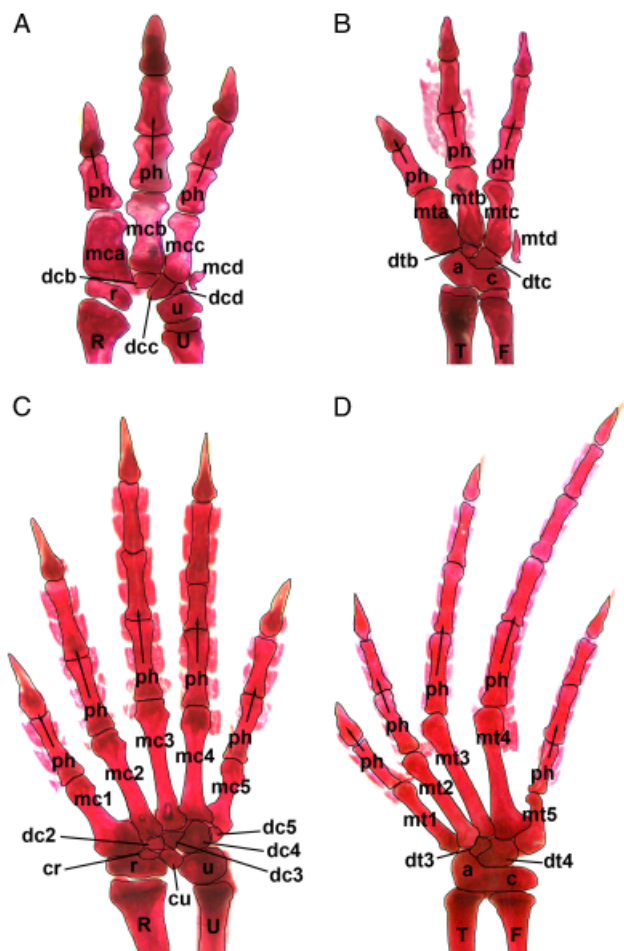


Fig. 3. Osteology of the fore- and hindlimbs of *Chalcides chalcides* (A and B; $n = 1$) and *Chalcides ocellatus* (C and D; $n = 1$). Shown are the cleared and Alizarin red stained adult fore- and hindlimbs. Skeletal elements are labeled R, radius; U, ulna; r, radiale; u, ulnare; cr, centrale radiale; cu, centrale ulnare; dc, distal carpal; mc, metacarpal; ph, phalanges; T, tibia; F, fibula; a, astragalus; c, calcaneum; dt, distal tarsal; mt, metatarsal.

whether the developmental genetic features associated with digit identity have shifted by documenting patterns of *Hoxd11* expression in the developing *C. chalcides* autopod.

MATERIALS AND METHODS

Embryo collection

The three-toed skink (*C. chalcides*) is a live-bearing, short-limbed, snake-like skink distributed in peninsular (south of the Po river) and insular Italy and North Africa (Tunisia and Libya) (Caputo 1993). Their very specialized ecology confines this lizard to humid meadows, where it lives as a “grass-swimmer.” Gravid *C. chalcides* females were wild-caught between 2005 and 2008 in a mountain area of central Italy (1000 m above sea level, 43°07′21.20″N; 12°58′50.70″E). All animals were collected by hand in grasslands.

The animals were either sacrificed within 24 h for embryo processing or were held in captivity to allow further embryo development before harvesting. Soon after collection, the embryos were fixed in 4% paraformaldehyde (pH 7.5) at +4°C for 16 h and then transferred to absolute methanol and stored at –20°C until use. The embryos were subsequently rehydrated through a descending methanol scale (95%, 85%, 75%, and 50% methanol, each for 5 min), washed five times in distilled water (5 min for three times and 25 min for two times) and then transferred to RNA later (Amersham, Milan, Italy) for shipping to the United States. At Yale, the specimens were again transferred to methanol and stored until use at –20°C.

Visualization of skeletal elements

Embryos were staged based on limb development using the alligator staging series developed by Ferguson (1985). Skeletal elements critical for digit identification (e.g., the primary axis) were documented as cartilaginous condensations and subsequently compared in the adult skeletal form with a closely related five-toed skink (*Chalcides ocellatus*). To visualize these cartilaginous and skeletal structures, we cleared and stained whole *C. chalcides* embryos at various developmental stages as well as adult *C. chalcides* and *C. ocellatus* fore- and hindlimbs with Alcian Blue and Alizarin Red using standard procedures (described previously in McLeod 1980; Hanken and Wasserug 1981). Cleared and stained limbs were photographed at various magnifications using a Spot Insight 3.2.0 camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) attached to a WILD M3Z microscope (Heerbrugg, Switzerland).

Cloning and sequencing of a fragment of the *C. chalcides Hoxd11* gene

Genomic DNA was extracted from muscle tissue excised from *C. chalcides* using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer’s instructions. Degenerate primers were designed by targeting conserved regions of exon 1 of the *Hoxd11* gene (Vargas et al. 2008).

1FD11: 5′-ATGAMCGASTTTGACGAKTGC-3′

D11IDGR: 5′-GSCTCGTARAACGGTCRAA-3′

Using these primers, a 476 nucleotide fragment of exon 1 of the *C. chalcides Hoxd11* gene was amplified. The resulting polymerase chain reaction (PCR) product was cloned into PCR—Blunt II—TOPO (Invitrogen, Carlsbad, CA, USA) and the inserted sequence was verified using SP6 and T7 primers for sequencing. Sequence identity as *Hoxd11* was confirmed using neighborhood joining and maximum parsimony analysis in PAUP* (Swofford 2002). To construct a probe for in situ hybridization, the exon 1 fragment of *C. chalcides Hoxd11* was cloned into a PCR-BluntII TOPO vector (Invitrogen) and then used as a template to transcribe a labeled antisense mRNA probe.

In situ hybridization

C. chalcides embryos were processed and stored as described above. Location of *Hoxd11* transcripts in the developing fore- and hindlimbs were visualized using digoxigenin labeled *C. chalcides* anti-sense mRNA probes. In situ hybridization was carried out using standard procedures (described in Nieto et al. 1996).

RESULTS

Osteology of the *C. chalcides* and *C. ocellatus* Autopods

The reduced autopodia of the adult *C. chalcides* contain almost half the number of independent, ossified elements as the closely related five-toed *C. ocellatus* (17 vs. 29 in the hand and 15 vs. 26 in the foot). Because the identity of the three fully formed fore- and hindlimb *C. chalcides* digits is in question we will refer to them, from anterior to posterior, as digits Da, Db, and Dc as we describe their morphology and assess the anatomical evidence of their identity. In *C. chalcides* the forelimb autopod consists of the radiale (r), ulnare (u), three distal carpals (dc), three digits with fully formed metacarpals (mc) with 2, 3, and 3 associated phalanges (ph) from anterior to posterior (digits Da, Db, and Dc), and an additional rudimentary metacarpals positioned posteriorly to the three fully formed digits (Fig. 3A). The *C. chalcides* hindlimb autopod consists of a fused astragalus (a) and calcaneum (c), two distal tarsals (dt), three digits with fully formed metatarsals (mt) with 2, 3, and 3 associated phalanges from anterior to posterior (digits Da, Db, and Dc), and a rudimentary metatarsal in the same posterior position as in the forelimb (Fig. 3B). In *C. ocellatus*, the forelimb autopod consists of: the radiale, ulnare, centrale radiale (cr), centrale ulnare (cu), four distal carpals, five metacarpals with 2, 3, 4, 4, and 3 associated phalanges from anterior to posterior (digits 1–5, Fig. 3C). The *C. ocellatus* hindlimb autopod consists of a fused astragalus and calcaneum, two distal tarsals, five metatarsals with 2, 3, 4, 5, and 4 associated phalanges from anterior to posterior (digits 1–5, Fig. 3D).

Shape and size characteristic of the digits vary across the autopod. Most apparent is the variation in the metacarpal of the most anterior digits of the forelimb (Fig. 3A: mc1). Unlike the more posterior metacarpals, mc1 has a broad and rounded morphology in *C. ocellatus* (Fig. 3C). In addition, as in all other lepidosaurier, the proximal articulation surface of the mc1 is more proximal than that of the mc2–5 because of the absence of the dc1. Hence mc1 directly articulates with the radiale instead of a distal carpal. The absence of dc1 and dt1 in typical *C. ocellatus* is likely due to the fusion of the dc1/dt1 to mc1/mt1 during development, because natural variation occasionally shows a rudimentary individualized bony element proximal to mc1 of *C. ocellatus* and other pentadactyl species (Caputo et al. 1995). The number of phalanges in the hand are 2, 3, 4, 4, 3 and in the foot 2, 3, 4, 5, 4. Comparing the three digits of *C. chalcides* we find that the greatest difference here also exists between digits Da and Db, that is the anterior most and the following one. Digits Da and Db of *C. chalcides* differ in the same characters from each other as digits 1 and 2 in *C. ocellatus*: phalange numbers are 2 and 3, respectively, the mc-a is much broader and shorter than mc-b, and the mc-a articulates more proximally with the radiale,

which mc-b is articulating with a distal carpal and the proximal articulation surface of mc-a is broad rather than round. Most significantly, however, there are not any great differences between digit 1 of *C. ocellatus* and digit Da from *C. chalcides*. The only real differences are size, but not relative size, and that mc-a is broader than mc-b, but this makes digit Da not more similar to any other digit. Hence we conclude that, based on osteological similarity, the *C. chalcides* digit Da is most likely homologous to digit 1 in *C. ocellatus*. Digit Db in *C. chalcides* is also quite similar to digit 2 in *C. ocellatus*.

The digit that is most problematic to identify in the autopod of *C. chalcides* is digit Dc. If one assumes continuity of digit identity then it should be called digit 3, but this conclusion is not supported by structural similarity. In fact, the only digit in *C. ocellatus* hand is similar to digit Dc in *C. chalcides* is digit 5: it has three phalanges, a slender mc articulated to a distal carpal and the mc is shorter than the mc-b. We thus decline to make a homology assessment of Dc here.

Embryology of the *C. chalcides* autopod

It is clear from adult morphology that *C. chalcides* has three fully formed digits (digits Da, Db, and Dc) and a rudimentary metapodial element on both the fore- and hindlimb (mc-d and mt-d); however, the positional origins of these digits cannot be determined based on adult morphology. There are two possible origins for these digits. As suggested under the FSH, digit Dc could develop from the primary axis (position 4) resulting in the development of mc/mt-d in position 5 and digits Da and Db develop in positions 2 and 3. Alternatively, the rudimentary mc/mt-d could develop from the primary axis (position 4) resulting in the development of the three anterior digits Da, Db, and Dc in positions 1, 2, and 3, respectively. To distinguish between these alternative hypotheses, we tracked limb formation with the goal of identifying whether the primary axis develops into fully formed digit Dc or the rudimentary metapodial elements mc/mt-d.

We tracked limb formation beginning at developmental stage 17 (Fig. 4, A and D). At this stage, the radius (R), ulna (U), radiale, ulnare, two distal carpals, four metacarpals, and four phalanges are visible in the forelimb (Fig. 4A). The tibia (T), fibula (F), astragalus, calcaneum, two distal tarsals, four metatarsals, and five phalanges are visible in the hindlimb (Fig. 4, A and D). While numerous autopodial elements are visible at this stage, the positioning of the condensations in the hand- and footplate identify the primary axis. Specifically, the most posterior carpal (dc-c) and its associated metacarpal (mc-c) are developing in line with the ulna and ulnare in the forelimb and the most posterior tarsal (dt-c) and its associated metatarsal (dt-c) are developing in line with the fibula and calcaneum in the hindlimb (Fig. 4, A and D). Moreover, the distal carpal d associated with rudimentary metacarpal

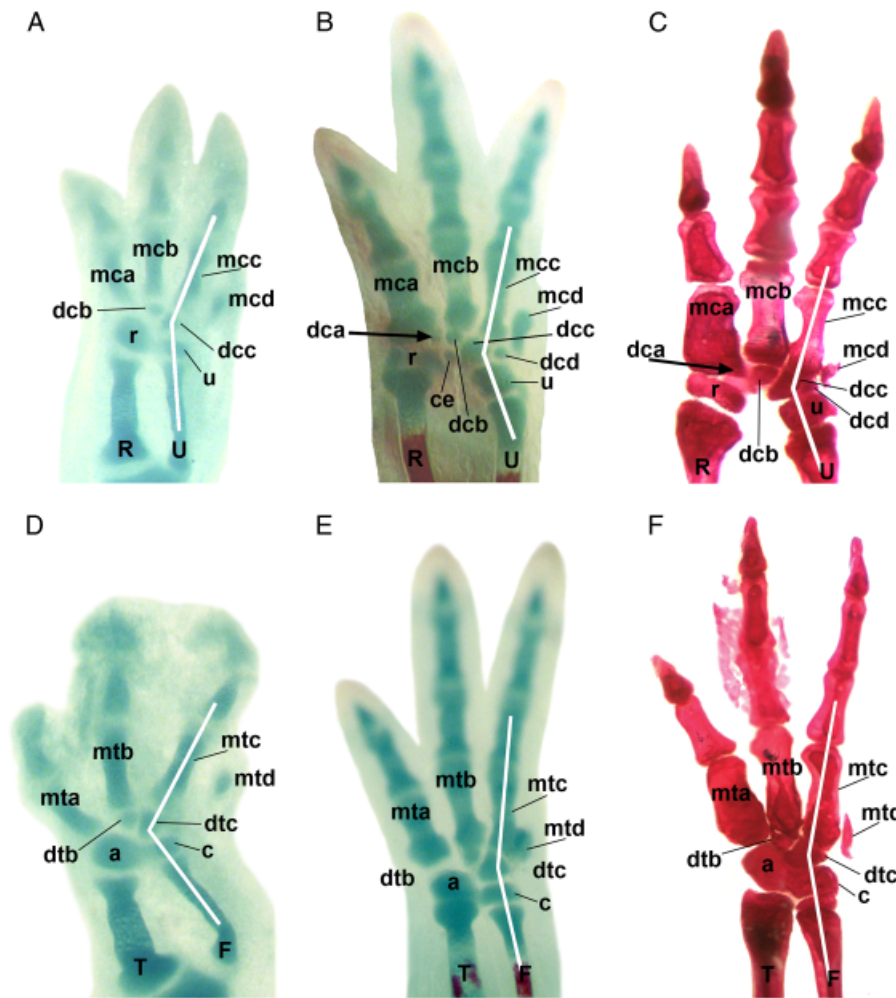


Fig. 4. Developmental series for *Chalchides chalcides* fore- and hindlimbs, see detailed description in text. Shown are stages 17 (A, forelimb; D, hindlimb; $n = 1$), 19 (B, forelimb; E, hindlimb; $n = 1$), and adult (C, forelimb; F, hindlimb; $n = 1$). White lines indicate the position of the primary axis. Black arrows point to dc-a before and after it fuses with mc-a (B and C, respectively). See Fig. 3 legend or text for labels of cartilaginous and ossified elements. Note that dcd is absent in stage 17 (A) and thus can not be part of the primary axis. For that reason the primary axis has to run through dcc and mcc and Dc.

d is not visible at stage 17 indicating that this rudimentary metacarpal cannot be part of the primary axis (Fig. 4A), distal carpal d is visible by stage 19 (Fig. 4B). Corroborating previous studies, these findings support the hypothesis that digit Dc develops from the primary axis (i.e., position 4) that the rudimentary metapodial element mc/mt-d is in position 5, and that anterior digits Da and Db are in positions 2 and 3.

By stage 19, several new elements appear in the forelimb (Fig. 4B). The centrale (ce) and two distal carpals (dc-a and dc-d) appear. Neither the centrale (ce) nor the anterior distal carpal (dc-a) are present in the adult. In the fully ossified hand, the centrale is fused with ulnare and the anterior distal carpal (dc-a) is fused with the first metacarpal (arrow in Fig. 4, B and C).

Also significant is the observation of a small round cartilage in the proximal-posterior position relative to the mc-a, which is likely to be the adult missing dc1. Remember that in *C. ocellatus* the existence of a transient dc1 is likely given the

occasional presence of a small bony element in the same relative position to mc-1 (Caputo et al. 1995).

Cloning and sequencing analysis

A genomic fragment of *Hoxd11* exon 1 of *C. chalcides* was amplified by PCR using degenerate primers designed to target conserved 5' regions of the gene (see "Materials and Methods"). These primers target a 21 nucleotide regions exhibiting high conservation among human, chicken, and alligator *Hoxd11* sequences. Using these primers, we amplified and sequenced a 476 nucleotide product. The translation of the sequenced region of *C. chalcides Hoxd11* has 73% sequence conservation with the corresponding region of *Hoxd11* in chicken (Fig. 5A). To confirm the identity of the amplified region as a *Hoxd11* ortholog, we performed phylogenetic analysis of the sequenced region in *C. chalcides* that included published sequences of both *Hoxd11* and two other paralogous group 11 Hox genes (*HoxA-11* and *HoxC-11*) in

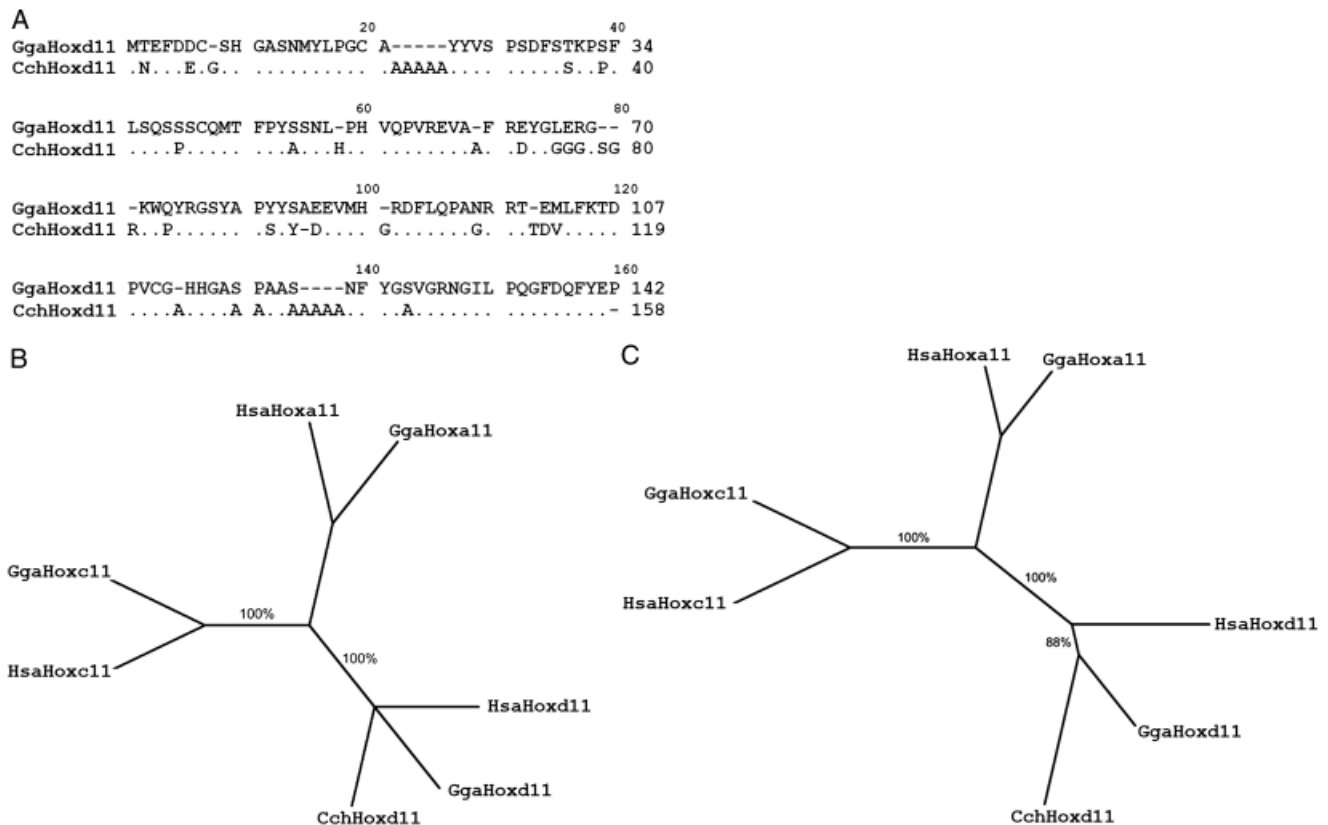


Fig. 5. Identification of *Chalcides chalcides* *Hoxd11* exon 1 sequence. (A) The amino acid sequence obtained from *C. chalcides* (Cch) is aligned with the chicken (*Gallus gallus*; Gga) *Hoxd11* sequence. In this exon 1 region of *Hoxd11*, the sequence conservation is 73%. Maximum parsimony and neighbor joining trees (B and C, respectively) were made from aligned sequences of exon 1 in *Hoxd11*, *Hoxc11*, and *Hoxa11* in chicken (Gga), human (Hsa), and *C. chalcides*. Numbers on the internal branches indicate bootstrap support values. For both trees, *Chalcides chalcides* *Hoxd11* forms a well-supported clade with human and chicken *Hoxd11* sequences indicating that the *C. chalcides* sequence is a *Hoxd11* homolog.

chicken and human. Supporting the hypothesis that our *C. chalcides* sequence is a *Hoxd11* ortholog, our sequence formed a well-supported clade with human and chick *Hoxd11* sequences (100% bootstrap support for both maximum parsimony and neighbor joining Trees; Fig. 5, B and C, respectively).

Expression of *Hoxd11* in embryonic limbs of *C. chalcides*

Hoxd11 expression patterns in the *C. chalcides* fore- and hindlimb at stage 17 ($n = 2$) are presented in Fig. 6 along with comparable stages in the alligator (*Alligator mississippiensis*) and chicken (*Gallus gallus*). Because of lack of embryonic samples, only stage 17 *C. chalcides* embryos were used. Similar to expression patterns in chicken, at stage 17 *Hoxd11* is expressed in the digit and interdigit of Db and Dc. Interestingly, at this stage, *Hoxd11* expression in *C. chalcides* extends to more anterior regions of the autopod. Specifically, as there

is no expression in digit Da, *Hoxd11* is mildly expressed in the interdigit region between digits Da and Db (Fig. 6, A and D, black arrows). A similar, but more pronounced, anterior expansion of *Hoxd11* occurs in mouse and alligator digit 1 a bit earlier in limb development (stage 10.5 in mouse, Panman et al. 2006; stage 14 in alligator, Vargas et al. 2008). By developmental stage 17 in the alligator, the anterior limit of *Hoxd11* expression is in the interdigit between 1 and 2 (Fig. 6B, black arrow; Vargas et al. 2008) as in *C. chalcides* between Da and Db (Fig. 6, A and D).

In addition to autopodial expression, *Hoxd11* has a known expression domain in the developing mesopod and zeugopod (“wrist” and “forearm,” respectively) of mouse, chicken, and alligator (Nelson et al. 1996; Chiang et al. 2001; Vargas et al. 2008). Expression of *Hoxd11* in the *C. chalcides* hindlimb mesopod and zeugopod is similar to chicken, there are some notable differences in forelimb expression. Specifically, the *C. chalcides* forelimb mesopod exhibits milder and more posteriorly restricted expression of *Hoxd11*.

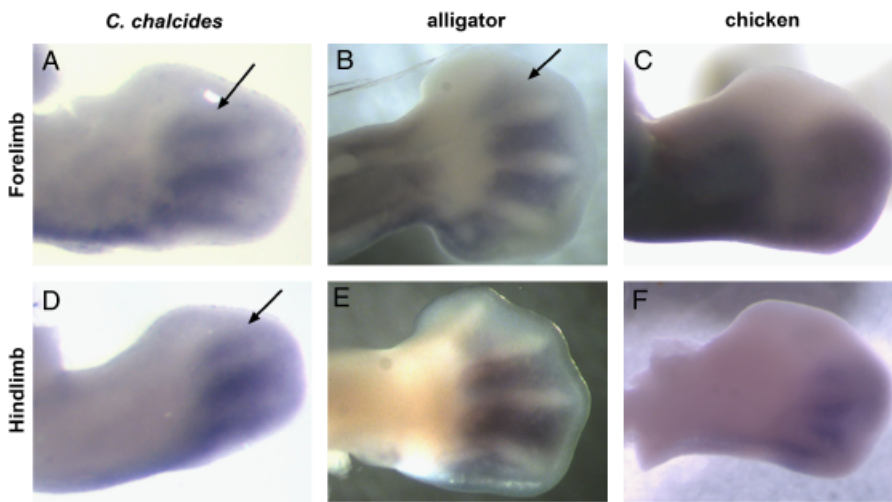


Fig. 6. Patterns of *Hoxd11* expression in stage 17 *C. chalcides* fore- and hindlimbs (A and D, respectively; $n = 2$). *Hoxd11* expression in similar stages of fore- and hindlimb development in the alligator (stage 17; B and E, respectively) and chicken (stage 29; C and F, respectively) fore- and hindlimb alligator (B and E, respectively) are provided for comparison. *Hoxd11* expression is restricted to the posterior digits (i.e., digits Db and Dc in *C. chalcides*, digits 2 and 3 in chicken, and digits 2–5 in alligator) and is absent in the anterior-most digit in all cases. In *C. chalcides* expression of *Hoxd11* extends into the interdigit between Da and Db (A and D, black arrows). Although this differs from expression patterns in the chicken, a similar expansion of *Ho-*

xd11 expression occurs in the interdigit between digits 1 and 2 in the alligator (B, black arrow; Vargas et al. 2008) as well as in the mouse (Panman et al. 2006).

DISCUSSION

Evidence of a frame shift of digit identity in *C. chalcides*

Our examination of embryology, anatomy, and gene expression patterns in the *C. chalcides* autopod suggest that a digit identity frame shift has occurred in this lineage. First, our results corroborate the previous finding that anatomical and embryological evidence of digit identity are in conflict. Based on the location of the primary axis (Fig. 4), we show that the embryological position of the *C. chalcides* digits are 2, 3, and 4, confirming findings of previous studies (Sewertzoff 1931; Raynaud et al. 1986). At the same time, characteristics of autopod anatomy suggest that the *C. chalcides* digits are 1, 2, and possibly 3 (previously described in: Fürbringer 1870; Steiner and Anders 1946; Renous-Lecuru 1973). The broad and round shape of the base of the anterior-most metacarpal is morphologically similar to that of metacarpal 1 in the closely related five-toed species *C. ocellatus* (Fig. 3, A and C). Moreover, in both the fore- and hindlimb, phalangeal formula of the two anterior digits of *C. chalcides* (2–3) is shared with digits 1 and 2 of *C. ocellatus* (Fig. 3). Although these morphological similarities suggest that the *C. chalcides* digits are digits 1, 2, and possibly 3, both morphology and number of phalanges are known to evolve and thus are not reliable indicators of digit identity. However, we show that the anterior-most distal carpal in *C. chalcides* fuses with the anterior-most metacarpal late in limb formation (Fig. 4, B and C, arrow). This carpal–metacarpal fusion is thought to be a characteristic of digit 1 in *C. ocellatus* as well as squamates in general (Steiner and Anders 1946; Rieppel 1992), and thus further supports the diagnosis of the anterior-most digit in *C. chalcides* as digit 1. Embryological evidence of this distal car-

pal–metacarpal fusion in *C. ocellatus* is currently investigated (Brandley, Young and Wagner in prep.).

As in the avian forelimb, associated with the shift in digit identities relative to embryological position is a shift in the developmental genetic determinants of digit identity, that is, expression of the posterior HoxD genes (reviewed in Vargas and Fallon 2005b). The posterior HoxD genes are expressed in two distinct phases of limb development (reviewed in Spitz et al. 2005; Tarchini and Duboule 2006; Deschamps 2007). While early phase HoxD expression likely plays an important role in establishing the anterior–posterior axis of the limb bud (Zákány et al. 2004), HoxD expression at this stage is independent of digit determination (Nelson et al. 1996). However, late phase expression of the posterior HoxD genes is strongly associated with digit identity, in particular with the difference between digits 1 and the more posterior digits. Specifically, lack of *Hoxd12–9* in the anterior-most digit is an evolutionary conserved determinant of “thumbness” or digit identity 1 (Montavon et al. 2008; Vargas et al. 2008). In *C. chalcides*, late phase expression of *Hoxd11* is restricted to the posterior digits and digit rudiment (Fig. 6). The lack of *Hoxd11* expression in embryological position 2 has several interesting implications. First, most important for the context of this study, it validates the identity of this most anterior digit as digit 1. Second, because *Hoxd11* is expressed in embryological position 2 of known five-digitated amniotes (Fig. 1A) (Vargas and Fallon 2005a; Vargas et al. 2008), such as alligator and mouse, it suggests that the shift in expression is a derived condition. While studies of HoxD expression in the avian forelimb and their outgroup taxa (alligator and mouse) have lead to the same conclusion (Vargas et al. 2008), unlike in birds, *C. chalcides* has extant, close relatives that maintain the ancestral five-digit morphology (Fig. 2; Carranza et al. 2008).

The lineages of the three-toed *C. chalcides* and the five-toed *C. ocellatus* diverged approximately 10.5 million years ago (Fig. 2; Carranza et al. 2008) indicating that modifications of posterior HoxD expression in the autopod can evolve relatively quickly. Moreover, future comparisons of autopod development and posterior HoxD expression patterns between these two taxa may provide insights critical for determining developmental mechanism underlying the digit identity frame shift.

An alternative interpretation of our results is that the primary axis has shifted from positions 4 to 3 and all digits maintain their ancestral identity and gene expression patterns (e.g., that both the identities and positions of digits Da, Db, and Dc are 1, 2, and 3). A similar hypothesis (the axis shift hypothesis) was proposed to resolve the conflict of digit homology in birds (Shubin 1994b; Chatterjee 1998; Garner and Thomas 1998). In the bird forelimb, this hypothesis has been falsified by developmental studies that stained the initial condensations of pre-chondrogenic cells allowing visualization of all five digit anlagen (Kundrat et al. 2002; Larsson and Wagner 2002; Welten et al. 2005). These studies reveal one pre-chondrogenic digit condensation posterior to the primary axis and three digit condensations anterior to the primary axis confirming the position of the primary axis as digit 3, position 4 in the bird hand (Fig. 1B). Localization of pre-chondrogenic condensations has not yet been carried out in *C. chalcides*, and it would be interesting to perform such a study in this species. However, both the evolutionary conservation of the primary axis position and the maintenance of the primary axis position in birds support our interpretation of a frame shift in digit identity (rather than an axis shift) in *C. chalcides*, and is consistent with the published consensus about the position of the three digits in this species (Sewertzoff 1931; Raynaud et al. 1986).

Implications for digit evolution

Limb reduction and more specifically digit loss is common in amniotes and as a result has been widely studied and discussed in the literature. Interestingly, loss of digits typically occurs in a consistent pattern across taxa—digits $1 > 5 > 2 > 3 > 4$ (Morse 1872; Sewertzoff 1931; Greer 1990, 1991). This pattern of digit loss likely reflects a developmental constraint caused by the highly conserved patterns and sequence of digit development (Burke and Alberch 1985; Shubin and Alberch 1986; Müller and Alberch 1990). Briefly, the primary axis, digit 4, is the first to appear during limb development. Following the primary axis, development proceeds anteriorly resulting in the development of digital arch and digits 3, 2, and 1 in that order. Digit 5 develops independent of the primary axis and digital arch. The digit identity frame shift may provide an escape from this developmental constraint by allowing loss of embryological positions 1 and 5, but maintenance of digit 1 morphology. In this case, a

frame shift of digit identity would only be predicted when digit loss is adaptive, and moreover, when digit 1 confers some functional advantage (Steiner and Anders 1946). In contrast, digit loss due to rudimentation should follow the typical $1 > 5 > 2 > 3 > 4$ pattern. In both the theropod ancestors of birds and *C. chalcides* and its three digit relatives, limb reduction and digit loss are thought to be adaptive (Steiner and Anders 1946; Wagner and Gauthier 1999; Wagner 2005). In the ancestors of birds, loss of digits occurred as a result of adaptive modifications for prey capture (Sereno 1999; Wagner and Gauthier 1999). In *C. chalcides*, while digit loss is associated with modifications of the limb that improve locomotor performance in this grass-swimming species, limbs are thought to maintain important functions to stabilize the animal while at rest and to push away vegetation when moving at slow speeds (Orsini and Cheylan 1981). Consistent with this view are observations that in fact signs of digit reduction are seen in the posterior digits of other *Chalcides* species, but not in the digit 1. For instance, *C. mionecton* includes both pentadactyl and tetradactyl individuals, the latter being definitely predominant and clearly lack the phalanges of digit 5 (Caputo et al. 1995), similar to the situation in the early theropod hand (Wagner and Gauthier 1999).

Implications for the assessment of digit homology

The research presented here and in previous studies (Wagner and Gauthier 1999; Larsson and Wagner 2002; Vargas and Fallon 2005a; Vargas et al. 2008; Vargas and Wagner 2009) is motivated by manifest conflicts between different established criteria for the assessment of homology. Specifically it is the conflict between embryological and anatomical criteria that led the FSH (Wagner and Gauthier 1999). Later research (Vargas and Fallon 2005a; Vargas et al. 2008) confirmed the homology assessment based on anatomical criteria because the anatomical digit identity has been found to be associated with transcription factor gene expression. Furthermore digit identity can experimentally be dissociated from digit position by manipulating Sonic hedgehog signaling (Vargas and Wagner 2009). These results are consistent with other experimental studies that show that the developmental genetic determinants of digit identity are not rigidly associated with digit position. Digit identity is determined much later in development than the initial patterning of condensations in the distal limb bud (Dahn and Fallon 2000; Drossopoulou et al. 2000; Zákány et al. 2004). This body of evidence implies that, during development, digit identity has a definite beginning that is later than the condensation of digit anlagen. The stage in development when digit identity arises is likely to be caused by the activation of a developmental program that bestows digit identity to the preformed digit condensations. Before the activation of this digit identity (or character identity) program, digit identity, and thus homology is not established and it is

meaningless to assign digit identity to a cell condensation that is not yet developmentally committed to a certain identity (Wagner 2007). In practical terms, criteria about digit or character homology based on position are useful in many circumstances, but in cases of conflict between position and structure, positional criteria are inferior to anatomical criteria. The latter more directly reflect the expression of the character specific developmental program and thus are closer to the mechanistic basis of character identity.

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