

DOLLO'S LAW AND THE IRREVERSIBILITY OF DIGIT LOSS IN *BACHIA*

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Several recent studies conclude that exceptions to Dollo's law are more common than used to be thought. If the claims are true this would change our view on the role of developmental constraints in the evolution of body plans. One study claims the reevolution of lost digits in the lizard genus *Bachia* (Kohlsdorf and Wagner 2006). We evaluate this claim. We conclude that the proposed molecular phylogenetic tree is in conflict with evolutionary mechanisms concerning the biogeography of lizards and with morphology-based phylogenies. A reanalysis of the molecular data does not support the topology of the published tree. Furthermore, two implicit assumptions, that digit numbers are fixed and that polydactyly evolves independently from other characters, are incorrect. We conclude that there is no convincing support for reevolution of digits in *Bachia*. We discuss our findings in the light of the current evidence for the reversal of losses of complex traits. We conclude that in metazoans, exceptions to Dollo's law are mainly found among meristic traits that originate relatively late during embryogenesis, when developmental systems are more compartmentalized. Finally, our study shows that phylogenetic analyses should incorporate evolutionary mechanisms including constraints, variation, and selection, not only for correct phylogenetic reconstruction, but also for correct evolutionary inference.

KEY WORDS: Evolutionary constraints, limb evolution, modularity, phylotypic stage, polydactyly, squamate lizards.

Dollo's law on the irreversibility of evolution states that "an organism never comes back exactly to its previous state due to the indestructible nature of the past, it always retains some trace of the transitional stages through which it has passed" (Dollo 1905; see also Dollo 1893; Simpson 1953; Gould 1970). Dollo's law is often cited in the context of the irreversibility of the loss of complex structures and as such continues to challenge evolutionary biologists. The rationale for the irreversibility of such losses is that they are thought to be statistically improbable if substantial genetic change has accumulated (Muller 1939; Simpson 1953; Marshall et al. 1994; Zufall and Rausher 2004). However, some examples show that evolutionary losses of complex traits can be reversed, for instance, the regaining of the second molar in the lynx (Kurtén 1964) and the fifth premolar in an anthracotheriid

(*Lybicosaurus*; Lihoreau et al. 2006). The number of nipples in mammals is extremely variable among species (e.g., Gilbert 1986; Sherman et al. 1999), indicating that reversals of changes are common. Other examples are the regaining of phalanges in several squamate taxa (Greer 1992). Perhaps not surprisingly nipples, phalanges and teeth are meristic traits. Marshall et al. (1994) have suggested that such serially repeated structures constitute a special case, because the genetic and developmental architecture to develop such structures continues to be fully present when decreases in number occur (see also Hennig 1966; Gould 1970; Collin and Miglietta 2008). It is perhaps more surprising that losses of meristic traits are so rarely reversed. For instance, there is no direct supporting evidence that reacquisition of limbs after loss has ever occurred in amniotes (e.g., Wright 1968; Lande 1978; Greer

1991; Galis et al. 2001, 2002; Fedak and Hall 2004; Polcyn et al. 2005).

Recently, a number of claims have been published for the reversal of evolutionary losses of complex characters, e.g., the reacquisition of wings in insects (Whiting et al. 2003), the reevolution of coiling in snails (Collin and Cipriano 2003), the regaining of digits in the lizard genus *Bachia* (Kohlsdorf and Wagner 2006), and the reevolution of sexuality in Oribatid mites (Domes et al. 2007). These claims are based on new phylogenetic trees and on tests of congruence for shared derived character states. Despite the publication of several criticisms (Trueman et al. 2004; contra Whiting et al. 2003; Urdy and Chirat 2005; contra Collin and Cipriano 2003; see also Polcyn et al. 2005), these claims have led to a reappraisal of the possibility of reversal of losses of complex features (Brandley et al. 2008; Collin and Miglietta 2008). Considering the moderate level of robustness of many phylogenies, it is realistic to assume that new phylogenies based on more and other traits will falsify at least some of these hypotheses. In addition, Goldberg and Igić (2008) conclude that there are “devastating methodological flaws” in these studies that challenge Dollo’s law based on statistical phylogenetic methods. Specifically, none of them has explicitly dealt with the implicit assumption of root state of the trees and its impact on character state-dependent differences in diversification rates. They conclude that more powerful tests of irreversibility require data beyond phylogenies and character states of extant taxa, and they highlight empirical work that incorporates additional information, such as geographic data and climate reconstructions.

In the light of this controversy, we have analyzed the robustness of a claim for the reversibility of digit losses in an influential recent study (Kohlsdorf and Wagner 2006) in the context of an understanding of evolutionary processes rooted in empirical evidence.

(Ir)Reversibility of Digit Loss in Bachia

Kohlsdorf and Wagner (2006) claim to provide strong statistical evidence for the reversibility of the evolutionary loss of digits in the lizard genus *Bachia*. If true, this would represent the first evidence of a gain of digits, so-called polydactyly, in amniotes at a species level, and, thus, evidence for a unique breaking of a constraint. Although mutations for polydactyly are frequent in amniotes, it is assumed that strong stabilizing selection due to pleiotropic effects prevents the establishment of such mutations in populations (Wright 1968; Lande 1978; Galis et al. 2001; see also Biesecker 2002). Ichthyosaurs were thought to form an exception, but current consensus is that no extra digits have evolved in ichthyosaurs. Instead, it is thought that not more

than four embryonal digit condensations were involved and that these condensations bifurcated during elongation leading to many supernumerary phalanges (Motani 1998; Fedak and Hall 2004). Polyphalangy, unlike polydactyly, is quite common among secondarily aquatic vertebrates (Richardson and Oelschläger 2002; Fedak and Hall 2004; Cooper et al. 2007). In extant whales, such branching of digit condensations is, for instance, regularly found among Beluga whales (*Delphinapterus leucas*, e.g., Kleinenberg et al. 1969). In contrast, Kohlsdorf and Wagner (2006) now claim, based on their *Bachia* study, that the likelihood of evolutionary digit gains in squamates has been drastically underestimated.

Kohlsdorf and Wagner (2006) do not follow the findings of Greer (1992) on the probability of the reversibility of evolutionary digit reduction. Greer (1992) estimated in a large study of squamates that the loss one or more of phalanges in squamates is about 5.3 times more common than the gain of a single phalanx. These findings led Whiting et al. (2003) and Kearney and Stuart (2004) to reject the parsimony of reversals of digit reduction for the lizard genera *Scelotes* and *Bipes*. In contrast, Kohlsdorf and Wagner (2006) concluded that digit gains must have occurred in *Bachia*, because phylogenetic reconstructions with only digit losses are only supported when assuming that evolutionary digit gains are at least 4.2 times more difficult than losses. They reject this possibility because 4.2 is larger than expected for a random phylogeny of *Bachia*. However, 4.2 is lower than the 5.3 value of Greer (1992) for phalanx gains versus losses. Kohlsdorf and Wagner (2006) indirectly state that they ignore Greer (1992) because he did not use rigorous phylogenetic methods. It may be noted that the estimate of Greer (1992) is conservative and the eight cases of hyperphalangia that he found include three that may have been environmentally induced. In addition, his estimate of 43 independent digit losses in squamates seems realistic, given that Wiens et al. (2006) have found with rigorous analytical phylogenetic techniques, that elongation of the body in squamates in combination with limb reduction has occurred at least 26 times and reversals (reduced elongation or less reduced limbs) less than one-fourth as often. The 26 cases exclude lineages in which limb reduction is not accompanied with obvious body elongation (involving less serious limb reductions), which according to Greer (1991) is the case for approximately 40% of the limb reduced lineages. In addition, the acquisition of extra phalanges in general will require fewer mutations than extra digits, given that less and/or shorter ectopic expression of *Sonic Hedgehog* is required (Tickle 1981; Hill 2007), and less activity of modifier genes (Forsthoefel 1968). In agreement with this, digit gains appear to be considerably more constrained than phalanx gains (Galis et al. 2001; Richardson and Oelschläger 2002; Fedak and Hall 2004). Hence, the conclusion that the value of 4.2 indicates parsimony of digit gains is doubtful.

Brandley et al. (2008) follow Kohlsdorf and Wagner (2006) in assuming that the likelihood of reversals of digit losses has been underestimated in squamates. Brandley et al. (2008) also dispute the rejections of the parsimony of digit gains of Whiting (2003) for *Scelotes* and Kearney and Stuart (2004) for *Bipes* that are based on the findings of Greer (1992). Furthermore, they claim significant phylogenetic statistical support for at least six cases of polydactyly in a new and large analysis of squamates. The claim for polydactyly in *Bachia* by Kohlsdorf and Wagner (2006) has, thus, led to a reappraisal of the likelihood of evolutionary digit gains (see also Collin and Miglietta 2008). If their claim for the multiple reversals of digit losses in *Bachia* is indeed true, this would overturn a long-held notion that digits disappeared many times, but were never gained in amniotes. This would throw a new light on the importance of developmental constraints in the evolution of body plans. Given the undisputedly high frequency of convergent digit losses in squamates (e.g., Greer 1991; Wiens and Slingluff 2001; Wiens et al. 2006) and the absence of any direct support for digit gains in amniotes at large, it is necessary to evaluate the robustness of their analyses and conclusions and to incorporate available empirical data on evolutionary mechanisms in the analysis of *Bachia*.

Kohlsdorf and Wagner (2006) propose an evolutionary scenario in which *Bachia* species with four toes on the hind limbs are nested at three positions among species with hind limbs with no or a single toe: (a) *B. heteropa trinitatis*, (b) *B. panoplia*, and (c) *B. heteropa alleni* and *B. scolecoides*. They assume that in at least two of these cases polydactyly has occurred (in the *B. scolecoides*/*B. heteropa alleni* clade and in *B. panoplia*). However, the position of *B. heteropa alleni* and *B. scolecoides* deserves our attention. The South East Caribbean *B. heteropa alleni* clusters among Peruvian and West Brazilian species (including *B. scolecoides*, Fig. 1 of Kohlsdorf and Wagner 2006), far apart from its highly morphologically similar subspecies *B. heteropa trinitatis* that occurs on a neighboring island (Fig. 2). This counter-intuitive positioning of the two *B. heteropa* subspecies is very important for the conclusions of Kohlsdorf and Wagner (2006) on polydactyly and deserves closer scrutiny.

The conclusion on polydactyly in *Bachia* of Kohlsdorf and Wagner (2006), furthermore, hinges on several statistical tests for character evolution reconstruction. The outcomes of the tests depend in part on several, mostly implicit, assumptions. One assumption is that the probability of the evolution of an extra digit in *Bachia* species can be considered as an isolated evolutionary event. However, changes in digit number in this genus, as in other Squamates, always co-occur with a suite of trait changes, assumed to be associated with the evolution of fossoriality. This indicates that the probability of a hypothesized reversal of digit loss should be seen in the light of the probability of the simultaneous reversal of the other traits of the complex. It is, thus, important to

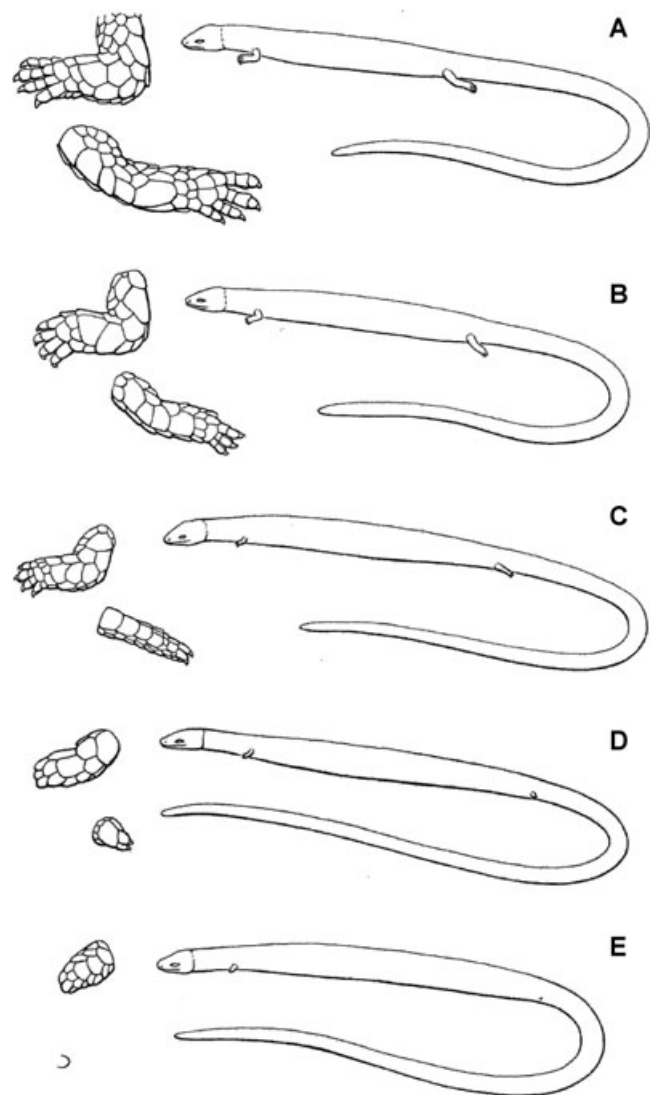


Figure 1. All *Bachia* species have reduced limbs, varying from very small limbs with four toes (A) to tiny stumps without toes (D, E); (A) *B. scolecoides*, (B) *B. pallidiceps*, (C) *B. heteropa lineata*, (D) *B. trisanale*, (E) *B. barbouri* (drawings by László Meszoli).

investigate which traits are involved and whether they belong to independent developmental pathways, i.e., can they plausibly be reversed in a coordinated way when fossoriality is lost and, thus, be treated as a single evolutionary event? Furthermore, is it plausible to assume a selective advantage for such a reversal? Another implicit assumption of the probability analyses is that the number of digits in the analyzed *Bachia* species is constant and that there is no intraspecific variation. However, the number of digits in *Bachia* species is, like in other squamates with reduced limbs, highly variable (Loveridge 1933; Dixon 1973; Presch 1975, 1980; Lande 1978; Kizirian and McDiarmid 1998; see also Krieg 1919; Camp 1923; Essex 1927). This affects the number of steps in character evolution reconstructions and the number of steps between species and, thus, deserves investigation.

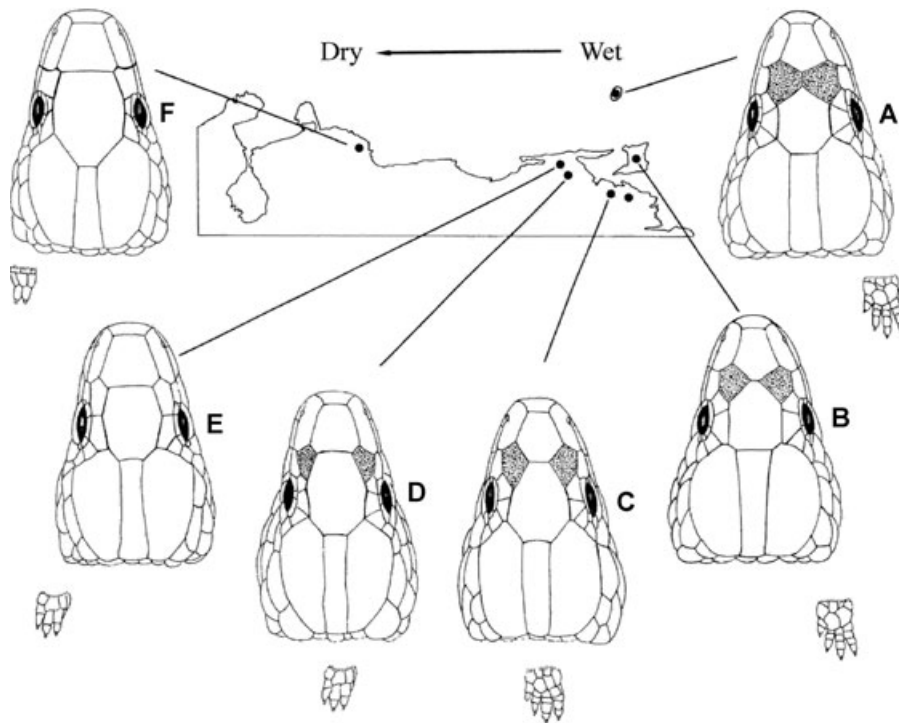


Figure 2. Progressive reduction in size and loss of hind limb toes and prefrontal scales in the *Bachia heteropa* subspecies in northern Venezuela. (A) *B. heteropa alleni*, (B) and (C) *B. heteropa trinitatis* (Trinidad and mainland), (D) *B. heteropa heteropa* x *B. heteropa trinitatis*, (E) *B. heteropa heteropa*, (F) *B. heteropa lineata*. Direction of arrow indicates trend from mesic to xeric habitat (reprinted from Dixon 1973).

There are substantial empirical data available on the morphology, physiology, ecology, taxonomy, and biogeographical distribution of *Bachia* (Loveridge 1933; Beebe 1945; Dixon 1973; Presch 1975, 1980; Lande 1978; Gasc 1981, 1984; Kizirian and McDiarmid 1998). Based on these studies, we have investigated: (a) Can digit gains be treated as single evolutionary events? (b) Does intraspecific variation of digit number in *Bachia* affect the analyses of digit evolution? (c) Is there a selective advantage for digit regain in species that have already lost most of their digits? We discuss whether our findings challenge the conclusion of Kohlsdorf and Wagner (2006) that digit evolution in *Bachia* forms an exception to Dollo's law and, more generally, the relevance for the many recent challenges of this law that are solely based on statistical phylogenetic analyses.

Robustness of the Phylogenetic Hypothesis

REASSESSMENT OF THE BAYESIAN ANALYSIS

Our molecular phylogenetic analysis, replacing an erroneous C-mos sequence for *Bachia dorbignyi* (see Castoe et al. 2004 and Appendix 1), yields a tree (Fig. 3) that differs considerably from the competing tree of Kohlsdorf and Wagner (2006; their Fig. 1; here-

under "KW-tree"). While the new tree is more likely than the KW-tree, the difference in likelihood scores is not statistically significant (Shimodaira–Hasegawa test in PAUP*; Swofford 1998). The tree topologies show a large Robinson–Foulds (1981) distance of 15. Since the maximum tree-to-tree distance for 15 taxa is 24, the topological dissimilarity is quantitatively high. Importantly, the rooting is different with *B. flavescens* as the most basal lineage (as in Castoe et al. 2004; their Fig. 6) instead of *B. bresslaui*. Second, *B. intermedia* and *B. monodactylus parkerii* are in the top of our tree and fairly basal in the KW-tree. The number of strongly supported clades ($P \geq 0.95$, ignoring the rooting branch) is four out of 13 while in the KW-tree 10 branches have strong support.

These marked differences between the trees are somewhat surprising, considering that both are derived from largely the same DNA sequence information. However, we have made several adjustments for our phylogenetic analysis, including (1) the use of a wide outgroup that represents the sister-group of *Bachia* instead of one, fairly distantly related taxon (*Eclepopus gaudichaudii*); (2) as mentioned above, the C-mos sequence for *Bachia dorbignyi* was replaced; and (3) third positions at the C-mos nuclear coding gene were not excluded (for details, see Appendix 1).

The differences in phylogenetic structure have serious consequences for the number of inferred character state changes: The KW-tree implies two-digit gains and four losses (or nine losses)

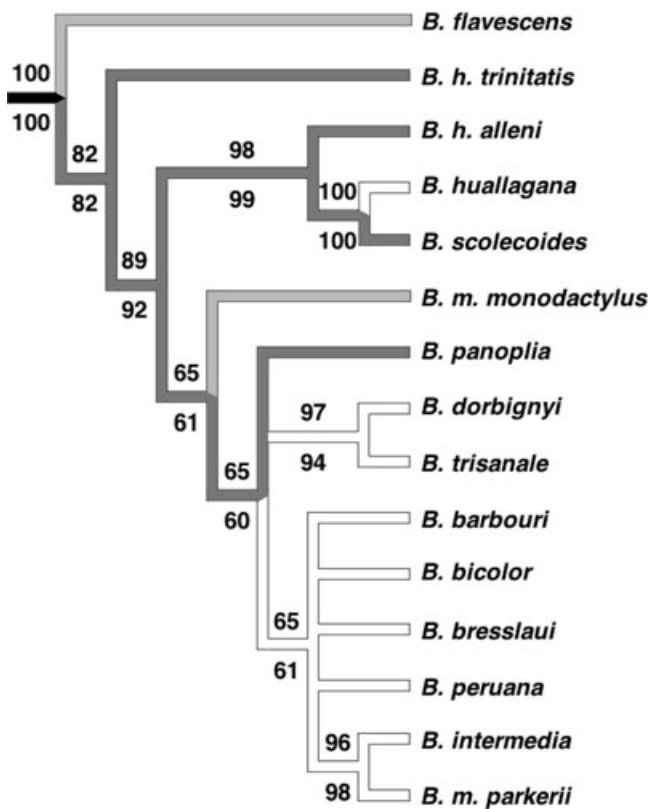


Figure 3. Molecular phylogenetic hypothesis on the lizard genus *Bachia* derived through a partitioned Bayesian analysis of DNA sequence data for the genes 12S, 16S, and C-mos. Figures above and below branches denote percent Bayesian posterior probability (pp) obtained with outgroup (1) and outgroup (2), respectively (for details, see Appendix 1). The analysis yields strong support for: the monophyly of the genus *Bachia* with a posterior probability (pp) of 1.0, the monophyletic grouping of *B. heteropoda alleni*, *B. huallagana*, and *B. scolecooides* (pp = 0.985) in which *B. huallagana* and *B. scolecooides* are sister-taxa (pp = 1) and the sister-taxon status of *B. dorbignyi* and *B. trisanala* (pp = 0.955) and *B. intermedia* and *B. monodactylus parkerii* (pp = 0.970). It also provides moderate support for the basal position of *B. flavescens* and *B. heteropoda trinitatis* through the monophyly of the remaining species (pp = 0.905). The distribution of states of the character “number of digits at the hind foot” is shown by shading as follows, black = 5 (outgroup), dark gray = 4, light gray = 1, and white = 0. This scenario involves the loss of one or more digits on five occasions.

for the hind limbs while the new phylogeny implies five losses of digits and no gains (Fig. 3). Furthermore, the different topology influences the number of steps that separate species. For instance, the two subspecies of *Bachia heteropoda* are separated over seven well-supported branches (pp > 0.95) in the KW-tree and just one such a step in our tree. Interestingly, the two *B. heteropoda* subspecies have identical (nuclear) C-mos sequences whereas they are apart by four well-supported branches in the (mitochondrial) 12S plus 16S data partition (results not shown). This suggests a scenario in which mtDNA may not be representative for the taxon

in which it is found through, e.g., hybridization and introgression (Ballard and Whitlock 2004; Currat et al. 2008). It is unlikely, however, that “mtDNA capture” would explain the phylogenetic separation of the two subspecies, because of their geographic distribution (see below).

MORPHOLOGICAL PHYLOGENY AND BIOGEOGRAPHY

The phylogenetic hypothesis of Kohlsdorf and Wagner (2006) concerning the two subspecies of *B. heteropoda* is in conflict with an earlier morphological phylogeny of Dixon (1973) in which these subspecies cluster together, in agreement with the findings of Thomas (1965). The two subspecies are morphologically highly similar and they occur on neighboring islands in the South East Caribbean. They appear to belong to a cline of five *B. heteropoda* subspecies that gradually change in morphology, including the number of digits on the hind limbs and the number of head shields (Fig. 2; Thomas 1965; Dixon 1973). *Bachia heteropoda alleni* occurs on the Grenadines, Grenada and Tobago and *B. heteropoda trinitatis* occurs on the neighboring island of Trinidad and on the north coast of Venezuela. In both molecular phylogenies, the Caribbean *B. heteropoda alleni* clusters among Peruvian and West Brazilian species approximately 2000 miles away. Hybridization cannot explain this problematic clustering, because hybridizing of a species from the South East Caribbean with species that are endemic to Peru and West Brazil is highly improbable. The position of these two subspecies is, furthermore, in conflict with well-supported hypotheses on evolutionary mechanisms concerning the migration of lizards from island to island (Carranza et al. 2000; Schoener et al. 2001; Calsbeek and Smith 2003; Glor et al. 2005; Thiel and Hays 2006). Rafting from the Pacific coast of Peru to the South Caribbean islands close to Venezuela or vice versa is virtually impossible, as is the migration over land, crossing both the Amazon and Orinoco rivers. On the other hand, rafting from Trinidad to the neighboring island of Tobago or the other way around is a much more probable event. Hurricanes appear to provide episodic opportunities for rafting between islands and between islands and the mainland coast in the Caribbean area (Censky 1998; Thiel and Hays 2006).

LIMITED SAMPLING OF BACHIA SPECIES

Given that only one individual appears to have been sampled for the two subspecies of *B. heteropoda* and also for most species for the molecular phylogenetic analysis, an accidental mix-up, either in the museum or in the laboratory, cannot, therefore, be entirely excluded. Sampling of more individuals, and in particular sampling of more of the five *B. heteropoda* subspecies that form the cline would, thus, be necessary for a firm conclusion regarding the phylogenetic relationship of these subspecies of *Bachia heteropoda*.

Robustness of the Digit Evolution Reconstruction

The outcomes of the probability analyses of character reconstruction of Kohlsdorf and Wagner (2006) depend on several implicit assumptions. One assumption is that the number of digits can be treated as fixed, despite the fact that Table 1 of Kohlsdorf and Wagner (2006) lists some variation, albeit incomplete. As mentioned above, the number of digits is highly variable in *Bachia* species with digits and a large variability has been observed both within and between populations of the same species (Dixon 1973; Presch 1975, 1980; Lande 1978; Kizirian and McDiarmid 1998). For example, Dixon (1973) reports that among 10 specimens of *B. panoplia* from Manaus (Brazil) eight have four digits on the hind limbs and two have three. Another example is *B. m. monodactylus*, which was observed to have usually one or two toes on their hind limbs, but more exceptionally also no toes and there is one observation of three toes on one hind limb, i.e., zero, one, two, or three toes. Dixon (1973) found that the average number of toes per hind foot for specimens from Guana is 1.41, Brasil 1.50, Surinam 1.62 (assigned one digit by Kohlsdorf and Wagner 2006), and Cayenne 1.83. Therefore, the minimum difference in digit number between *B. panoplia* and *B. m. monodactylus* is one instead of three. This and variation in other species will appreciably affect the statistical analyses.

DIGIT GAINS AND INTRASPECIFIC VARIATION

A second implication of the high intraspecific variation in digit number in *Bachia* species is that if indeed the average number of digits has increased in species, it seems more probable that

this has happened via selection for still present variation for the ancestral, higher number of digits (Lande 1978). Mutations for polydactyly presumably occur at a low frequency, lower than the observed frequency of variation in *Bachia* species that still have digits and where even samples of less than 30 specimens regularly display variation (Loveridge 1933; Dixon 1973; Presch 1975, 1980; Kizirian and McDiarmid 1998). Thus, selection for still existing variation for the ancestral condition may well be a more plausible mechanism to explain a possible gain in the average number of digits than the reversal of an evolutionary loss of digits.

DIGIT LOSSES AND ASSOCIATED CHARACTER CHANGES

The conclusion of Kohlsdorf and Wagner (2006) that evolutionary loss of digits has been reversed at least twice in *Bachia* hinges on the assumption that digit gains and losses can be regarded as single evolutionary events. In contrast, digit loss in *Bachia*, as in other squamates with limb reduction, *always* coincides with a suite of other changes, presumably associated with the evolution of fossoriality (Dixon 1973; Presch 1975, 1980; Kizirian and McDiarmid 1998; see also Thomas 1965). The hypothesized reversal of digit loss should, thus, be accompanied by the reversal of other changes and all these changes should be in the ancestral direction, for instance, there should be a decrease in the number of presacral vertebrae (Fig. 4) and an increase in the number of head shields (Fig. 2). Furthermore, the changes associated with digit loss include the loss of other hind limb bones, the reduction of the pelvic girdle, front limbs and pectoral girdle, reduction

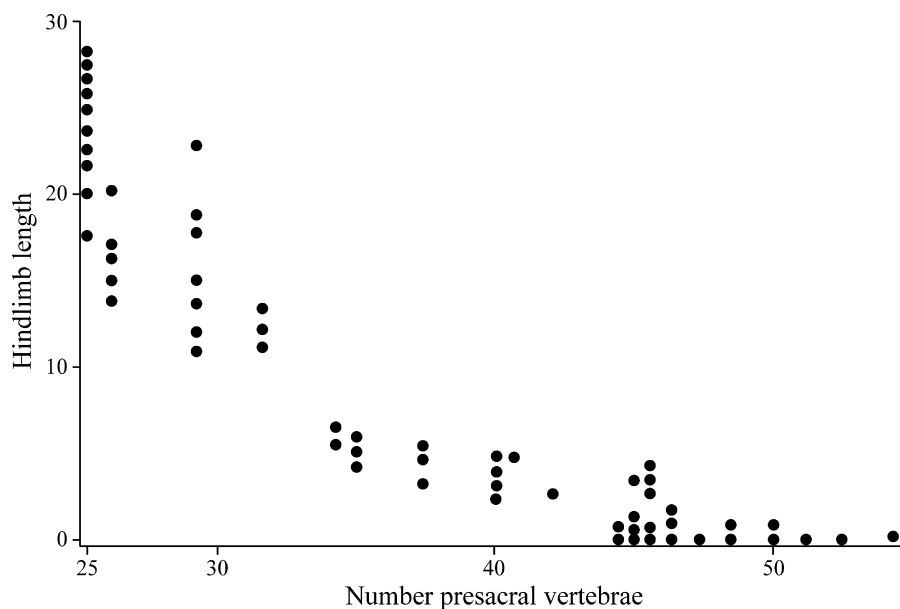


Figure 4. The number of presacral vertebrae plotted against the length of the hind limb (mm) of 13 from a total of 16 species of *Bachia*. Note that the number of presacral vertebrae strongly increases with reduction of the hind limb length (reprinted from Presch 1975).

of the right lung and elongation of the left lung, elongation of the liver and stomach, asymmetry of the elongated kidneys, the modification of the atlas-axis and anterior cervical vertebrae, reduction and loss of the sacral diapophyses, the loss of a rib on the last presacral vertebra, a shift toward an underslung lower jaw, and a shift in locomotory mode (Thomas 1965; Dixon 1973; Presch 1975, 1980; Gasc 1984; Klein et al. 2005). Digit reduction in *Bachia*, thus, co-occurs with changes in virtually all organ systems and, hence, the hypothesized reversals of digit losses as well.

The changes in these different organ systems involve different genetic and developmental pathways. For example, the developmental pathway that is involved in the determination of the number of presacral vertebrae is independent from that of the determination of digit number and both pathways are independent from dermal plate development (e.g., Gilbert 2006). The compartmentalization of developmental pathways implies that many mutations would be necessary to accomplish the reversals of digit losses and associated traits. Especially, such extreme increases in asymmetry of internal organs are rare in the animal kingdom and expected to require multiple new mutations. In addition, in the cases where characters are lost, degeneration in one or more of the involved pathways would make it even more unlikely that the characteristics would reevolve, because multiple simultaneous mutations would be required for the reversal of even one character (Zufall and Rausher 2004). Therefore, selection for a change of all associated characters in the ancestral direction seems highly improbable.

SELECTIVE DISADVANTAGES TO LOSS OF THE SERPENTINE LOCOMOTION

Selection for a reversal of limb reduction may also be unlikely from the point of view of locomotory efficiency. In *Bachia* species, the body elongation that accompanies digit loss is relatively strong (Fig. 1) and, for instance, stronger than in scincids (Presch 1975; Gasc 1984). This implies that the distance between the front and hind limbs increases rapidly with digit loss (Fig. 1), leading to a smaller contribution to the propulsive force of the limbs and a higher contribution from the trunk during the lateral undulation (Gans 1986; Renous et al. 1998). The increased elongation accelerates the locomotory shift from force propulsion predominantly by the limbs (walking) to predominantly by the trunk (lateral undulation). The diminution of the limbs presumably decreases the resistance of the limbs to the forward movement of the body during lateral undulation. One of us (RL) observed in the field in Ecuador a specimen of *B. intermedia* with three digits on front limbs and no digits on hind limb; locomotion in its leaf litter habitat was primarily by undulation with front limbs folded next to the body, but at rest the front limbs were used to prop up the body

and elevate the head when viewing surroundings. Gasc (1984) reported that in experiments *B. flavescens* (formerly *B. cophias*) the front and hind limbs were dragged along by the body during lateral undulation. The hind limbs in this species are tiny, with only one digit, but the front limbs are larger and have three toes and yet were dragged along. Gasc (1984) also reported a reduced coordination between the left and right limbs in these experiments. In agreement with these results, Beebe (1945) observed in the field for *B. heteropa heteropa*, which has forelimbs with four toes and hind limbs with three toes (Fig. 1), that locomotion is very similar to that of *B. flavescens* and that individuals of *B. heteropa heteropa* are difficult to distinguish in the field from the juveniles of the burrowing snake *Atractus trilineatus*, unless caught and checked for the presence or absence of limbs. Hence, lateral undulation is also the common locomotory mode for *Bachia* species with the least limb reduction.

Lateral undulation in a lizard (*Bipes biporus*) was estimated to be approximately one-third more energetically efficient than walking with four limbs in lizards (Dial et al. 1987). The elongated shape that is associated with lateral undulation may, however, lead to an increased loss of heat compared to less elongated body shapes, however heat loss is presumably relatively unimportant in cold-blooded animals and in particular in the tropical habitats where *Bachia* occurs. This suggests that solely from the point of view of locomotory efficiency, the advantage of selection for a reversal of limb reduction and body elongation is doubtful. Losos and Irschick (1994) showed for *Anolis* that such selective constraints also play a role in explaining irreversibility in lizard evolution, even for quantitative traits (see also Bull and Charnov 1985).

REGAINING DIGITS IN BACHIA IS NOT PARSIMONIOUS

The inconsistencies that we find in the tree topology and the assumptions underlying the statistical analyses for the character evolution demonstrate that Kohlsdorf and Wagner (2006) have not presented conclusive evidence for an evolutionary reversal of digit loss in *Bachia*.

Our conclusions on *Bachia* also are more generally relevant for the interpretation of digit loss and digit gain in squamates. Digit loss has happened many times independently in squamates and it has always been correlated with a set of other traits, most often associated with fossoriality. This implies that hypothesized reversals of digit losses in squamates should not be considered as isolated evolutionary events, but in concert with reversals in other traits. In addition, the extremely high intraspecific variation for digit number is a general phenomenon in squamates with reduced limbs and, it is therefore important that the variation is carefully documented and included in the analyses.

Evolutionary Constraint on Polydactyly in Vertebrates

The findings of Kohlsdorf and Wagner (2006), thus, do not overturn the long-held notion that evolutionary reversals of digit losses appear to be severely constrained in amniotes. Polydactyly mutations are among the most common in many vertebrate species, yet there is no direct support for the establishment in amniotes, other than in artificial populations of domestic animals under reduced natural selection. The constraint appears to be due to extremely strong selection against polydactyly mutations and it was found that in humans approximately 90% of all individuals with extra digits are dead at birth (Opitz et al. 1987; Galis and Wijnaendts, pers. data; see also Wright 1968 and Lande 1978).

The extremely strong selection against polydactyly mutations is thought to be due to associated pleiotropic effects (Wright 1968; Lande 1978; Galis et al. 2001). Indeed, polydactyly is associated with many medical problems (Grüneberg 1963; McKusick et al. 1964; Opitz et al. 1987; Cohn and Bright 1999; Galis et al. 2001) and there are at least 110 syndromes associated with polydactyly in humans (Biesecker 2002). The pleiotropic effects appear to be due to the extremely strong interactivity during the early organogenesis stage (also called phylotypic stage) when the determination of the number of long bones and digits takes place in amniotes. As a result of the strong interactivity, mutations with an effect during this stage have many deleterious pleiotropic effects in at least rodents and humans (Galis and Metz 2001; Galis et al. 2006). The deleterious pleiotropic effects severely reduce the chance of such mutations being successful. Such pleiotropic constraints are thought to be responsible for the conservation of the number of other structures as has been documented most clearly for the number of cervical vertebrae in mammals (Galis and Metz 2003; Galis et al. 2006; see also Hansen and Houle 2004 on pleiotropic constraints). Rare incomplete expression of atavistic traits demonstrates the persistence of genetic and developmental pathways, but genetic mutations will tend to reduce the fitness of such underdeveloped traits and underutilized traits, reducing the possibility of evolutionary reversals (Zufall and Rausher 2004).

In contrast, decreases in the number of digits are common in evolution. Nonetheless, oligodactyly mutations also appear to be constrained because of associated pleiotropic effects (Grüneberg 1963; Galis et al. 2001; see also Lande 1978). However, there is an alternative mode for digit reduction, i.e., the slow continued evolution of earlier developmental arrest followed by degeneration (Lande 1978; Galis et al. 2002). Such an alternative is not possible for gaining digits. However, the acquisition of extra digit-like structures appears to form an alternative; examples are the panda's thumb and the mole's thumb (for a discussion, see Galis et al. 2001).

Reversals of Digit Loss in Amphibians

The support for polydactyly in amphibians is stronger than for amniotes and digit gains have probably taken place in frogs, in particular in leptodactylid frogs (Galis et al. 2001). Interestingly, this is in agreement with the above-mentioned hypothesis on the pleiotropic constraint against polydactyly. In amphibians that undergo metamorphosis, limb development is delayed and occurs only during larval stages, long after the vulnerable early embryogenesis stages. This late determination during a less interactive and more modular stage leads to the expectation of a weaker pleiotropic constraint and weaker stabilizing selection against polydactyly. The hypothesized weaker pleiotropic constraint and weaker stabilizing selection are in agreement with the apparent relaxation of the evolutionary constraint on polydactyly in frogs (Galis et al. 2001; see also Galis et al. 2003).

Dollo's Law and the Timing of Development of Meristic Traits

For meristic structures of which the determination of the number occurs after the early organogenesis stage, when development has become more compartmentalized, the evolutionary constraint on number changes appears to be relaxed (Galis and Metz 2007; see also Galis and Metz 2001). Good examples are the number of teeth, nipples, phalanges, carpal and tarsal elements, and caudal vertebrae. Galis et al. (2006) demonstrated that changes of the number of thoracic vertebrae were indeed associated with fewer pleiotropic effects and with weaker selection than changes of the earlier determined number of cervical vertebrae. For many structures, however, the number is determined during the vulnerable early organogenesis stage (e.g., the number of eyes, ears, nasal capsules, lungs, kidneys, limbs, long bones, digits). In agreement with the early determination, these numbers are highly conserved. Exceptions to Dollo's law, thus, appear to be common for meristic traits of which the number determination occurs late. Well-documented examples are the regaining of the second molar in the lynx (Kurtén 1964), the fifth premolar in an anthracotheriid (*Lybicosaurus*; Lihoreau et al. 2006), and hyperphalangia in squamates (Greer 1992). Possibly reversals of losses of other complex traits also have taken place in the evolution, however, most of the claims for such reversals are only based on phylogenetic statistical evidence and their conclusions have been challenged (Trueman et al. 2004; Polcyn et al. 2005; Urdy and Chirat 2005; Goldberg and Igić 2008). Dollo seems to be right in concluding that such reversals are rare, at least in metazoans. Where there are exceptions they are about single characters and not regarding the whole organism, as originally suggested by Dollo. It may well

be that evolutionary reversals and other saltational changes are more common in plants, where embryonal selection against variations appears to be less strong (Bateman and DiMichele 2002). Simple metazoans may also deserve more attention in this regard (Hoeksema 1990).

Incorporation of Evolutionary Mechanisms in Phylogenetic Analyses

The success of the comparative phylogenetic method will probably imply that more exceptions to Dollo's law will be hypothesized in the near future. However, as Wiens et al. (2007) have convincingly demonstrated, phylogenetic reconstructions of character evolution in natural systems can sometimes generate misleading results with strong statistical support (see also Waegele et al. 2009). We agree with them that such results do not call for the abandonment of phylogenetic comparative methods, but underscore the need for their continued testing, improvement, and appropriately cautious interpretation. In particular, it is important, as we have shown in this study, that evolutionary mechanisms including constraints, variation, and selection are incorporated. This is not only necessary for correct phylogenetic reconstruction, but also for correct inference regarding character evolution.

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Appendix 1

MOLECULAR PHYLOGENETIC STUDY OF THE GENUS *BACHIA*

Three published molecular phylogenetic analyses that include the genus *Bachia* are by Pellegrino et al. (2001), Castoe et al. (2004), and Kohlsdorf and Wagner (2006) with 3, 3, and 15 *Bachia* (sub)species, respectively. The partial genes that these studies have in common are the nuclear C-mos and the mitochondrial 12S and 16S ribosomal RNA. We downloaded *Bachia* sequences from GenBank and replaced the erroneous sequence of C-mos for *B. dorbignyi* (AF420861; see Castoe et al. 2004) with:

```
GCATCAAGACAAAGCTTCTGGGCAGAGCTCAATGTA
GCATGTCTTGAGCACAAAATGTTGTACGTGTTATTGCT
GCTAGCACATGCACTCCTTCCAGTCAAGATAGTATGGGC
ACAATAATCATGGAGTATGCAGGTAATAGTACTCTGCACC
ATATTATCTATGGGACTGAATGTGCAAAAACAAAAGGGG
AGGGCGATGGACTTGGATATAGGCATGCCAATTTGAACT
TAGTGCAGTATCTGGCATACTCCTGTAGCATTGTGTCAGG
CTTAGTTTTTCTTCATTCTCACCTGATAGTCCACTTGGAT
```

```
TTAAAACCTGCCAACATATTCATCACTGAACAAAATGTT
TGCAAGATAGGAGACTTTGGATGCTCTCAGAACTGGA
AAATAATACTTCAAGACCTCAACTTTGTCAA (data courtesy K. Pellegrino, 26/06/2009, GenBank submission pending).
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Outgroups in the phylogenetic analysis were (1) the non-*Bachia* species in “Clade III” of Pellegrino et al. (2001; their Fig. 4) in which we swapped two labels of species names for 12S and 16S following Castoe et al. (2004; Appendix 2) or (2) the “Cercosaurini” sensu Castoe et al. (2006; their Fig. 6). Outgroup sequence data were provided in aligned format by K. Pellegrino and T. Castoe, since the published links to their websites were not any longer functional. Outgroup taxa with data missing for one or more genes were excluded. The *Bachia* sequences were aligned among themselves with the subroutine ClustalW in DAMBE 5.0.48 (Xia 2000) and then aligned en block to the outgroup, with the removal of sections in which alignment had been ambiguous. Substitution saturation at C-mos third positions was qualified with DAMBE as “little,” which was confirmed by the visual inspection of plots with the estimated number of transitions and transversions versus Kimura’s two-parameter distance from pairwise comparisons. Given the result C-mos third positions were not excluded. Inferred insertions/deletions (indels) corresponding to codons were coded as gaps and other indels were coded as missing data. The size of the dataset (1) was 351, 319, and 377 characters for C-mos, 12S, and 16S, respectively, while the corresponding numbers were 351, 300, and 375 characters in dataset (2). The most appropriate models for sequence evolution were estimated with MrModelTest 2.3 (Nylander 2004). The model for C-mos was GTR + G and for 12S and 16S it was GTR + I + G. Bayesian phylogenetic analyses were performed with MrBayes 3.1 (Huelsenbeck and Ronquist 2001) on the concatenated data file with two partitions, each with its own independent model of evolution and model parameters. Running conditions were such that stability was reached in terms of likelihood scores and parameter estimation. Discarding a “burn-in” of 50% and with one tree sampled in every 1000 the remaining “forest” of 5000 trees was converted into a majority rule consensus tree, in which the frequency of clades represents their posterior probability (pp). Posterior probability values $50 < pp < 0.90$, $0.90 < pp < 0.95$ and $pp \geq 0.95$ were interpreted as respectively (very) weak, moderate, and strong support for the clades under consideration.