DATA AND DATA INTERPRETATION IN THE STUDY OF LIMB EVOLUTION: A REPLY TO GALIS ET AL. ON THE REEVOLUTION OF DIGITS IN THE LIZARD GENUS *BACHIA*

Tiana Kohlsdorf,[1] Vincent J. Lynch,[2] Miguel T. Rodrigues,[3] Matthew C. Brandley,[2] and Günter P. Wagner[2,4]

[1]*Department of Biology, FFCLRP, University of São Paulo, São Paulo, Brazil*

[2]*Department of Ecology and Evolutionary Biology, Yale University, New Haven, Connecticut 06520-8106*

[3]*Department of Zoology, University of São Paulo, São Paulo, Brazil*

[4]*E-mail: gunter.wagner@yale.edu*

Galis and collaborators (2010) claim that our recent paper (Kohlsdorf and Wagner 2006), presenting statistical evidence for the reevolution of digits in the genus *Bachia,* may be flawed. Their reanalysis of the data does not support the possibility of a reevolution of digits and the authors also argue that such a reevolution would be implausible on functional and developmental grounds. In response, we reanalyzed our data with additional outgroup species. Our results differ from the one published in 2006, but this incongruence is not statistically significant. In contrast, the hypothesis presented by Galis et al. is significantly worse. An analysis of digit number evolution, using novel techniques to test for irreversibility of character loss (Goldberg and Igic 2008), confirmed our original conclusion that there is strong evidence for reevolution of digits in *Bachia*. We also point out that this result is not in conflict with the hypothesis by Galis and Metz (2001) that mutations affecting the initial digit patterning are associated with strong negative pleiotropic effects and thus unlikely to be fixed in evolution. An important avenue of future research will be to directly test whether reevolved digits develop from conserved digit condensations retained after digit loss.

KEY WORDS: Comparative method, digit loss, Dollo's law, limb evolution.

Konrad Lorenz once said: "the greatest honor is bestowed upon a scientist by critically engaging with his/her work." In that spirit, we appreciate the scrutiny that Galis et al. (2010) have given to our recent paper (Kohlsdorf and Wagner 2006) (KW06), which, to our knowledge, was the first paper that tested the Dollo's law statistically. Irreversibility of limb and digit loss is a broadly accepted assumption in studies of limb evolution, but this widely held assumption had not been tested rigorously before our study. We agree with Galis et al. that this issue is important and has many consequences for our understanding of developmental evolution and the role of developmental constraints in evolution.

Galis et al.'s (2010) criticism centers on an apparent belief that the results of Kohlsdorf and Wagner (2006) are incompatible with existing hypotheses of digit evolution (e.g., Galis et al. 2001, 2005) and are therefore implausible. Moreover, Galis et al. question Kohlsdorf and Wagner's phylogenetic analysis, method of ancestral state reconstruction, and calculation of the rate of digit loss/gain, in addition to questioning the developmental plausibility of digit gain and the relationship between digit gains and changes in overall body form.

Here, we critically reevaluate the KW06 study including additional data and newly developed phylogenetic tools. We demonstrate that, not only are Galis et al. (2010) analyses of these data

flawed, but our phylogenetic reanalyses and ancestral state reconstruction presented here reenforce the KW06 results that digits have been regained in *Bachia*. We also show that at least one of Galis et al.'s (2010) criticisms of this study is based on a fundamental misunderstanding of Kohlsdorf and Wagner hypothesis that digits may reevolve via different genetic pathways. Finally, we will address some of the theoretical concerns raised by Galis and collaborators unrelated to the specific data we published.

Below we start with a brief discussion of the underlying theoretical issue, because this topic seems to be the driving motivation to challenge our results. The second more important question is whether the data in fact support reevolution of digits. This question is addressed in the next section. Finally, we address a number of peripheral issues about limb function, character evolution, and adaptive scenarios.

Developmental Biology of Digit Reevolution

While we agree with Galis et al.'s model of developmental constraints on digit evolution (2010), we do not think that our result of digit regain contradicts that model. Galis's model suggests that pattern formation in the embryonic limb bud is hard to change because it is part of the phylotypic stage, which has been shown to be particularly sensitive to perturbations, as changes occurring during this stage are more likely to affect the development of the whole embryo, thus constraining evolutionary change. Indeed, our main motivation to examine digit loss and possible reevolution was the finding that digit condensations are maintained for many millions of years after the definite digit has been lost. This was demonstrated for the anterior most digit condensation in the avian hand, which in the chicken has a putative metacarpal condensation that never expresses cartilage extracellular material (Feduccia and Nowicki 2002; Kundrát et al. 2002; Larsson and Wagner 2002; Welten et al. 2005). This finding is consistent with the model of Galis and Metz, that early pattern of digit condensation is likely to be constrained because of deleterious pleiotropic effects (Galis and Metz 2001; Galis et al. 2001, 2002). Hence if digit condensations are retained long after the ossified digit has been lost, then it should be easier to reevolve lost digits than generally assumed. Apparently, this seems to be the case in the genus we studied (*Bachia*), confirming the prediction. The constraints on digit repatterning, hypothesized by Galis and Metz (2001), explain why digit condensations should be maintained after digit loss, making the reevolution of digits likely. In the light of these considerations, it seems that the disagreement between us and Galis et al. mainly is whether their model applies to digits or to digit condensations. We think that their model explains the retention of digit condensations even in the absence of manifest digits in the adult, which in turn should make reevolution of digits easier than generally assumed.

Based on our model, that digit reevolution is probably based on the maintenance digit condensations after digit loss and their "reuse" for reevolution of digits, we reject the comparison of digit reevolution with polydactyl mutations as done by Galis et al. (2010). Galis and collaborators argue that polydactylous mutations are invariably associated with severely negative pleiotropic effects and are thus unlikely to contribute to evolutionary change. We agree with this rationale concerning polydactyl mutations, but disagree about the implications on the reevolution of digits. Crown group tetrapods possess at most five digit condensations in each limb bud, thus polydactyl phenotypes have to involve the repatterning of early limb development. In digit-reduced limbs, however, retention of the ancestral pattern has been documented (e.g., Larsson and Wagner 2002). Hence polydactyl limbs arise from more radical developmental changes, ones that likely are under severe constraints as predicted by Galis et al., but reevolution of digits may take advantage of a conserved five-digit ground state of the amniote limb bud. We acknowledge that the reevolution of digits from conserved condensation patterns has not been formally demonstrated. The reason for this is the difficulty of obtaining embryo material from critical taxa, in particular from the genus *Bachia.* To our knowledge, there are no published studies on the condensation patterns of squamate taxa with digit reduction, and one of us (MTR) is currently working on the first table with embryo stages for a gymnophthalmid species. We welcome any efforts to test our model of retention and reuse of digit condensation as a mode for the reevolution of digits.

Reanalysis of Bachia Species Phylogeny and Digit Variation

Galis et al. (2010) reanalyzed our data (with the addition of closer outgroup taxa) and presented an alternative phylogeny in which all of the digit-reduced species are in a derived position, suggesting that the most parsimonious evolutionary scenario is multiple losses of digits. Despite finding no support to statistically reject the KW06 phylogeny, Galis et al. nonetheless use their poorly resolved phylogeny to claim no support for or even a possibility of digit reevolution. However, upon closer scrutiny, we demonstrate that rigorous phylogenetic analyses support a phylogeny statistically indistinguishable from the original KW06 tree, yet can statistically reject the phylogeny inferred by Galis et al. (2010).

BACHIA PHYLOGENY

The results of our partitioned Bayesian phylogenetic analysis of *Bachia* are shown in Figure 1. Unlike the Galis et al. phylogeny,
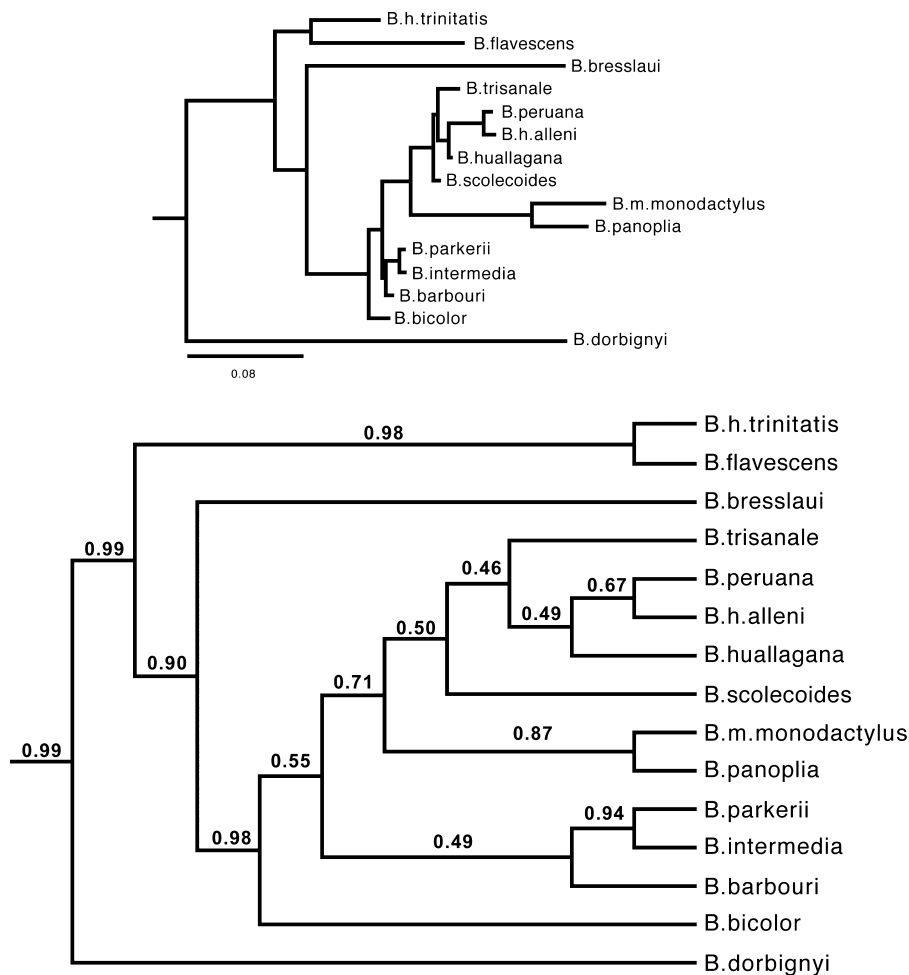
Figure 1. Bayesian phylogeny of *Bachia*. For simplicity, the outgroups are not shown. Numbers above branches indicate posterior probabilities.

which places reduced digit species in a derived position implying only losses of digits, we find that species with more ancestral digit patterns (e.g., *B. peruana* and *B. huallagana*) are nested within species that lost most digits. This result implies reevolution of digits, and a statistical test is presented below. Thus, our phylogeny of *Bachia* is consistent with the conclusions of Kohlsdorf and Wagner (2006) that there is phylogenetic evidence for reversal of digit loss. Interestingly, however, the *Bachia* phylogeny inferred in this analysis is different from both the original phylogeny proposed by Kohlsdorf and Wagner (2006) and the phylogeny presented by Galis et al. (2010). The source of these different results is not completely clear. One potential explanation of the slight difference between our new phylogeny and the original KW06 tree is that our reanalysis includes additional outgroup taxa and also includes the third codon positions of *C-mos* which were excluded in the KW06 study (but the comparison between this and the KW06 phylogeny shows that they are statistically indistinguishable, see below). Why Galis et al. infer a radically different phylogeny despite analyzing the same data is not entirely

clear, but may be due to inadequate data partitioning by gene instead of by gene and codon position and stems and loops as we did here (see Brandley et al. 2005), or that their Bayesian analyses did not adequately sample the true posterior distribution of trees.

To determine if the differences in tree topology are statistically significant we directly compared the log-likelihood ($-lnL$) of each tree using the approximately unbiased (AU) test (Shimodaira 2002). While the tree inferred in this study has a better $-lnL$ score than both the KW06 phylogeny (16.7 $-lnL$ units worse) and the Galis et al. phylogeny (60.2 $-lnL$ units worse), we can only statistically reject the Galis et al. phylogeny as being significantly worse than our current phylogeny (AU test, $P = 0.017$). Thus, while there are differences between our current phylogeny and the original phylogeny proposed by KW06, these differences are statistically not significant. However, both phylogenies are significantly better than the Galis et al. tree when compared with the AU test. Indeed, the phylogeny inferred in this study is significantly better than the Galis et al. tree in 5/6 of the tree comparison metrics shown in Table 2.

NEW TREE, NEW METHOD, SAME OLD STORY

In their critique of our previous study, Galis et al. rightly point out that the methods we used were later found to be potentially flawed (Goldberg and Igic 2008). In particular, they note problems with the implicit assumption of the root state and also the phenomenon where diversification rates between species are correlated with a character state. For example, if digitless species diversify at a significantly greater rate than species with digits, then given enough time, species with digits will be "surrounded" by digitless species and only appear to have reevolved them using standard character evolution models. However, accounting for differential diversification rates corrects this problem (Goldberg and Igic 2008).

Although Galis et al. invoke these potentially "devastating methodological flaws" to discount the results of Kohlsdorf and Wagner (2006), they did not actually reanalyze the data despite the recent availability of software that addresses these potential sources of error. Therefore, we reanalyzed these data using a model of character evolution (BiSSE) that explicitly accounts for differences in both the speciation and extinction rate between species with digits (defined as having four digits) and those with reduced digit numbers (species with one or no digits) to test for the reversibility of digit loss.

To account for phylogenetic uncertainty, we summarized parameters of the BiSSE model and tested irreversibility across the 100 trees from the posterior distribution of the Bayesian analysis. While ancestral character reconstructions were inconclusive (Fig. 2), we could easily reject the irreversible model for all 100 trees (mean $-\ln L$ reversible model $= -72.22$, mean $-\ln L$ irreversible model $= -77.52$; mean $\Delta AIC = 14.60$; Fig. 2). In addition, the mean $-\ln L$ of the reversible model is significantly better than the irreversible model ($P = 0.0003$; Fig. 2). Thus, we conclude that there is significant phylogenetic signal and statistical support indicating that digit loss is reversible.

Parsimony of Digit Reevolution

In our paper, we used a variety of methods to determine whether there is a signal for reevolution of digits. In one of them, we compared the Dollo scenario of irreversible evolution with one that allows digit regain by comparing how much a regain of digits needs to be penalized in the parsimony score to make Dollo the
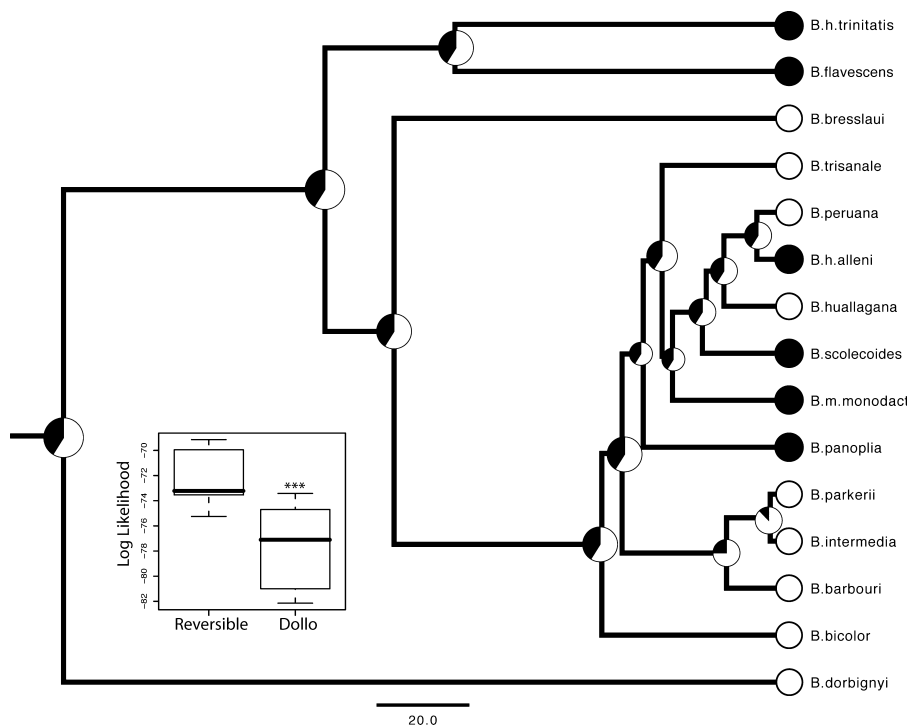
Figure 2. Evolution of digit number in *Bachia*. This figure shows the *Bachia* phylogeny from Figure 1 transformed to an ultrametric tree with an arbitrary root age of 100. Pie charts at internal nodes show the proportional likelihood of the ancestor being digitless (state 0, white) or having digits (state 1, black). Terminal nodes show the character state in that taxon. The boxplot (inset) shows the median and quartiles for the log-likelihood under the reversible and irreversible (Dollo) models, respectively, calculated over 100 trees from the Bayesian posterior distribution. Note that the reversible model is significantly better than the irreversible model for each tree individually (mean log-likelihood reversible model $= -72.22$, mean log-likelihood irreversible model $= -77.52$; mean $\Delta AIC = 14.60$) and that the distribution of likelihood scores is significantly higher for the reversible model than for the irreversible model.

more parsimonious scenario. We determined that a model where gains and losses of digits are equally possible gives the most parsimonious result. More specifically, the reevolution of digits needs to be 4.2 times more difficult than a loss of digits to make the Dollo scenario more parsimonious than reevolution of digits. We compared this value with those obtained from a distribution of random phylogenies (by permutating the terminal taxa on a fixed tree) and found that in random phylogenies the reevolution penalty is smaller than in our data. Thus, we concluded that our phylogeny has a stronger signal for reevolution than expected by chance.

Galis et al. (2010) compared our "critical regain penalty" of 4.2, derived from our quantitative, phylogenetic analyses using digit data from almost all species of *Bachia* included in the phylogeny, with one estimated by Greer (1992). Greer (1992) focused on the rates of gain and loss of phalanges (instead of digits), and has argued that a gain of an additional phalange is five times more difficult than the loss of phalanges. Based on that number, Galis and collaborators claim that the reevolution of digits is not unusually more difficult than expected. We respectfully disagree with that conclusion, because although an important contribution to our understanding of squamate limb reduction, it is unclear how Greer's squamate-wide "global" rate of loss of phalanges, estimated without a phylogenetic framework, would relate to the specific rates of evolution we measured in *Bachia.* Greer compared the number of times additional phalanges have been added in the evolution of squamates to the ancestral amniote phalangeal formula and compared it with the number of times phalanges have been lost from the ancestral phalangeal formula. Unfortunately, there was no well-supported phylogeny of squamates at the time, thus Greer used hyperphalangea to count events of phalange gain. Hence, for Greer hyperphalangea was the only way to reliably infer phalange gain. It is questionable whether addition of phalanges to the ground plan of the amniote hand is comparable to regain of lost phalanges. Intuitively, one would expect that true hyperphalangea is less likely than the regain of lost phalanges, but this remains to be determined by more adequate data. But more importantly, our results were generated using a resolved phylogeny and actual data for most species of *Bachia.* In our opinion, each dataset has to be evaluated on its own terms and comparing numbers that were generated in different ways on different species may be meaningless.

Selective Disadvantages to Loss of the Serpentine Locomotion

In their paper, Galis et al. (2010) question if it is plausible to assume a selective advantage for digit reversals in *Bachia*, and explicitly state that there would be no "biological reason" for re-

versals in digit loss in the group, based on biomechanical predictions that assume increase in the distance between hind and front limbs due to body elongation. We first note that there are very few biomechanical studies of limb-reduced squamates and extrapolating biomechanical "biological reason" for limb reduction or reevolution in a specific clade of lizards should be approached with a measure of skepticism. Nonetheless, as discussed in the next section, body elongation may have occurred at the origin of the *Bachia* clade rather than in association with digit loss, and the body of *Bachia* species with fewer digits may not be necessarily longer than the trunk of *Bachia* species with more digits. But even if body length has gradually increased with digit loss during the history of the genus, evolutionary reversals could be triggered both because of selective pressures related to biomechanics of movement in specific ecological contexts or due to sexual selection.

The biomechanical argument against digit reevolution is based on some studies (e.g., Presch 1975) and personal observations suggesting that *Bachia* locomote mostly by body undulation, when the limbs are dragged close to the trunk and apparently have no role in locomotion. As pointed out by personal observations of RL in the Galis et al. paper, which agrees with TK's and MR's experience with *Bachia* individuals in the lab and personal communications by A. Camacho, some *Bachia* species are not entirely fossorial, and they also locomote on the surface or in the layer of dead leaves between surface and soil of the rain forests. It is possible that in alternative ecological contexts, for example, when moving from one layer (e.g., underground) to another (e.g., surface), *Bachia* lizards use their tiny legs as a lever to elevate their center of mass to the next surface level. Also, when exploring the environment in a nonthreatening situation, some *Bachia* exhibit a behavior of elevating their head and anterior part of their trunk above the ground and support their body only with the posterior part of the trunk (pelvic girdle, legs, and tail) in contact with the ground (A. Camacho, pers. comm.; behavior also described by Galis et al. 2010). In this specific ecological context, hind limbs that present higher number of toes likely improve stability during trunk elevation.

Another misleading assumption is that *Bachia* species inhabiting similar habitats would have similar morphologies and would also perform similarly. Two recently described species, *Bachia psamophila* and *Bachia oxyrhina* (Rodrigues et al. 2007), are found in ecologically similar habitats that are geographically very close. These species have a striking pattern of morphological evolution: while they are overall morphologically similar and present a highly projecting snout that is hypothesized to be an adaptation to subaerial locomotion in sandy habitats (Rodrigues 1991; Rodrigues et al. 2007), their hind limb morphology is completely different. According to Rodrigues et al. (2008), the divergent evolution of hind limb morphology in species ecologically

similar and likely morphologically adapted to similar habitats suggests that other causes than biomechanics of locomotion should be invoked to account for the patterns of morphological variation observed in *Bachia* limbs.

One alternative source of selective pressures acting on the evolution of limb morphology in *Bachia* could be related to reproductive strategies and male–male interactions (Rodrigues, unpubl. obs.). For example, the vestigial hind limbs ("pelvic spurs") of some snakes are claimed to have been maintained due to sexual selection (Stickel and Stickel 1946), as in several species the pelvic spurs are longer and thicker in males than in females, and are used by males to manipulate the female's tail prior to copulation (Barker et al. 1979; Slip and Shine 1988). Also, there is some evidence that pelvic spurs may be used during male–male interactions in snakes (Barker et al. 1979). Some species of boas engage in combat bouts by entwining their tails and posterior trunk regions, when pelvic spurs are actively being flexed against the scales of the opponent (Carpenter et al. 1978). Together, these data suggest a behavioral function, instead of a biomechanical one, for the retention of vestigial hind limbs in some snake lineages (Carpenter et al. 1978). It is interesting to point out that, in several recent phylogenies, lineages that present species with vestigial hind limbs, as Boinae and Pythoninae, are placed in intermediate or derived positions (Forstner et al. 1995; Lee et al. 2007), and Brandley et al. (2008) estimated that this vestigial condition has persisted between 41 and 63 million years for two lineages of snakes. Thus, a possible scenario for the reevolution of pelvic elements in snakes is due to selective pressures related to reproduction and male–male interactions. The investigation of reproductive behaviors and social interactions in *Bachia*, as well as a better description of their locomotor behavior and body posture in different ecological contexts, would definitely clarify which sources of selective pressures may have shaped limb morphology in this group.

Digit Losses and Associated Character Changes

One argument against reversibility of digit loss in *Bachia* raised by Galis et al. (2010) claims that reevolution of digits would imply that several other features associated with digit loss (and presumably fossoriality, but see comments on semi-fossoriality of *Bachia* above) had also to be reversed. One critical point raised by Kohlsdorf and Wagner (2006) is that the toes of derived *Bachia* species predicted to be reevolved exhibit a completely different morphology than the ancestral digits in the clade. They represent a reversal of the trait "digit number" but not a reversal at the level of digit morphology. In the reevolved digits, digit identity is absent and all toes have two phalanges. Therefore, the reversals to the an-

cestral state of four toes may have occurred through evolution of alternative developmental pathways rather than simply reversing the process by which limbs were reduced and the body elongated. If this is the case, then reversals of digit loss would not demand reversals of all characters changed during limb reduction but, instead, would have involved the evolution of alternative pathways.

Some of the morphological traits typical of *Bachia* species, as an elongated body, may have evolved at the origin of the genus, instead of gradually evolving in association with digit loss. As pointed out by Gasc (1984) and Presch (1975), body elongation is a typical feature of the genus *Bachia*, but the absence of phylogenetic methods in the traditional studies limits our ability to dissect the effects of phylogenetic signal (given by common ancestry) from adaptation. Therefore, at this point it is not possible to conclude whether body elongation is an ancestral characteristic of *Bachia* or if this trait has gradually evolved in conjunction with digit loss. Only in the latter case would one expect body shape to be reversed when toes reevolve. Patterns of morphological evolution in the entire Gymnophthalmidae family are currently under investigation in one of our labs (TK), and a large dataset based on external measurements and x-rays suggests that body elongation seems in fact be associated with decrease of limb *length* (Grizante and Kohlsdorf, unpubl. ms.); a pattern commonly seen throughout squamate reptiles (Wiens et al. 2006; Brandley et al. 2008). However, apparently once the species in a clade became elongated (at the origin of the lineage), the trunk length did not necessarily increase even more when digit loss occurred (Grizante and Kohlsdorf, unpubl. ms.).

Conclusions

While we agree that better sequence evolution models and closer outgroups affect the reconstructed phylogeny of the *Bachia* species as well as inferences about digit evolution, our new phylogeny still strongly supports the conclusion that digits in the hind limb have reevolved in at least two instances. Hence, the main conclusion of the KW06 paper is not affected and is robust to improvements of the phylogenetic hypothesis. Parenthetically, the phylogeny proposed by Galis et al. (2010), which is to a large degree unresolved, is statistically much worse than our new phylogeny, but the −lnL difference between our new phylogeny and the one proposed in KW06 is statistically insignificant. We also note that our result of reevolution of digits does not contradict Galis et al.'s model of strong constraints against polydactyl mutations (Galis and Metz 2001). Based on developmental evidence from the bird hand, we suggest that digit loss does not necessarily lead to a loss of the corresponding digit condensations (Larsson and Wagner 2002; Welten et al. 2005), and thus reevolution of digits might not be under the same constraints as polydactylous

Table 1. Statistical tests of alternative partitioning strategies for the *Bachia* DNA dataset. The seven-partition model (7×) (shown in bold) is a statistically superior explanation of our data and was the model used for our phylogenetic analyses.

Model	Prms.	Description	$-\ln L$	ΔAIC
1×	9	Single GTR+Γ (4) model extended to entire dataset	-17265.23	1959.64
2×	*18*	*Independent GTR+Γ (4) models for CMOS, 12s+16s**	*-17137.52*	*1724.22*
3×	27	Independent GTR+Γ (4) models for CMOS, 12s, 16s	-17076.78	1622.74
4×	36	Independent GTR+Γ (4) models for 1st+2nd and 3rd codon position of CMOS; 12s and 16s	-17005.60	1500.38
5×	45	Independent GTR+Γ (4) models for each codon position of CMOS; 12s and 16s	-16992.01	1493.20
7×	**63**	**Independent GTR+Γ (4) models for each codon position of CMOS; stems and loops of 12s and 16s**	**-16225.41**	**0.00**

mutations. Finally, we believe that many of the remaining arguments brought forward by Galis, Arntzen, and Lande are solely based on plausibility rather than real data and hold little weight as evidence. As noted above, many of these arguments can be reversed with equal plausibility. We have demonstrated that the balance of the available evidence supports digit reevolution in *Bachia* lizards. We also emphasize that such evidence also exists in other groups of squamate reptiles. Using the same phylogeny of Wiens et al. (2006) that Galis et al. (2010) claims was "found with rigorous phylogenetic techniques," Brandley et al. (2008) inferred statistically significant support for at least six examples of digit reevolution in addition to *Bachia*. Moreover, this study used an ancestral state reconstruction analyses that used an explicitly ordered model of digit transition and an empirically derived biased rate of digit loss. Now it will be important to investigate limb development of digit reduced and putatively reevolved digits to gain insights into the developmental mechanisms involved. In particular, it would be interesting to test whether in fact reevolved digits derive from digit condensations that were retained after digit loss.

Methods

PHYLOGENETIC ANALYSIS

Genes for *C-Mos, 12s,* and *16s* were downloaded from GenBank and aligned with MUSCLE and adjusted manually; the alignments are available from VJL by request. Following Galis et al.

(2010), we corrected the *B. dorbignyi* sequence and included the same additional outgroups as Galis et al.; thus, our datasets are equivalent. We used a combined maximum likelihood and Bayesian approach to infer the phylogeny of *Bachia*. Maximum likelihood was used to infer the best partitioning scheme for the phylogenetic analysis using RAxML version 2.2.3 (Stamatakis 2006). Partition models ranged in complexity from a single partition that applied a single GTR+Γ substitution model with six discrete rate categories across all sites and genes (1× model) to a partitioning scheme that applied a separate GTR+Γ substitution model to each codon position of *C-Mos*, each stem and loop of *12s*, and each stem and loop of *16s* (7×). Based on differences in Akaike information scores (ΔAIC), the 7× model was preferred (Table 1).

Bayesian phylogenetic analyses were performed with MR.BAYES version 3.0 (Huelsenbeck and Ronquist 2001) using the 7× partitioning scheme. Two independent Bayesian analyses were run for 10,000,000 generations each with two runs of four chains sampled every 50,000 generations and a burnin of 100 trees. Run progress was visually checked with TRACER version 1.4 by plotting the log-likelihoods of sampled generations, and the stability of parameter estimates (chain convergence) checked by ensuring that the standard deviation of split likelihood frequencies was below 0.01 while the potential scale reduction factor (PSRF) was close to 1.0 for all parameters. The Bayesian consensus tree was generated from the post burnin sample of 100 trees.

Table 2. Results *Bachia* phylogeny topology tests. Tree topologies are ordered by decreasing likelihoods (shown as Rank). ΔlnL, difference in likelihood score between the best tree (rank 1) and this tree; AU, the *P*-value of the approximately unbiased test; NP, bootstrap probability of the tree; PP, Bayesian posterior probability (calculated from BIC); KH, the *P*-value of the Kishino–Hasegawa test; SH, the *P*-value of the Shimodaira–Hasegawa test. The tree inferred from this study is shown in bold and is ranked 1st, the Kohlsdorf and Wagner tree (KW) is ranked 2nd, and the Galis et al. tree (GAL) is ranked 3rd. Significant results are shown in italics (*P*<0.05).

Tree	Rank	ΔlnL	AU	NP	BP	PP	KH	SH
Current	**1**	**0.00**	**0.691**	**0.669**	**0.661**	**1.00**	**0.657**	**0.784**
KW06	2	16.7	0.347	0.325	0.334	*6×10^{-8}*	0.343	0.454
GAL09	3	60.2	*0.017*	*0.005*	*0.005*	*7×10^{-27}*	*0.034*	0.486

TESTS OF TREE TOPOLOGIES

The likelihoods of three alternate *Bachia* phylogeneies, identified by Kohlsdorf and Wagner (KW), Galis et al. (GAL) and this study, were directly compared to determine if the inferred phylogenetic relationships within *Bachia* were significantly different. Briefly, the Bayesian consensus tree identified from our phylogenetic analysis was used as the backbone tree keeping outgroup relationships constant while the ingroup *Bachia* sub-tree was modified to match the branching order identified by Kohlsdorf and Wagner, and Galis et al., respectively. Note that the Galis et al. tree shown in their Figure 2 is majority rule consensus and contains polytomies; to generate a resolved version of their tree we inferred the entire tree following the methods of Galis et al. Site-wise log-likelihoods under these three alternate tree topologies were calculated with PAML using BASEML under a GTR+Γ model with model parameters directly estimated from the data, followed by *P*-value estimation using CONSEL. Although we rely on the AU test for our tree tests, we show the results for several other tree selection methods implemented in CONEL for comparison purposes only.

CHARACTER EVOLUTION

Recent studies indicate that character associated changes in diversification rate can lead to erroneous rejection of irreversible models (Goldberg and Igic 2008); therefore, we tested for character associated diversification using BiSSE, which models character associated diversification rates and is implemented in Mesquite version 2.01 (Maddison et al. 2007). For the BiSSE models, a stationary or "uninformative" prior was used to infer the state of the root node under reversible model while the irreversible model fixed the state of the root node at 0. We accounted for uncertainty in the phylogeny and branch length estimates by summarizing parameters of the BiSSE model and testing irreversibility across the 100 trees used to generate the Bayesian consensus tree. Prior to BiSSE analysis, phylograms were transformed into ultrametric trees with an arbitrary root age of 100 using the penalized likelihood method implemented in r8s version 1.7 (Sanderson 2003).

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