

EVOLUTION OF MOUTHBROODING AND LIFE-HISTORY CORRELATES IN THE FIGHTING FISH GENUS *BETTA*

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Abstract.—The origin of and evolutionary transitions among the extraordinary diverse forms of parental care in teleost fish remain largely unknown. The “safe harbor” hypothesis predicts that the evolution from a “guarding” to a “brooding” form of care in teleost fish is associated with shifts in reproductive and life-history features such as reduced fecundity, and increased egg volume with higher parental investment. Robust phylogenetic hypotheses may help to identify evolutionary changes in key traits associated with differences in the form of parental care. Here, we used reconstruction of ancestral character states to study the evolution of the two forms of parental care, bubble nesting and mouthbrooding in the fighting fish genus *Betta*. We also applied a comparative analysis using the phylogenetic generalized least-squares method to test the “safe harbor” hypothesis by evaluating differences between the two forms of parental care in standard length, life-history traits, and three habitat variables. Evolutionary hypotheses were derived from the first molecular phylogeny (nuclear and mitochondrial DNA sequence data; 4448 bp) of this speciose group. Ancestral character state reconstructions of the evolution of the form of parental care in the genus *Betta*, using the methods of unweighted parsimony and maximum likelihood, are uncertain and further indicate a high rate of evolutionary transitions. Applying different weights for the suspected directionality of changes, based on the consistent phenotypic and behavioral differences found between bubble nesters and mouthbrooders, recurrent origin of mouthbrooding in the genus *Betta* is favored using parsimony. Our comparative analyses further demonstrate that bubble nesters and mouthbrooders do not have a consistent set of life-history correlates. The form of parental care in *Betta* is correlated only with offspring size, with mouthbrooders having significantly bigger offspring than bubble nesters, but is not correlated with egg volume, clutch size, and broodcare duration, nor with any of the three habitat variables tested. Our results thus challenge the general predictions of the “safe harbor” hypothesis for the evolution of alternative brood care forms in the fighting fish genus *Betta*.

Key words.—Brood care, bubble nesting, comparative method, mitochondrial DNA, phylogenetic generalized least-squares, phylogeny, RAG1.

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Teleost fish offer a wide variety of opportunities for the comparative study of mating systems and patterns of parental care. Of the approximately 422 families of teleost fish, 89 (21%) show some form of parental care (Blumer 1982). In contrast to many other vertebrate groups in which female-only parental care dominates, in fish male-only parental care is a dominant feature (Blumer 1979, 1982; Gross and Sargent 1985). In addition to differences in the sex of the caregiver, teleosts also exhibit a high diversity in the forms of parental care. Mouthbrooding, one of the most specialized forms of parental care is only found in nine teleost families (Blumer 1982). The evolution from a “guarding” form of care, such as substrate spawning or bubble nesting, to “mouthbrooding” is generally assumed to be linked with shifts in reproductive and life-history features as predicted by Shine’s “safe harbor” hypothesis (Shine 1978). Shine’s safe harbor hypothesis states that propagule (i.e., egg) size is expected to evolve in response to relative survivorship rates during and after the propagule stage depending on the form and quality of parental care. Natural selection is most likely to favor large egg size when the egg stage can be considered a safe harbor due to parental care investment. The transitions from no parental care to parental care, and also changes in the form and quality of parental care, such as from “guarding” to “brooding,” are therefore expected to result in shifts in reproductive

and life-history features. These shifts include, for example, reduced fecundity, increased egg size, and higher rates of juvenile survival with higher parental investment (Kühme 1961; Fishelson 1966; Chardon and Vandewalle 1971; Shine 1978; Noakes and Balon 1982; Gross and Sargent 1985; Sargent et al. 1987). Mouthbrooders further show a general reduction or loss of egg and larval attachment systems (Peters and Berns 1982). However, broad comparative analyses within robust phylogenetic frameworks are still needed to identify the factors that shaped the evolution of alternative forms of parental care in teleost fish.

The perciform suborder Anabantoidei, or labyrinth fishes, includes about 127 species with a remarkable diversity of reproductive behaviors ranging from free spawners without parental care, to substrate spawners with male parental care, bubble-nesters with male or biparental care, and male or rarely female mouthbrooders (e.g., Forselius 1957; Berns and Peters 1969; Cambay 1990, 1997; Vierke 1991; Britz 1995; Britz and Cambay 2001). Among labyrinth fishes, mouthbrooding occurs in all species of the closely related genera *Ctenops*, *Sphaerichthys*, and *Luciocephalus* (Osphronemidae, Luciocephalinae), and in some species of the fighting fish genus *Betta* (Osphronemidae, Macropodinae). With more than 40 species, the latter genus is the largest among anabantoids. In roughly 70% of the total number of species with-

TABLE 1. Differences of phenotypic and behavioral character states between bubble nesting and mouthbrooding species of the genus *Betta*. Mean values for several life-history traits for bubble nesters and mouthbrooders are given in the Appendix.

Character	Bubble nesters	Mouthbrooders
Egg surface structure ^a	wrinkled eggs	knob-like projections ^b
Sexual dimorphism ^c	pronounced	little or no
Spawning embrace ^c	females turned upside down	reduced spawning embrace ^d
Head shape (in dorsal view) ^e	narrow with a parallel outline	broad with a conical outline
Larval head attachment cells ^f	present	unknown
Ability to produce mucous coated bubbles ^f	yes	unknown

^a Britz (1995; 2001); ^b Not known for *B. macrostoma*; ^c Schmidt (1996); ^d With the exception of *B. foerschi* (no data available for *B. strohi*); ^e Witte and Schmidt (1992); ^f Britz and Cambray (2001).

in the genus *Betta*, males take spawned eggs into their mouths and incubate them for up to four weeks (Schmidt 1996). The remaining 30% of the species within the genus exhibit bubble nesting, the dominant reproductive style and plesiomorphic condition among osphronemid taxa (Britz and Cambray 2001), in which eggs and larvae are guarded and defended by the male. Bubble nesting and mouthbrooding *Betta* species differ from each other in several phenotypic and behavioral aspects (Table 1). The most prominent features include differences in head shape, spawning embrace, egg surface structure, and in the degree of sexual dimorphism. Vierke (1991) hypothesized that mouthbrooding in the genus *Betta* is not only an adaptation to increased predation risk, but more importantly to increased water currents that would inhibit the construction of a bubble nest on the water surface.

Vierke (1991) and Schmidt (1996) suggested, without an explicit phylogenetic methodology, that mouthbrooding evolved several times independently within the genus. Britz (1995, 2001) studied the egg surface structures among bettas and found numerous evenly distributed knob-like projections on the eggs of mouthbrooders. This egg surface structure, which is unique among anabantoids, was interpreted as a synapomorphy of the mouthbrooding bettas and hence as evidence for a single origin of mouthbrooding in the genus. In contrast, all bubble-nesting bettas show a wrinkled egg surface without projections, the plesiomorphic state, which is also found in other macropodines.

To resolve among competing hypotheses on the origin of the form of parental care and their associated life-history correlates in the genus *Betta*, a detailed phylogenetic hypothesis is needed. Here we provide a phylogenetic framework for the fighting fishes based on an analysis of both nuclear and mitochondrial DNA sequences. We use our molecular phylogeny to address three points: (1) monophyly of the genus *Betta*, (2) the hypotheses of a single versus a multiple origin of mouthbrooding in the genus, and finally, (3) whether changes in the form of parental care are related to changes in life-history variables, as predicted by the “safe harbor” hypothesis, as well as to shifts in habitat characteristics as hypothesized by Vierke (1991). There is no consensus about what evolutionary forces have influenced the different forms of parental care (bubble nesting and mouthbrooding) and comparative analyses based upon robust phylogenetic hypotheses may help to identify evolutionary changes in key traits that are correlated with differences in the form of parental care.

MATERIALS AND METHODS

DNA Sources and Extraction

To assess the molecular phylogeny of the genus *Betta*, DNA samples of 32 individuals representing 30 species were obtained. In addition, DNA samples of five species of other Macropodinae and two species of Luciocephalinae were gathered, and the latter were used as outgroups in the phylogenetic analyses. Whole fish were preserved in 70–100% ethanol and total genomic DNA was isolated from white muscle tissue or fin clips by proteinase K/SDS digestion, phenol-chloroform extraction, and ethanol precipitation (Kocher et al. 1989).

PCR Amplification and Sequencing

A 2100-bp fragment that includes the mitochondrial 12S rRNA, tRNA Val, and 16S rRNA genes was obtained by polymerase chain reaction (PCR) amplification of three overlapping fragments with the primers shown in Table 2. The complete mitochondrial cytochrome *b* gene (1140 bp) and 1500-bp of the nuclear RAG1 gene were PCR amplified with the primers given in Table 2. PCR amplification of the complete cytochrome *b* in the *Betta splendens* group required specific primers and was accomplished by obtaining three overlapping fragments (Table 2). PCR products were either cloned into pGEM-T vectors (Promega, Madison, WI) and sequenced using M13 universal primers or were sequenced directly after PCR purification by ethanol/sodium acetate precipitation. Sequencing reactions were performed with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (V3.0) following manufacturer's instructions (Applied Biosystems, Foster City, CA). Cycle sequencing products were analyzed on an ABI Prism 3700 DNA Analyzer (Applied Biosystems). Details on PCR and sequencing reactions followed protocols given by Rüber et al. (2003). Sequences have been deposited in GeneBank under accession numbers AF519624–AF519662 (12S rRNA, tRNA Val, 16S rRNA), AF519663–AF519701 (cytochrome *b*), and AF519702–AF519740 (RAG1).

Phylogenetic Analyses

DNA sequences were aligned with CLUSTAL X (Thompson et al. 1997) using the default settings, and alignments were further optimized by eye. Ambiguous alignment positions were excluded from the analyses because of uncertainty

TABLE 2. Polymerase chain reaction primers used for the amplification of the mitochondrial 12S rRNA-tRNA Val-16S rRNA, cytochrome *b*, and the nuclear RAG1 in the fighting fish genus *Betta*.

Primer	Sequence 5'–3' (this study) or reference
12S rRNA, tRNA Val, and 16S rRNA	
L1091	(Kocher et al. 1989)
H1478	(Kocher et al. 1989)
fish-12F1	(Rüber et al. 2003)
fish-16SR1	(Rüber et al. 2003)
fish-12SF2 (internal sequencing primer)	(Rüber et al. 2003)
16Sar-L	(Palumbi et al. 1991)
16Sbr-H	(Palumbi et al. 1991)
cytb	
DonGlu F	AAC CAC CGT TGT ATT CAA CTA CAA
DonThr R	ACC TCC GAT CTT CGG ATT ACA AGA CCG
cytb (<i>Betta splendens</i> group)	
Fish ND6 F	TAA GGA GAC GGA TTA GAA GC
Betta-cbout R	TAT CAG AGG TRT AGT GTA T
Betta-cytb F	CAC CCC CTA TTA AAA ATT GCT AA
Betta-cytb R	TCT ACR GGY ATR CCT CCA ATT CA
Betta-cbout F	ATT AAA CCT GAR TGR TAT TTY TTA TT
TDK-D	(Kocher et al. 1993)
RAG1	
R1-2533F	(Chen and Ortí, unpubl. ms.)
R1-4090R	(Chen and Ortí unpubl. ms)

in homology assignments. To maximize phylogenetic information, the mitochondrial and nuclear gene sequences were combined into a single data set. MODELTEST version 3.06 (Posada and Crandall 1998) was used to determine the evolutionary model that best fitted the data set. The hierarchical likelihood ratio tests (LRT) implemented in MODELTEST selected the TrN + I + Γ model (proportion of invariable sites = 0.44; α = 0.57, empirical base frequencies: A = 0.32; C = 0.28; G = 0.17; T = 0.23; substitution rates: A–C = 1.00; A–G = 5.25; A–T = 1.00; C–G = 1.00; C–T = 10.58; G–T = 1.00). These settings were subsequently used for maximum-likelihood (ML) analyses and to estimate ML distances for minimum evolution (ME) analyses. Maximum-parsimony (MP) analyses were conducted with heuristic searches (TBR branch swapping, MULTREES option effective, and 10 random stepwise additions of taxa). Transversions (Tv) were given four times the weight of transitions (Ts) based on an empirical Ts:Tv ratio of 3.72 that was estimated from the ML tree. Robustness of the inferred trees was tested using nonparametric bootstrapping (Felsenstein 1985) with 1000 pseudoreplicates. All previous phylogenetic analyses were conducted with PAUP* version 4.0b10 (Swofford 2002). A Bayesian inference (BI) of *Betta* phylogeny was performed with MrBayes v2.01 (Huelsenbeck and Ronquist 2001) through Metropolis coupled Markov chain Monte Carlo (MCMCMC) sampling for 1,000,000 generations (four simultaneous MC chains; sample frequency 100; burn-in 100,000 generations; chain temperature 0.2) under the GTR + I + Γ model.

Alternative phylogenetic hypotheses were tested using the Shimodaira-Hasegawa test (SH; Shimodaira and Hasegawa 1999) and parametric bootstrapping (SOWH test; Swofford et al. 1996; Goldman et al. 2000). For the SOWH tests we first constructed the ML tree under a given constrained to-

pology (our null hypothesis). For each hypothesis we simulated 100 replicate DNA sequence datasets with a length of 4448 bp along the constrained ML topology using Seq-Gen version 1.2 (Rambaut and Grassly 1997) under the GTR + I + Γ model. Each of the simulated datasets was subjected to heuristic parsimony searches to find the MP tree and the MP tree compatible with the constraint. Differences in length between the constrained and unconstrained trees were used as the null distribution and compared against the value obtained from the empirical data.

Reconstruction of Ancestral Character States

MacClade version 4.03 (Maddison and Maddison 2001) was used to trace the evolution of mouthbrooding on different phylogenetic hypotheses with unweighed parsimony reconstruction, a method that assumes that transitions in either direction are equally probable. However, this assumption might not be realistic when considering gains and losses of complex characters. In such cases unequal weighting may represent a more realistic model for the directionality of changes (Cunningham et al. 1998; Omland 1997). Therefore, we also applied a sensitivity analysis (Donoghue and Ackerly 1996) by increasing weights to one of the two transformations (holding constant the weight of the reverse transformation, i.e., mouthbrooding to bubble nesting) to find the minimum additional weight (in 0.1 increments) needed to reverse the results obtained under the assumption of equal transition costs. In addition, a maximum-likelihood approach to reconstructing ancestral character states was conducted with Discrete version 4.0 (Pagel 1999a).

Comparative Analyses

It is now generally recognized that species values do not provide independent data points for comparative analyses

because samples are taken across phylogenetically related taxa (e.g., Felsenstein 1985; Harvey and Pagel 1991; Martins and Hansen 1996; Nunn and Barton 2001; Pagel 1999b; but see, e.g. Westoby et al. 1995; Ricklefs and Starck 1996; Björklund 1997). A variety of methods that incorporate phylogenetic information such as Felsenstein's (1985) phylogenetic independent contrast (PIC), phylogenetic autocorrelation (PA; Cheverud et al. 1985), and phylogenetic generalized least-squares (PGLS; Grafen 1989) have been proposed to account for the historical nonindependence among taxa. Furthermore, several methods are now available to a priori test the assumption of phylogenetic independence (e.g., Abouheif 1999; Blomberg et al. 2003; Gittleman et al. 1996; Pagel 1999a), and for the optimal transformation and scaling of phylogenies (Pagel 1997, 1999b; Blomberg et al. 2003).

Here, we used the PGLS method to determine whether the two forms of parental care (bubble nesters vs. mouthbrooders) are associated with differences in standard length (SL), clutch size, egg volume, broodcare duration, and offspring size (see Appendix). PGLS is a phylogenetic regression method (Grafen 1989), in which the covariance among taxa due to phylogeny is expressed in the regression error term, and is thus accounted for during the analysis. It is a standard statistical approach with versatile applications in a variety of statistical designs, and it has been shown that the widely used PIC method is a special case of PGLS (Rohlf 2001).

The program Continuous version 1.0d13 (Pagel 2002) was used to assess the required amount of phylogenetic correction of the life-history data based on three alternative ML trees (see Results for more details), and to obtain phylogenetic covariance matrices for the PGLS analyses (Pagel 1997, 1999b). Continuous version 1.0d13 implements maximum-likelihood estimates of three parameters (λ , δ , and κ) that can be used to optimally transform and scale the phylogeny. The parameter λ is a quantitative measure of phylogenetic dependence that assesses the amount of the phylogenetic component in character variation (Pagel 1999b; Freckleton et al. 2002). Its value ranges from zero (species are independent) to one (default phylogeny). The parameter δ scales overall path length in the phylogeny as well as the shared path length, and thus, can be used to detect whether the rate of trait evolution has accelerated or slowed over time. Values of $\delta < 1$ indicate a rapid early evolution followed by slower rate of change among closely related species (signature of an adaptive radiation), values of $\delta = 1$ indicate default gradualism, and $\delta > 1$ indicate an accelerating rate of trait evolution through time. The parameter κ defines the relationship between the lengths of individual branches and the probability that a character changes state and thus, allows one to stretch or compress individual branches. In the extreme case of $\kappa = 0$, trait evolution is independent of the branch lengths (punctuational evolution), $\kappa < 1$ compresses longer branches more than shorter ones, $\kappa = 1$ indicates default gradualism, and $\kappa > 1$ stretches longer branches more than shorter ones, indicating that longer branches contribute more to trait evolution.

Phylogenetic covariance matrices were obtained in two ways. First, by setting λ to its estimated ML value and constraining δ and κ to one, and second by setting all three parameters to their estimated ML values. The simultaneous

use of the parameters λ , δ , and κ are sought for the optimal scaling of phylogenies and are used to test the tempo, mode, and phylogenetic associations of trait evolution (Pagel 1997, 1999b). However, because there is very little theoretical and empirical data available on the application of the parameters δ , and κ we decided to also perform the analyses using λ only as it has been done in an extensive review on comparative analyses by Freckleton et al. (2002).

In order to assess whether SL had to be taken into account as a covariate in the between group (bubble nesters vs. mouthbrooders) comparisons we first tested for significant correlation between SL and each of the dependent variables taking phylogeny into account as described above. For illustrative reasons only, we also conducted the analyses from trait values of taxa with no phylogenetic correction (TIP data). As there was no evidence of heteroscedasticity (*F*-test; Sokal and Rohlf 1995), we used the untransformed values for all the analyses. PGLS analyses, using the phylogenetic covariance matrices obtained with Continuous version 1.0d13, were performed using the MULREG module in NTSYS-pc version 2.1 (Rohlf 2000).

Data for the life-history analyses were gathered from the literature (e.g., Vierke 1991; Britz 1995, 2001; Schmidt 1996) for 21 species, and included: breeding mode (bubble nester vs. mouthbrooders); standard length (SL) of adult males (in mm); number of spawned eggs (clutch size); egg volume [in (mm)³] which was calculated directly from measures of egg diameter (in mm) because this provides a more suitable estimate of egg size (Elgar 1990); broodcare duration in days; and total length (TL) of young (in mm) at the end of the parental broodcare period, when yolk sacs are resorbed and first exogenous feeding begins. All raw data including data sources, mean values for the bubble nesting and mouthbrooding species, and details on the phylogenetic hypotheses used for the comparative analyses are given in the Appendix.

We also tested for correlated evolution between the form of parental care and some available habitat characteristics based on the three alternative ML trees (see Results for more details) using Discrete version 4.0 (Pagel 1999a), which compares the likelihoods of the models of independent and dependent evolution via a LRT. Three habitat variables (occurrence in lowland vs. highland streams, occurrence in fast vs. slow water current, and occurrence in peat swamp forests vs. absence in this habitat type) used for 24 species were gathered for this study from field observations (see Appendix).

RESULTS

Phylogenetic Analyses

The alignment of the two mitochondrial gene regions and the nuclear RAG1 gene for the 39 taxa consisted of 4839 positions. A total of 4448 positions were analyzed (1134/1835/1479 for the cytochrome *b*/12S rRNA, tRNA Val, and 16S rRNA/ RAG1 datasets, respectively) of which 2573 (469/1034/1070) were invariant and 1388 (563/550/275) were phylogenetically informative sites under the parsimony criterion. The cytochrome *b* in the analyzed taxa consists of 378–387 amino acids. In all nonbeta taxa and some bettas the stop codon is either T— TA— or TAA. In those species that

show an incomplete stop codon (T— or TA—), completion of the stop codon TAA occurs posttranscriptionally by polyadenylation of the mRNAs (Ojala et al. 1981). Interestingly, the remaining bettas show an ORF of 378 amino acids, and AGR stop codons (Fig. 1).

The 50% majority-rule consensus tree recovered from the Bayesian analysis of the combined mitochondrial and nuclear data set is depicted in Figure 2, and represents our best hypothesis for the phylogenetic relationships within the fighting fish genus *Betta*. Seven major clades within the genus *Betta* were recovered, that is, *pugnax* (clade A), *albimarginata* (clade B), *coccina* (clade C), *foerschi* (clade D), *splendens* (clade E), *unimaculata* (clade F), and *macrostoma* (clade G) (Fig. 2). Phylogenetic relationships among these clades were fully resolved: (G,(F,(E,((D,C),(B,A))))). Phylogenetic analyses of the mitochondrial dataset alone recovered the same clades A–G with identical interrelationships. In contrast, RAG1-based trees were unresolved at deeper nodes, and failed to recover clades A, C, and E.

The ML topology ($-\ln$ likelihood = 41380.33) is shown in Figure 3a. MP analyses with a 4:1 Tv:Ts weighting scheme resulted in four shortest trees of 14,823 steps (CI = 0.372, RI = 0.543; Fig. 4a). Different Tv:Ts weighting schemes (e.g. 1:1 and 2:1; not shown) resulted in topologies that differed from the MP analyses with a 4:1 Tv:Ts weighting scheme only in a few internal nodes within clades A and C. Likewise, the topologies obtained with the BI, ML, and MP analyses differed from each other only in a few internal nodes within clades A and C. ME recovered one tree (score = 2.60) which differed from the BI, ML, and MP analyses in the relative position of the *albimarginata*-clade (clade B) which was placed as sister group to the *pugnax*-, *coccina*-, and *foerschi*-clades (clades A, C, and D; not shown).

Phylogenetic Hypotheses Testing

We tested four alternative phylogenetic hypotheses: (1) mouthbrooders: all mouthbrooders are monophyletic following the results of Britz (2001), (2) *splendens*: at least one bubble nesting clade (*splendens*-clade, clade E) is resolved as sistergroup to the remaining bettas (clades A–D and F), which would hence define bubble nesting as the ancestral condition for the genus (3) *macrostoma*: *B. macrostoma* is the sistergroup to the *unimaculata*-clade (clade F) to which it has been traditionally assigned (Vierke 1991), and (4) *simorum*: *B. simorum* is part of a monophyletic group consisting of the *pugnax*-, *albimarginata*-, *coccina*-, and *foerschi*-clades (clades A–D) as suggested by differences in the *cytb* stop codons among the bettas (Fig. 1). The SH- and the SOWH tests used to test different topologies both failed to reject the hypotheses (3) and (4) whereas hypotheses (1) and (2) were clearly rejected (Fig. 3).

Transitions between Bubble Nesting and Mouthbrooding

Figure 4 illustrates the most parsimonious character state reconstructions for the evolution of the two reproductive modes onto the unconstrained topology as well as onto the alternative phylogenetic hypotheses (*macrostoma* and *simorum*), assuming either equal or differential transition weighting. Unweighted character state reconstruction for the un-

constrained MP tree inferred mouthbrooding as the ancestral condition in bettas with one gain and two losses of mouthbrooding (and two associated gains of bubble nesting; Fig. 4a). Delayed transformation (DELTRAN) of equivocal reconstructions in the two alternative phylogenetic hypotheses inferred bubble nesting as the ancestral condition in bettas with three and four gains of mouthbrooding for the *macrostoma* and *simorum* hypotheses, respectively (Fig. 4b and 4c).

Using a sensitivity analysis for the parsimony character state reconstruction on the unconstrained tree, we found that a transition cost from bubble nesting to mouthbrooding of ≤ 0.6 resulted in bubble nesting as the ancestral condition with four independent gains of mouthbrooding (Fig. 4a). In the two alternative hypotheses (*macrostoma* and *simorum*), increasing or decreasing the cost of the transition from bubble nesting to mouthbrooding by 0.1 resolved the equivocal reconstructions either as mouthbrooding or bubble nesting, respectively (Fig. 4b and 4c). Differences in the number of transitions for the *macrostoma* hypothesis ranged from one (and two losses of mouthbrooding with associated gains of bubble nesting) to three (and no associated losses) independent gains of mouthbrooding (Fig. 4b) and in the *simorum* hypothesis from one (and three losses with associated gains of bubble nesting) to four (and no associated losses) independent gains of mouthbrooding (Fig. 4c). Maximum-likelihood character state reconstructions indicate that the ancestral conditions of the internal nodes leading to the major clades for any of the three alternative topologies tested are uncertain (Fig. 4). For example ML ancestor states for the common ancestor of bettas only weakly supported mouthbrooding (supported 1.32:1/1.77:1/1.50:1 over bubble nesting for the unconstrained/*macrostoma*/*simorum* hypotheses, respectively; note that only a support of 7.4:1 or higher would be considered significant (Schluter et al. 1997)). This lack of significant support for ancestral states may be due to the high rate of evolutionary transitions throughout the history of the clade (Schluter et al. 1997), as well as due to the relatively short branch lengths early on in the evolutionary history of the genus *Betta*.

Comparative Analyses

No significant effect of SL on the four dependent variables was detected in the PGLS data (Table 3). A significant effect, however, was observed in the TIP data for egg volume, brood-care duration, and offspring size (Table 3). In those latter cases we proceeded with ANCOVA to test for between group differences (bubble nesters vs. mouthbrooders). Table 4 shows the results from the ANOVA/ANCOVA analyses for the PGLS and TIP data. The phylogenetic covariance matrices were obtained by setting either λ to its ML value, or setting all three scaling parameters (δ , κ , and λ) to their ML values (see Materials and Methods). The results from the PGLS analyses were congruent regardless of the way the phylogenetic covariance matrices were obtained. The only significant difference between the two forms of parental care was offspring size with mouthbrooders showing bigger offspring than bubble nesters (Table 4; see also Appendix). Table 4 further indicates that the phylogenetic component of

	ORF	Stop	tRNA Thr
<i>Betta anabatooides</i>	AAGACCCATTTC	AGACGCCGCC	GCCCTA
<i>Betta chloropharynx</i>	..AG.....	...A.TCT.....
<i>Betta hipposideros</i>	..G.....	...G.CT.....
<i>Betta waseri</i>	..G.T.....	...C.....
<i>Betta pi</i>	..AG.G.....	...CT.....
<i>Betta breviobesus</i>	..AG...G.....
<i>Betta pugnax</i>	..A...C.....	...G...G.....
<i>Betta fusca</i>	G.....G.....	...TT.....
<i>Betta simplex</i>	..G...C.....
<i>Betta cf. picta</i>	..G...C.....
<i>Betta dimidiata</i>	..AG...G.C.....	...G.A..AGGCACCCCCCCC.....
<i>Betta prima</i>	..A...T.G.....	...G...A.TCCGCCGCC.....
<i>Betta edithae</i>	..A...T.....	...G...CCGCCGCC.....
<i>Betta cf. albimarginata</i> "Pampang"	..AG...GC.....	...G.....	...T.....
<i>Betta cf. albimarginata</i> "Malinau"	..A...GC.....	...A.C.GGCC.....
<i>Betta cf. burdigala</i>	...T..CC.....	...AA.....
<i>Betta tussya</i>	...C.....	...G..A.A..GCCTGC.....
<i>Betta coccina</i>	..A..G.GC.....
<i>Betta coccina</i>	..A..G.AC.....
<i>Betta brownorum</i>	..A.T...C.....	...G.C.T.....
<i>Betta rutilans</i>	..A...C.....	...C.....
<i>Betta miniopinna</i>	..AG...CC.....
<i>Betta strohi</i>	..AG...C.....	...A.....
<i>Betta foerschi</i>	..AG...C.....
<i>Betta simorum</i>	..AATT..C.....	...A.C..CACCCC.....
<i>Betta smaragdina</i>	..AGT..... AAACACACCACA	T-----
<i>Betta splendens</i>	G.A.TTG.T.. AAGCACATTACC	T-----
<i>Betta imbellis</i>	G.A.TTA.T.. AAACACATCACC	T-----	...T.....
<i>Betta patoti</i>	..A.TT..T.. TAAAACCCGC	T-----
<i>Betta ocellata</i>	..A.T..T.. TAAATACCCGC	T-----
<i>Betta unimaculata</i>	..A.TT..T.. AAACGCCGC	T-----
<i>Betta macrostoma</i>	...TG.ACGAAAA.....	TA.TCAAT--	...T.....
<i>Trichopsis vittata</i>	...TT..T.. TAAA	TA.CAT----	...T.....
<i>Parosphromenus deissneri</i>	..A.TAT.C.C. AAA	TA..CT----	...A.....
<i>Macropodus opercularis</i>	..A.TT..T.. AAAAATACAAAACGACGAA	TA-----
<i>Pseudosphromenus cupanus</i>	..ATTT..T.. AAACTAGACGAATTAAAAGCAAACAAAT	-----	...ATC.....
<i>Malpulutta kretseri</i>	..ATGT..TC. AAAAC TTATGAG.....	TA.ATAAA.TCCGC.....	...ATC.....
<i>Trichogaster leerii</i>	..A.TT..C.. AAC	TA-----	...T.....
<i>Colisa chuna</i>	..A.TTT.C.. ACACTTCCTAGAT	TA-----	...T.....

FIG. 1. Alignment of the nucleotide sequences of the 3' end of the cytochrome *b*, the stop codon, noncoding region, and the beginning of the tRNA Thr for the taxa used in this study. The limits of the ORF (open reading frame), the stop codon, and the beginning of the tRNA Thr are boxed in different greyscales. The second codon position of the lysine (AAA), where an A–G transition likely occurred leading to AGR stop codons is highlighted in bold. Hyphens denote gaps that were introduced to improve the alignment and dots indicate sequence identity with *B. anabatooides*. In species with an incomplete stop codon (T– or TA–) the TAA stop codon is completed by the addition of 3' A residues to the mRNA (Ojala et al. 1981).

character variation was strong ($\lambda = 1.0$ for all comparisons) in the *Betta* dataset.

Compared to the PGLS analyses, the species level comparisons (TIP data) revealed significant differences between mouthbrooders and bubble nesters in egg volume (mouthbrooders having marginally significant bigger eggs than bubble nesters), broodcare duration (mouthbrooders having longer broodcare periods than bubble nesters), offspring size, and SL (mouthbrooders tend to be bigger than bubble nesters) (Table 4; see also Appendix). We found no significant correlation between the two forms of parental care and any of the habitat variables tested (Table 5).

DISCUSSION

Intra- and Interrelationships of the Fighting Fishes

A robust molecular phylogeny of the fighting fish genus *Betta* was recovered based on a combined sequence dataset

including complete mitochondrial cytochrome *b*, 12S rRNA, tRNA-Val, and 16S rRNA genes, as well as partial nuclear RAG1 gene. Phylogenetic analyses of combined mitochondrial and nuclear sequence data provided resolution not achieved by each type of data separately. Overall, RAG1 gene was the least phylogenetically informative partition within the data set.

Using representatives of all macropodine genera the monophyly of the genus *Betta* is supported with our molecular data (Fig. 2). This is in agreement with a recent morphological study (Britz 2001) that identified three morphological synapomorphies to support the monophyly of the genus *Betta*, namely: (1) presence of a cartilaginous process at the base of the labyrinth, (2) complete loss of teeth on pharyngo-branchial two, and (3) loss of serration of the preopercular.

Within the genus *Betta*, we found in all phylogenetic analyses that the *macrostoma* (clade G), *unimaculata* (clade F), and *splendens* (clade E) clades are placed in a basal position

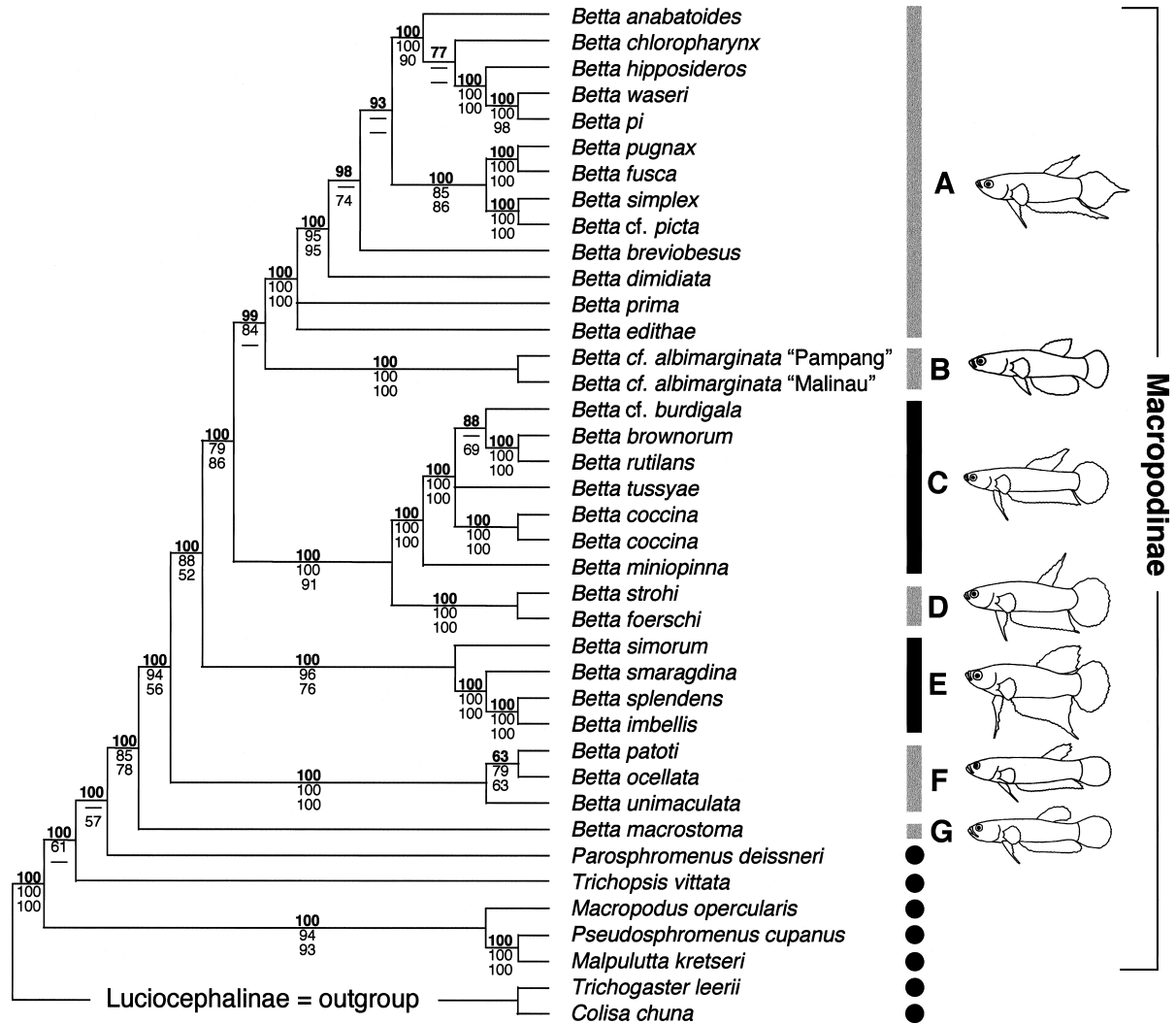


FIG. 2. Reconstructed phylogeny of the fighting fish genus *Betta* using a Bayesian phylogenetic approach. The number above each branch refers to the Bayesian posterior probability (shown as percentage) of the node derived from 9000 MCMCMC sampled trees on the basis of a 4448 bp dataset using both nuclear and mitochondrial DNA sequence data. The two Luciocephalinae taxa were used as outgroups. Bootstrap values (≥ 50) for MP and ME (upper and lower values, respectively) are shown below branches. Major *Betta* clades are highlighted with boxes: A, *pugnax*-; B, *albimarginata*-; C, *coccina*-; D, *foerschi*-; E, *splendens*-; F, *unimaculata*-; G, *macrostoma*-clade. The breeding mode is shown by differently colored boxes for the *Betta* species and in circles for the nonbettas (bubble nesting, black; mouthbrooding, grey).

relative to the more derived clades A–D. This finding is further supported by the *cytb* stop codon data (Fig. 1). The *macrostoma*, *unimaculata*, and *splendens* (excluding *B. simorum*) clades showed the plesiomorphic T–/TAA stop codon, whereas AGR stop codons can be considered as a synapomorphy of more derived betta clades (Fig. 1). Among these more derived clades, an unexpected sister group relationship between the *foerschi* (clade D) and *coccina* (clade C) clades was recovered. Previously, *B. foerschi* has been considered to be closely related to the *B. splendens* group (clade E without *B. simorum*), given its similarities in spawning behavior and body shape (Schmidt 1996; Vierke 1991), or to other mouthbrooders (Britz 2001), based on a similar egg surface structure.

Our data did not reject two of the four alternative phylogenetic hypotheses tested (Fig. 3). *B. macrostoma* (clade G)

may be sister group to the remaining betta species or the sister group of the *unimaculata* clade (clade F). *B. simorum* may be part of the *splendens* clade (clade E) or the sistergroup of more derived *Betta* clades (clades A–D; Fig. 3). Statistical support for alternative topologies over the short basal branches within *Betta* may be taken as evidence for a rapid divergence i.e. radiation of lineages early in the history of the genus.

The Evolution of Brood Care and Egg Surface Structure

Our results reject the monophyly of the mouthbrooding species (Fig. 3b), thus opposing the results of a previous morphological study (Britz 2001). However, these results do not allow us to distinguish, using unweighted parsimony, whether mouthbrooding evolved once in the ancestor of bet-

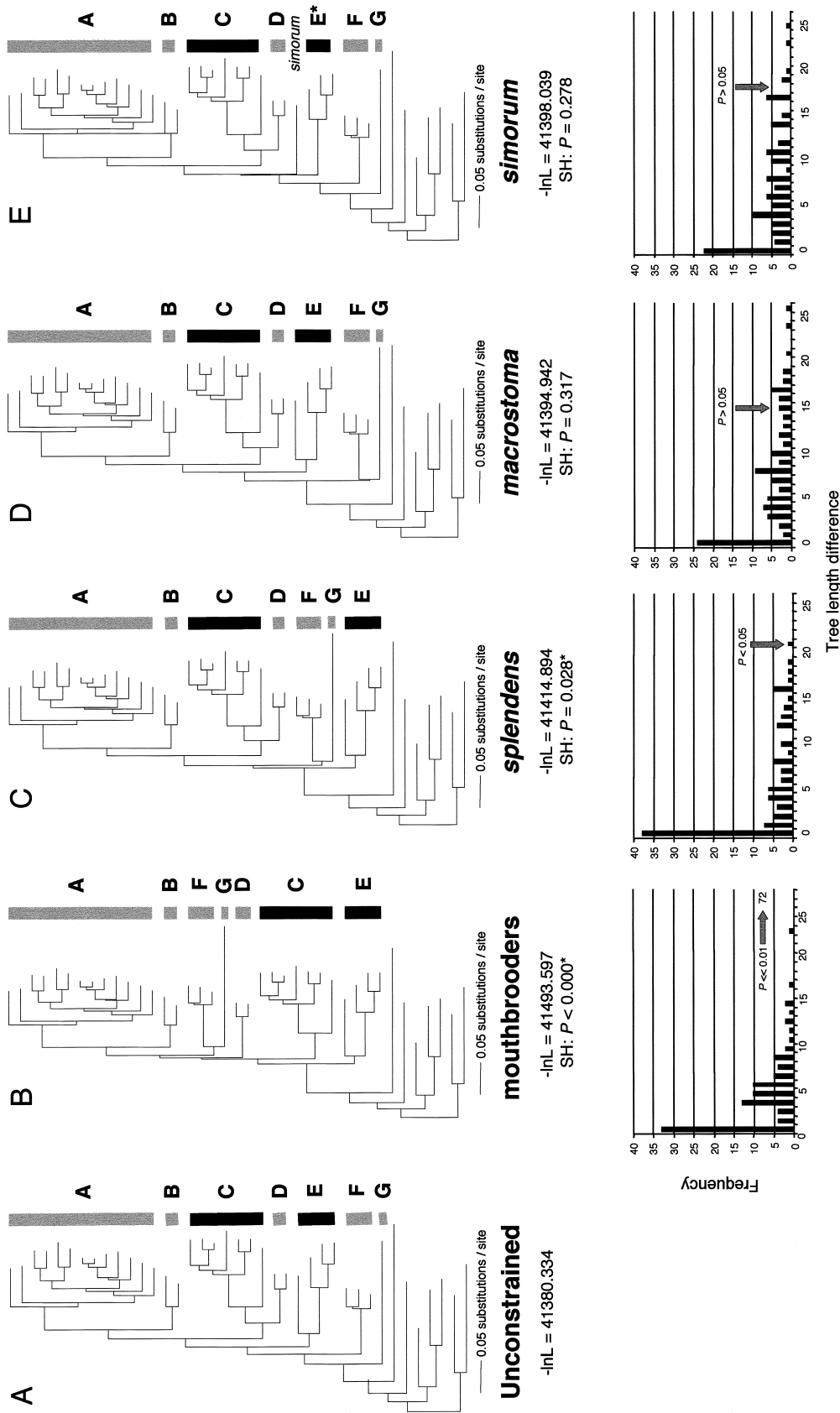


FIG. 3. Constrained and unconstrained maximum-likelihood trees (with likelihood scores indicated) used for the SH-tests and the SOWH-tests. The four constrained trees represent null hypotheses about monophyly and sistergroup relationships (see text for details of constraints). Results from the SH- and the SOWH-tests for the four alternative hypotheses are shown below the trees. For the SOWH-tests the differences in length between the constrained and unconstrained trees from 100 simulated trees were used as the null distribution and compared against the value obtained from the actual data (indicated by an arrow). Clade designation follows Figure 2 except E* which is the *splendens*-clade without *B. simorum*.

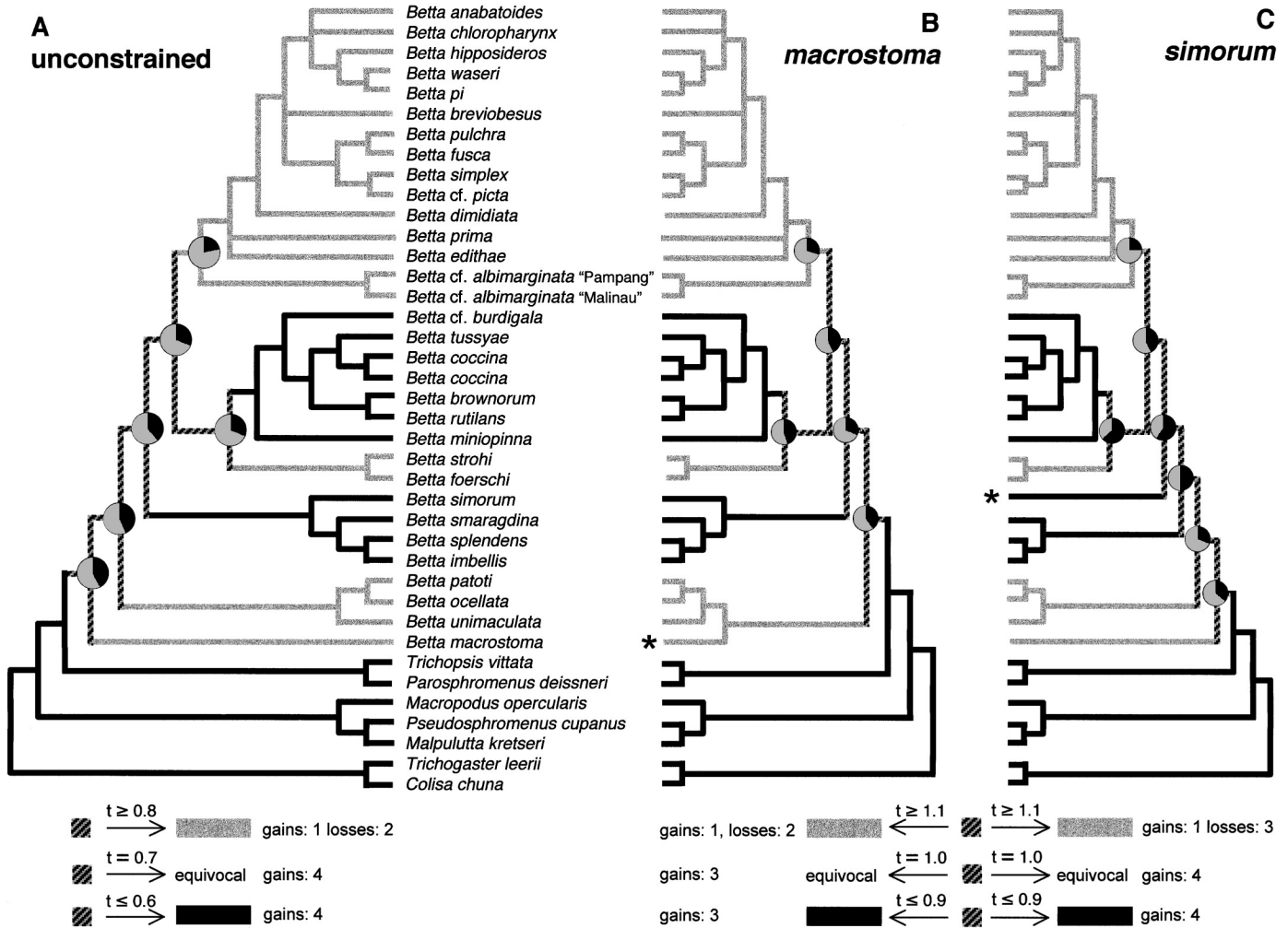


FIG. 4. Character evolution for bubble nesting (black) and mouthbrooding (grey) in the fighting fish genus *Betta*. (A) Parsimony character state reconstruction on the strict consensus tree of four maximum-parsimony trees obtained with a 4:1 Tv:Ts weighting scheme. (B) and (C) Parsimony character state reconstruction for the alternative *macrostoma*- and *simorum*-hypotheses, respectively. Stars indicate taxa that changed positions on the alternative phylogenetic hypotheses that were not rejected by our data using the SH- and SOWH-tests (*simorum*- and *macrostoma*-hypotheses; Fig. 3). Ancestral character state reconstruction along the stripped branches changes under different models of transformation costs (t) from bubble nesting to mouthbrooding while holding the reverse transformation cost constant (= 1). Below the trees the three different resolutions (black, bubble nesting; equivocal; and grey, mouthbrooding) of the reconstruction of ancestral character states along the stripped branches are given in relation to the transformation costs (t) from bubble nesting to mouthbrooding. In addition, the inferred number of gains and losses of mouthbrooding are given for the different phylogenetic hypotheses and the different models of transformation costs. For the equivocal reconstructions the inferred number of gains and losses are given under DELTRAN. Maximum-likelihood character state reconstructions (one-rate model, local estimates) performed using DISCRETE 4.0 (Pagel 1999a) based on the ML trees in Figure 3 (A, D, E) are shown in the pie diagrams. The differently colored areas in the pies represent the relative support for the two reconstructions (black, bubble nesting; grey, mouthbrooding).

tas (with two to three transitions from mouthbrooding to bubble nesting), or whether mouthbrooding evolved three to four times independently and therefore that bubble nesting is the ancestral state for the genus *Betta* (Fig. 4). This result is compatible with the ML approach that showed considerable uncertainty in the ancestral reconstructions (Fig. 4), and also indicates a high rate of evolutionary transition in the form of parental care in the genus *Betta*.

However, there are several independent lines of evidence consistent with the hypothesis that bubble nesting may be the plesiomorphic condition and, hence, mouthbrooding may have evolved several times independently within the genus *Betta* compared to a scenario of independent gains of bubble

nesting from a mouthbrooding condition (see also Table 1): (1) All bubble nesting bettas, as well as the mouthbrooding *B. foerschi* (no information available for *B. strohi*), show a complex spawning embrace which is the plesiomorphic condition for anabantoids (Britz 2001) while mouthbrooders show a reduced spawning embrace (Schmidt 1996). (2) Unique characters that are associated with bubble-nesting include complex physiological processes required for the production of the mucus for constructing a bubble nest, as well as behavioral elements that are essential for the construction and maintenance of the bubble nest. However, it is not known if the mouthbrooding species still maintain the ability to produce mucous coated bubbles (Table 1). (3) All Macropodinae,

TABLE 3. Correlation analyses between standard length (SL) and each of the four dependent variables under the three different phylogenetic hypotheses (see Fig. 3). Analyses were conducted with no phylogenetic correction (TIP data) and alternatively using phylogenetic generalized least-squares (PGLS). The phylogenetic covariance matrices used for the PGLS analyses were obtained in two ways with Continuous vers. 1.0d13 (Pagel 2002) first by setting λ to its maximum-likelihood value while constraining κ and δ to one, and second by setting all scaling parameters (λ , κ , and δ) to their ML values, respectively. All $df = 19$.

Trait	Hypothesis	TIP	PGLS		PGLS			
		F (P)	F (P)	$\lambda = ML$	F (P)	$\lambda = ML$	$\kappa = ML$	$\delta = ML$
Clutch size	unconstrained	3.54 (0.075)	3.34 (0.083)	1.000	1.41 (0.249)	1.000	0.194	1.367
	<i>macrostoma</i>	3.54 (0.075)	3.36 (0.082)	1.000	2.15 (0.158)	1.000	0.368	1.145
	<i>simorum</i>	3.54 (0.075)	3.21 (0.089)	1.000	1.68 (0.209)	1.000	0.529	1.325
Egg volume	unconstrained	7.79 (0.012)*	2.83 (0.108)	0.961	0.91 (0.351)	0.961	0.447	1.546
	<i>macrostoma</i>	7.79 (0.012)*	2.80 (0.110)	0.975	1.26 (0.274)	0.975	0.563	1.336
	<i>simorum</i>	7.79 (0.012)*	2.90 (0.104)	0.944	0.99 (0.332)	0.944	0.462	0.944
Broodcare duration	unconstrained	11.21 (0.003)**	3.59 (0.073)	0.800	1.13 (0.300)	0.800	0.000	1.842
	<i>macrostoma</i>	11.21 (0.003)**	3.51 (0.075)	0.794	2.85 (0.107)	0.794	0.362	1.646
	<i>simorum</i>	11.21 (0.003)**	3.60 (0.073)	0.804	2.10 (0.163)	0.804	0.160	1.874
Offspring size	unconstrained	8.48 (0.009)**	0.89 (0.355)	1.000	0.54 (0.471)	1.000	1.800	0.305
	<i>macrostoma</i>	8.48 (0.009)**	0.87 (0.363)	1.000	0.35 (0.561)	1.000	1.670	0.246
	<i>simorum</i>	8.48 (0.009)**	0.88 (0.359)	1.000	0.50 (0.489)	1.000	2.260	0.327

* significant at 0.05, ** significant at 0.01.

with the exception of *Trichopsis* but including all bubble-nesting bettas, show a pronounced sexual dimorphism, whereas most of the mouthbrooding bettas show little or no sexual dimorphism. It is possible that sexual dimorphism as found in the bubble nesters may play an important role in the territorial defense of the bubble nest during the brood care period. (4) Two recent studies on cichlids showed that mouthbrooding evolved several times (9–14) independently from substrate brooding with only a few possible cases (0–3) of a transition in the reverse direction (Goodwin et al. 1998; Klett and Meyer 2002). This analogy highlights that there is little evidence that a transition to the ancestral mode of breeding behavior has occurred in any of the teleost families in which mouthbrooding has evolved. Furthermore, there are differences in the number, size, and distribution of the knob-like projections on the egg surface found in the different mouthbrooding clades, which might be taken as evidence for the recurrent evolution of mouthbrooding. Unfortunately, eggs of the key species *B. macrostoma* remain undescribed because of unavailability of material (Britz 2001).

Therefore, the loss of several complex traits in the mouthbrooding bettas may provide tentative support for bubble nesting as the plesiomorphic condition, and further may suggest that the recurrent evolution of mouthbrooding represents a more realistic model for the directionality of evolutionary transformations in the form of brood care among fighting fishes. This is also supported by our sensitivity analysis that accounted for the suspected biases in transformation probabilities. We found that with only a slightly lower cost for the transition from bubble nesting to mouthbrooding than vice versa, the hypothesis of recurrent evolution of mouthbrooding was favored with three to four independent gains of mouthbrooding with no transition from mouthbrooding to bubble nesting (Fig. 4). Oral transport of eggs to the bubble nest and the observed oral uptake of eggs or juveniles in cases of disturbance in some bubble-nesting species (Schmidt 1996) may have provided the first steps toward the evolution of mouthbrooding in bettas through prolongation of oral protection. However, a very conservative interpretation of our results of the sensitivity analysis would be the observation

TABLE 4. Results of the ANOVA/ANCOVA analyses for the comparisons between bubble nesters and mouthbrooders. ANCOVAs were performed based on the results shown in Table 3. The analyses follow the procedures outlined in Table 3.

Trait	Hypothesis	TIP	PGLS		PGLS			
		F (P)	F (P)	$\lambda = ML$	F (P)	$\lambda = ML$	$\kappa = ML$	$\delta = ML$
Clutch size	unconstrained	0.36 (0.555)	1.15 (0.296)	1	0.51 (0.482)	1	1.826	0.586
	<i>macrostoma</i>	0.36 (0.555)	1.15 (0.297)	1	0.38 (0.546)	1	1.705	0.465
	<i>simorum</i>	0.36 (0.555)	1.46 (0.241)	1	0.34 (0.566)	1	2.246	0.609
Egg volume	unconstrained	4.42 (a)*	0.88 (0.358)	1	0.62 (0.441)	1	1.572	0.577
	<i>macrostoma</i>	4.42 (a)*	0.89 (0.357)	1	0.64 (0.434)	1	1.448	0.464
	<i>simorum</i>	4.42 (a)*	0.93 (0.348)	1	0.56 (0.462)	1	1.967	0.639
Broodcare duration	unconstrained	10.59 (a)**	1.94 (0.192)	1	1.37 (0.256)	1	1.159	0.624
	<i>macrostoma</i>	10.59 (a)**	1.94 (0.180)	1	1.13 (0.301)	1	1.070	0.497
	<i>simorum</i>	10.59 (a)**	2.13 (0.160)	1	1.32 (0.265)	1	1.599	0.704
Offspring size	unconstrained	553.05 (a)***	395.22 (0.000)***	1	518.48 (0.000)***	1	3.000	0.103
	<i>macrostoma</i>	553.05 (a)***	394.41 (0.000)***	1	442.65 (0.000)***	1	3.000	0.099
	<i>simorum</i>	553.05 (a)***	424.22 (0.000)***	1	518.85 (0.000)***	1	3.000	0.109
SL	unconstrained	8.76 (0.008)**	0.92 (0.348)	1	0.60 (0.448)	1	1.852	0.419
	<i>macrostoma</i>	8.76 (0.008)**	0.90 (0.354)	1	0.48 (0.496)	1	1.705	0.347
	<i>simorum</i>	8.76 (0.008)**	0.92 (0.349)	1	0.54 (0.469)	1	2.312	0.448

a, ANCOVA, $F[0.05]_{(1,18)} = 4.41$; * significant at 0.05, ** significant at 0.01, *** significant at 0.001.

TABLE 5. Results from the test for correlated evolution between the form of parental care and three selected habitat characteristics (see Appendix for their definitions) for three alternative phylogenetic hypotheses using Discrete version 4.0 (Pagel 1999a). The likelihoods of the models of independent and dependent evolution were compared via likelihood ratio tests.

Trait	Hypothesis	δ	df	P
high/low	unconstrained	5.34	4	>0.1
	<i>macrostoma</i>	4.99	4	>0.1
	<i>simorum</i>	5.24	4	>0.1
slow/fast	unconstrained	3.70	4	>0.1
	<i>macrostoma</i>	3.79	4	>0.1
	<i>simorum</i>	3.87	4	>0.1
swamp/no swamp	unconstrained	2.20	4	>0.5
	<i>macrostoma</i>	2.54	4	>0.5
	<i>simorum</i>	3.35	4	>0.5

that the reconstruction of ancestral states in bettas is not consistent across different weighting schemes.

Omland (1997) advocated the use of step matrices to account for known or suspected biases in transformation probabilities when examining the loss or gain of a complex character (see also Wray and Raff 1991; Cunningham and Buss 1993; Maddison 1994). This might seem subjective, but Swofford and Maddison (1992) argued that different transition weighting does not imply that unweighted transformations are more objective, and Schulz et al. (1996) further showed that failing to account for transformation biases increases the probability of error in ancestral reconstructions. Nevertheless, a recent study suggests that it is possible that complex morphological, physiological, and behavioral features may be lost and later recovered within an evolutionary lineage, and that this reacquisition of complex features may play an important role in evolutionary diversification (Whiting et al. 2003; although applying different transition costs would challenge this conclusions). Therefore, we cannot discard the possibility that transitions from mouthbrooding to bubble nesting have occurred within the genus *Betta*. More information from genetics, developmental biology, ecology, and functional morphology are needed to understand the developmental mechanisms and possible adaptive value of the phenotypic and behavioral differences found between bubble nesting and mouthbrooding bettas (Table 1). This information is ultimately needed to better understand the evolutionary transitions in the form of parental care in the genus *Betta* and to test whether mouthbrooding has evolved once (resulting in two to three transitions from mouthbrooding to bubble nesting) or recurrently (three to four times) with no transition from mouthbrooding to bubble nesting.

The larvae of the studied bubble nesting bettas possess wartlike attachment cells distributed on the head to the anterior trunk, which are used to attach the larvae to the air bubbles of the bubble nest, a character complex shared with most other macropodines (Britz 2001). Unfortunately, there is no information available on this complex for any of the mouthbrooding *Betta* species (Table 1). In cichlids, where mouthbrooding evolved several times independently, (Goodwin et al. 1998; Klett and Meyer 2002) differential regression of the multicellular larval headglands in various lineages of mouthbrooders has been reported (Peters and Berns 1982,

1983). In this context, it would be interesting to investigate if the different mouthbrooding lineages in the genus *Betta*, that we hypothesize to have independently evolved, also display lineage-specific stages of regressions in their larval attachment cells, if remnants of the attachment cells are still present in mouthbrooders.

The Influence of Phylogenetic Correction

We observed striking differences in the results obtained from the species level comparisons (TIP) and the PGLS analyses taking shared ancestry into account (Table 3 and 4). The groups differed in four of five variables in the TIP analyses, whereas with the PGLS method they differed only in offspring size (Table 4). Evidence of significant phylogenetic correlation in our data was indicated by the ML values of $\lambda = 1$ for all ANOVA analyses for between group comparisons (Table 4). This agrees with two recent studies exploring phylogenetic signal in several published studies (Freckleton et al. 2002, Blomberg et al. 2003), which found a prevalence of phylogenetic signal in empirical data sets. Freckleton et al. (2002) also found that in 16% of the studied cases the statistical significance of correlations differed between phylogenetic and nonphylogenetic analyses. The high degree of phylogenetic signal in our data as indicated by λ , and the different outcomes of the phylogenetic and nonphylogenetic analyses indicate that conclusions based upon nonphylogenetic analyses might be misleading in understanding life-history correlates in the genus *Betta*.

Life-History and Habitat Correlates

The evolution of life-history correlates in teleost fish has received extensive attention in the literature (e.g., Fishelson 1966, Shine 1978; Noakes and Balon 1982; Sargent et al. 1987). However, few comparative analyses controlling for phylogenetic effects have been carried out to test the basic assumptions and predictions of Shine's "safe harbor" hypothesis, or related life-history models (e.g., Sargent et al. 1987). The fighting fish genus *Betta* provides a good opportunity to test these predictions because of the occurrence of different forms of parental care within a single genus. Our phylogenetic analyses provide the necessary information on evolutionary relationships that is essential for such a phylogenetic comparative method.

Vierke (1991) hypothesized that mouthbrooding in the genus *Betta* is not only an adaptation to increased predation risk, but more importantly to increased water currents that would hinder the construction of a bubble nest on the water surface. Although bubble nesters might be more constrained than mouthbrooders in their choice of habitat due to exclusive conditions needed to construct bubble nests, we did not find significant correlations between the form of parental care and habitat preferences. Only six of 15 mouthbrooding species listed in the Appendix are found in highland streams and only three of them are associated with increased water currents. On the other hand peat swamp forests are unique habitats characterized by very shallow, oligotrophic blackwaters with a pH < 5 that seem to be well suited for bubble nesting. Indeed, several nonbeta Macropodinae from the genus *Trichopsis* and *Pseudosphromenus*, and a high proportion of bub-

ble nesting bettas species can be found in these habitats, but also more than half of the mouthbrooders occur in these habitat types as well (Appendix). The numerous observed evolutionary transitions in the form of parental care (Fig. 4) may have facilitated the exploitation of distinct microhabitats of bubble nesters and mouthbrooders under sympatric conditions. This may explain the lack of habitat correlates between mouthbrooders and bubble nesters, a possibility that needs to be tested when more detailed field data become available.

A central prediction of the “safe harbor” hypothesis and related life-history models is that species that protect their offspring by egg brooding or live bearing produce larger offspring, at the expense of fecundity, than do related species which show a lower quality of parental care or do not protect their offspring at all (Shine 1978; Sargent et al. 1987). Consequently, when offspring survivorship is increased by parental care, selection may favor the female making fewer and larger eggs, which result in bigger initial juvenile size. Egg size should increase until the proportional gain in offspring survival equals the female’s cost in decreased fecundity due to larger eggs.

In teleost fish there are many species level comparisons in support of the predictions from the “safe harbor” hypothesis. For instance, increased offspring size and/egg size has been found with intensified parental care when comparing live bearers with egg layers (Wourms and Lombardi 1992), mouthbrooders with substrate or bubble nest guarders (Kühme 1961; Fishelson 1966; Noakes and Balon 1982; Peters and Berns 1982), and species that exhibit prolonged parental care with those that show no changes in parental care form (Gross and Sargent 1985). Unfortunately, only few phylogenetic comparative studies have thus far been carried out to test some of the predictions of the “safe harbor” hypothesis. For example, Goodwin et al. (2002) found that live bearing fish produce larger offspring than egg layers, but they were not able to detect a significant decrease in fecundity in live bearers. And in another study, the origin of semelparity in salmonids was found to be associated with a substantial increase in egg weight and increased maternal care (Crespi and Teo 2002).

Our results only partly support the hypothesized shifts in life-history characters associated with the evolution from bubble nest guarding to mouthbrooding. We found that mouthbrooders evolve larger offspring, but we did not detect a decrease in fecundity, neither an increase in egg volume, nor a higher investment in brood care time associated with this form of parental care. This lack of a consistent set of life-history correlates between the two forms of parental care may be the result of the high rate of evolutionary transitions between the two forms of parental care leading to alternative reproductive strategies within major lineages.

Implicit in most life-history models is that the relative survivorship rates during and after the guarding or brooding period are critical in the evolutionary determination of alternative strategies. Juvenile size is often associated with increased survival during the juvenile stage (Gross and Sargent 1985; Sargent et al. 1987). We expect new insights into the evolution of alternative forms of parental care in the genus *Betta* by studying survival rates under natural conditions that

may differ between bubble nesters and mouthbrooders due to predation and environmental factors. Most life-history models assume that juvenile size is a simple function of egg size (albeit Sargent et al. (1987) noted that this general assumption may not hold in species that exhibit extended parental care), and that egg size determines egg quality (Shine 1978; Sargent et al. 1987). Although egg volume and brood-care duration do not significantly differ between bubble nesting and mouthbrooding bettas, offspring size does (Table 4). The latter is measured at the termination of the parental care period, when yolk sacs are completely resorbed and first exogenous feeding begins (there is no form of exogenous feeding including parental provisioning during the parental care phase in bubble nesters and mouthbrooders). Therefore, given that the yolk is the only source of food for the larvae during the brood care period these findings seem to contradict each other. Nevertheless, there are several possible explanations that could account for similar egg volumes resulting in significant differences in offspring size. (1) Larger offspring size found in mouthbrooders is accomplished through differences in yolk quality (e.g., lipid content, chemical composition, hydration; for examples in cichlids, see Noakes and Balon (1982)) between mouthbrooding and bubble nesting species. (2) Mouthbrooders show a more efficient metabolism than bubble nesters. (3) Higher energy loss in bubble nesters during the larval phase due to elevated larval activity while attached to the bubble nest compared to their brooded counterparts. Although at present merely speculative, we feel that future research toward this direction could provide new insights that might help us to better understand life-history correlates in bettas.

Our understanding of the evolution of alternative forms of brood care and their life-history correlates in teleost fish is largely based on model predictions underlying sometimes simplified assumptions (Shine 1978; Sargent et al. 1987), or empirical species level data (e.g., Kühme 1961; Noakes and Balon 1982; Gross and Sargent 1985; but see e.g. Crespi and Teo 2002; Goodwin et al. 2002). In this study we used phylogenetic comparative analyses to study life-history correlates in mouthbrooding and bubble nesting species of the fighting fish genus *Betta*. Our results seem to challenge the general predictions of the “safe harbor” hypothesis that the evolution from a “guarding” to a “brooding” form of care in teleost fish is associated with shifts in reproductive and life-history features such as reduced fecundity, and increased egg volume with higher parental investment. Robust phylogenetic hypotheses supplemented with an increased knowledge of basic life-history data, that are still missing for many taxa, will doubtlessly help us to broaden our view on the evolutionary mechanisms shaping life-history correlates in teleost fishes.

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APPENDIX
Life-history and habitat data used for the comparative analyses. Data are from Schmidt (1996) unless otherwise stated.

Species	Life-history data						Habitat data		
	Parental care ^a	SL male (mm)	Number of eggs	Egg diameter (mm) ^b	Broodcare in days ^c	Size of young (TL in mm)	low/high ^{de}	slow/fast ^{ef}	swamp/no ^{dg}
<i>B. brownorum</i>	0	25.9 ^d	50	1.20 ^p	5	2.5	0	0	1
<i>B. burdigala</i>	0	25.2 ^d	80	1.40	6	2.5	0	0	1
<i>B. coccina</i>	0	32.1 ^d	80	1.20 ^p	6	2.5	0	0	1
<i>B. imbellis</i>	0	36.8 ^d	160 ⁿ	1.15 ^p	4 ⁿ	2.0	0	0	0
<i>B. rutilans</i>	0	23.0 ^k	80	1.40	6	2.5	0	0	1
<i>B. simorum^h</i>	0	68.5 ^d	300 ⁿ	1.40 ⁿ	5	2.0	0	0	1
<i>B. smaragdina</i>	0	34.5 ^d	300 ⁿ	1.30 ⁿ	4 ⁿ	2.0	0	0	0
<i>B. splendens</i>	0	42.4 ^d	400 ⁿ	1.30 ⁿ	4 ⁿ	2.0	0	0	0
<i>B. tussyaie</i>	0	36.5 ^d	60	1.20 ^p	5	2.5	0	0	1
Mean ± SD (bubble nesters)		36.1 ± 13.7	167.8 ± 131.1	1.28 ± 0.1	5 ± 0.8	2.3 ± 0.3			
<i>B. cf. albimarginata</i> “Malinau”	1	34.0 ^l	50 ^o	1.60 ^{p,q}	9 ^o	7.0 ^o	0	0	1
<i>B. anabatoides</i>	1	90.0 ^k	250	1.45 ^{p,r}	16	7.0	0	0	1
<i>B. dimidiata</i>	1	40.0 ^d	150	1.55 ^r	14	7.0	0	0	1
<i>B. edithae</i>	1	56.2 ^d	150	1.40	12	7.0	0	0	1
<i>B. foerschi</i>	1	51.0 ^k	100	1.60 ^p	10	6.0	0	0	1
<i>B. fusca</i>	1	59.7 ^d	80	1.80 ^{p,r,s,t}	30	7.0	0	0	1
<i>B. macrostoma</i>	1	69.8 ^d	250	1.80	22	8.0	1	1	0
<i>B. ocellata^l</i>	1	79.8 ^d	100 ⁿ	1.20 ^p	10 ⁿ	7.0	0	0	0
<i>B. cf. picta</i>	1	36.4 ^d	60 ⁿ	1.50 ⁿ	12	7.0	1	0	0
<i>B. prima</i>	1	N/A	N/A	N/A	N/A	N/A	0	0	0
<i>B. pugnax</i>	1	67.3 ^d	100 ⁿ	1.90 ^{p,r}	15 ⁿ	7.0	1	1	0
<i>B. simplex</i>	1	38.4 ^m	150	1.40	12	7.0	1	0	0
<i>B. strohi</i>	1	N/A	N/A	N/A	N/A	N/A	0	0	1
<i>B. unimaculata</i>	1	N/A	N/A	N/A	N/A	N/A	1	1	0
<i>B. waseriⁱ</i>	1	90.8 ^d	250	2.00	23	7.0	0	0	1
Mean ± SD (mouthbrooders)		59.4 ± 20.4	140.8 ± 73.5	1.60 ± 0.2	15.4 ± 6.4	7 ± 0.4			

^a 0, Bubble nesters; 1, mouthbrooders; ^b egg diameter was converted into egg volume [in (mm)³] and egg volume was used for the calculations. In cases where the eggs were not spherical the average from the two diameters, measured on the equator and from pole to pole, respectively, were used; ^c period from spawning till the termination of parental care, when yolk sacs are resorbed and first exogenous feeding begins; ^d H. H. Tan, pers. obs.; ^e 0, absent in highland habitats (only occurs in lowland habitats); 1, present in highland habitats; ^f 0, absent in fast flowing waters; 1, present in fast flowing waters; ^g 0, absent in peat swamp forest habitats; 1, present in peat swamp forest habitats; ^h data for *Betta bellica*; ⁱ as *Betta unimaculata* in Vierke (1991) and Schmidt (1996); ^j as *Betta macrophthalma* in Schmidt (1996); ^k (Witte and Schmidt 1992); (Grams and Dieckmann 1997); ^m Kottelat (1994); ⁿ Vierke (1994); ^o Linke (1995, 2001); ^p Britz (1995, 2001); ^q cf. *albimarginata* “Pampang”; ^r egg not spherical (see Britz 1995, 2001); ^s data for *Betta cf. fusca*; ^t bigger size class of eggs used for the analyses, see Britz (1995, 2001). The phylogenetic hypotheses used were the ML trees given in Figure 3A, D, E. Trees in NEXUS format used for the phylogenetic generalized least-squares analyses are available from the authors upon request.