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Short Communication

Mitochondrial variation of the "eyed" turtles (*Sacalia*) based on known-locality and trade specimens

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1. Introduction

The decimation of Chinese turtle populations has pre-dated molecular studies, resulting in a paucity of phylogenetic studies of turtles from this region. What studies do exist uncover deep genetic lineages obscured by present nomenclature (Stuart and Parham, 2004; Fong et al., 2007). One sensitive and poorly known group of freshwater turtles from southern China is the genus *Sacalia* Gray, 1870. Their shell is smooth, gray, and mottled so that it resembles stream cobbles, an effective camouflage for their preferred habitat of rocky streams. Species of *Sacalia* have four distinctive "eye" spots on the back of the head (e.g., Fig. 1A and B) that give them the common name "four-eyed," "eyed," or "occelated" turtles. Presently, the genus is composed of two valid species, *S. quadriocellata* (Siebenrock, 1903) and *S. bealei* (Gray, 1831a). Historically, there has been some confusion over the status of *S. quadriocellata*, with some authors considering it a junior synonym of *S. bealei*. Fu and Zhao (1990) justify the separation of these taxa on morphological grounds, and this appears reasonable based on limited genetic evidence from two non-vouchered pet trade samples (Spinks et al., 2004). A third putative species described from the pet trade, "*S. pseudocellata*", was recently shown to be an artificially produced hybrid, rendering it invalid (Stuart and Parham, 2007).

Geographically, the genus *Sacalia* is restricted to southern China, Laos, and Vietnam (Fig. 1). Currently, *Sacalia quadriocellata* occupies the western part of the range, including Hainan, while *S. bealei* occupies the eastern part of the range. Based on known distributions, the area of contact probably is/was in the vicinity of Hong Kong. However, this area is now heavily developed, and there is a lack of reliable data for *Sacalia* and other turtle species within this region (Fong et al., 2007). According to the IUCN Red list (2006), there are modest populations of *S. quadriocellata* in Laos and Vietnam, but the Chinese populations of this species, as well as *S. bealei*, are certainly endangered. In light of the serious conservation status of these turtles, the lack of any genetic data tied to geography, and overall poor understanding of the genetic structure of the genus, we undertook a phylogeographic survey of the genus *Sacalia*. This study includes the first-ever field-collected genetic samples of the genus, including a topotypic specimen, and supplemented with trade samples from throughout its range.

2. Methods and materials

2.1. Samples and laboratory methods

Twenty-nine total sequences of *Sacalia quadriocellata* and *S. bealei* were added to 10 GenBank sequences (three outgroup and seven ingroup). Individuals unique to our study came from both the field (18 samples, 11 from one site) and markets (11 samples); known-locality samples of *S. quadriocellata* are from China (Hainan and Guangdong Provinces), Vietnam, and Laos, while a single known-locality sample of *S. bealei* is from Hong Kong (Appendix A). One specimen from Vietnam (#20; Appendix A) was purchased from a local farmer who found the turtle stranded after heavy

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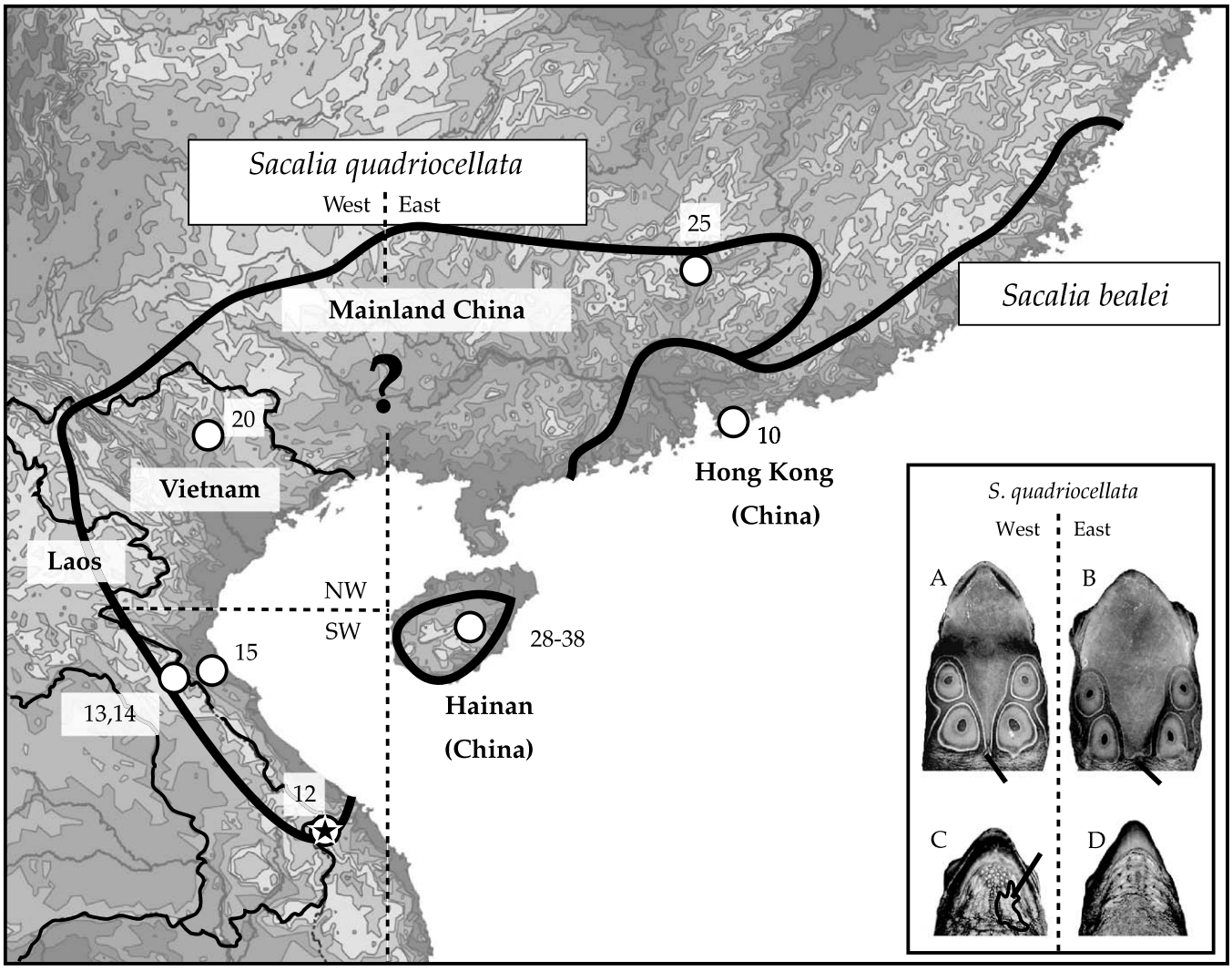

Fig. 1. Map showing the distribution of *Sacalia* species and localities mentioned in the text. The dots refer to the known-locality genetic samples. The dot with a star is the type locality of *S. quadriocellata*. NW and SW refer to the northwest and southwest clades of *S. quadriocellata* (see Fig. 2). Insets A–D: morphological differences between male *Sacalia quadriocellata* from different parts of the range. A and B: dorsal view of the head showing the eye spots. C and D: ventral view of the head showing the chin. A and C: in western populations, the space between the eye spots forms a "V" posteriorly and there are two light spots (red in life) on the chin. B and D: in eastern populations, the space between the posterior spots is distinctly wider (not V-shaped) and there are no large spots on the chin.

rains, so we assume it is from that region. For this study, we collected, to our knowledge, every known-locality sample available for study. Tissue samples in the form of blood, tail tip, or liver, were preserved in a DMSO/EDTA salt buffer, RNA*later* (Ambion), or 95% ethanol. DNA was extracted using a standard salt extraction protocol (Sambrook and Russell, 2001). Each sample was sequenced for 1140 bp of mtDNA, a nearly complete Cytochrome *b* (*cytb*) gene, with standard PCR conditions and the primers GLUDGE (Palumbi et al., 1991) and THR-8 (Spinks et al., 2004). Purified PCR products were sequenced using PCR primers and internal sequencing primers (CBJSi and CBJSr, Spinks et al., 2004). Sequences were aligned by eye and translated into amino acids to check for erroneous stop codons using the online software EMBOSS Transeq (Rice et al., 2000). When it was necessary to clone samples for sequencing, we used a TOPO TA Cloning Kit (Invitrogen) following the included instructions.

2.2. Phylogenetic analyses

Phylogenies were constructed using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI). For all analyses, sequences of *Heosemys depressa* (AY434607), *Cyclemys dentata* (AY434579), and *Cuora trifasciata* (AY434627) were used as outgroups. For MP analyses, PAUP* v.4.0b10 (Swofford, 2003) was implemented using a heuristic search with all characters equally weighted and a tree-bisection–reconnection branch-swapping algorithm. The topology was reconstructed using a majority rule, and support values were assessed using 1000 non-parametric bootstrap pseudoreplicates (Felsenstein, 1985). For ML analyses, RAxML v.2.2.3 (Stamatakis, 2006) was implemented. To find the best-known likelihood tree, the "d" (default) hill-climbing algorithm, GTRMIX model of sequence evolution, and 100 runs were selected. Bootstrap proportions were calculated using the "d" hill-climbing algorithm, GTRCAT model of sequence evolution, and 1000 replicates (Stamatakis, 2006). For BI analyses, the program MrBayes v.3.1.2 was used. These data were partitioned into 1st, 2nd, and 3rd codon positions, and the appropriate model for each partition was selected using the hLRT criteria in MrModeltest v.2.2 (Nylander, 2004). Searches were run using four chains, four million generations, and sampling every 1000th tree. Burn-in was estimated in the program AWTY (Wilgenbusch et al., 2004) by plotting the cumulative pos-

terior probability of nodes against the generation time. The BI analysis was run twice with random starting trees and the results were compared. Resultant trees (minus the burn-in) were summarized to calculate posterior probabilities.

3. Results

One thousand one hundred and forty base pairs of *cytb* were sequenced for almost all samples, with some individuals having truncated sequences due to poor sequencing with internal primers. Products from sequencing produced single peaks and there were no erroneous stop codons (with one exception for *Sacalia pseudocellata*, discussed in more detail below). The average nucleotide frequencies were A = 0.295, C = 0.306, G = 0.122, T = 0.277, showing the typical mtDNA bias against guanine (Kocher et al., 1989). In the dataset, 380 characters are variable and 265 characters are parsimony-informative. Uncorrected pair-wise differences within the ingroup ranged from 0% to 9.7%. The MP analysis resulted in six most parsimonious trees (length = 636, CI = 0.654, RI = 0.859). ML analyses were run under the GTRMIX model of nucleotide substitution, with the final tree evaluated and optimized under the GTR + Γ model. The result was a single tree ($-\ln L = -4505.618$, $a = 0.2861$). Under the partitioned dataset for BI, the following models were selected: 1st position-HKY + Γ, 2nd position-HKY + Γ, 3rd position-GTR + Γ. For both independent BI runs, likelihood estimates and tree topologies were essentially identical, and runs were combined for calculation of posterior probabilities (with the first 500 generations discarded as burn-in). All three analyses (MP, ML, BI) resulted in similar topologies, with minor differences being rearrangements in the terminal taxa (Fig. 2).

The analyses resulted in a monophyletic *Sacalia* genus, as well as reciprocally monophyletic *S. quadriocellata* and *S. bealei* (Fig. 2) that differ by an uncorrected pair-wise difference of 9.0–9.7%. The distinctiveness of *S. bealei* from *S. quadriocellata* suggested by Fu and Zhao (1990) is therefore confirmed. Within *S. quadriocellata*, there are three distinct clades differing by an uncorrected pair-wise difference of 2.1–5.2%. One group corresponds to known-locality samples from Vietnam and Laos, the second group corresponds to *S. quadriocellata* from the wild in Northern Vietnam and from markets in western China, while the third group contains all known-locality samples from Hainan and Guangdong Provinces in addition to "*S. pseudocellata*" (Fig. 2).

After preliminary phylogenetic reconstruction, we found the branch connecting the "*Sacalia pseudocellata*" sequence reported by Spinks et al. (2004, AY434614) to be unusually long. When we attempted to duplicate the sequence of Spinks et al. (2004) from the same genetic tissue sample, our sequences had multiple peaks, indicating heterozygosity in what should be a single copy marker. We cloned and recovered four unique sequences for "*S. pseudocellata*". Three of the four clone sequences had premature stop codons (#s 2, 4, 27; Appendix A). All four sequences were included in the phylogenetic analyses, one which fell into the Hainan/Guangdong *S. quadriocellata* clade, while the other three fell outside the *Sacalia* clade, differing by an uncorrected pair-wise difference up to 14.2%.

4. Discussion

4.1. Previously unrecognized lineages among endangered Chinese populations

Our genetic survey reveals that the two species of *Sacalia* contain four distinct mitochondrial clades. One of these clades corresponds to *S. bealei*, the other three correspond to two morphologically diagnosable populations of *S. quadriocellata*. Samples from the western part of the range, in Laos and Vietnam, form sister clades that are 2.1–2.7% different. We do not know of any morphological characters that diagnose these mitochondrial clades. The third clade of *S. quadriocellata* includes field-collected samples from Hainan and Mainland China, the eastern part of its range. Our ongoing morphological studies of the genus *Sacalia* (Shi and Fong, unpublished data) reveal diagnostic morphological characters that correspond to this eastern mitochondrial clade (Fig. 1A–D), supporting a distinction already reported in the hobbyist literature (Vetter and van Dijk, 2006). If further studies demonstrate a congruence of morphological and genetic data sets, it may be warranted to divide *S. quadriocellata* into two species. In this case, *S. quadriocellata* would be restricted to the two western clades (that include the type locality, Fig. 1) and the available name *Sacalia insulensis* (Adler, 1962) comb. nov. would be used for the eastern clade. However, we highlight the need for careful research to avoid premature taxonomic inflation (Parham et al., 2006).

The tripartite division of *S. quadriocellata* matches that found in co-distributed taxa such as the *Cuora galbinifrons* (Bourret, 1939) (Stuart and Parham, 2004) and *Mauremys mutica* (Cantor, 1842; Fong et al., 2007) species complexes. Especially similar to *C. galbinifrons*, *S. quadriocaellata* shows a lineage endemic to Hainan and southern China, a lineage in northern Vietnam, and a third lineage in central Vietnam and adjacent Laos. The pattern in the *M. mutica* complex is less clear because of a lack of known-locality samples. Other co-distributed taxa, such as *Platysternon* Gray, 1831b and *Geoemyda* Gray, 1834, have not yet been analyzed.

From a conservation perspective, the discovery of previously unknown genetic diversity in *Sacalia* and other turtles seriously undermines the wisdom of *ex situ* captive breeding efforts (Rahbek, 1993; Fong et al., 2007). These well-intentioned efforts may be inadvertently mixing genetic lineages that are discrete in nature. This is a serious problem given the well-documented ability of Asian turtles to hybridize in captivity (Parham et al., 2001). Since *Sacalia* is known to interbreed with other genera of turtles (Buskirk et al., 2005; Stuart and Parham, 2007), the likelihood of intrageneric breeding is equally high.

4.2. "*Sacalia pseudocellata*"

"*Sacalia pseudocellata*" is a known F1 hybrid between *Sacalia quadriocellata* and *Cuora trifasciata* (Bell, 1825) (Stuart and Parham, 2007). Although it translates perfectly, we cannot be certain that the sequence reported by Spinks et al. (2004; AY434614) is genuine. As stated previously and shown in Fig. 2, the branch connecting this sample to the others is unusually long. We sequenced four unique haplotypes by cloning the sample, three of which had premature stop codons. The fourth clone sequence (#1; Appendix A) had no stop codons, but is not closely related to either of the parental species and does not match any turtle sequence in Genbank. Therefore we conclude that we were unable to recover genuine sequence from our sample of "*S. pseudocellata*" and identify all four clones as nuclear pseudogenes. In our study, only the blood sample of "*S. pseudocellata*" consistently yielded pseudogene sequences; all the other samples were tail tips, muscle, or liver. Red blood cells of turtles (and other reptiles including birds) are nucleated and low in mtDNA, consequently they are especially susceptible to nuclear contamination during amplification (Sorenson and Quinn, 1998). In addition to the example noted here, blood samples have yielded numerous nuclear pseudogenes in other turtles (Spinks and Shaffer, 2007) where studies on the same taxa using liver or muscle encountered much fewer or none (Stuart and Parham, 2004; Parham et al., 2004). Consequently, we conclude that blood samples are problematic for mitochondrial surveys and results from turtle studies that rely on blood samples need to be verified by sequences from other tissue types or markers.

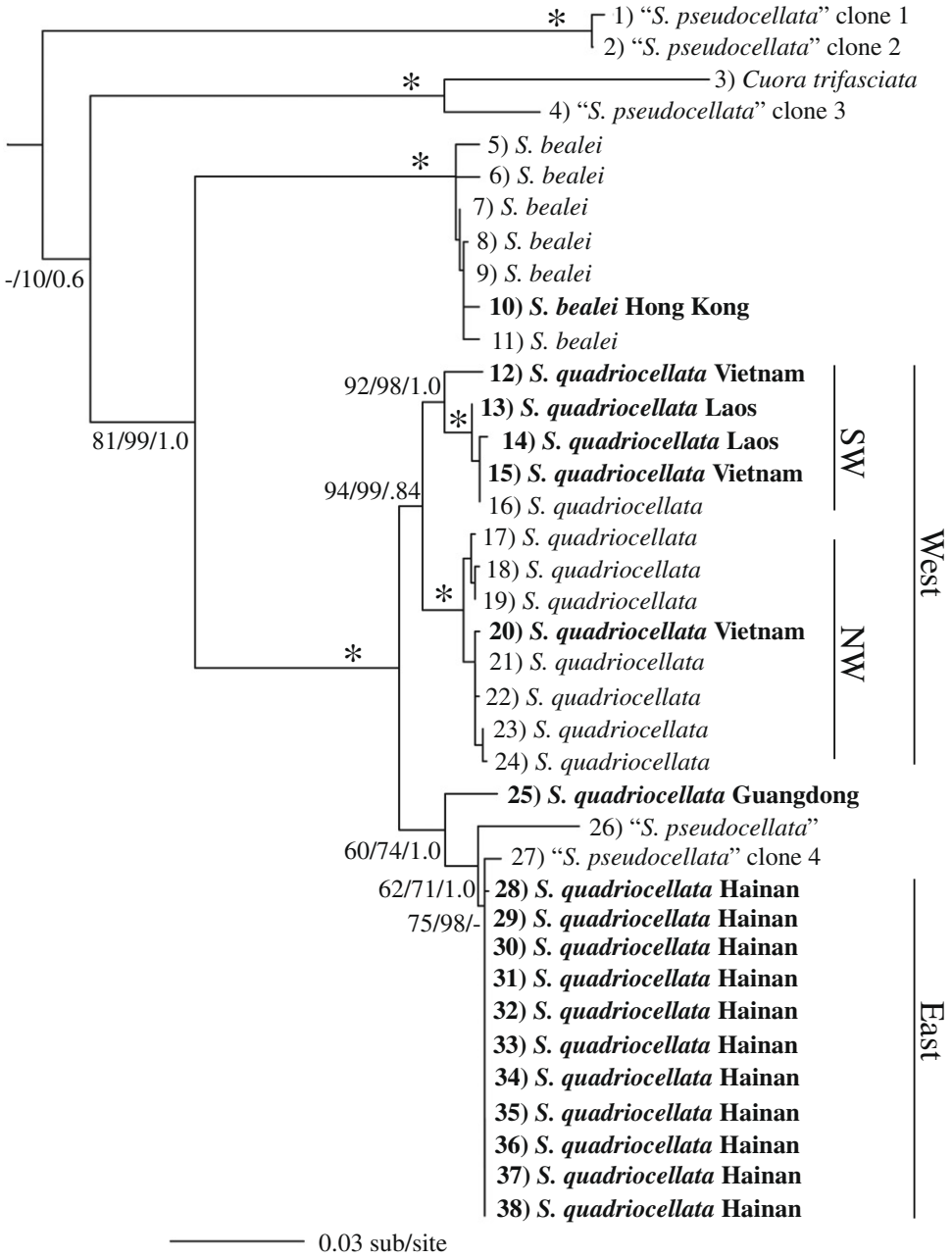

Fig. 2. Phylogram from Maximum Likelihood (ML) analysis of mitochondrial DNA (*cytb*) of the genus *Sacalia*. Trees from Maximum Parsimony (MP) and Bayesian Inference showed nearly identical topologies. The support values for each node are in the order of MP bootstrap/ML bootstrap/Bayesian Posterior Probability. Values denoted with a "–" indicates no support under that method, and an "∗" indicates high support of ⩾95. A single "∗" indicates a node with all three support values ⩾95. Numbers in front of the specimen label correspond to Appendix A. Individuals with reliable collection locality are in bold font. The resultant two clades of *S. quadriocellata* are labeled with their relative geographic localities: east and west. NW and SW refer to the northwest and southwest clades of *S. quadriocellata* (see Fig. 1).

5. Conclusions

Our mitochondrial survey of the genus *Sacalia* reveals four mitochondrial clades that differ between 2.1% and 9.7% within the two currently recognized species. One of these clades corresponds to samples of *S. bealei*, while the other three clades are currently referred to as *S. quadriocellata*. These three clades correspond to two morphologically diagnosable *S. quadriocellata* groups. The western group includes known-locality samples from Laos and Vietnam, including the type locality. The eastern group occurs in Hainan and Mainland China (Guangdong Province). This eastern clade of *S. quadriocellata* is diagnosable from the western clade based on morphological characters and might represent a distinct species. Our research emphasizes why studies of turtle mitochondrial variation should eschew blood samples (because of the pseudogene problem) from trade specimens (because of the need for geographic data).

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Appendix A

The following is a list of the voucher information followed by the Genbank accession numbers for 37 samples (Institutional voucher or tissue #/GenBank). The outgroup sequences and ingroup sequences #s 2–4, 11, 21, 23, 25 are from Spinks et al. (2004) and #s 9, 15 from Barth et al. (2004). Note specimen #32 (R0520) is a voucher at Hainan Normal University, and #19 has no voucher because it was used in a study that required destructive sampling of the specimen. Institutional abbreviations: FMNH, Field Museum of Natural History; HBS, Brad Shaffer's tissue collection at U.C. Davis [no voucher]; HNU, Hainan Normal University; MTD, Museum fur Tierkunde Dresden; ROM, Royal Ontario Museum). Numbers correspond to the numbers on the phylogenetic tree in Fig. 2. Outgroups: *Heosemys depressa* (HBS 38425/AY434607); *Cyclemys dentata* (HBS 38397/AY434579); (3) *Cuora trifasciata* (HBS 38445, AY434627). Ingroup: (1) "*S. pseudocelalta*" clone 1 (HBS 38432/EU910996); (2) "*S. pseudocelalta*" clone 2 (HBS 38432/EU910999); (4) "*Sacalia pseudocelalta*" clone 3 (HBS 38432/EU910997); (5) *S. bealei* (HNU TSB0/EU910982); (6) *S. bealei* (HNU TSB28/EU910983); (7) *S. bealei* (HNUTSB19/EU910984); (8) *S. bealei* (MTD 41583/AJ519501); (9) *S. bealei* (HNU TSB25/EU910981); (10) *S. bealei* (MVZ 257748/EU910992); (11) *S. bealei* (HBS 38403/AY434585); (12) *S. quadriocellata* (MVZ 258203/FJ211058); (13) *S. quadriocellata* (FMNH 256542/EU910995); (14) *S. quadriocellata* (FMNH 256543/EU910994); (15) *S. quadriocellata* (ZFMK 81536/FJ211059); (16) *S. quadriocellata* (ZFMK 81535/FJ211060); (17) *S. quadriocellata* (HNU TSQ11/EU910974); (18) *S. quadriocellata* (MTD 42442/AJ564465); (19) *S. quadriocellata* (HNU TSQ8/EU910973); (20) *S. quadriocellata* (ROM 28458/EU910993); (21) *S. quadriocellata* (n/a/EU910990); (22) *S. quadriocellata* (HBS 38436/AY434614); (23) *S. quadriocellata* (HNU TSQ4/EU910988); (24) *S. quadriocellata* (HNU TSQ3/EU910987); 25) *S. quadriocellata* (MVZ 257747/EU910991); (26) "*S. pseudocellata*" (HBS 38432/AY434614); (27) "*S. pseudocellata*" clone 4 (HBS 38432/EU910998); (28) *S. quadriocellata* (HNU TSQ281/EU910985); (29) *S. quadriocellata* (HNU TSQ224/EU910975); (30) *S. quadriocellata* (HNU TSQ61/EU910989); (31) *S. quadriocellata* (HNU TSQ264/EU910978); (32) *S. quadriocellata* (R0520/EU910986); (33) *S. quadriocellata* (HNU TSQ273/EU910980); (34) *S. quadriocellata* (HNU TSQ231/EU910976); (35) *S. quadriocellata* (MVZ 230485/EU911001); (36) *S. quadriocellata* (MVZ 230484/EU911000); (37) *S. quadriocellata* (HNU TSQ239/EU910977); (38) *S. quadriocellata* (HNU TSQ284/EU910979).

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