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EVALUATING AND RECONSTRUCTING THE GENETIC DIVERSITY OF BUTTERFLY LIZARDS OF THE GENUS *Leiolepis* CUVIER, 1829 (REPTILIA: SQUAMATA: AGAMIDAE) IN CENTRAL VIETNAM

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Studies on genetic relationships and phylogenetic origins, and mutations in nucleotide of *Leiolepis* in central Vietnam are limited. In this study, thirty-five representative samples of four species (*Leiolepis reevesii*, *L. guttata*, *L. guentherpetersi*, and *L. rubritaeniata*) from multiple provinces in central Vietnam were collected for identification based on 16S rRNA sequences. The results from phylogenetic analyses showed that *L. rubritaeniata* is highly genetically conserved and was unique for the Central Highland areas. Patterns, colors, and genetic characteristics of the population of *L. reevesii* in Thanh Hoa Province exhibited differences between the populations in Thua Thien Hue Province. The population of *L. guentherpetersi* had the closest sister relationship to the population of *L. guttata* found in the same province, supporting a hypothesis that the origin of the triploid *L. guentherpetersi* from *L. guttata*. *Leiolepis reevesii* populations found in Thanh Hoa and Thua Thien Hue provinces were not in the same clade for both morphology and genetics. Lastly, the overall similarity between *L. rubritaeniata* and *L. reevesii* populations further suggesting that both species were originally from the same clade with a diversification occuring to adapt to the ecological conditions.

Keywords: butterfly lizards; Central Vietanm; phylogenetics; *Leiolepis*; morphology; genetics.

INTRODUCTION

Lizards belonging to the genus *Leiolepis* or butterfly lizards of the family Leiolepidinae are a diverse and widely distributed group throughout Indochina. To date, the genus *Leiolepis* Cuvier, 1829 has recorded ten species, six sexual species of *L. belliana*, *L. guttata*, *L. peguensis*, *L. reevesii*, *L. rubritaeniata*, and *L. ocellata* (Hardwicke and Gray, 1827; Cuvier, 1829; Gray, 1831; Mertens, 1961; Peters, 1971); four asexual species of *L. boehmei*, *L. guentherpetersi*, *L. ngovantrii*, and *L. triploida* (Peters, 1971; Darevseky and Kupriyanova, 1993; Grismer and Grismer, 2010). Butterfly lizards have a wide distribution from Southern China throughout Southeast Asia (Grismer et al., 2014). They are terrestrial lizards, preferring to live in empty lands, arid areas, open grassland habitats (Phimphan et al., 2013; Jantarat et al., 2018).

In Indochina, the genus *Leiolepis* Cuvier, 1829 has recorded nine species, five sexual species, and four asexual (Grismer and Grismer, 2010; 2014; Jantarat et al., 2018). Among these species, four species have been identified in central Vietnam, including one asexual triploid *L. guentherpetersi* and three sexual diploids of *L. guttata*, *L. reevesii*, and *L. rubritaeniata*. Both *L. guttata* and *L. reevesii* are distributed mainly in central Vietnam, however, *L. rubritaeniata* is only found in Gia Lai Province (Hartmann et al., 2012). *Leiolepis guentherpetersi* was recorded in Thua Thien Hue, Quang Tri, Da Nang, and Quang Nam provinces (Nguyen et al., 2009). All species are medium-sized, omnivorous, daytime activities, creating long, and interconnected burrows used for escape. The four asexual species have similar natural histories, each occurs in a unique ecosystems (Grismer et al., 2008).

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In the past, *Leiolepis* species were identified through the characteristics of morphological data. However, identification of such species with similar morphologies has become difficult. Through the process of hybridization, new species appeared with appearance similar to the previous species, which has caused confusion about taxonomy for zoologists when classifying based on morphological characteristics. Morphological observation indicates that *L. rubritaeniata* is similar to *L. reevesii* in both color and pattern. A previous study by Ngo et al. (2012) identified the species occurring in Yok Don National Park (Dak Lak) as *L. reevesii*. However, based on the results of Hartmann et al. (2012) on the morphological characteristics of these two species, Ngo et al. (2020) identified the species occurring in Yok Don National Park as *L. rubritaeniata*. Researchers have focused on identifying species using molecular techniques to study the sister relationship between species. As a result, the taxonomy and distribution of *Leiolepis* are being reliable and accessible. *Leiolepis guentherpetersi* has been demonstrated as sexual reproduction between *L. guttata* and *L. reevesii* (Schmitz et al., 2001; Grismer et al., 2010). In addition, Grismer and Grismer (2010) also clarified that *L. guttata* is the ancestor of asexual species (*L. guentherpertersi*, *L. boehmei* and *L. ngovantrii*), while *L. boehmei* is the ancestor of *L. triploida*.

Vietnam is a part of Indochina and is one of the world's most threatened biodiversity hotspots (Myers et al., 2000; Malysheva et al., 2006; Lin et al., 2010). Focused studies on species occurring throughout these areas of Vietnam have been limited and this area potential represents as an area of needed conservation. Although there have been studies on relationships among *Leiolepis* lizards in Vietnam and neighboring countries (Grismer and Grismer, 2010; Grismer et al., 2014; Lin et al., 2010; Zhu et al., 2020), there has been no report on the sister relationship among species and distribution of species in central Vietnam. This study aims to further clarify the sister relationship between species and populations, and the dispersal of *Leiolepis* in central Vietnam using molecular identification approaches combined with morphological data. Based on the phylogenetic trees, the origin of L. guentherpeter is elucidated. Furthermore, the identification between *L. rubritaeniata* and *L. reevesii* is clearly clarified.

MATERIAL AND METHODS

Sample collection. *Leiolepis* samples were selected and collected by trap or noose from five populations in four provinces belonging to central Vietnam (Fig. 1). The samples were labeled, put in bags, and taken to the laboratory. Next, the samples were photographed and measured to determine morphological parameters before tail muscles were extracted. Muscle tissue of each individual was collected for molecular analysis. The tail muscle was extracted to approximately 3 cm length with a sterilized scalpel and stored in 100% ethanol.

External morphology. We measured individuals with standard calipers (Prokits, Taipei, Taiwan) to the nearest 0.1 mm for snout-vent length (SVL) and tail length (TL). We measured head length (HL), head width (HW), head height (HH), height of ear (HE), foream length (FA), distance between nares (DN), forelimb length (FL), axilla-groin length (AG), hind limb length (HB), and tibia length (TiB) using digital calipers (Mitutoyo Corporation, Kawasaki, Japan) to the nearest 0.1 mm. Body mass (BM) was weighed using an electronic balance (Prokits, Taipei, Taiwan) to the nearest 0.01 g. We used some ratios of HL/SVL, HW/HL, FL/SVL, TiB/HB, and HB/SVL to calculate relative measurements. To test significant difference of snout-vent length and body mass among species, we used a one-way analysis of variance (ANOVA) using MINITAB 16.0 software. All data are presented as mean ± 1 SD (unless otherwise noted) with a significance level of $P < 0.05$ considered to be statistically significant.

Genomic DNA extraction. Total DNA extraction was performed as described by Grismer and Grismer (2010) with a minor modification. The skin was removed and 0.2 g of the muscle was washed twice in distilled water. Samples were milled in liquid nitrogen, then transferred to a 1.5 ml tube containing 800 µl extraction buffer, 100 µl of 10% SDS, and 2 µl of 20 mg/ml proteinase K. The mixture was mixed well for 30 sec, incubated at 65°C for 2 h and supplemented with 300 µl of 6 M NaCl. The solution was then incubated at –30°C for 20 min and centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant solution was transferred into a new 1.5 ml tube and an equal volume phenol:chloroform: isoamyl alcohol (25:24:1) was added and mixed well. The mixture was centrifuged at 14,000 rpm for 15 min at 4°C. The upper phase was transferred into a new 1.5 ml tube and equal volumes of isopropanol were added and incubate at –30°C for 2 h. DNA was precipitated by centrifuge at 14,000 rpm for 15 min at 4°C, washed twice with 70% ethanol, and eluted in 30 µl of sterile distilled water.

DNA amplification and sequencing. Total DNA was used to amplify an approximately 400 bp of the 16S rRNA region using pair primer L52 (5'-CGT GCA AAG GTA GCA CAA TC-3'), and H455 (5'-CGG ACC CTT GAT AGC TTC TG-3') (Cobb et al., 2016). The PCR protocol was adapted from a previous report (Cobb et al.,

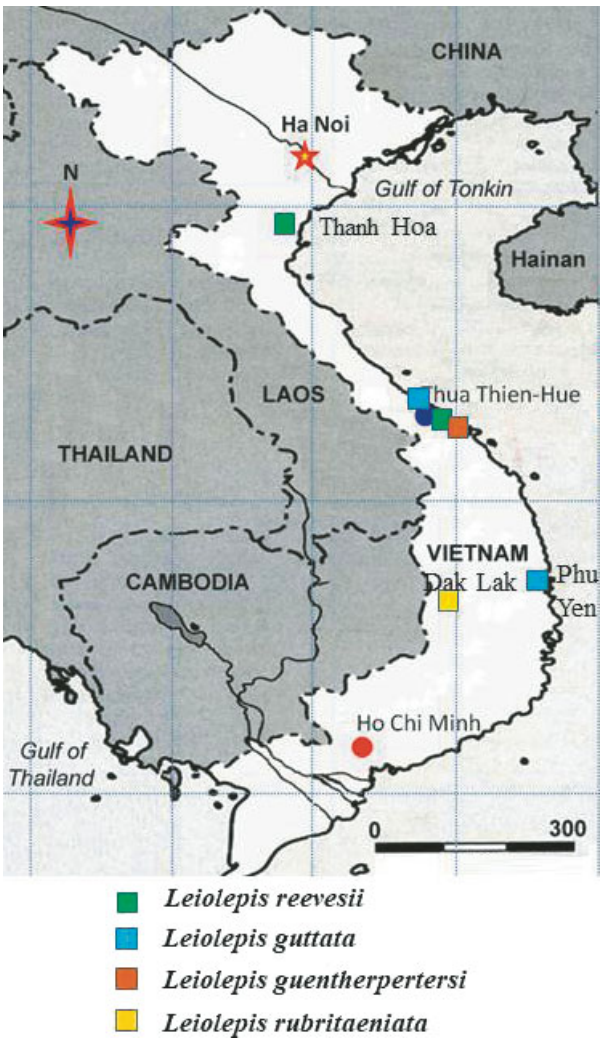

Fig. 1. Map of sampling localities for the species of the genus *Leiolepis* in central Vietnam used in this study.

2016). PCR products were visualized with 1.2% agarose gel electrophoresis before performing DNA sequencing (FirstBase, Malaysia). The nucleotide sequences were aligned and searched BLAST against the nucleotide sequences database available on GenBank.

Phylogenetic analysis. Nucleotide sequences of all samples were processed according to different evolutionary models to reach the best fit result. The sequences were aligned in MEGA X software (Kumar et al., 2018) based on the MUSCLE algorithm (Edgar, 2004). The phylogenetic relationships between species were generated by reconstructing the phylogenetic trees with the criteria of Maximum Likelihood (ML), Maximum Parsimony (MP), and UPGMA approaches. The confidence level of the tree was estimated using the bootstrap method with 1000 replicates. Furthermore, the phylogenetic trees were validated using BEAST v. 1.10.4, TreeAnnotator v. 1.10.4, and FigTree v. 1.4.4 (Marc et al., 2018) following the author instructions. Meanwhile, the corresponding nucleotide sequences showing high similarity of each species were downloaded from GenBank. The phylogenetic tree between the present study and previous studies was built through the MP approach based on the MUSCLE alignment algorithm by MEGA X software.

RESULTS

Morphological characteristics. Based on external morphological features and previous studies on the distribution of species of the genus *Leiolepis*, we chose four provinces in central Vietnam from which to collect samples. The selected provinces are geographically far apart to study the differences in morphological and genetic characteristics among populations (Fig. 1). Thirty-five individuals of *Leiolepis* were collected (Table 1). The individuals observed in Thanh Hoa, Phu Yen, and Dak Lak areas are unique species, while Thua Thien Hue obtained three species. We found that all individuals obtained in the Chan May area were females with the pattern and position of the stripes on the body that showed high similarity with *L. guttata* males (Fig. 2). However, these individuals exhibited high relevance to *L. guentherpetersi* through a comparison of 16S rRNA sequences (Figs. 3 and 4). Interestingly, populations collected in Yok Don National Park, Dak Lak Province belonged to *L. rubritaeniata* species, which has not previously been recorded in that location. *L. rubritaeniata* and *L. reevesii* have the most similar external morphology (Fig. 2).

Adult *L. rubritaeniata* males have alternating black and red-orange streaks on half of the front region of the ribs while the rear half of the flank is only reddish-orange running down to the groin. These features are consistent with the original description by Mertens (1961) and Hartmann et al. (2012). Adult *L. reevesii* males have alternating black and red-orange (fainter) streaks from the armpit down to the groin and yellow-orange patterned spots on the head with a back and tail that are also darker and showier than that of *L. rubritaeniata*. In the same species, *L. reevesii*, the population in Thanh Hoa Province exhibited a darker orange-yellow pattern, spreading to the cheeks and chin, while the population of *L. reevesii* in Thua Thien Hue Province showed lighter patterns with no patterned spots on the bottom of the chin.

The largest *L. guttata* was 173.1 mm SVL and the largest *L. rubritaeniata* 122.0 mm SVL (Table 2). Among these species, *L. guttata* exhibited different mor-

phological characteristics and a bigger body size than that of the other three species, which is easy to identify (SVL: 139.1 ± 14.3 mm; BM: 77.2 ± 23.7 g). *Leiolepis rubritaeniata* had a smaller body size than that of the other three species (SVL: 105.6 ± 9.4 mm; BM: 31.9 ± 10.7 g). The average SVL and BM of adults were significantly different among species (SVL: $F_{3,33} = 18.41$, $P < 0.0001$; BM: $F_{3,33} = 13.14$, $P < 0.0001$).

Molecular identification. To identify the species using molecular biology approaches, various primer sets for 12S rRNA, 16S rRNA, and ND2 were designed according to previous studies. The primer set for 16S rRNA amplified all thirty-five samples with a PCR product size of 400 bp in length. Meanwhile, the 12S rRNA and ND2 amplicons only observered in a few samples. The nucleotide sequence comparison indicated that all populations belonged to the genus *Leiolepis*. Four individuals in Thanh Hoa Province were *L. reevesii*. Meanwhile, seventeen samples collected from Thua Thien Hue Province consisted of six individuals of *L. reevesii*, six individuals of *L. guttata*, and five individuals of *L. guentherpetersi*. The population in Dak Lak Province was identified to *L. rubritaeniata*. All nucleotide sequences were deposited on GenBank (Table 2).

Phylogenetic tree construction among populations. To analyze the genetic relationship among species, the phylogenetic trees were constructed using two different software (MEGA v. 7.0, and BEAST v. 1.10.4). The results were represented in Figs. 3 and 4. The tree structures and brands showed different in these analyses.

TABLE 1. List of Butterfly Lizard Samples in This Study with Codes Were Deposited on GenBank

Species	Sample symbols	Locality	Accession No.
Leiolepis rubritaeniata	DK01	Dak Lak	MZ190176
Leiolepis rubritaeniata	DK02	Dak Lak	MZ190177
Leiolepis rubritaeniata	DK03	Dak Lak	MZ190178
Leiolepis rubritaeniata	DK04	Dak Lak	MZ190179
Leiolepis rubritaeniata	DK05	Dak Lak	MZ190180
Leiolepis rubritaeniata	DK06	Dak Lak	MZ190181
Leiolepis rubritaeniata	DK07	Dak Lak	MZ190182
Leiolepis rubritaeniata	DK08	Dak Lak	MZ190183
Leiolepis rubritaeniata	DK09	Dak Lak	MZ190184
Leiolepis rubritaeniata	DK10	Dak Lak	MZ190185
Leiolepis reevesii	TA07	Thuan An, Thua Thien Hue	MZ190143
Leiolepis reevesii	TA08	Thuan An, Thua Thien Hue	MZ190144
Leiolepis reevesii	TA09	Thuan An, Thua Thien Hue	MZ190145
Leiolepis reevesii	TA10	Thuan An, Thua Thien Hue	MZ190146
Leiolepis reevesii	TA11	Thuan An, Thua Thien Hue	MZ190147
Leiolepis reevesii	TA12	Thuan An, Thua Thien Hue	MZ190148
Leiolepis reevesii	TH01	Thanh Hoa	MZ190139
Leiolepis reevesii	TH02	Thanh Hoa	MZ190140
Leiolepis reevesii	TH03	Thanh Hoa	MZ190141
Leiolepis reevesii	TH04	Thanh Hoa	MZ190142
Leiolepis guentherpetersi	CM01	Chan May, Thua Thien Hue	MZ190165
Leiolepis guentherpetersi	CM02	Chan May, Thua Thien Hue	MZ190166
Leiolepis guentherpetersi	CM03	Chan May, Thua Thien Hue	MZ190167
Leiolepis guentherpetersi	CM04	Chan May, Thua Thien Hue	MZ190168
Leiolepis guentherpetersi	CM05	Chan May, Thua Thien Hue	MZ190169
Leiolepis guttata	TA01	Thuan An, Thua Thien Hue	MZ190155
Leiolepis guttata	TA02	Thuan An, Thua Thien Hue	MZ190156
Leiolepis guttata	TA03	Thuan An, Thua Thien Hue	MZ190157
Leiolepis guttata	TA04	Thuan An, Thua Thien Hue	MZ190158
Leiolepis guttata	TA05	Thuan An, Thua Thien Hue	MZ190159
Leiolepis guttata	TA06	Thuan An, Thua Thien Hue	MZ190160
Leiolepis guttata	PY02	Phu Yen	MZ190151
Leiolepis guttata	PY03	Phu Yen	MZ190152
Leiolepis guttata	PY04	Phu Yen	MZ190153
Leiolepis guttata	PY05	Phu Yen	MZ190154

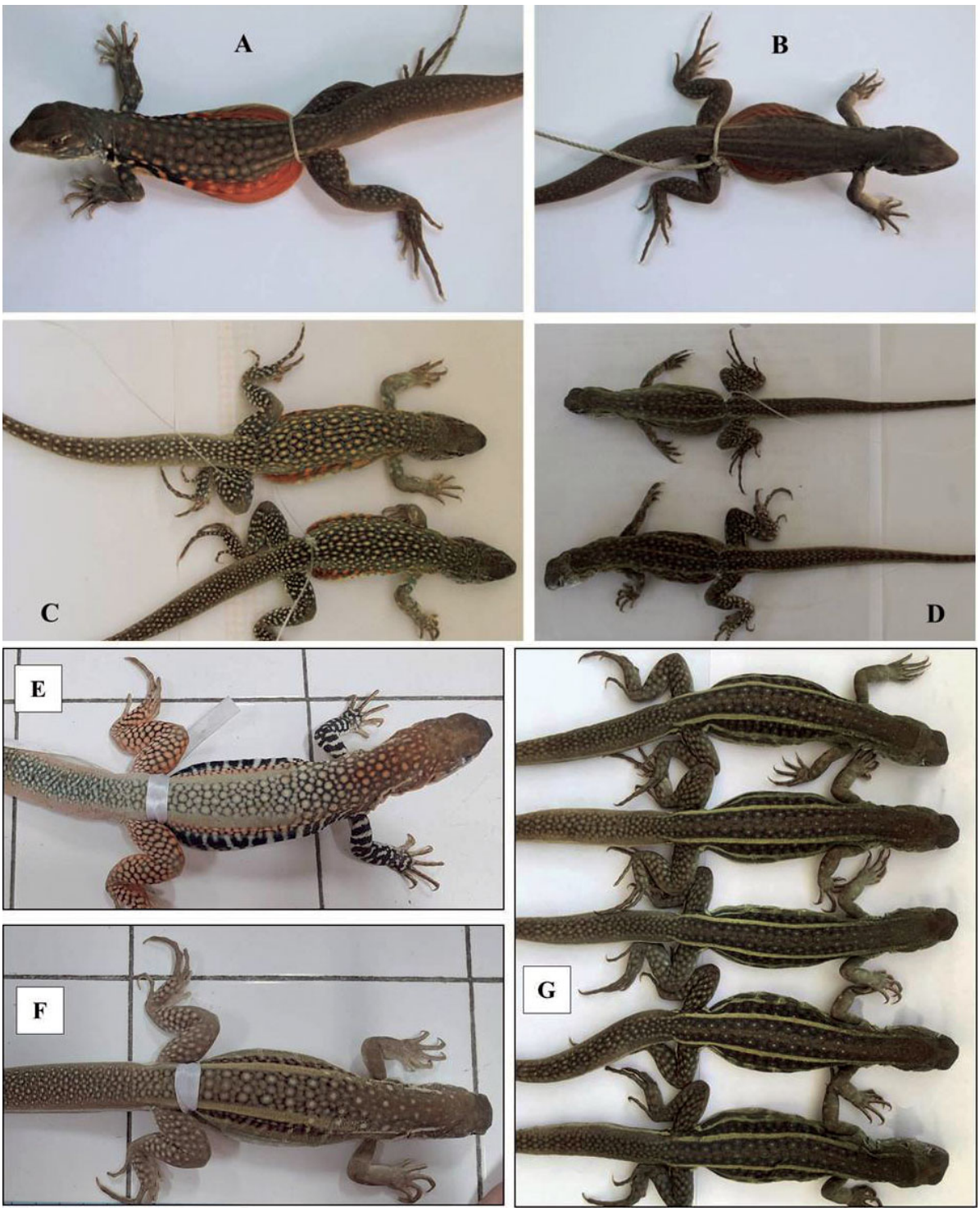

Fig. 2. External morphology of four species of the genus *Leiolepis*: A, male *L. rubritaeniata*; B, female *L. rubritaeniata*; C, male *L. reevesii*; D, female *L. reevesii*; E, male *L. guttata*; F, female *L. guttata*; G, *L. guentherpetersi*.

However, the results indicated each species is grouped into the same brand as well as showing a close relationship to other species. The phylogenetic trees strongly supported the population divided into two groups. Group

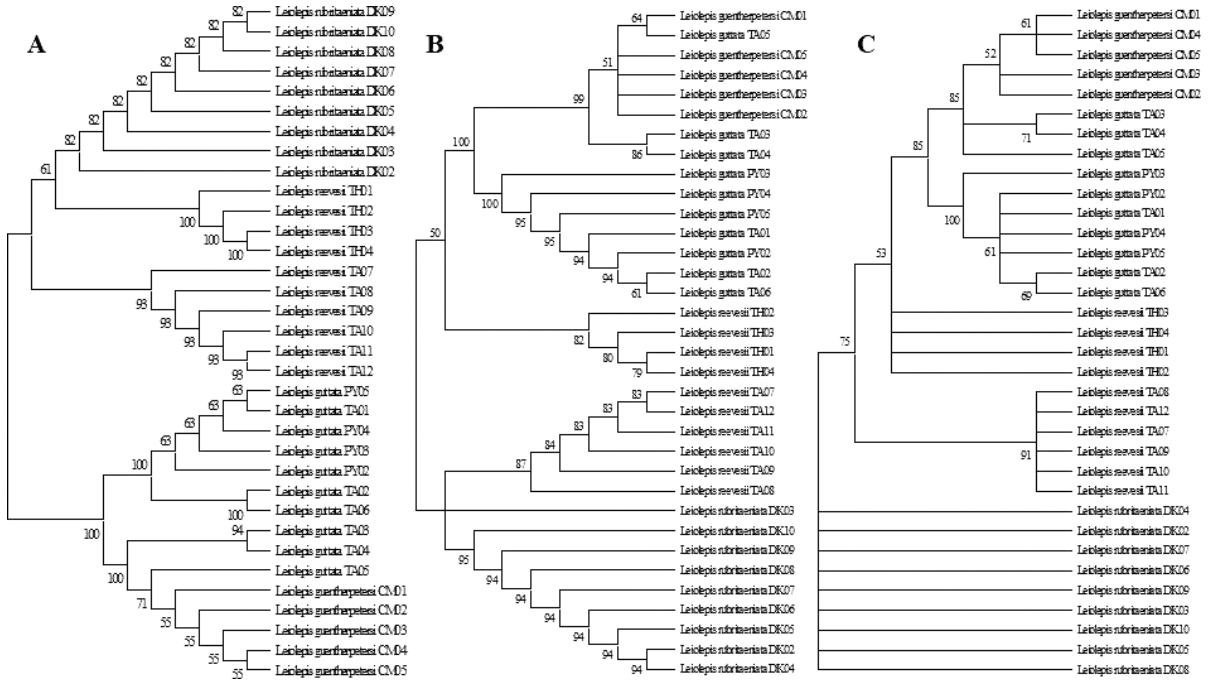

Fig. 3. The phylogenetic trees among *Leiolepis* species using the maximum parsimony (MP; A), maximum likelihood (ML; B), and (UPGMA; C) approaches from the MEGA analysis of thirty-five individuals. Numbers above nodes represent bootstrap proportions for 1000 replicates. Bootstrap proportions of less than 50% are not shown.

TABLE 2. Morphological Characteristics and Some Ratios of Measurements of the Genus *Leiolepis* in Central Vietnam [mean ± SD (min – max)]

Trait	*L. rubritaeniata* (*n* = 10)	*L. reevesii* (*n* = 10)	*L. guttata* (*n* = 12)	*L. guentherpetersi* (*n* = 5)
SVL, mm	105.63 ± 9.4 (92.02 – 122.03)	127.3 ± 20.8 (94.08 – 167.12)	139.09 ± 14.33 (128.0 – 173.0)	132.20 ± 7.08 (125.0 – 143.0)
TL, mm	197.50 ± 40.96 (88.0 – 238.0)	212.5 ± 71.45 (53.0 – 309.0)	267.64 ± 35.39 (222.0 – 352.0)	271.80 ± 12.51 (257.0 – 293.0)
HL, mm	22.37 ± 2.13 (19.07 – 25.71)	25.38 ± 2.84 (20.66 – 30.32)	25.99 ± 2.02 (23.61 – 30.11)	25.42 ± 0.91 (24.21 – 26.92)
HW, mm	16.55 ± 2.19 (12.87 – 19.05)	21.72 ± 3.95 (17.94 – 29.08)	21.05 ± 1.71 (18.02 – 24.47)	18.44 ± 1.20 (17.23 – 20.65)
HH, mm	12.57 ± 1.19 (10.64 – 14.13)	15.22 ± 2.02 (12.32 – 18.99)	15.87 ± 1.26 (13.95 – 17.65)	17.85 ± 0.38 (17.36 – 18.54)
MW, mm	13.99 ± 1.41 (12.01 – 16.14)	17.44 ± 2.16 (14.53 – 21.12)	17.44 ± 1.76 (14.74 – 20.87)	16.67 ± 0.83 (15.73 – 18.21)
HE, mm	4.32 ± 0.76 (2.39 – 5.33)	6.04 ± 0.94 (5.06 – 8.16)	6.38 ± 0.69 (4.94 – 7.48)	4.75 ± 0.59 (4.28 – 5.87)
DE, mm	3.18 ± 0.41 (2.41 – 3.99)	3.06 ± 0.56 (2.23 – 4.15)	4.29 ± 0.47 (3.48 – 5.02)	3.49 ± 0.29 (3.12 – 3.92)
DiE, mm	5.83 ± 0.46 (5.01 – 6.41)	6.98 ± 0.64 (5.93 – 8.29)	7.41 ± 0.48 (6.61 – 8.16)	6.91 ± 0.49 (6.31 – 7.74)
DN, mm	5.33 ± 0.41 (4.73 – 6.12)	5.59 ± 0.44 (5.15 – 6.27)	6.03 ± 0.66 (5.13 – 7.31)	6.03± 0.61 (5.55 – 7.21)
AG, mm	47.51 ± 5.82 (39.36 – 47.28)	64.82 ± 7.06 (50.51 – 76.56)	72.27 ± 8.45 (59.79 – 88.62)	74.16 ± 5.35 (65.62 – 79.78)
FL, mm	21.43 ± 2.49 (18.49 – 25.64)	26.97 ± 2.92 (22.82 – 32.14)	31.88 ± 3.67 (25.81 – 40.03)	26.55 ± 2.62 (22.14 – 29.37)
FA, mm	12.89 ± 2.49 (10.83 – 15.15)	15.32 ± 1.72 (13.12 – 18.31)	18.32 ± 2.51 (15.49 – 24.35)	16.58 ± 1.14 (14.76 – 17.63)
HB, mm	34.37 ± 2.84 (28.93 – 37.31)	43.92 ±6.11 (34.38 – 54.72)	50.57 ± 9.75 (37.29 – 74.8)	42.95 ± 2.28 (39.51 – 46.29)
TiB, mm	22.91 ± 10.68 (19.67 – 25.02)	24.35 ± 3.87 (17.41 – 30.51)	28.59 ± 3.61 (24.41 – 36.04)	25.78 ± 1.92 (23.24 – 28.32)
BM, gam	31.88 ± 10.68 (14.74 – 43.41)	66.19 ± 15.76 (48.62 – 84.33)	77.22 ± 23.71 (48.61 – 124.12)	62.58 ± 9.91 (48.38 – 73.73)
HL/SVL	0.21 ± 0.23	0.19 ± 0.14	0.18 ± 0.14	0.19 ± 0.13
HW/HL	0.74 ± 1.03	0.86 ± 1.39	0.81 ± 0.85	0.72 ± 1.33
FL/SVL	0.21 ± 0.26	0.21 ± 0.14	0.23 ± 0.26	0.20 ± 0.36
TiB/HB	0.67 ± 0.78	0.55 ± 0.63	0.57 ± 0.37	0.60 ± 0.84
HB/SVL	0.33 ± 0.30	0.34 ± 0.29	0.36 ± 0.68	0.32 ± 0.32

I consisted of *L. rubritaeniata* and *L. reevesii*, whereas group II were *L. guentherpetersi* and *L. guttata*. These two groups exhibited highly genetic mutations to each other (Table 3).

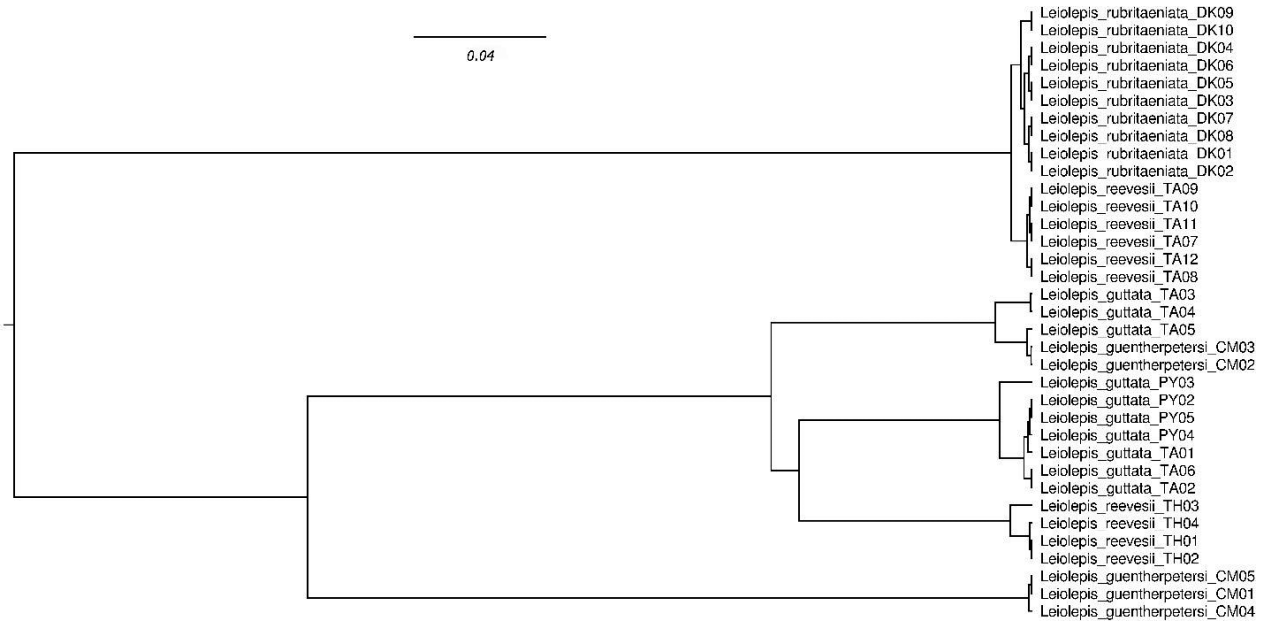

Fig. 4. The phylogenetic tree among *Leiolepis* species using Bayesian evolutionary analysis with a standard recommendation. The tree was built by TreeAnnotator following FigTree software.

Based on the MEGA analysis, the phylogenetic trees strongly supported that the population of *L. guenthepetersi* in the Chan May area exhibited the closest sister relationship with the population of *L. guttata* in the Thuan An area than that of the population of *L. guttata* in Phu Yen (Fig. 3). An individual of *L. guttata* TA05 had the closest sister relationship to *L. guentherpetersi* CM01. An individual of *L. guttata* PY02 had the high relationship with *L. guttata* TA02 and *L. guttata* TA06 (Fig. 3A – C). Thus, two populations of *L. guttata* in Thua Thien Hue and Phu Yen have genetic interferences. Bayesian phylogenetic analysis by BEAST strongly matched with MP and ML analyses (Fig. 4). These trees show the *L. reevesii* population in Thanh Hoa had a close sister relationship with *L. guttata* population in Phu Yen than that of *L. guttata* population in Thua Thien Hue. We observed that individual relationships within the population have a slight difference between the two models. However, the population relationship showing in the two models is highly in accordance. Meanwhile, the *L. guentherpetersi* population was more closely related to the *L. guttata* population in the Thuan An area than in Phu Yen, confirming a previous study's hypothesis that triploid *L. guentherpetersi* originated from *L. guttata* by Schmitz et al. (2001) and Grismer and Grismer (2010).

The analysis results of MP, ML, and UPGMA were relatively similar in structure. MP analysis showed that *L. rubritaeniata* and *L. reevesii* populations in Thua Thien Hue have a close relationship. Both groups have a sister relationship with other groups (*L. reevesii* population in Thanh Hoa, *L. guentherpetersi* and *L. guttata* populations in Thua Thien Hue and Phu Yen). In the UPGMA analysis tree, *L. rubritaeniata* group in Dak Lak and *L. reevesii* group in Thanh Hoa are highly similar. This branch had a sister relationship with other groups (*L. reevesii* population in Thua Thien Hue, *L. guentherpetersi* and *L. guttata* populations in Thua Thien Hue, Phu Yen). On the contrary, the BEAST analysis exhibited similarity to the MP and ML analyses. Thus, based on the morphology, biogeography, natural development history of *Leiolepis* and genetic distance, we support the hypothesis of *L. rubritaeniata* population is closely related to *L. reevesii* than other populations (*L. guttata* and *L. guenthepetersi*). *Leiolepis rubritaeniata* population in Dak Lak had a genetic relationship with *L. reevesii* population in Thua Thien Hue than that of the same population in Thanh Hoa. Interestingly, *L. reevesii* populations in Thanh Hoa and Thua Hue Thien showed a high genetic distance. Meanwhile, *L. guttata* populations in Phu Yen and Thua Thien Hue were more close (Figs. 3 and 4). We propose that the genetic mutations occurring between *L. reevesii* and *L. guttata* populations are timely different.

Phylogenetic relationships among populations in central Vietnam and other countries. The available 16S rRNA nucleotide sequence on GenBank of four species were downloaded and used to generate the phylogenetic

TABLE 3. Nucleotide Mutations of 16S rRNA Among Individuals of *Leiolepis* Populations in Central Vietnam

Sample symbols	Mutation position																		
	26	36	38	51	56	67	80	83	90	96	102	123	131	168	174	176	178	180	181
L. rubritaeniata DK1	T	A	G	C	T	T	G	T	G	C	T	A	T	T	A	A	A	A	A
L. rubritaeniata DK2	T	A	G	C	T	T	G	T	G	C	T	A	T	T	A	A	A	A	A
L. rubritaeniata DK3	T	A	G	C	T	T	G	T	G	C	T	A	T	T	A	A	A	A	A
L. rubritaeniata DK4	T	A	G	C	T	T	G	T	G	C	T	A	T	T	A	A	A	A	A
L. rubritaeniata DK5	T	A	G	C	T	T	G	T	G	C	T	A	T	T	A	A	A	A	A
L. rubritaeniata DK6	T	A	G	C	T	T	G	T	G	C	T	A	T	T	A	A	A	A	A
L. rubritaeniata DK7	T	A	G	C	T	T	G	T	G	C	T	A	T	T	A	A	A	A	A
L. rubritaeniata DK8	T	A	G	C	T	T	G	T	G	C	T	A	T	T	A	A	A	A	A
L. rubritaeniata DK9	T	A	G	C	T	T	G	T	G	C	T	A	T	T	A	A	A	A	A
L. rubritaeniata DK10	T	A	G	C	T	T	G	T	G	C	T	A	T	T	A	A	A	A	A
L. reevesii TA7	T	A	G	C	T	C	G	T	G	C	T	A	T	T	A	A	A	A	A
L. reevesii TA8	T	A	G	C	T	C	G	T	G	C	T	A	T	T	A	A	A	A	A
L. reevesii TA9	T	A	G	C	T	C	G	T	G	C	T	A	T	T	A	A	A	A	A
L. reevesii TA10	T	A	G	C	T	C	G	T	G	C	T	A	T	T	A	A	A	A	A
L. reevesii TA11	T	A	G	C	T	C	G	T	G	C	T	A	T	T	A	A	A	A	A
L. reevesii TA12	T	A	G	C	T	C	G	T	G	C	T	A	T	T	A	A	A	A	A
L. reevesii TH1	T	A	G	C	T	C	G	T	G	C	T	A	T	C	A	A	G	A	A
L. reevesii TH2	T	A	G	C	T	C	G	T	G	C	T	A	T	C	A	A	G	A	A
L. reevesii TH4	T	A	G	C	T	C	G	T	G	C	T	A	T	C	A	A	G	A	A
L. reevesii TH3	T	A	G	C	T	C	G	T	G	C	T	A	T	C	A	A	G	A	A
L. guttata PY3	T	G	G	T	A	T	A	C	A	T	C	G	A	C	C	A	G	C	G
L. guttata PY2	T	G	G	T	A	T	A	C	A	T	C	G	A	C	C	A	G	C	G
L. guttata PY4	T	G	G	T	A	T	A	C	A	T	C	G	A	C	C	A	G	C	G
L. guttata PY5	T	G	G	T	A	T	A	C	A	T	C	G	A	C	C	A	G	C	G
L. guttata TA1	T	G	G	T	A	T	A	C	A	T	C	G	A	C	C	A	G	C	G
L. guttata TA2	T	G	G	T	A	T	A	C	A	T	C	G	A	C	C	A	G	C	G
L. guttata TA6	T	G	G	T	A	T	A	C	A	T	C	G	A	C	C	A	G	C	G
L. guttata TA3	C	A	A	C	T	T	G	C	A	C	T	A	A	C	C	G	G	T	G
L. guttata TA4	C	A	A	C	T	T	G	C	A	C	T	A	A	C	C	G	G	T	G
L. guentherpetersi CM1	C	A	A	C	T	T	G	C	A	C	T	A	A	C	C	G	G	T	G
L. guentherpetersi CM4	C	A	A	C	T	T	G	C	A	C	T	A	A	C	C	G	G	T	G
L. guentherpetersi CM5	C	A	A	C	T	T	G	C	A	C	T	A	A	C	C	G	G	T	G
L. guentherpetersi CM2	C	A	A	C	T	T	G	C	A	C	T	A	A	C	C	G	G	T	G
L. guentherpetersi CM3	C	A	A	C	T	T	G	C	A	C	T	A	A	C	C	G	G	T	G
L. guttata TA5	C	A	A	C	T	T	G	C	A	C	T	A	A	C	C	G	G	T	G

TABLE 3 (continued)

Sample symbols	Mutation position																		
	185	186	191	193	195	200	202	203	205	207	209	210	213	214	218	222	225	229	230
L. rubritaeniata DK1	G	—	T	G	T	C	A	C	A	G	A	G	A	C	A	C	C	C	A
L. rubritaeniata DK2	G	—	T	G	T	C	A	C	A	G	A	G	A	C	A	C	C	C	A
L. rubritaeniata DK3	G	—	T	G	T	C	A	C	A	G	A	G	A	C	A	C	C	C	A
L. rubritaeniata DK4	G	—	T	G	T	C	A	C	A	G	A	G	A	C	A	C	C	C	A
L. rubritaeniata DK5	G	—	T	G	T	C	A	C	A	G	A	G	A	C	A	C	C	C	A
L. rubritaeniata DK6	G	—	T	G	T	C	A	C	A	G	A	G	A	C	A	C	C	C	A
L. rubritaeniata DK7	G	—	T	G	T	C	A	C	A	G	A	G	A	C	A	C	C	C	A
L. rubritaeniata DK8	G	—	T	G	T	C	A	C	A	G	A	G	A	C	A	C	C	C	A
L. rubritaeniata DK9	G	—	T	G	T	C	A	C	A	G	A	G	A	C	A	C	C	C	A
L. rubritaeniata DK10	G	—	T	G	T	C	A	C	A	G	A	G	A	C	A	C	C	C	A
L. reevesii TA7	G	—	T	G	T	C	A	C	A	G	A	G	A	C	A	C	C	C	A
L. reevesii TA8	G	—	T	G	T	C	A	C	A	G	A	G	A	C	A	C	C	C	A
L. reevesii TA9	G	—	T	G	T	C	A	C	A	G	A	G	A	C	A	C	C	C	A
L. reevesii TA10	G	—	T	G	T	C	A	C	A	G	A	G	A	C	A	C	C	C	A
L. reevesii TA11	G	—	T	G	T	C	A	C	A	G	A	G	A	C	A	C	C	C	A
L. reevesii TA12	G	—	T	G	T	C	A	C	A	G	A	G	A	C	A	C	C	C	A
L. reevesii TH1	G	G	T	G	T	C	A	C	A	G	A	G	A	C	A	C	C	T	A
L. reevesii TH2	G	G	T	G	T	C	A	C	A	G	A	G	A	C	A	C	C	T	A
L. reevesii TH4	G	G	T	G	T	C	A	C	A	G	A	G	A	C	A	C	C	T	A
L. reevesii TH3	G	G	T	G	T	C	A	C	A	G	A	G	A	C	A	C	C	T	A
L. guttata PY3	A	T	A	T	G	A	C	T	G	A	G	A	C	A	C	T	A	T	G
L. guttata PY2	A	T	A	T	G	A	C	T	G	A	G	A	C	A	C	T	A	T	G
L. guttata PY4	A	T	A	T	G	A	C	T	G	A	G	A	C	A	C	T	A	T	G
L. guttata PY5	A	T	A	T	G	A	C	T	G	A	G	A	C	A	C	T	A	T	G
L. guttata TA1	A	T	A	T	G	A	C	T	G	A	G	A	C	A	C	T	A	T	G
L. guttata TA2	A	T	A	T	G	A	C	T	G	A	G	A	C	A	C	T	A	T	G
L. guttata TA6	A	T	A	T	G	A	C	T	G	A	G	A	C	A	C	T	A	T	G
L. guttata TA3	A	T	A	T	G	A	C	T	G	A	G	A	C	A	C	T	—	T	G
L. guttata TA4	A	T	A	T	G	A	C	T	G	A	G	A	C	A	C	T	—	T	G
L. guentherpetersi CM1	A	T	A	T	G	A	C	T	G	A	G	A	C	A	C	T	A	T	G
L. guentherpetersi CM4	A	T	A	T	G	A	C	T	G	A	G	A	C	A	C	T	A	T	G
L. guentherpetersi CM5	A	T	A	T	G	A	C	T	G	A	G	A	C	A	C	T	A	T	G
L. guentherpetersi CM2	A	T	A	T	G	A	C	T	G	A	G	A	C	A	C	T	—	T	G
L. guentherpetersi CM3	A	T	A	T	G	A	C	T	G	A	G	A	C	A	C	T	—	T	G
L. guttata TA5	A	T	A	T	G	A	C	T	G	A	G	A	C	A	C	T	—	T	G

TABLE 3 (continued)

Sample symbols	Mutation position																		
	239	254	258	259	260	261	262	263	264	265	266	269	270	271	273	274	293	312	402
L. rubritaeniata DK1	C	T	C	T	A	—	T	T	G	A	T	G	A	T	A	T	T	A	C
L. rubritaeniata DK2	C	T	C	T	A	—	T	T	G	A	T	G	A	T	A	T	T	A	C
L. rubritaeniata DK3	C	T	C	T	A	—	T	T	G	A	T	G	A	T	A	T	T	A	C
L. rubritaeniata DK4	C	T	C	T	A	—	T	T	G	A	T	G	A	T	A	T	T	A	C
L. rubritaeniata DK5	C	T	C	T	A	—	T	T	G	A	T	G	A	T	A	T	T	A	C
L. rubritaeniata DK6	C	T	C	T	A	—	T	T	G	A	T	G	A	T	A	T	T	A	C
L. rubritaeniata DK7	C	T	C	T	A	—	T	T	G	A	T	G	A	T	A	T	T	A	C
L. rubritaeniata DK8	C	T	C	T	A	—	T	T	G	A	T	G	A	T	A	T	T	A	C
L. rubritaeniata DK9	C	T	C	T	A	—	T	T	G	A	T	G	A	T	A	T	T	A	C
L. rubritaeniata DK10	C	T	C	T	A	—	T	T	G	A	T	G	A	T	A	T	T	A	C
L. reevesii TA7	C	T	C	T	A	—	T	T	G	A	T	G	A	T	A	T	T	A	C
L. reevesii TA8	C	T	C	T	A	—	T	T	G	A	T	G	A	T	A	T	T	A	C
L. reevesii TA9	C	T	C	T	A	—	T	T	G	A	T	G	A	T	A	T	T	A	C
L. reevesii TA10	C	T	C	T	A	—	T	T	G	A	T	G	A	T	A	T	T	A	C
L. reevesii TA11	C	T	C	T	A	—	T	T	G	A	T	G	A	T	A	T	T	A	C
L. reevesii TA12	C	T	C	T	A	—	T	T	G	A	T	G	A	T	A	T	T	A	C
L. reevesii TH1	C	T	C	T	A	—	T	T	G	A	T	G	A	T	A	T	T	A	C
L. reevesii TH2	C	T	C	T	A	—	T	T	G	A	T	G	A	T	A	T	T	A	C
L. reevesii TH4	C	T	C	T	A	—	T	T	G	A	T	G	A	T	A	T	T	A	C
L. reevesii TH3	C	T	C	T	A	—	T	T	G	A	T	G	A	T	A	T	T	A	T
L. guttata PY3	A	C	C	T	A	T	G	A	—	C	C	A	T	A	T	C	T	G	T
L. guttata PY2	A	C	C	T	A	T	G	A	—	C	C	A	T	A	T	C	T	G	C
L. guttata PY4	A	C	C	T	A	T	G	A	—	C	C	A	T	A	T	C	T	G	C
L. guttata PY5	A	C	C	T	A	T	G	A	—	C	C	A	T	A	T	C	T	G	C
L. guttata TA1	A	C	C	T	A	T	G	A	—	C	C	A	T	A	T	C	T	G	C
L. guttata TA2	A	C	C	T	A	T	G	A	—	C	C	A	T	A	T	C	T	G	C
L. guttata TA6	A	C	C	T	A	T	G	A	—	C	C	A	T	A	T	C	T	G	C
L. guttata TA3	A	C	A	A	C	C	A	T	G	A	C	G	A	T	A	A	C	A	T
L. guttata TA4	A	C	A	A	C	C	A	T	G	A	C	G	A	T	A	A	C	A	T
L. guentherpetersi CM1	A	C	A	A	C	T	A	T	G	A	C	G	A	T	A	A	C	A	C
L. guentherpetersi CM4	A	C	A	A	C	T	A	T	G	A	C	G	A	T	A	A	C	A	C
L. guentherpetersi CM5	A	C	A	A	C	T	A	T	G	A	C	G	A	T	A	A	C	A	C
L. guentherpetersi CM2	A	C	A	A	C	T	A	T	G	A	C	G	A	T	A	A	C	A	C
L. guentherpetersi CM3	A	C	A	A	C	T	A	T	G	A	C	G	A	T	A	A	C	A	C
L. guttata TA5	A	C	A	A	C	T	A	T	G	A	C	G	A	T	A	A	C	A	C

Note. DK, Dak Lak; TA, Thuan An; CM, Chan May; PY, Phu Yen; TH, Thanh Hoa.

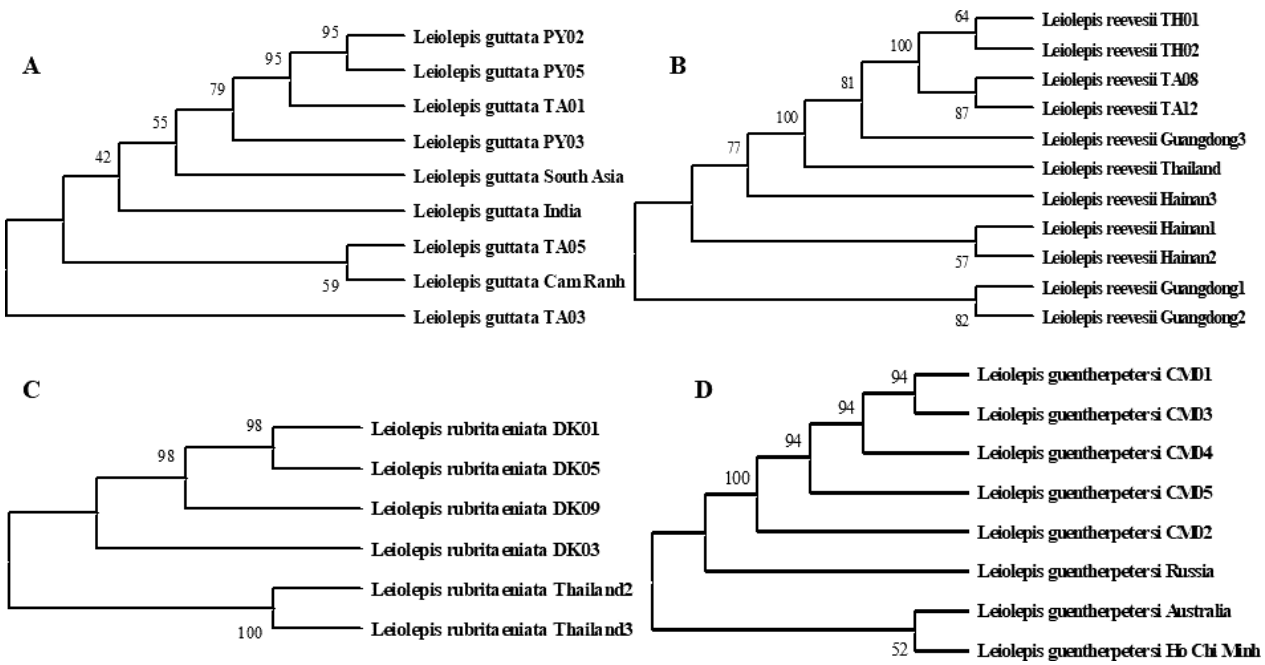

Fig. 5. The phylogenetic trees among individuals of *Leiolepis* species in this study and from other countries identified through the GenBank database using the maximum parsimony (MP) approach: A, *L. guttata*; B, *L. reevesii*; C, *L. rubritaeniata*; D, *L. guentherpetersi*. The numbers above the nodes represent the bootstrap rate for 1000 replicates for parsing and probability analysis, respectively. A bootstrap scale of less than 50% is not displayed.

trees (Fig. 5, Table 4). The results indicated the populations of *L. guttata* in Phu Yen and Thua Thien Hue have a close relationship with populations in Southeast Asia. *L. guttata* found in the Cam Ranh area (Khanh Hoa Province, Vietnam) is more closely related to *L. guttata* in Thua Thien Hue Province than in Phu Yen Province (Fig. 5). All *L. guttata* populations are sisters to *L. guttata* in India (Fig. 5A). The *L. reevesii* populations found in this study were more similar to each other than the population in other countries, which is closer to the *L. reevesii* populations in Thailand, following by populations in China. However, there is a branch of the population in Guangdong, China that is closer to the populations in Thailand and Vietnam than the populations in Hainan of China (Fig. 5B). Of the four species, *L. rubritaeniata* is the least commonly encountered. Thus, data on this species is limited, especially on the kinship between domestic and foreign populations. From the collected data, at present, the *L. rubritaeniata* population in Vietnam (Dak Lak) has the closest sister relationship in the population in Thailand (Fig. 5C). The population of *L. guentherpetersi* from Thua Thien Hue Province was grouped into the same branch and was more closely related to individuals reported by a Russian research group than individuals found by a research group in Australia.

DISCUSSION

The present study found that the nucleotide sequences of the *L. rubritaeniata* population in Dak Lak are highly conserved. The *L. rubritaeniata* population may live in a stable and unique ecosystem rather than other regions in Vietnam and other countries. Thus, the *L. rubritaeniata* population evolved independently, forming a new population. Supporting this hypothesis, nucleotide sequence mutation did not occur among *L. rubritaeniata* but was found in *L. rubritaeniata* from Thailand. Hartmann et al. (2012) only recorded the presence of *L. rubritaeniata* in Chu Pong, Gia Lai, Vietnam which has similar ecology habitats to Dak Lak. This study added a new area for *L. rubritaeniata* as well as suggesting this species may be distributed only in Central Highland areas of Vietnam. The *L. rubritaeniata* with a narrow distribution has been identified as native to Eastern Thailand (Peters, 1971; Darevsky and Nguyen, 2004; Hartmann et al., 2012) which is closely related to the *L. rubritaeniata* population in Dak Lak. Thus, the appearance of *L. rubritaeniata* in the Central Highlands of Vietnam and Thailand may be due to the dispersal or migration of this species between the two countries. It is mapped that

TABLE 4. List of 16S rRNA Nucleotide Sequences of *Leiolepis* Species Available on GenBank

Sample symbols	Location	Accession No.	References
L. guttata South Asia	—	AB266888	Amer et al., 2007
L. guttata Cam Ranh	Vietnam	AF378377	Schmitz et al., 2001
L. guttata India	India	AB476400	Okajima et al., 2010
L. rubritaeniata Thailand[1]	Thailand	AB516970	Srikulnath et al., 2010
L. rubritaeniata Thailand[2]	Thailand	AB480293	Srikulnath et al., 2009
L. rubritaeniata Thailand[3]	Thailand	AB537553	*
L. reevesii Guangdong[1]	China	FJ599654	Lin et al., 2010
L. reevesii Guangdong[2]	China	FJ599655	Lin et al., 2010
L. reevesii Guangdong[3]	China	JN869377	Lin et al., 2010
L. reevesii Thailand	—	LC365671	*
L. reevesii Hainan[1]	China	EU305003	Lin et al., 2010
L. reevesii Hainan[2]	China	EU305019	Lin et al., 2010
L. reevesii Hainan[3]	China	KJ530718	Lin et al., 2010
L. guentherpetersi Russia	—	AY847662	Martirosyan et al., 2006
L. guentherpetersi Australia	—	AF137529	*
L. guentherpetersi Ho Chi Minh	Vietnam	DQ340733	Hugall et al., 2008

—, Unknown location; *, data available only on NCBI.
Note. The name of *Leiolepis* species from the unknown locations was named based on the author's country address on NCBI.

L. rubritaeniata may be found in other Southeast Asia countries such as Laos and Cambodia.

The populations of *L. reevesii* in Thanh Hoa and *L. reevesii* in Thua Thien Hue exhibited a genetic distance as shown by both analyses (Figs. 3 and 4). It seems that mutations between *L. reevesii* populations may be influenced by different ecological environments. It is observed that the population in Thua Thien Hue did not have a mutation, but the Thanh Hoa population exhibited a mutation in the 16S rRNA nucleotide sequence. We propose that the *L. reevesii* population in Thanh Hoa was spread from the same population in Thua Thien Hue. Although *L. reevesii* from both provinces show differences in morphology and exhibit genetic distance. However, the phylogenetic analysis indicated mutations are not enough to form a new species (Fig. 3B). The morphological difference between two *L. reevesii* populations may be due to environmental factors such as climate, temperature, and habitat in two different regions.

Data on *L. guttata* and *L. guentherpetersi* populations from Thua Thien Hue indicated that there were three *L. guttata* individuals in the Thuan An area consisting of substitution mutation as same as the *L. guentherpetersi* population in the Chan May area. The phylogenetic analysis indicated that *L. guentherpetersi* is genetically closest to the species *L. guttata* and a high similarity in color and pattern to the female of species *L. guttata* was observed. Schmitz et al. (2001) and Grismer and Grismer (2010) reported the origin of triploid *L. guentherpetersi* is from multiple crosses between *L. guttata* and at least one intermediate species (the third species) (Reeder et al., 2002; Sites et al., 2011). Therefore, the origin of the *L. guentherpetersi* population may be the result of repeated hybridization between two sexual species *L. guttata* and *L. reevesii*, or between *L. guttata* and an intermediate species (third species) found in the same province with a distance of 70 km. The mutation evidence also strongly supports this hypothesis.

Although *L. rubritaeniata* and *L. reevesii* populations share many morphological and genetic similarities and are more closely related than *L. guentherpetersi* and *L. guttata* species. *L. rubritaeniata* and *L. reevesii* are two separate species (Grismer et al., 2014). Hartmann et al. (2012) hypothesized that the appearance of *L. rubritaeniata* in the Central Highland areas of Vietnam may have occurred originally from an individual from a clade of *L. reevesii* in Southern Indochina. The two species have different habitats, *L. reevesii* is found in undisturbed coastal habitats, dunes; while *L. rubritaeniata* is found in undisturbed habitats in forest areas.

The individuals of *L. reevesii* populations found in this study have the closest relationship to each other. Thus, the populations would have the same origin. The current populations are formed through a long history of geographical distributions and development. Furthermore, these populations are more closely related to populations in Thailand than to populations in China. It is hypothesized that *L. reevesii* in Thailand spread to Vietnam, then to the northern region of Hainan, Guangdong, and Guangxi of China (Lin et al., 2010; Zhu et al., 2020).

However, the data in Figure 3 do not support this hypothesis and suggest that populations in Thailand and China are closer. However, the number of samples for the phylogenetic tree is small, limiting the ability to draw strong conclusions. It is recommended that the relationships among *L. reevesii* populations in Vietnam, Thailand, and China should be investigated for further clarification.

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