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Molecular phylogeny and biogeography of the *Cyrtodactylus angularis* group: diversification in a global biodiversity hotspot

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Abstract

Cyrtodactylus is the third-largest genus of vertebrates, with nearly 400 nominal species. Comprising at least 21 members, the *C. angularis* species group represents a diverse clade in Indochina, a main center of the genus' radiation. Recent works, which have attempted to resolve the phylogenetic relationships among the congeners but primarily used a couple of mitochondrial loci and sparse taxon sampling, could not provide a robust phylogenetic hypothesis. In addition, important aspects of its evolutionary and biogeographic history have not been addressed in previous research. To fill these gaps, data from three mitochondrial and four nuclear loci were analyzed for all recognized taxa. The largest dataset to date for the group helps produce highly congruent phylogenetic relationships among the species and recover, for the first time, six evolutionarily independent clades with high confidence (except for the bootstrap value in Group III). Using this well-corroborated phylogenetic framework to infer the species group's evolutionary history, this study offers novel hypotheses on its origin and colonization and speciation events in Indochina. The results suggest that the group first emerged in the Oligocene, while most of its lineages started to diverge in the Miocene, when the East Asian summer monsoon developed and might have brought about large-scale vegetation change. Moreover, the Mekong Lowlands are likely the ancestral area of the *C. angularis* group, whereas most of the remaining members, except for Group II, originate from the Annamite Mountain Range. The study once again highlights the importance of the Annamite landscape, a global biodiversity hotspot, in maintaining evolutionary processes for many endemic and evolutionarily distinct vertebrates.

Key Words

Ancestral area, Annamite Mountain Range, bent-toed gecko, Indochina, Miocene

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Introduction

Cyrtodactylus is by far the most speciose and ecologically diverse genus of the family Gekkonidae (Grismer et al. 2021a, 2021b, 2022; Uetz et al. 2026). It currently contains 398 nominal species (Uetz et al. 2026) and is distributed from Tibet and tropical South Asia through Indochina, the Philippines, and the Indo-Australian Archipelago to the Solomon Islands in the east (Wood et al. 2012; Grismer et al. 2015, 2021b). New species discoveries have surged in recent years, as only 70 species were known to science at the turn of this century (Uetz et al. 2026). The use of molecular data has helped uncover many cryptic species across its range. Although the current number of taxa makes *Cyrtodactylus* the third-largest vertebrate genus on Earth, its diversity remains largely underestimated, with many undescribed forms yet to be named (Grismer et al. 2021a, 2021b; Ngo et al. 2022, 2023).

Members of the genus inhabit a broad array of habitat types, ranging from intertidal, arboreal, cave, karst, granite, and swamp habitats to coffee plantations (Luu et al. 2017; Grismer et al. 2020, 2021a, 2022). Bent-toed geckos' remarkable adaptive abilities have resulted in numerous shifts in habitat preference. Specifically, Grismer et al. (2020) documented at least 39 transitions from the general habitat preference—the ancestral trait of the genus—to eight other specific types, 11 events in reverse, and 16 others between different specific habitats. With this remarkable adaptability, *Cyrtodactylus* has evolved into one of the most successful vertebrate groups on the planet, with several major radiations in Indochina, Sundaland, Wallacea, and the Philippines since the Eocene from its ancestral area in the proto-Himalaya. Among these regions, Indochina and Sundaland form two centers of group diversity (Wood et al. 2012; Grismer et al. 2022).

The *Cyrtodactylus angularis* group is one of the most speciose Indochinese lineages, with at least 21 nominal species, excluding *C. zhaoi* because of its taxonomic invalidity, and several undescribed forms, as reported in Grismer et al. (2021b). Even though the type species was discovered in the early 20th century (Smith 1921), as with other species groups within *Cyrtodactylus*, most of its diversity remained unrecognized until the early 21st century (Uetz et al. 2026). A large portion of the genus occurs in a special ecotype—karst (Grismer et al. 2020, 2021a). The color patterns of these species are also diverse and include dorsal patterns formed by blotches (e.g., *C. teyniei*), irregularly shaped separate spots (e.g., *C. multiporus*), and bands (e.g., *C. bansocensis* and *C. darevskii*) (David et al. 2011; Nazarov et al. 2012; Luu et al. 2016a, 2016b). Most species of the group are geographically restricted and are known only from their type localities, i.e., *C. angularis* and *C. chanhomeae* in Thailand; *C. bansocensis*, *C. calamei*, *C. darevskii*, *C. hinnamnoensis*, *C. jaegeri*, *C. khammouanensis*, *C. lomyenensis*, *C. multiporus*, *C. muangfuangensis*, *C. pageli*, *C. sommerladi*, *C. soudthichaki*, *C. thathomensis*, and *C. teyniei* in Laos; and *C. hangvaensis*, *C. nigriocularis*, and *C. phongnhakebangensis* in Vietnam, while *C. jarujini* is found in Laos and Thailand, and *C. roesleri* is recorded in both Laos and Vietnam (Ulber 1993; Stuart 1999; Teynié and David 2010; Luu et al. 2013, 2016a, 2016b).

Interestingly, aside from two species known only from Thailand, all other species, except for the Black-eyed Bent-toed Gecko (*C. nigriocularis*), occupy the northern Annamite Mountain Range, a region that extends across the border between Laos and Vietnam and is well known for its high level of endemism and evolutionary distinctiveness (Sterling et al. 2006; Bain and Hurley 2011; Nicolas et al. 2012; Le et al. 2015; Stenger et al. 2023). On the Vietnamese side, the northern Annamites, despite their relatively small geographic extent, harbor 115 species of reptiles, representing approximately 24% of the country's reptile diversity, including 12 endemic species (Stenger et al. 2023).

In addition to reptiles, other endemic and flagship species in the landscape include such Critically Endangered vertebrates as the Saola (*Pseudoryx vuquangensis*), the Large-antlered Muntjac (*Muntiacus vuquangensis*), the Southern White-cheeked Gibbon (*Nomascus siki*), and Edwards's Pheasant (*Lophura edwardsi*) (Timmins et al. 2016; Bird Life International 2018; Nguyen et al. 2020; Timmins et al. 2020). The small mammal fauna of the region is represented by microendemic species, e.g., the Laotian Rock Rat (*Laonastes aenigmamus*) and Paulina's Limestone Rat (*Saxatilomys paulinae*), which are confined to karst habitats (Nicolas et al. 2012; Kennerley and Laginha 2016). In particular, the presence of the Laotian Rock Rat, often considered a living fossil because all of its relatives found throughout Asia went extinct approximately 11 million years ago, clearly shows that the Annamites have served as an ancient refuge for diverse taxa throughout the history of Indochina (Dawson et al. 2006; Le et al. 2015, 2016). However, little research has been undertaken to elucidate the tempo and mode of evolutionary processes driving species radiations in the karst habitats of the Annamites (but see Nicolas et al. 2012; Le et al. 2015, 2016). Therefore, further studies on the evolution and biogeography of the *Cyrtodactylus angularis* group could provide valuable insights into the processes underlying diversification in the karst habitats of the Annamite region.

Although several studies (Wood et al. 2012; Grismer et al. 2021a, 2022; Duong et al. 2024) have been carried out to recover the phylogeny, estimate divergence times, and reconstruct the historical biogeography of *Cyrtodactylus*, previous work has neither specifically addressed the phylogenetic relationships, evolutionary history, and biogeography of the *C. angularis* group nor included all currently recognized representatives of the group. Additionally, previous phylogenetic analyses of the group have relied primarily on one or two mitochondrial genes, hindering their ability to resolve relationships within the group (Grismer et al. 2022; Sitthivong et al. 2019; Duong et al. 2024). To improve the understanding of the phylogenetic relationships and evolutionary radiation of this group, especially

in the Annamite biodiversity hotspot, the phylogeny of 21 species of this group was reconstructed using three mitochondrial genes—cytochrome c oxidase subunit I (COI), cytochrome *b* (*Cytb*), and NADH dehydrogenase subunit 2 (*ND2*) (including tRNA)—and four nuclear genes (*Cmos*, *PDC*, *Rag1*, and *Rpl35*). This dataset represents the largest molecular dataset assembled for the species group to date, both in terms of the number of loci and the length of the concatenated matrix. Divergence times of the group congeners were also estimated using a Bayesian relaxed-clock approach, and biogeographic patterns were reconstructed to infer the historical diversification of this group.

Materials and methods

Taxon sampling

In total, 36 samples representing 21 described taxa of the *C. angularis* group in Thailand, Laos, and Vietnam and one sample of an undescribed taxon were included in the present study. Four outgroup species, namely *C. ayeyarwadyensis*, *C. brevidactylus*, *C. condorensis*, and *C. grismeri*, were selected (Grismer et al. 2021a, 2021b). In total, 199 new sequences spanning seven genes from 36 samples were incorporated into this study (Table 1). In addition, 68 published sequences were included in the analyses. Detailed information and GenBank accession numbers for both newly generated and previously published sequences used in the study are provided in Table 1.

DNA extraction, PCR, and DNA sequencing

Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Germany) or GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Lithuania), following the manufacturers' instructions. Seven molecular markers, comprising three mitochondrial loci, *COI*, *Cytb*, and *ND2* (including tRNA), and four nuclear loci, *Cmos*, *PDC*, *Rag1*, and *Rpl35*, were employed in this study. The primers used to amplify the fragments are listed in Table 2. PCR amplification was performed in a total volume of 21 μL containing 2 μL of template DNA, 2 μL of each primer, 10 μL of DreamTaq Master Mix (Thermo Fisher Scientific, Lithuania) or HotStarTaq Master Mix (Qiagen, Germany), and 5 μL of nuclease-free water. The reaction was carried out with an initial denaturation at 95 °C for 15 min with HotStarTaq Master Mix or 5 min with DreamTaq Master Mix, followed by 35 cycles of amplification (denaturation at 95 °C for 30 s, annealing at 48–58 °C for 45 s, and extension at 72 °C for 1 min), with a final extension at 72 °C for 10 min. Negative and positive controls were included in all DNA extractions and PCR amplifications to check for possible contamination. The PCR products were visualized by agarose gel electrophoresis and stored at –4 °C after visualization. The PCR products were purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific, Lithuania), in accordance with the manufacturer's instructions. Forward and reverse strands were sequenced for all samples using the sequencing service provided by 1st Base (Malaysia).

Table 1. Sequences used in this study.

Species	Voucher number	Genbank accessions							References
		COI	*Cytb*	*ND2*	*Rpl35*	*Rag1*	*PDC*	*Cmos*	
C. ayeyarwadyensis	CAS216446	–	EU268380	EU268348	GU458039	EU268287	EU268317	JQ945550	(Bauer et al. 2008; Siler et al. 2010; Gamble et al. 2012)
C. brevidactylus	CAS214104	MF169905	–	JX440527	GU458037	JX440687	JX440636	–	(Siler et al. 2010; Wood et al. 2012; Brennan et al. 2017)
C. condorensis	UNS0431	MF169910	–	MF169958	–	–	–	–	(Brennan et al. 2017)
C. grismeri	LSUHC8638	–	–	JX440538	–	JX440698	JX440647	–	(Wood et al. 2012)
*C. angularis**	FMNH265815	–	–	JX041340	GU458041	JQ945301	JX440632	JQ945549	(Wood et al. 2012; Siler et al. 2010; Gamble et al. 2012)
C. bansocensis (1)	VFU R.2015.20	KU175573	**PV463231**	MT953469	–	**PV463332**	**PV463286**	**PV463272**	This study
C. bansocensis (2)*	VNUF R.2016.4	ON145865	**PV463232**	**PV475491**	**PV463382**	**PV463331**	**PV463283**	**PV463273**	This study
C. calamei (1)	NUOL R.2015.22	KX064043	–	**PV475485**	**PV463378**	**PV463350**	**PV463294**	**PV463266**	This study
C. calamei (2)*	VNUF R.2015.28	KX064044	–	**PV475486**	**PV463379**	**PV463338**	**PV463293**	**PV463265**	This study
C. chanhomeae (2)	AP018117	AP018117	AP018117	AP018117	–	–	–	–	(Areesirisuk et al. 2018)
C. chanhomeae (1)*	CUMZ2003.62	MF169908	–	JX440529	–	JX440688	JX440637	–	(Wood et al. 2012; Brennan et al. 2017)
C. darevskii (1)*	VNUF R.2016.306	PV454675	**PV463243**	**PV475483**	**PV463375**	**PV463336**	**PV463309**	**PV463254**	This study
C. darevskii (2)	VNUF R.2016.342	PV454676	**PV463244**	**PV475484**	**PV463377**	**PV463321**	**PV463310**	**PV463264**	This study
C. darevskii (3)	ZISP FN187	HQ967223	–	–	–	–	–	–	NCBI

Species	Voucher number	Genbank accessions							References
		COI	*Cytb*	*ND2*	*Rpl35*	*Rag1*	*PDC*	*Cmos*	
C. darevskii (4)	ZISP FN256	HQ967221	–	–	–	–	–	–	NCBI
*C. hangvaensis**	DBN000132	PP865549	–	PP853075	–	–	–	–	(Duong et al. 2024)
C. hinnamnoensis (1)*	IEBR A.2013.89	KX064045	**PV463239**	**PV475479**	**PV463373**	**PV463323**	**PV463292**	**PV463269**	This study
C. hinnamnoensis (2)	VNUF R.2017.11	PV454679	**PV463240**	**PV475480**	**PV463376**	**PV463322**	**PV463311**	**PV463259**	This study
C. hinnamnoensis (3)	VNUF A.2015.3	KX064046	**PV463241**	**PV475481**	**–**	**PV463342**	**PV463302**	**PV463263**	This study
C. hinnamnoensis (4)	VNUF R.2014.489	PV454678	**PV463242**	**PV475482**	**PV463374**	**PV463337**	**PV463296**	**PV463253**	This study
C. jaegeri (1)*	NUOL R.2013.1	KT004365	**PV463228**	**PV475489**	**PV463380**	**PV463333**	**PV463285**	**PV463268**	This study
C. jaegeri (2)	VFU TK.914	KT004366	**PV463229**	**–**	**PV463381**	**PV463334**	**PV463284**	**PV463262**	This study
C. jarujini (1)	FMNH255472	–	–	JX440541	–	JQ945303	JX440651	JQ945552	(Gamble et al. 2012; Wood et al. 2012)
C. jarujini (2)	VNUF R.2015.7	KX077907	–	**PV475476**	**PV463370**	**PV463345**	**PV463300**	**PV463252**	This study
C. jarujini (3)*	VNUF R.2016.01	ON145876	**PV463236**	**PV475477**	**PV463369**	**PV463346**	**PV463291**	**PV463261**	This study
C. khammouanensis (1)*	ZISP FN191	HM888467	–	–	–	–	–	–	NCBI
C. khammouanensis (2)	ZISP FN192	HM888468	–	–	–	–	–	–	NCBI
C. lomyenensis (1)*	VFU R.2015.14	KU175572	**PV463233**	**PV475490**	**–**	**–**	**PV463287**	**PV463255**	This study
C. lomyenensis (2)	UNS0527	ON145866	**–**	**PV475471**	**–**	**–**	**PV463289**	**PV463257**	This study
C. lomyenensis (3)	UNS0534	–	–	MF169966	–	–	–	–	(Brennan et al. 2017)
C. multiporus (1)*	ZMMU R–13985–2	PV454680	**PV463237**	**PV475474**	**PV463371**	**PV463347**	**PV463297**	**PV463270**	This study
C. multiporus (2)	ZMMU R–13985–3	PV454681	**PV463238**	**PV475475**	**PV463372**	**PV463348**	**PV463301**	**PV463271**	This study
C. muangfuangensis (1)*	VNUF R.2018.32	MN395826	**PV463222**	**PV475492**	**PV463365**	**PV463351**	**PV463312**	**PV463281**	This study
C. muangfuangensis (2)	NUOL R.2018.33	MN395827	**PV463223**	**–**	**PV463366**	**PV463352**	**PV463313**	**PV463282**	This study
C. nigriocularis (1)	VNMN 2184	ON145793	–	MT953485	**PV463353**	**PV463319**	**PV463317**	**PV463247**	This study
C. nigriocularis (2)*	VNMN 2189	ON145794	**PV463246**	**PV475466**	**PV463354**	**PV463320**	**PV463318**	**PV463248**	This study
C. pageli (1)*	IEBR A.2010.1	KJ817431	**–**	**–**	**PV463362**	**PV463339**	**PV463314**	**PV463249**	This study
C. pageli (2)	NQT 2010.37	KX077902	**–**	**–**	**PV463363**	**PV463341**	**PV463315**	**PV463250**	This study
C. pageli (3)	ZFMK91827	KX077903	–	MT953487	**PV463364**	**PV463340**	**PV463316**	**PV463251**	This study
*C. phongnhakebangensis**	PNKN2011.19	KF929528	–	–	–	–	–	–	(Nguyen et al. 2014)
C. roesleri (1)	ZFMK89377	ON145862	–	MW792057	**PV463359**	**PV463324**	**PV463308**	**PV463274**	This study
C. roesleri (2)*	ZFMK89378	ON145863	–	–	**PV463358**	**PV463325**	**PV463307**	**PV463275**	This study
C. sommerladi (1)	HNN68	KJ817437	**PV463224**	**PV475467**	**PV463356**	**PV463329**	**PV463303**	**PV463276**	This study
C. sommerladi (2)*	IEBR A.2015.57	KX064040	**PV463225**	**PV475468**	**PV463355**	**PV463328**	**PV463306**	**PV463277**	This study
C. sommerladi (3)	VNUF R.2017.28	PV454673	**PV463226**	**PV475469**	**PV463357**	**PV463326**	**PV463305**	**PV463278**	This study
C. sommerladi (4)	VNUF R.2017.206	PV454674	**PV463227**	**PV475470**	**PV463360**	**PV463327**	**PV463304**	**PV463279**	This study
C. soudthichaki (1)*	VFU R.2015.18	KX077905	**PV463230**	**PV475487**	**PV463383**	**PV463335**	**PV463299**	**PV463260**	This study
C. soudthichaki (2)	IEBR A.2015.34	KX077906	**–**	**PV475488**	**PV463384**	**PV463330**	**PV463288**	**PV463258**	This study
Cyrtodactylus sp*	IEBR KM2012.54	KJ817436	**PV463245**	**PV475478**	**PV463361**	**PV463349**	**PV463290**	**PV463280**	This study
C. thathomensis (1)	ZMMU R14919.2	MG791875	–	–	–	–	–	–	(Nazarov et al. 2018)
C. thathomensis (2)*	ZMMU R14919.3	MG791874	–	–	–	–	–	–	(Nazarov et al. 2018)
C. teyniei (1)*	IEBR KM2012.77	KJ817430	**PV463234**	**PV475472**	**PV463368**	**PV463343**	**PV463295**	**PV463267**	This study
C. teyniei (2)	VNUF R.2016.302	ON145872	**PV463235**	**PV475473**	**PV463367**	**PV463344**	**PV463298**	**PV463256**	This study

* samples used in BEAST analysis; GenBank accession numbers shown in bold identify sequences generated in this study.

Table 2. PCR primers used in this study.

Gene	Primer	Primer sequences (5' – 3')	Reference
COI	VF1d	TTCTCAACCAACCACAARGAYATYGG	(Nazarov et al. 2012)
	VR1d	TAGACTTCTGGGTGGCCRAARAAYCA	
Cytb	L14910	GACCTGTGATMTGAAAACCAYCGTTGT	(Burbrink et al. 2000)
	H16064	CTTTGGTTTACAAGAACAATGCTTTA	
ND2 + tRNA	MetF1	AAGCTTTCGGGCCCATACC	(Macey et al. 1997)
	COIR1	AGRGTGCCAATGTCTTTGTGRTT	(Arevalo et al. 1994)
Rpl35	N66	GCTAAACAAGCACAGAGTTGATCC	(Siler et al. 2010)
	N67	TCAGGCTCAGAAAGRACTATTATGG	
Rag1	R13	TCTGAATGGAAATTCAAGCTGTT	(Groth and Barrowclough 1999)
	CyrRag1	CTCCTTGTGRCTAGAAAGAT	(Nguyen et al. in press)
PDC	PHOF1	AGATGAGCATGCAGGAGTATGA	(Bauer et al. 2007)
	PHOR1	TCCACATCCACAGCAAAAAACTCCT	
Cmos	G73	GCGGTAAAGCAGGTGAAGAAA	(Saint et al. 1998)
	G74	TGAGCATCCAAAGTCTCCAATC	

Sequence alignment and phylogenetic analyses

Three datasets containing mitochondrial fragments (hereafter referred to as mt) (*COI* + *Cytb* + *ND2* + tRNA), nuclear genes (hereafter referred to as nu) (*Cmos* + *Rag1* + *PDC* + *Rpl35*), and combined loci (*COI* + *Cytb* + *ND2* + tRNA + *Cmos* + *Rag1* + *PDC* + *Rpl35* sequences) were analyzed separately. The combined dataset was divided into 21 partitions based on loci and codon positions (first, second, and third). Each gene was initially aligned separately using ClustalX v. 2.1 (Thompson et al. 1997) under the default settings for complete alignment and subsequently concatenated into the three datasets. Data were analyzed using maximum likelihood (ML), as implemented in IQ-TREE v. 2.3.6 (Minh et al. 2020), Bayesian inference (BI), as implemented in MrBayes v. 3.2.7 (Ronquist et al. 2012), and maximum parsimony (MP), as implemented in PAUP v. 4.0b10 (Swofford 2001), to estimate the phylogenetic relationships of the *C. angularis* group. Genetic divergences between members of the group were calculated using uncorrected p-distance in PAUP.

For BI and ML analyses, optimal nucleotide substitution models were determined using the corrected Akaike information criterion (AICc), as implemented in jModelTest v. 2.1.10 (Darriba et al. 2012) and PartitionFinder v. 1.1.1 (Lanfear et al. 2012), with parameters estimated by MrBayes. All the best models selected for the three datasets are shown in Table 3. For BI, two simultaneous analyses with four Markov chains (one cold and three heated) were run for 10^7 generations with a random starting tree, sampling every 1,000 generations. Log-likelihood scores of sample points were plotted against generation time to determine the stationarity of Markov chains. The cutoff point for the burn-in function was set to 25% of the total number of trees generated in the Bayesian analyses. For ML analyses, 10,000 ultrafast bootstrap replications (UFB) were run. For MP analysis, a heuristic analysis was conducted for the combined dataset with 100 random taxon addition replicates using a tree-bisection and reconnection (TBR) branch-swapping algorithm, with no upper limit set for the maximum number of trees saved. Bootstrap (BP) support was calculated using 1,000 pseudoreplicates and 100 random taxon additions. Nodal support was evaluated using UFB, as estimated in IQ-TREE v. 2.3.6, posterior probability (PP), as calculated in MrBayes v. 3.2.7, and BP, as computed in PAUP v. 4.0b10. UFB > 95%, PP ≥ 0.95, and BP ≥ 70% were regarded as strong support for a clade (Hillis and Bull 1993; Ronquist et al. 2012; Hoang et al. 2018).

Time divergence analysis

To calibrate time divergences at the nodes using the relaxed molecular clock method, one operational taxonomic unit (OTU) for each described species and one OTU for the undescribed species were selected. A combined dataset consisting of 26 taxa, including 22 ingroup taxa (21 described species and one undescribed species) and four outgroups, was employed as input for BEAST v. 2.7.6 (Bouckaert et al. 2014), with the substitution models unlinked and the molecular clock and trees linked for each gene partition. Since valid fossil records are presently unavailable for this lineage, secondary calibrations derived from prior studies were utilized (Wood et al. 2012; Grismer et al. 2022). In particular, one calibration point, approximately 27 million years ago (MYA) (log-normal distribution with a median of 27.1), was set at the node representing the split between *C. angularis* + *C. chanhomeae* and all other species within the *C. angularis* group, except for *C. nigriocularis* (Wood et al. 2012). Another calibration point, approximately 33 MYA (log-normal distribution with a median of 33.1), was used for the node representing the split between *C. nigriocularis* and all other species within the *C. angularis* group (Grismer et al. 2022). The use of secondary calibrations might carry an inherent risk of propagating estimation errors.

The substitution models applied to the data matrix were determined using jModelTest v. 2.1.10 (Darriba et al. 2012) (Table 4). As for priors, all parameters were set to their default settings, i.e., log-normal and standard

Table 3. Best-fit models for all the datasets in MrBayes.

Gene			Model		
Mitochondrial dataset					
COI + cytb + ND2 + tRNA					GTR+I+G
Nuclear dataset					
Rpl35 + Rag1 + PDC + Cmos					GTR+G
Combined dataset					
COI + cytb + ND2 + tRNA + *Rpl35 + Rag1 + PDC + Cmos*					GTR+I+G
Partitioned datasets					
COI					
Codon position 1	GTR+I+G	Codon position 2	K81uf+I	Codon position 3	GTR+G
cytb					
Codon position 1	GTR+G	Codon position 2	TVM+I+G	Codon position 3	TIM+I+G
ND2					
Codon position 1	TVM+I+G	Codon position 2	GTR+I+G	Codon position 3	GTR+I+G
Rpl35					
Codon position 1	K81uf+G	Codon position 2	K80+I	Codon position 3	K81uf+G
Rag1					
Codon position 1	TrN	Codon position 2	HKY+G	Codon position 3	TVM+G
PDC					
Codon position 1	TVM+G	Codon position 2	TrN	Codon position 3	F81+I
Cmos					
Codon position 1	TrN	Codon position 2	F81+I	Codon position 3	TVM+G

Table 4. Best-fit models for each gene in BEAST.

Gene	Model	Gene	Model
COI	GTR+I+G (gamma category count = 4, shape = 1.107, proportion invariant = 0.554, rate AC = 0.4791, rate AG = 9.4493, rate AT = 0.4121, rate CG = 0.1979, rate CT = 3.0282, rate GT = 1.0)	*ND2*	GTR+I+G (gamma category count = 4, shape = 0.948, proportion invariant = 0.34, rate AC = 0.3961, rate AG = 3.9842, rate AT = 0.4257, rate CG = 0.2887, rate CT = 2.7116, rate GT = 1.0)
Cytb	TIM1+I+G (gamma category count = 4, shape = 1.589, proportion invariant = 0.481)	*Rag1*	TPM1uf+G (gamma category count = 4, shape = 0.501)
Rpl35	HKY+G (kappa = 2.0, gamma category count = 4, shape = 0.906)	*PDC*	K80+G ((gamma category count = 4, shape = 0.207)
		Cmos	TrN

deviation, except that the Yule process was selected for the tree prior, as recommended in the program manual for a species-level phylogeny. The time calibration analysis was run for 300,000,000 generations, with trees sampled every 5,000 generations. After the dataset with the above settings was analyzed in BEAST, the resulting likelihood profile was then examined using Tracer v. 1.7.1 to confirm that ESS > 200 for all parameters. The final tree with date estimates was computed using TreeAnnotator v. 2.7.6 (Bouckaert et al. 2014), as recommended by the program manual.

Inferring historical biogeography

Bayesian binary MCMC (BBM) analysis (Ronquist and Huelsenbeck 2003; Ali et al. 2012), as implemented in Reconstruct Ancestral State in Phylogenies (RASP v. 4.3) (Yu et al. 2020), and BioGeoBEARS, as implemented in R (Matzke 2013; R Core Team 2024), were used to reconstruct the historical biogeography of the *C. angularis* group. The tree generated from the BEAST program was used as the input data for the analysis. The geographic distribution of the *C. angularis* group was assigned to three areas: (A) northern Annamite, (B) Mekong Lowland, and (C) West Indochina (Bain and Hurley 2011). For BioGeoBEARS, all biogeographic models, including DIVALIKE (a likelihood implementation of the processes assumed by DIVA—Dispersal-Vicariance Analysis), dispersal-extinction-cladogenesis (DEC), and Bayesian analysis of biogeography when the number of areas is large (BayAreaLike), with and without the jump dispersal parameter (j), were run in this study (Ronquist 1997; Matzke 2014). The total number of areas each species was allowed to occupy was set to three.

Results

Phylogenetic analysis

The combined dataset (*COI* + *Cytb* + *ND2* + tRNA + *Cmos* + *Rag1* + *PDC* + *Rpl35*) consisted of 5,275 aligned characters (*COI*: 657 + *Cytb*: 1,031 + *ND2* and tRNA: 1,247 + *Cmos*: 354 + *PDC*: 395 + *Rag1*: 1,038 + *Rpl35*: 553), of which 3,534 were constant, 314 were variable and parsimony-uninformative, and 1,427 were parsimony-informative. In the MP analysis,

after 4,360,791 rearrangements were attempted, a single most-parsimonious tree with 5,417 steps was recovered (consistency index = 0.48; retention index = 0.52). The phylogenetic estimates recovered by ML, MP, BI, and Bayesian partitioned analyses based on the combined dataset are shown in Fig. 1. The aligned mt dataset (*COI* + *Cytb* + *ND2* + tRNA) comprised 2,935 positions, of which 1,476 were constant, 187 were variable and parsimony-uninformative, and 1,272 were parsimony-informative. In the MP analysis, after 4,076,905 rearrangements were attempted, a single most-parsimonious tree with 5,070 steps was recovered (consistency index = 0.45; retention index = 0.55). The phylogenetic results obtained using ML, MP, and BI analyses based on the mt dataset are shown in Fig. 2. The nu matrix (*Cmos* + *Rag1* + *PDC* + *Rpl35*) comprised 2,340 characters, of which 2,058 were constant, 127 were variable and parsimony-uninformative, and 155 were parsimony-informative. In the MP analysis, a single most-parsimonious tree with 342 steps was recovered (consistency index = 0.87; retention index = 0.89). The phylogenetic relationships inferred from the ML, MP, and BI analyses based on the nu dataset are shown in Fig. 3.

Overall, phylogenies generated from the combined (hereafter referred to as mt-nu) dataset were very similar to those produced by the mt dataset and significantly different from those of the nu dataset, especially in terms of the number of unresolved nodes in the trees from the nu dataset. However, there were some areas of agreement among the three datasets. For example, almost all trees from the mt-nu, mt, and nu datasets strongly corroborated the monophyly of the *C. angularis* group, except for the trees from the ML and MP analyses of the mt dataset (UFB = 81 and BP = 65). In addition, analyses of all three datasets consistently recovered Group I, Group II, and Group III as monophyletic, as well as the clades encompassing Groups II–VI, III–VI, and IV–VI, even though MP did not support the monophyly of Group III or the clade consisting of Groups III–VI. Furthermore, the branches in the trees inferred from both analyses of the nu dataset generally received low nodal support. Nonetheless, the monophyly of Groups IV–VI was not corroborated by the nu dataset, in contrast to the phylogenetic signals derived from trees based on the mt and combined matrices (Figs 1–3).

In cases where analyses of both the mt and mt-nu datasets recovered the same deeper nodes, trees based on the mt-nu matrix generally showed higher statistical support for those nodes (Suppl. material 1: file 1). For example, nodes representing the *C. angularis* group, Group II, Group III, and Groups III–VI recovered based on the mt-nu dataset all received higher support values across the analyses compared with those resulting from the mt dataset (Figs 1, 2). When analyzed separately, significant support values for Groups IV–VI were observed only in analyses based on the mt data, whereas analyses of the nu dataset resulted in largely unresolved or weakly corroborated phylogenetic relationships. Moreover, the results recovered based on the nu dataset showed that four species, *C. darevskii*, *C. jaegeri*, *C. roesleri*, and *C. sommerladi*, are paraphyletic, while *C. hinnamnoensis* is polyphyletic (Fig. 3).

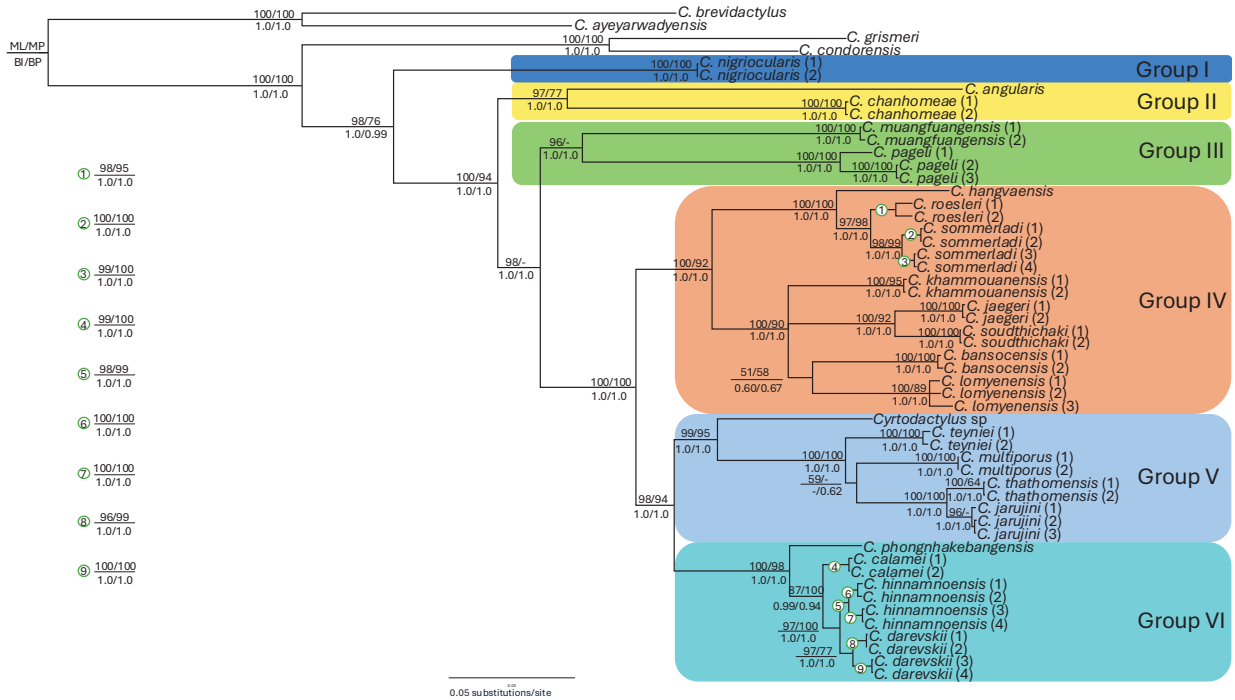

Figure 1. Phylogram inferred from the mixed-model Bayesian analysis, with branch lengths estimated by MrBayes based on three mitochondrial loci, *COI*, *Cytb*, and *ND2*, and four nuclear markers, *Cmos*, *PDC*, *Rag1*, and *Rpl35*. Numbers above and below the branches are ML ultrafast bootstrap/MP bootstrap values and Bayesian single-model posterior probabilities/Bayesian mixed-model posterior probabilities, respectively.

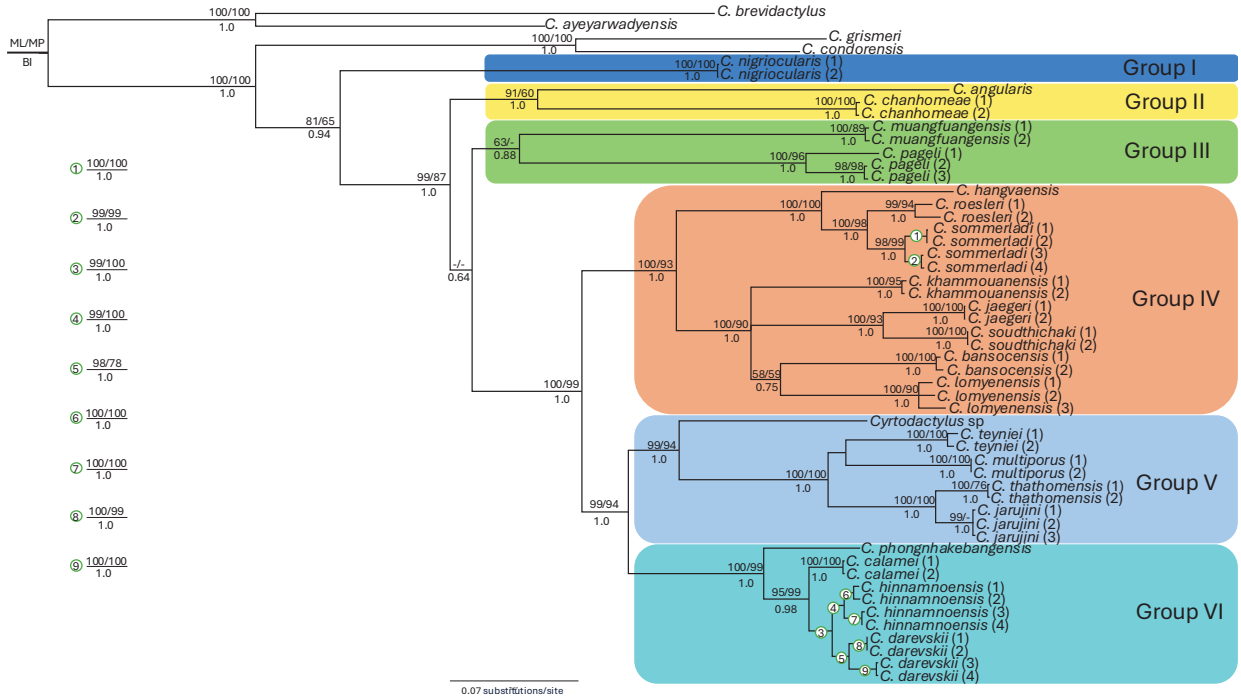

Figure 2. Phylogram inferred from ML and Bayesian single-model analyses, with branch lengths estimated by MrBayes based on three mitochondrial genes, *COI*, Cytb, and *ND2*. Numbers above and below the branches are ML ultrafast bootstrap/MP bootstrap values and Bayesian posterior probabilities, respectively.

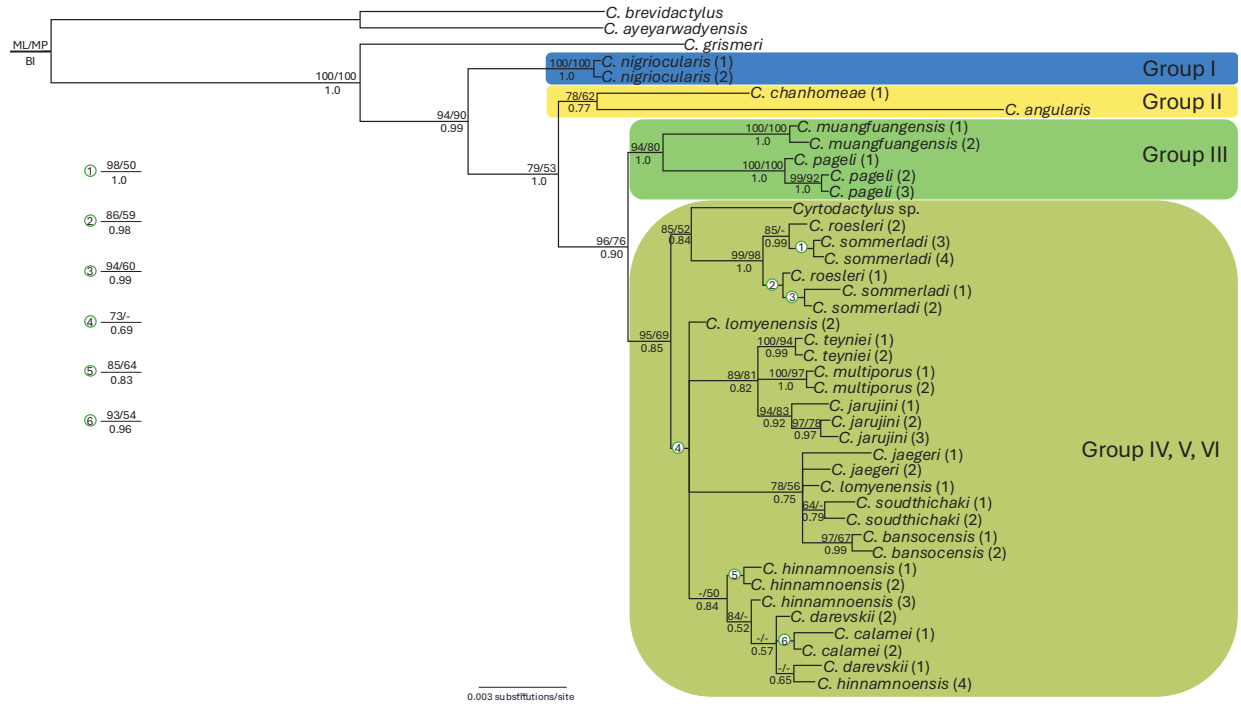

Figure 3. Phylogram inferred from ML and Bayesian single-model analyses, with branch lengths estimated by MrBayes based on four nuclear loci, *Cmos*, *PDC*, *Rag1*, and *Rpl35*. Numbers above and below the branches are ML ultrafast bootstrap/MP bootstrap values and Bayesian posterior probabilities, respectively.

In general, all nodes in the trees from the mt-nu dataset were well corroborated by at least three analyses, except for three poorly supported nodes, i.e., the placements of *C. bansocensis*, *C. khammouanensis*, and *C. multiporus*. *C. nigriocularis* was recovered as the sister species to all other members of the *C. angularis* group. The remaining *C. angularis* species clustered into five major subclades with significant statistical support, except for Group III,

for which the MP analysis did not recover the same relationship: Group II (UFB = 97, BP = 77, PP = 1.0 and 1.0), Group III (UFB = 96, PP = 1.0 and 1.0), Group IV (UFB = 100, BP = 92, PP = 1.0 and 1.0), Group V (UFB = 99, BP = 95, PP = 1.0 and 1.0), and Group VI (UFB = 100, BP = 98, PP = 1.0 and 1.0). When combined with geographic data, the five distinct phylogenetic groups were found to be primarily distributed in distinct areas. Specifically, the earliest-diverging group, Group I, is restricted to Ba Den Mountain, southern Vietnam, and Group II consists of two species from eastern Thailand (*C. angularis* and *C. chanhomeae*). Groups III–VI occur almost exclusively in the northern Annamites, where Group III consists of two species from northern Laos (*C. muangfuangensis* and *C. pageli*), Group IV occupies central Vietnam and eastern Laos (*C. bansocensis*, *C. hangvaensis*, *C. jaegeri*, *C. lomyenensis*, *C. roesleri*, *C. sommerladi*, and *C. soudthichaki*), Group V extends from eastern Thailand (*C. jarujini*) to central and northern Laos (*C. jarujini*, *C. multiporus*, *C. teyniei*, *C. thathomensis*, and one undescribed species), and Group VI ranges from central Laos to north-central Vietnam (*C. calamei*, *C. darevskii*, *C. hinnamnoensis*, and *C. phongnhakebangensis*) (Figs 1, 4A). Within the five groups, the lowest and highest pairwise genetic divergences range from 3.65% (between *C. darevskii* and *C. hinnamnoensis*) to 21.01% (between *C. khammouanensis* and *C. muangfuangensis*) based on fragments of the COI gene; from 3.10% (between *C. darevskii* and *C. hinnamnoensis*) to 22.41% (between *C. chanhomeae* and *C. teyniei*) based on fragments of *Cytb*; and from 2.31% (between *C. darevskii* and *C. hinnamnoensis*) to 22.99% (between *C. angularis* and *C. lomyenensis*) based on fragments of the ND2 gene (Suppl. material 1: file 2).

Divergence dating analysis

The results of divergence time estimation indicated that *C. nigriocularis* diverged from the other members of the *C. angularis* group (Node 3) in the early Oligocene, approximately 29.46–35.48 MYA (Fig. 5, Table 5). Group II, Group III, and Groups IV–VI began to emerge in the Oligocene (Nodes 4 and 5; Fig. 5, Table 5). Two species within Group II also diverged in the late Oligocene (Node 6; Fig. 5, Table 5). The remaining lineages of the *C. angularis* group diverged in the Miocene (23.03–5.3 million years ago) (Fig. 5, Table 5). The most recent speciation event, i.e., the split between *C. hinnamnoensis* and *C. darevskii*, took place in the Pleistocene approximately 2.06 MYA (Node 25; Fig. 5, Table 5). In addition to *C. hinnamnoensis* and *C. darevskii*, *C. calamei*, *C. jaegeri*, *C. jarujini*, *C. roesleri*, *C. soudthichaki*, *C. sommerladi*, and *C. thathomensis* are the youngest members of the group, which started to speciate from the early Pliocene, ca. 4.8–2.75 MYA (Nodes 21–24; Fig. 5, Table 5).

Inferring historical biogeography

The historical biogeographic scenarios inferred from analyses using RASP are presented in Fig. 4B. In the reconstruction of the group's ancestral geographic range, several areas of endemism contributed differentially, with the Mekong Lowland (purple) receiving the highest probability of 73.67%, followed by West Indochina at 12.55% and northern Annamite at 10.91% (Node 43 in the biogeographic tree—Fig. 4B). The most probable ancestral areas for the remaining five groups (Node 42

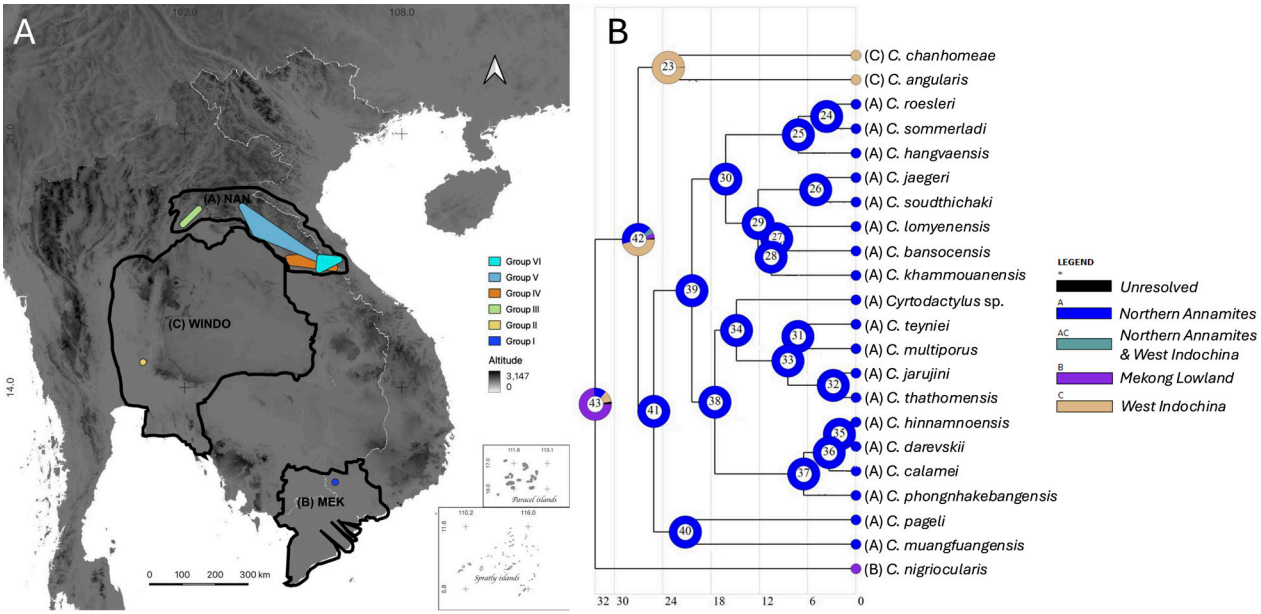

Figure 4. A. Distribution of the *C. angularis* species group (altitude data based on the GADM database of Global Administrative Areas 2021). MEK: Mekong Lowland; NAN: northern Annamites; WINDO: West Indochina; **B.** Divergence time estimation and ancestral area reconstruction of the *C. angularis* species group using the combined dataset. Each node is labeled with a number, which can be used to check statistical details in Suppl. material 1: file 3.

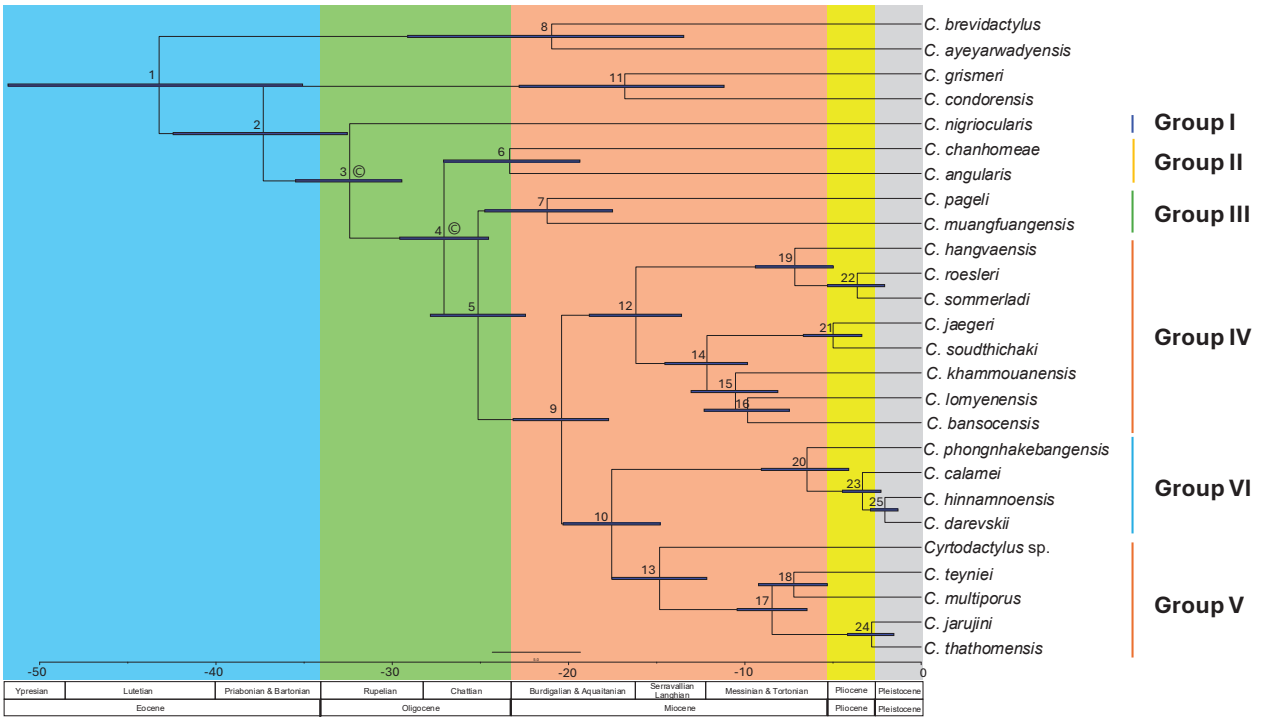

Figure 5. Time calibration using BEAST. The blue bars show 95% highest posterior densities (HPD). (C) represents the calibration points (Nodes 3 and 4).

Table 5. Time calibration for nodes in the phylogeny. Node numbers defined in Fig. 5.

Node	Age estimate (million years)	95% HPD (million years)	Node	Age estimate (million years)	95% HPD (million years)
1	43.22	35.09–51.79	13	14.84	12.16–17.54
2	37.32	32.55–42.43	14	12.15	9.85–14.54
3	32.42	29.46–35.48	15	10.53	8.13–13.05
4	27.07	24.54–29.58	16	9.85	7.48–12.31
5	25.13	22.44–27.84	17	8.46	6.48–10.44
6	23.34	19.36–27.09	18	7.22	5.32–9.23
7	21.21	17.51–24.76	19	7.17	4.98–9.41
8	20.96	13.47–29.12	20	6.48	4.11–9.07
9	20.40	17.73–23.15	21	5.00	3.37–6.68
10	17.55	14.80–20.31	22	3.62	2.07–5.31
11	16.81	11.18–22.80	23	3.33	2.28–4.46
12	16.18	13.59–18.82	24	2.81	1.55–4.18
			25	2.06	1.31–2.88

in the biogeographic tree—Fig. 4B) were northern Annamite (45.66%) and West Indochina (42.43%). The ancestral area with the highest probability for Group II (Node 23 in the biogeographic tree—Fig. 4B) was West Indochina (99.35%). Northern Annamite was recovered as the most probable ancestral area for Groups III, IV, V, and VI (≥99.85%) (Nodes 30, 34, 37, and 40 in the biogeographic tree—Fig. 4B).

The BioGeoBEARS analysis recovered the DIVALIKE model as the best fit to the data and the most likely to infer the correct ancestral range at each node, with the highest LnL and the lowest AIC and AICc (Suppl. material 1: file 4). However, the time-calibrated biogeographic reconstruction using the DIVALIKE model did not provide informative results for the *C. angularis* group (Suppl. material 1: file 5).

Discussion

Phylogenetic relationships

Using both mitochondrial and nuclear markers, which together constitute the largest dataset assembled to date for the species group, a well-corroborated phylogeny was recovered for all members of the *C. angularis* group, except for a few shallow nodes that received low statistical support across all analyses. For the first time, this study provides strong support for six distinct phylogenetic subgroups within the *C. angularis* species group. It is also important to note that while mitochondrial markers produce well-resolved topologies, combining mt and nu data results in significantly enhanced statistical nodal support, especially for the deep nodes due to the slower rate of evolution of nuclear markers, i.e., nodes representing the monophyly of the *C. angularis* species group, the monophyly of Group III, and the monophyly of Groups III–VI.

Consistent with the findings of Grismer et al. (2021a, 2022) using *ND2* and Brennan et al. (2017) and Duong et al. (2024) using a combination of *COI* and *ND2*, the phylogenetic analyses of the combined dataset in this study support the monophyly of the *C. angularis* group. In contrast, in the review study based on the *COI* gene by Ngo et al. (2022), the group was rendered paraphyletic, possibly due to the relatively limited number of base pairs

provided by this marker compared with other genes. This study therefore confirms that, even though *COI* is a suitable marker for DNA barcoding in *Cyrtodactylus*, its relatively short sequence length limits the number of informative characters available to resolve deeper nodes. Overall, the addition of nuclear data substantially improves the support level at the deeper nodes compared with support values reported in previous studies. In particular, unlike previous studies based on limited mitochondrial data or sparse taxon sampling, the results clearly show that all six subgroups are strongly corroborated by at least three independent analyses.

Several discrepancies remain between the results of this study and those reported by Grismer et al. (2021a) and Duong et al. (2024). Specifically, according to the analyses of the mt-nu dataset in this study, *C. pageli* is placed as a sister taxon to *C. muangfuangensis*, and the two species together form a distinct lineage, Group III. Nevertheless, Grismer et al. (2021a) and Duong et al. (2024), using *ND2* and *COI* or a combination of *ND2*, *Rag1*, *MXRA5*, and *PDC*, suggested that *C. pageli* clusters with members of Group II, including *C. angularis* and *C. chanhomeae*. In addition, despite the similar taxon compositions of Groups IV–VI, the phylogenetic positions of *C. bansocensis*, *C. teyniei*, and *C. jaegeri* + *C. soudthichaki* differ among the three studies.

Grismer et al. (2021a), Duong et al. (2024), and the analyses in this study illustrate that the current taxonomy underestimates the diversity of the group. At least six populations, two from Thailand and four from Laos, were shown to be potential new species in the three studies (this study; Grismer et al. 2021a; Duong et al. 2024). Additionally, the broad geographic area across central Indochina suggests that other undescribed species likely occur between the ranges of Groups I and II and those of the remaining groups (Fig. 4A). Furthermore, this study reveals the presence of genetically distinct lineages within several species, including *C. darevskii*, *C. hinnamnoensis*, *C. pageli*, and *C. sommerladi*. Interestingly, the analyses of the nu dataset suggest that at least five species—*C. darevskii*, *C. hinnamnoensis*, *C. jaegeri*, *C. roesleri*, and *C. sommerladi*—are either paraphyletic or polyphyletic (Fig. 3). While the relationships observed in *C. jaegeri* may reflect incomplete lineage sorting, those of the other species may be attributable to historical gene flow between closely distributed taxa. Future studies using genome-wide single nucleotide polymorphisms (SNPs) and microsatellites will be able to shed more light on these issues. The inclusion of genome-wide SNPs can also help resolve the weakly supported shallow nodes identified in this study.

Time calibration and biogeography

The biogeographic reconstruction provides novel hypotheses regarding the origin of the species group and its subsequent colonization and speciation events. In a large-scale biogeographic analysis of the genus, Grismer et al. (2022) suggested that the group, along with seven others, i.e., the *C. chauquangensis*, *C. irregularis*, *C. intermedius*, *C. linnwayensis*, *C. oldhami*, *C. sadansinensis*, and *C. sinyineensis* groups, originated broadly within the Indochinese region but did not specify a more precise ancestral area within Indochina. By assigning the distributions of six strongly supported subgroups to three well-established areas of endemism—the northern Annamites, Mekong Lowland, and West Indochina—the Mekong Lowland was determined to be the most likely ancestral area of the *C. angularis* species group. The time-calibrated molecular results reveal that *C. nigriocularis* and three other clades, Groups II, III, and IV + V + VI, emerged in the Oligocene. The group might have subsequently colonized West Indochina and the Annamites during the late Oligocene, as the two distribution ranges were reconstructed as ancestral areas of Group II and Groups II–VI, respectively. The most recent common ancestors of Groups II–VI, representing a majority of the nodes within the species group (65% or 13/20 nodes), emerged in the Miocene (Figs 4B, 5).

The burst of speciation events within the *C. angularis* group corresponds remarkably well to the development of the East Asian summer monsoon around the Oligocene–Miocene boundary (Li et al. 2021, 2022). During this period, increased precipitation as a result of the monsoon likely accelerated the dissolution of the limestone substrates and deeply influenced the karst landscape (Li et al. 2021, 2022). The formation of open habitats within the newly developed evergreen broadleaf forests may have facilitated the range expansion and diversification of the *C. angularis* group, especially in the northern Annamites. Assuming that the habitat preference of *C. nigriocularis* represents its ancestral state, the group may have undergone a transition from granite caves to karst specialization during the lineage's early evolutionary history. However, this scenario remains hypothetical and requires formal ancestral state reconstruction testing in future studies. The *C. angularis* species group represents one of the two largest karst-associated radiations in the genus *Cyrtodactylus*. The other groups comprise the *C. chauquangensis*, *C. oldhami*, *C. sadansinensis*, and *C. yathepyanensis* species groups, which occur across karst landscapes in China, Laos, Myanmar, Thailand, and Vietnam (Grismer et al. 2021a).

As the *C. angularis* group began dispersing out of the Mekong Lowland to the northern Annamites and eastern Thailand (West Indochina) during the late Oligocene, other lineages of animals, fungi, and plants were also colonizing karst landscapes across subtropical East Asia (Loria et al. 2022). During the Miocene, these geckos appear to have adapted successfully to the new habitat and subsequently undergone multiple speciation events (Fig. 5). The Miocene must also have been a significant epoch for other taxa endemic to the Annamites, as several distinctive and endemic species evolved in the mountain range during this time, including the cavernicolous genus of scorpions, *Aemngvantom*, in the early Miocene,

ca. 18 MYA (Loria et al. 2022); Saola in the mid-Miocene, approximately 16–12 MYA (Hassanin et al. 2012; Garcia-Erill et al. 2025); and the Laotian rock rat in the late Miocene, approximately 7 MYA (Nicolas et al. 2012; Le et al. 2016). As the favorable climatic conditions characterized by higher rainfall diminished and vegetation cover shifted approximately 4 MYA (Li et al. 2022), diversification within the *C. angularis* species group also declined. Nonetheless, the central Annamites continue to receive higher levels of rainfall than the northern and southern regions and remain dominated by evergreen forests, conditions that may help sustain ongoing ecological and evolutionary processes for many endemic and evolutionarily distinct species (Sterling et al. 2006; Le et al. 2015).

Conclusion

Using multiple molecular markers, including mitochondrial and nuclear loci, this study has provided new insights into the evolutionary and biogeographic history of one of the largest radiations within the speciose gecko genus *Cyrtodactylus* in Indochina. In particular, multiple speciation events within the *Cyrtodactylus angularis* species group during the Miocene may have been strongly influenced by physical factors, i.e., changes in paleoclimate patterns and the limestone substrate, and the subsequent large-scale expansion of evergreen forest habitats in the Miocene. Although previous studies have reported similar results showing that several unique taxa, including mammals and insects, in the Annamites emerged in the same geological era, none has formally evaluated changes in speciation events during this period. Further research on other ancient groups, both animals and plants, in the region is expected to reveal additional evolutionary patterns in this remarkable yet understudied mountain range. The findings also add to a growing body of evidence highlighting the importance of the landscape in maintaining the region's unique fauna and flora.

Acknowledgments

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Artificial Intelligence (AI) use

The authors accept full responsibility for the content of the manuscript, including the disclosure of any use of AI.
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Author contributions

Hanh Thi Ngo: Conceptualization (equal), data curation (lead), formal analysis (lead), methodology (equal), visualization (lead), writing—original draft (equal), writing—review and editing (equal). Duy Nghia Pham: Methodology (equal), writing—review and editing (equal). Anh Thi Ngoc Ho: Data curation (supporting), methodology (equal), writing—review and editing (equal). Vinh Quang Luu: Investigation (equal), writing—review and editing (equal). Van Thi Hong Nguyen: Methodology (equal), writing—review and editing (equal). Truong Quang Nguyen: Investigation (lead), writing—review and editing (equal). Cuong The Pham: Investigation (equal), writing—review and editing (equal). L. Lee Grismer: Writing—review and editing (equal). Thomas Ziegler: Writing—review and editing (equal). Minh Duc Le: Conceptualization (lead), data curation (equal), formal analysis (equal), methodology (equal), visualization (equal), writing—original draft (equal), writing—review and editing (equal), funding acquisition (lead).

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

Supplementary material 1

Additional information

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Data type: pdf

Explanation note: **file 1.** Comparison of nodal support between analyses based on different datasets. **file 2.** Uncorrected p-distance of species in the *C. angularis* group (highlighted in bold are the lowest and highest percentages). **file 3.** Detailed output from the S-DIVA analysis. **file 4.** Model testing for the BioGeoBEARS analysis with and without found-event speciation (+J). **file 5.** Time-calibrated biogeographic reconstruction of the DIVALIKE model from the BioGeoBEARS analysis.

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