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Colorful patterns indicate common ancestry in diverged tiger beetle taxa: Molecular phylogeny, biogeography, and evolution of elytral coloration of the genus *Cicindela* subgenus *Sophiodela* and its allies ☆

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ABSTRACT

We investigated the phylogenetic relationships among tiger beetles of the subtribe Cicindelina (=*Cicindela* s. lat.; Coleoptera: Cicindelidae) mainly from the Oriental and Sino-Japanese zoogeographic regions using one mitochondrial and three nuclear gene sequences to examine the position of the subgenus *Sophiodela*, currently classified in the genus *Cicindela* s. str., their biogeography, and the evolution of their brilliant coloration. The subgenus *Sophiodela* was not related to the other subgenera of *Cicindela* s. str. but was closely related to the genus *Cosmodela*. In addition, the Oriental genus *Calochroa* was polyphyletic with three lineages, one of which was closely related to *Sophiodela* and *Cosmodela*. The clade comprising *Sophiodela*, *Cosmodela* and two *Calochroa* species, referred to here as the *Sophiodela* group, was strongly supported, and most species in this clade had similar brilliant coloration. The *Sophiodela* group was related to the genera *Calomera*, *Cicindela* (excluding *Sophiodela*) and *Cicindelidia*, and these were related to *Lophyra*, *Hipparidium* and *Calochroa*, except species in the *Sophiodela* group. Divergence time estimation suggested that these worldwide Cicindelina groups diverged in the early Oligocene, and the *Sophiodela* group, which is found in the Oriental and Sino-Japanese zoogeographic regions, in the mid Miocene. Some components of the elytral pattern related to maculation and coloration in the Cicindelina taxa studied contained weak, but significant, phylogenetic signals and were partly associated with habitat types. Therefore, the brilliant coloration of the *Sophiodela* was related to both phylogeny and habitat adaptation, although the function of coloration needs to be studied.

1. Introduction

Diversity in insects is partly represented by extensive variation in color patterns. Although similarities in color patterns are the first clue to recognizing relatedness among species, it has also been well documented that distantly related species exhibit similar color patterns as a result of convergence (e.g., mimicry; Wickler, 1968). In contrast, genital characters, especially of males, are often more reliable clues for discriminating closely related species and for discriminating groups of related species (e.g., genera) based on homologous genital characters. However, identifying homologous characters and correctly evaluating character states for complex genitalia are difficult tasks given that genital morphology can diversify rapidly under sexual selection (Eberhard, 1985; Hosken and Stockley, 2004). Therefore, classifications based on genital characters may not necessarily result in correct groupings of species. Thus, molecular phylogenetic analysis is ultimately needed to resolve phylogenetic relationships among diverse species of insects.

Tiger beetles (Coleoptera: Cicindelidae) are an iconic group of the hyper-diverse insect order Coleoptera, comprising approximately 2300 species (Pearson and Vogler, 2001). They provide intriguing materials for the study of character evolution, including body coloration and biogeography (Cassola and Pearson, 2000; Pearson and Vogler, 2001). Previous molecular phylogenetic studies have revealed that basal groups of Cicindelidae are confined to different continents and generally possess dark coloration, whereas the most derived group of this family, the subtribe Cicindelina (=*Cicindela* s. lat.), is widely distributed worldwide and comprises some 1000 species of various coloration (Vogler

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and Barraclough, 1998). The genus *Cicindela* is a major group in Cicindelina, and some species exhibit brilliant coloration with a metallic luster, which is a structural coloration produced by multiple transparent reflecting layers (Shelford, 1917; Schultz, 1991). Such a brilliant metallic coloration may confuse or deceive predators in habitats with contrasting patches of illumination (Schultz, 1986, 1991). A distinct group in *Cicindela* with brilliant coloration is the subgenus *Sophiodela* confined to Asia in the Oriental and Sino-Japanese zoogeographic regions (Wiesner, 1992; zoogeographic regions follow Holt et al., 2013). A molecular phylogenetic study of Cicindelina (=*Cicindela* s. lat.) revealed that *Cicindela* (*Sophiodela*) is not related to other *Cicindela* species studied, but is sister to *Cosmodela* (Pons et al., 2004). *Cosmodela* is centered in the Oriental region and generally has brilliant coloration like that of *Sophiodela*. Although taxon sampling by Pons et al. (2004) was limited (one species from each of *Sophiodela* and *Cosmodela*; six species from *Cicindela* excluding *Sophiodela*), this result suggests that *Sophiodela* and *Cosmodela* are sister groups, distinct from *Cicindela*. Further, among diverse Oriental species of Cicindelina, the genus *Calochroa* includes species with similar coloration to that of *Sophiodela*/*Cosmodela*. *Calochroa* appears to be a heterogeneous group, considering that Rivalier (1961) recognized six groups within this genus, and it may be polyphyletic.

In this study, we aimed to reveal the phylogenetic relationships of *Sophiodela* with its related groups in the subtribe Cicindelina in terms of molecular phylogeny as suggested by clade I in Pons et al. (2004). Clade I consists of *Cicindela* (s. str.), *Cicindelidia*, *Lophyridia* (=*Calomera*), *Cosmodela*, *Calochroa*, *Hipparidium* and *Lophyra*, in addition to *Cicindela* (*Sophiodela*). Specifically, we intended to assess whether *Sophiodela* was most closely related to *Cosmodela* and a part of *Calochroa* with similar color patterns compared with the other *Cicindela* species, thus determining how similarity in color pattern in these tiger beetles reflects phylogeny rather than parallel evolution due to adaptation to habitat. In addressing the evolution of color pattern we also estimated divergence time and discussed the biogeography of clade I, which includes *Sophiodela*.

2. Materials and methods

2.1. Taxon sampling

We analyzed samples from genera/subgenera in clade I of Pons et al. (2004), namely *Cicindela*, including the subgenus *Sophiodela*, *Cicindelidia*, *Lophyridia* (=*Calomera*), *Cosmodela*, *Calochroa*, *Hipparidium* and *Lophyra*; clade I was sister to a group comprising *Jansenia*, *Chaetotaxis* and *Taenidia*, which can be regarded as outgroup taxa. We analyzed samples from all taxa except *Taenidia*; we also used *Cylindera* species (clade II in Pons et al., 2004) as potential outgroup taxa in addition to *Jansenia* and *Chaetotaxis*. Together, we used a total of 178 samples from 75 species (Table 1; see Supplementary Table S1 for details). The subgenus *Sophiodela* includes three species, *C. cyanea*, *C. chinensis* with four subspecies and *C. ferriei*. We could not include *C. cyanea* in our analysis as this species has not been collected recently. Adult beetles preserved in 99% ethanol were used for DNA extraction.

2.2. DNA sequencing

Total genomic DNA was extracted from the thorax muscles using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Regions of the mitochondrial cytochrome oxidase subunit I (COI), nuclear 28S rDNA (28S), elongation factor 1a F2 copy (EF-1a) and wingless (wg) genes were PCR-amplified, and the PCR products were sequenced using an ABI 3031xl sequencer (Applied Biosystems, Foster City, CA, USA). Primers used for PCR and direct sequencing were as follows. COI: forward COS2183N (5′-CAR CAY YTA TTY TGR TTY TTY GG-3′) and reverse COA3107 (5′-TCT ATT ARD GGD GAD GCD CTA TCT TG-3′) (Sota and Hayashi, 2004) or forward C1J-2195 (5′-TTG ATT TTT TGG TCA TCC AGA AGT-3′) and reverse TN2N-3014 (5′-TCC AAT GCA CTA ATC TGC CAT ATT A-3′) (Simon et al., 1994); EF-1a: forward EF-1a-cicindela-F (5′-GGA CAC AGA GAT TTC ATC AAR AA-3′) and reverse EF-1a-cicindela-R (5′-CAA AGC TTC RTG RTG CAT TT-3′) (this study); 28S: forward 28S-01 (5′-GAC TAC CCC CTG AAT TTA AGC AT-3′) and reverse 28S-01R (5′-GAC TCC TTG GTC CGT GTT TCA AG-3′) (Kim et al., 2000); wg: forward LepWg1 (5′-GAR TGY AAR TGY CAY GGY ATG TCT GC-3′) and reverse ModLepWg2 (5′-ACT ICG CAR CAC CAR TGG AAT GTR CA-3′) (Brower and DeSalle, 1998). Sequences used in this study were deposited in the DNA Data Bank of Japan (DDBJ; accession numbers: AB821879–AB821950, LC020251–LC020264, LC020266–LC020396).

2.3. Phylogenetic analysis

The COI and wg gene sequences were manually aligned unambiguously, and 28S and EF-1a sequences were aligned using the program MUSCLE ver. 3.8.31 (Edgar, 2004). A maximum-likelihood (ML) analysis of the combined matrix was conducted using RAxML version 8.0.0 (Stamatakis, 2014). The sequence data were divided into 11 partitions, including three codon positions each of COI, wg and EF-1a exon, along with EF-1a intron and the entire 28S sequence. The substitution model GTR+G (general time reversible model with gamma distribution for rate heterogeneity) was applied to each partition because this model is the most general and versatile model, and no computational problems due to over-parameterization occurred in our study (see also The RAxML v.8.0.X Manual by A. Stamatakis, 2014). We used PartitionFinder v1.1.1 (Lanfear et al., 2012) to select the optimal partition scheme for 11 partitions. We used four "user schemes" with 4–11 partitions to avoid the combination of partitions from different genes. The substitution model was limited to GTR+G. All analyses using the AIC, AICc, and BIC criteria resulted in the maximally partitioned scheme. RAPID ML analysis with 1000 bootstrapping analyses was used to obtain an ML tree and bootstrap percentages of nodes. In addition, Bayesian inference (BI) of phylogeny was conducted using MrBayes version 3.2 (Ronquist et al., 2012) with the same partitioning scheme and substitution models as in the RAxML analysis. Two runs of four Metropolis-coupled Markov chain Monte Carlo (MCMC) iterations were conducted for 10 million generations with sampling of trees every 1000 generations. We ensured that the potential scale reduction factor (PSRF) approached 1 for all parameters and that the average standard deviation of split frequencies was less than 0.01. We also used Tracer version 1.6 (Rambaut et al., 2014) to ensure that convergence was reached for the posterior distributions of the parameter estimates, and the effective sample size (ESS) of these estimates was >200. A 50% majority rule consensus tree after initial 25% generation data were discarded as burn-in.

To estimate divergence time and construct a ultrametric tree for analyzing the evolution of elytron color pattern, we constructed a calibrated species tree using BEAST ver. 1.8.0 (Drummond et al., 2012). This analysis included 59 samples representing different taxa with data for all genes (except a few taxa that had no EF-1a data). Sequence partitions and substitution models were the same as in the previous ML analysis, different partitions were unlinked for the substitution models, and different genes were unlinked for the clock models, but linked for topology. For the tree prior, we used a speciation model following the Yule process. To enable calibration of divergence time, the mean clock rate of the COI gene was set to 0.0177 referring to a divergence rate of 3.54% per million years for insect mitochondrial genes, estimated based on a

Table 1
List of study taxa, distribution range, habitat type, sample locality and sequenced samples. See Table S1 for more information about sequences.

Taxon	Distribution	Habitat	Sample locality	No. sequenced[a]
Calochroa assamensis	S, SE Asia	Moist forest floor	Bangladesh	2*
Calochroa bicolor	S, SE Asia	Moist forest floor	Bangladesh	2*
Calochroa bramani	SE Asia	Moist forest floor	Thailand	2*
Calochroa carissima	SE Asia	Sandy water edge	Laos	3*
Calochroa corbetti	Myanmar	Moist forest floor	Myanmar	1⁻
Calochroa elegantula	SE Asia	Moist forest floor	Thailand	3*
Calochroa flavomaculata	E, SE, S Asia	Muddy water edge	Bangladesh, Laos, Nepal	3*
Calochroa harmandi	SE Asia	Moist forest floor	Laos	1*
Calochroa interruptofasciata	E, SE Asia	Moist forest floor	Thailand	2*
Calochroa mariae	Myanmar	Moist forest floor	Myanmar	1⁻
Calochroa myinthlaingi	Manmar	Moist forest floor	Myanmar	2⁻
Calochroa octonotata	S, SE Asia	Sandy water edge	Nepal	2*
Calochroa pseudosiamensis	SE Asia	Moist forest floor	Laos	1*
Calochroa salvazai	SE Asia	Moist forest floor	Laos	1*
Calochroa schillhammeri	Myanmar	Moist forest floor	Myanmar	2⁻
Calochroa chatthinensis	Myanmar	Moist forest floor	Myanmar	2⁻
Calomera angulata	E, SE, S Asia	Sandy water edge	Japan, Philippines	4*
Calomera chloris	E, S Asia	Sandy water edge	Nepal	3⁻
Calomera cristipennis	Madagascar	Sand dune	Madagascar	2*
Calomera fimbriata imperatrix	Africa	Sand dune	Zambia	1*
Calomera funerea	S, SE Asia	Sandy water edge	Nepal	1*
Calomera funerea multinotata	Indonesia	Sandy water edge	Indonesia (Sulawesi)	1⁻
Calomera lacrymosa	Philippine	Sandy water edge	Philippines	2⁻
Calomera plumigera	India to China	Sandy water edge	Nepal	1*
Chaetodera laetescripta	Far East, Japan	Sand dune	Japan, South Korea	4⁻
Cicindela (*Cicindela*) *coerulea nitida*	E Asia	Stream edge	China	1*
Cicindela (*Cicindela*) *gemmata*	E Asia	Stream edge	Japan	2*
Cicindela (*Cicindela*) *hirticollis*	N America	Stream edge	USA	1*
Cicindela (*Cicindela*) *hybrida raparia*	Europe; E Asia	Stream edge?	Italy	1*
Cicindela (*Cicindela*) *japana*	E Asia	Open forest floor	Japan	2*
Cicindela (*Cicindela*) *lewisii*	E Asis	Saline flat	Japan, South Korea	3*
Cicindela (*Cicindela*) *repanda*	N America	Stream edge	Canada	2*
Cicindela (*Cicindela*) *restricta*	E Asia	Stream edge?	Mongolia	1*
Cicindela (*Cicindela*) *sachalinensis*	E Asia	Alpine grassland	Japan, Russia	2*
Cicindela (*Cicindela*) *sexguttata*	N America	Moist forest floor	Canada	2*
Cicindela (*Cicindela*) *sylvatica*	Europe; E Asia	Open forest floor?	Mongolia	1*
Cicindela (*Cicindela*) *transbaicalica*	E Asia	Sandy water edge	China, Japan, South Korea	5*
Cicindela (*Pachydela*) *scutellaris*	N America	Sand dune	USA	2*
Cicindela (*Sophiodela*) *chinensis chinensis*	E Asia	Open forest floor	China	7*
Cicindela (*Sophiodela*) *chinensis flammifera*	E Asia	Open forest floor	South Korea	4⁻
Cicindela (*Sophiodela*) *chinensis japonica*	E Asia (Japan)	Open forest floor	Japan	9*
Cicindela (*Sophiodela*) *chinensis okinawana*	E Asia (Japan)	Open forest floor	Japan (Okinawa I.)	3*
Cicindela (*Sophiodela*) *ferriei ferriei*	E Asia (Japan)	Open forest floor	Japan (Amami-oshima I., Amami Is,)	2*
Cicindela (*Sophiodela*) *ferriei indigonacea*	E Asia (Japan)	Open forest floor	Japan (Tokunoshima I., Amami Is,)	3*
Cicindela (*Tribonia*) *tranquebarica*	N America	Open forest floor	USA	2*
Cicindelidia carthagena jamaicana	N, C America	Ocean beach	Jamaica	2*
Cicindelidia obsoleta	N America	Grassland	USA	1*
Cicindelidia ocellata	N America	Stream edge	USA	2*
Cicindelidia punctulata chihauhuae	N America	Sandy water edge	USA	1⁻
Cicindelidia punctulata punctulata	N America	Stream edge	USA	2*
Cicindelidia rufiventris	N America	Open forest floor	USA	1*
Cosmodela aurulenta aurulenta	E, SE, S Asia	Stream edge	Malaysia	4*
Cosmodela aurulenta juxtata	E, SE, S Asia	Stream edge	China, Viet Nam, Laos	10⁻
Cosmodela batesi	E Asia	Open forest floor	Taiwan, Japan (Iriomote I.)	5*
Cosmodela duponti bramanica	Thailand	Open forest floor	Thailand	2*
Cosmodela duponti duponti	E, SE, S Asia	Open forest floor	Bangladesh, Viet Nam	4⁻
Cosmodela fleutiauxi	S Asia	Stream edge	Nepal	2*
Cosmodela intermedia	Afghanistan to India, Nepal	Stream edge	India	2*
Cosmodela separata	E Asia,	Stream edge	China	4*
Cosmodela setosomalaris	E Asia	Stream edge	China	1*
Cosmodela sp. cf. separata	SE Asia	Stream edge	Viet Nam	2*
Cosmodela virgula	E, SE, S Asia	Stream edge	Nepal	2*
Cylindera elisae	Far East	Sandy water edge	Japan	1*
Cylindera humerula	Japan (Okinawa)	Moist forest floor	Japan	1*
Cylindera viduata	India to Papua New Guinea	Sandy water edge	Nepal	1*
Hipparidium alluaudi	Madagascar	Open forest floor	Madagascar	1*
Hipparidium conturbatum	Madagascar	Open forest floor	Madagascar	1*
Hipparidium equestre	Madagascar	Open forest floor	Madagascar	2*
Jansenia myanmarensis	Myanmar	Moist forest floor	Myanmar	1*
Lophyra (*Lophyra*) *cancellata*	S. SE Asia	Sandy water edge	Laos	2⁻
Lophyra (*Lophyra*) *fuliginosa*	S. SE Asia	Sandy water edge	Thailand	2*
Lophyra (*Lophyra*) *abbreviata*	Madagascar	Sandy water edge	Madagascar	2⁻
Lophyra (*Lophyra*) *neglecta*	Africa	Sandy water edge	Zambia	2⁻
Lophyra (*Lophyra*) *tetradia*	Madagascar	Sandy water edge	Madagascar	1⁻

(*continued on next page*)

Table 1 (*continued*)

Taxon	Distribution	Habitat	Sample locality	No. sequenced[a]
Lophyra (*Lophyra*) *perrieri*	Madagascar	Sandy water edge	Madagascar	2⁻
Lophyra (*Spilodia*) *atkinsonii*	Myanmar	Moist forest floor	Myanmar	1⁻
Lophyra (*Spilodia*) *lineifrons*	S. SE Asia	Sandy water edge	Laos, Myanmar	2⁻
Lophyra (*Spilodia*) *striolata*	E, SE, S Asia	Moist forest floor	Bangladesh, Viet Nam, Japan	4*
Lophyra (*Spilodia*) *vittigera*	S Asia	Stream edge	Nepal	1*

[a] Asterisks indicate taxa included in species tree.

relatively ancient geographic event (9–12 mya) compared to other estimations (Papadopoulou et al., 2010). This rate is comparable with a COI divergence rate of 3.34% per million years for a tiger beetle group (Pons and Vogler, 2005). For comparison, we used the estimated node age of *Cicindela* clade I (Pons et al., 2004). Following the range of the estimated node age, 8.4–10.9 mya, we set the node age prior as a normal distribution with mean = 9.65 and *SD* = 0.965. Note that Pons et al. (2004) used a recent Pleistocene geographic event for calibration, and this could give a markedly different age estimation compared with our first method. We attempted analyses with both a strict clock and an uncorrelated lognormal relaxed clock and compared the results using AICM (Baele et al., 2012) in TRACER ver. 1.6. In all cases, the latter clock model gave a better fit, so we report the results for that model. A MCMC run was conducted for 50 million generations, sampling every 5000 generations, and the results were checked using TRACER version 1.6. A species tree was obtained as a most credible tree after removing the initial 1000 trees as burn-in using TreeAnnotator version 1.8.0 in BEAST.

2.4. Color pattern

To investigate the evolution of elytral color pattern in relation to habitat type, we took a digital image of a representative specimen from each taxon using a digital microscope (Keyence VHX, Osaka, Japan) under standardized conditions and analyzed the image using ImageJ ver. 1.49 (U.S. National Institutes of Health, Bethesda, MD, USA, http://imagej.nih.gov/ij/). From each image, we cut out the left elytron and determined the number of maculations and the proportion of maculation area. In addition, for the entire elytra area, we obtained the mean and standard deviation of the gradation level for the primary colors red, blue and green from the single-channel histograms (the frequency distribution of gradation level [0–255] at pixels for each primary color). We conducted a principal component analysis using the statistical package JMP ver. 10.0.0 (SAS Institute, Carry, NC) to summarize elytral coloration with principal components from the six RGB gradation level variables.

To examine whether the diversity in the elytral maculation and coloration contains a phylogenetic signal (i.e., the tendency for related species to resemble each other more than they resemble species drawn at random from a phylogenetic tree; Blomberg et al., 2003), we calculated the *K*-statistic of Blomberg et al. (2003) using the package phytools with APE (Paradis et al., 2004) in R using the BEAST tree obtained above. A randomization test with 1000 simulations was used to test the null hypothesis of $K = 0$. The *K* value can range from zero to infinity: a *K* that is significantly greater than zero 0 indicates that there is a significant phylogenetic signal. In particular, $K = 1$ means that there is a strong phylogenetic signal expected when the trait has evolved according to the Brownian motion model of evolution.

The main habitat type of each species was classified following Pearson et al. (1988) and Vogler and Goldstein (1997) with some modifications. To examine the association of elytral maculation and coloration with habitat type, we conducted a comparative phylogenetic analysis with the generalized estimating equation (GEE; Paradis and Claude, 2002) using the BEAST tree obtained above. We used the compar.gee function implemented in the R-package APE (Paradis et al., 2004).

3. Results

3.1. Phylogenetic relationships

We obtained a combined data matrix with 2944 aligned positions for data comprising 739 bp COI, 966 bp 28S, 501 bp wg, 530 bp EF-1a exon and 208 bp EF-1a intron. The ML and BI analyses resulted in similar topologies (Fig. 1, ML tree; Supplementary Fig. S1, BI tree). These trees recovered monophyly of *Cosmodela*, *Sophiodela*, *Cicindelidia* and *Hipparidium* with high branch support values, and *Calomera* with a weak value, but not of the genera *Cicindela*, *Calochroa* and *Lophyra*. Although *Sophiodela* is a subgenus of *Cicindela*, it was not related to the other subgenera of *Cicindela* evaluated, namely *Cicindela*, *Pachydela* and *Tribonia*; these three formed a clade including the genus *Cicindelidia*. Within *Sophiodela* in East Asia and Japan, two Japanese subspecies of *Cicindela chinensis*, *C. c. japonica* and *C. c. okinawana*, were sister to each other, derived from the mainland *C. chinensis*. *Calochroa* was polyphyletic, divided into three groups, assigned as groups A–C in Fig. 1. Group C, which formed a strongly supported clade with *Sophiodela* and *Cosmodela*, comprised *Calochroa octonotata* and *C. carissima* and corresponds to group II of Rivalier (1961). The other two *Calochroa* lineages were related to *Lophyra*, *Hipparidium* and *Chaetodela*. *Calochroa* group B comprised 10 species from Rivalier's (1961) groups III, IV and V, and group A comprised four species from Rivalier's (1961) groups I and V. We found that the subgenus *Sophiodela*, genus *Cosmodela*, and part of *Calochroa* form a monophyletic clade and refer it hereafter as the *Sophiodela* group.

3.2. Divergence time

The divergence time estimation with the uncorrelated lognormal relaxed clock model was based on the mitochondrial COI gene evolutionary rate (0.0177 per million years); the resultant mean evolutionary rates (ucld means) for other gene partitions were 0.00122 for wg, 0.00126 for EF-1a exon, 0.00211 for EF-1a intron, and 0.00146 for 28S. This analysis gave the age of the most recent common ancestor (MRCA) of the *Sophiodela* group as 12.5 mya (mid Miocene; 95% HPDI [highest probability density interval], 8.8–16.6 mya), and the age of clade I MRCA as 33 mya (early Oligocene; 95% HPDI, 25–43 mya) (Fig. 2). These estimated ages are much older than the divergence time estimation based on the clade I age prior according to Pons et al. (2004); the age of MRCA of the *Sophiodela* group was estimated to be 3.2 mya (Pliocene; 95% HPDI, 2.1–4.4 mya), with the clade I age of 8.9 mya (mid Miocene; 95% HPDI, 7.0–11.0 mya) (Fig. S2).

3.3. Evolution of color pattern

There was a significant phylogenetic signal for the proportion of maculation area ($K = 0.22$, $P = 0.012$), but not for the maculation number ($K = 0.16$; $P = 0.084$). The principal component analysis of

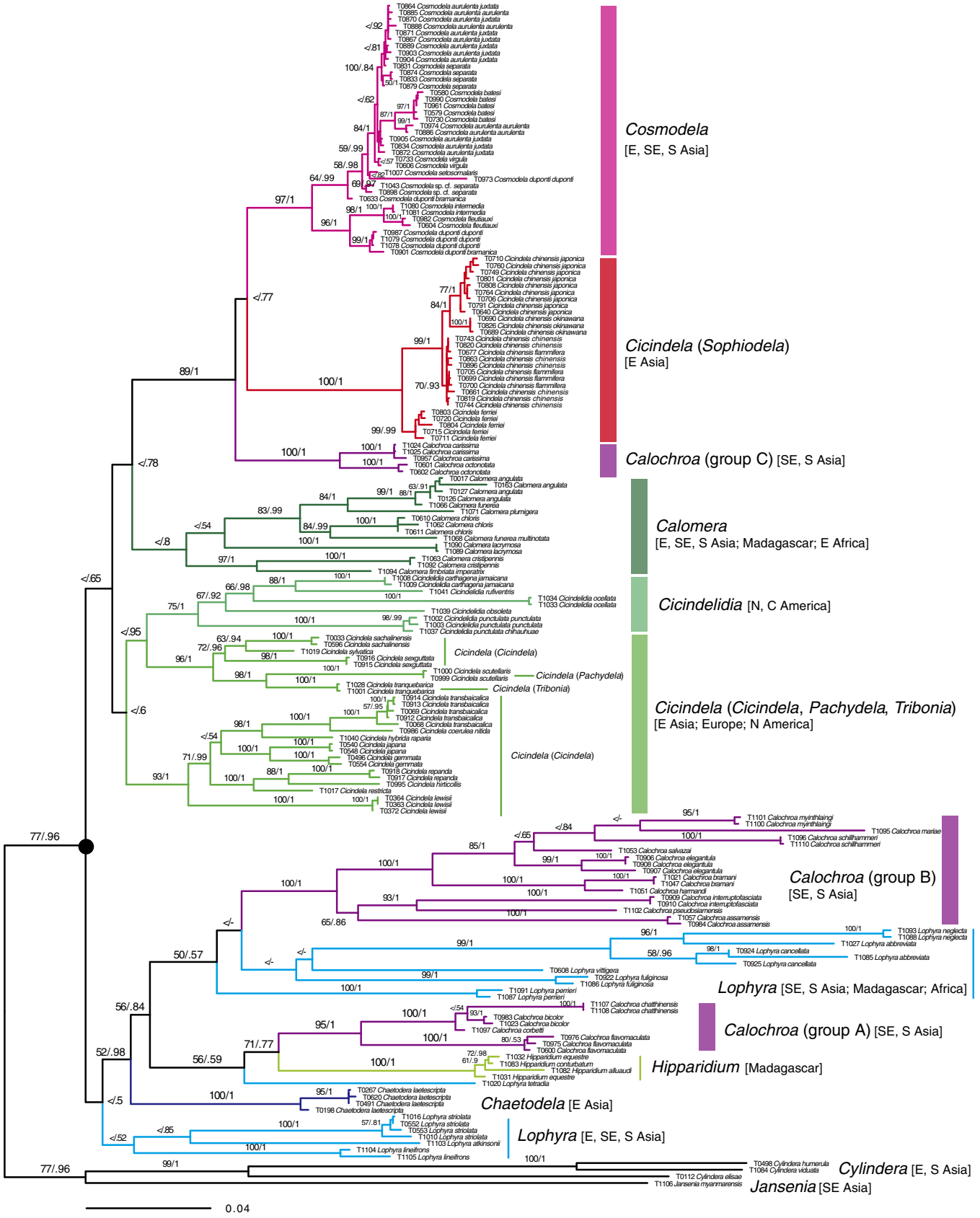

Fig. 1. Maximum-likelihood tree resulting from the combined analysis of four genes. The black circle indicates the ingroup node corresponding to clade I of Pons et al. (2004).

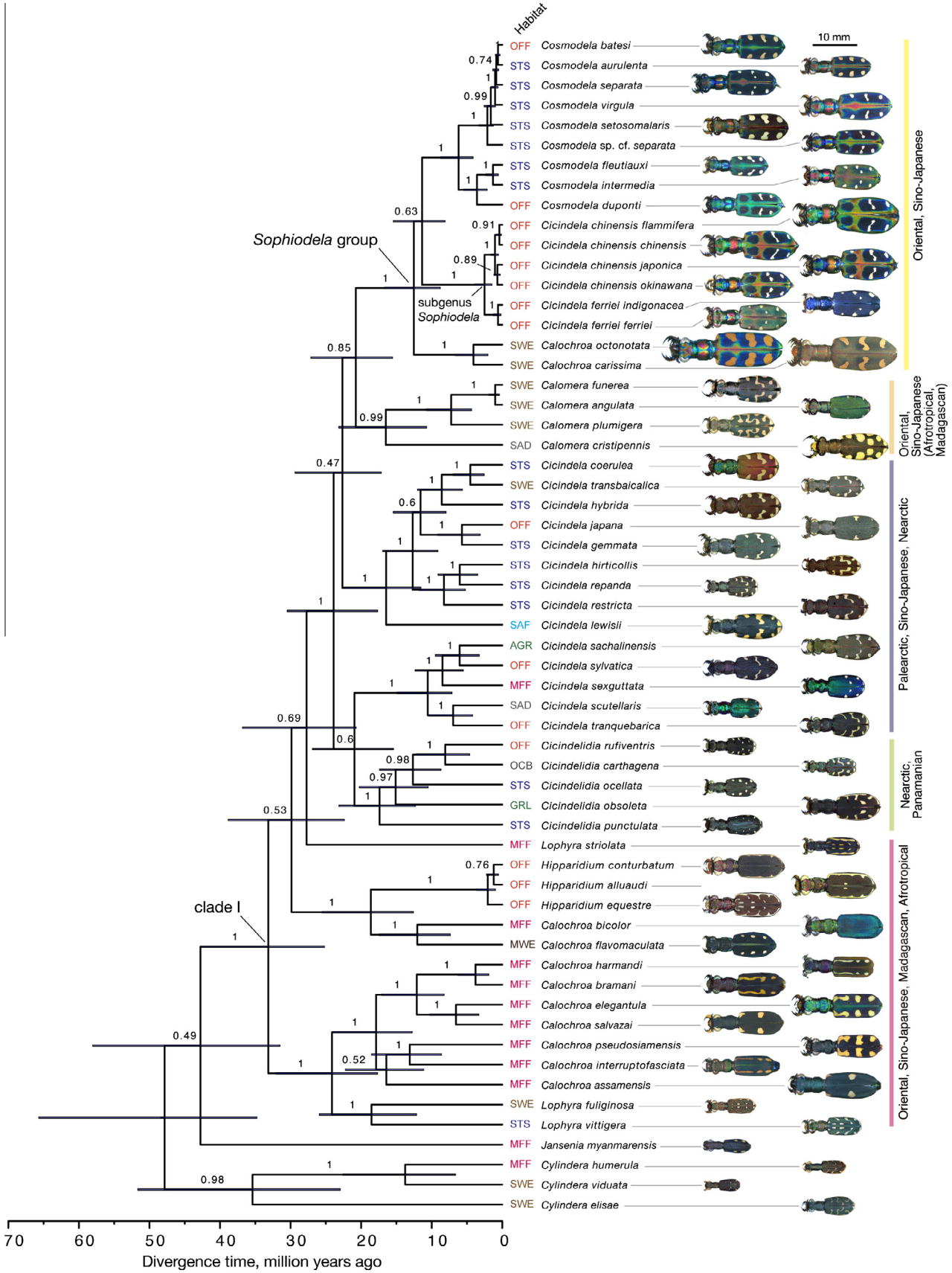

Fig. 2. Phylogenetic tree with divergence times for the 59 taxa used in the analysis of color pattern resulting from a Bayesian relaxed clock analysis with a mean COI clock rate of 0.0177/my. Posterior probabilities of nodes are shown on the branches. Blue bars at nodes show 95% highest probability density intervals (HPDIs) of node ages. For each taxon, habitat types are indicated. Habitat code: STS, streamside; OFF, open forest floor; SWE, sandy water edge; SAD, sand dune; SAF, saline flat; AGF, alpine grassland; MFF, moist forest floor; OCB, ocean beach; GRL, grassland; MWE, muddy water edge. Zoogeographic regions indicated on the right follow Holt et al. (2013). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

elytral coloration based on RGB scores resulted in four significant principal components (PC1–PC4; Fig. 3), which together explained 96.4% of total variance (Table S3). PC1 had positive loadings for all variables, particularly for the *SD* of the RGB gradation levels, and reveals overall vividness. PC2 gave high scores for vivid blue and green, with PC3 for vivid blue, and PC4 for quiet colors with blue and red. These PCs had significant phylogenetic signals, except for PC3 (PC1, $K = 0.22$, $P = 0.008$; PC2, $K = 0.20$, $P = 0.017$; PC3, $K = 0.16$, $P = 0.099$; PC4, $K = 0.18$, $P = 0.039$). Note that the observed K values are far below unity (0.22 at most), suggesting that the phylogenetic signals are not as strong as expected by the Brownian motion evolutionary model. The species of the *Sophiodela* group generally had high PC1–PC3 scores, indicating their colorfulness.

Phylogenetic comparative analyses using a GEE showed that the maculation number, proportion of maculation area, PC1, and PC2 scores were not related to habitat type (i.e., GEE analysis returned no significant effect of habitat type; Table S4). However, the PC3 score was positively related to moist forest floor. Further, PC4 was negatively related to moist forest floor, muddy water edge, open forest floor, and saline flat (Table S4).

4. Discussion

4.1. Phylogenetic relationships

Our results are largely consistent with Pons et al. (2004) for the relationships among taxonomic groups involved in clade I of Pons et al. (2004); *Sophiodela* is sister to *Cosmodela*, and these are more closely related to *Calomera* (=*Lophyridia*) than *Cicindela*. By extending taxonomic sampling, however, our study revealed problems in the grouping of oriental taxa. Most importantly *Calochroa* was polyphyletic, containing at least three different phylogenetic groups, and one of them, comprising *C. octonotata* and *C. carissima*, was closely related to *Cosmodela* and *Sophiodela*. *Cosmodela*,

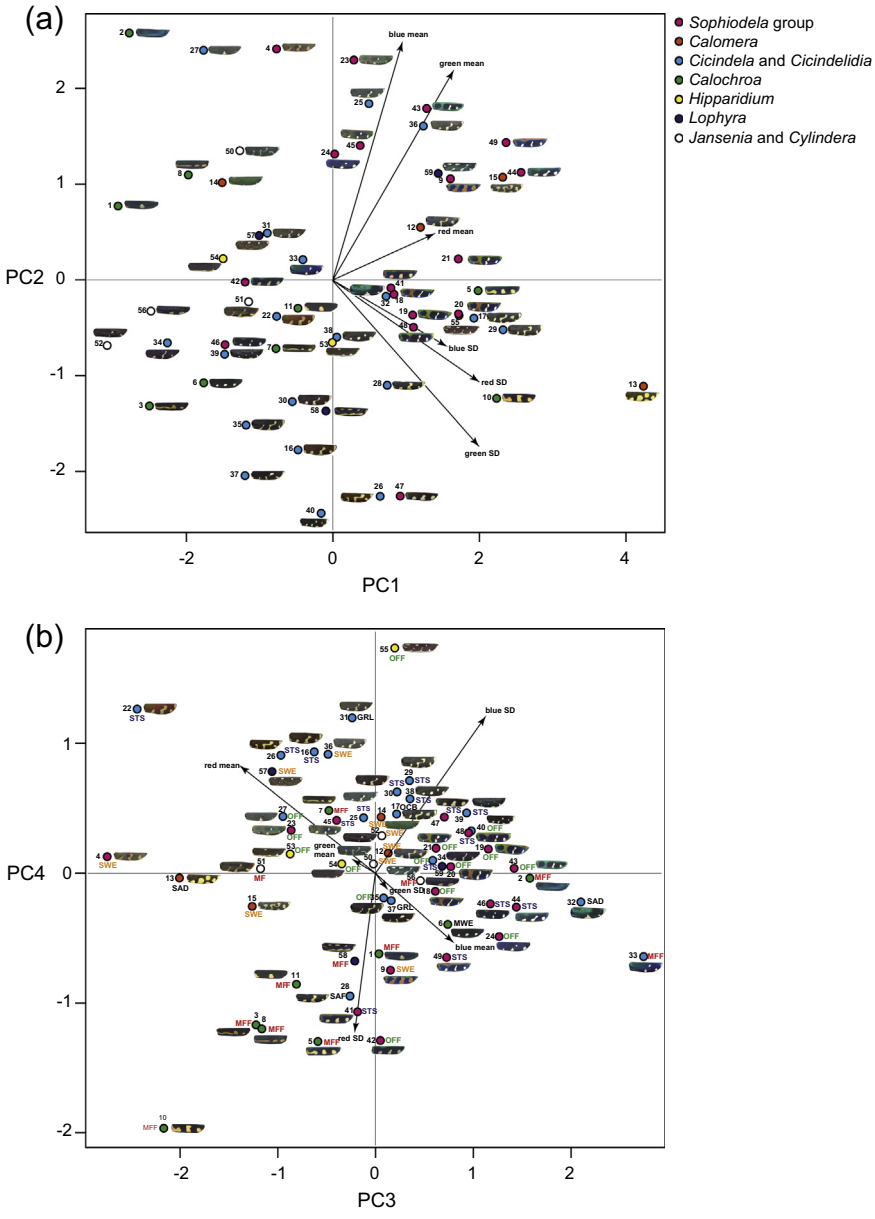

Fig. 3. Plots of the principal component scores for the elytral color pattern. (a) PC1 vs. PC2; (b) PC3 vs. PC4. Pictures of the left elytra used in the analysis are shown. In (b), habitat types are indicated by the code given in the legend of Fig. 2. Numbers are the taxon codes given in Table S2. Arrows from the origin show eigenvectors. For clarity, vector values are multiplied by 4 and 2 in (a) and (b), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Sophiodela and one group of *Calochroa* constituted a well-supported monophyletic group, which is referred to here as the "*Sophiodela* group".

The current systematics of Cicindelina evaluated here largely depends on Rivalier (in 1950–1963) who divided Cicindelidae into more than 50 genera based on male genital morphology. Rivalier emphasized the parts of the endophallus, especially flagella, which vary in shape and length among species. Species of *Sophiodela* were classified in the subgenus *Sericina* (Rivalier, 1961; synonym of *Sophiodela*, Nakane, 1955) in *Cicindela*, as they possess short flagella considered to be similar to the basic form of *Cicindela* (s. str.). Meanwhile, *Cosmodela* was treated as an independent genus because of its developed and characteristic flagella. The genus *Calochroa* comprised species whose flagella are intermediate between *Cicindela* and *Cosmodela*, and it was further divided into six subgroups. Our results suggest that Rivalier's analysis of male genital morphology was not always correct, probably because of misidentification of homologous characters or character states. Therefore, the classification of higher taxa by Rivalier (1961) needs to be revised. Although classification is beyond the scope of our study, it is possible to treat *Sophiodela* (Nakane, 1955) as a genus that includes *Cosmodela* (Rivalier, 1961) and part of *Calochroa*.

We could not investigate the enigmatic species *Sophiodela cyanea*, which has not been found recently (Acciavatti and Pearson, 1989). Unlike the other members of *Sophiodela* in East Asia, this species is from the eastern part of the Indian subcontinent (Fowler, 1912; Acciavatti and Pearson, 1989). Although Rivalier (1961) assigned this species to *Sericina* (=*Sophiodela*) based on its short, hooked flagellum, other characters of *cyanea* such as color pattern and body shape (Horn, 1915; Supplementary Fig. S2) show resemblance to species of the *Calochroa* group A, rather than the other two species of *Sophiodela*. Our preliminary observation of male genitalia of museum specimens of *cyanea* showed that its aedeagus and endophallus differ largely from those of the other *Sophiodela* species. Therefore, *cyanea* may be a member of *Calochroa* outside the *Sophiodela* group. The following discussion is made along the hypothesis that *Calochroa* is polyphyletic and *cyanea* is not a member of *Sophiodela*.

4.2. Biogeography

Our study dealt mainly with species included in clade I of Pons et al. (2004), which comprised species of Cicindelina (=*Cicindela* s. lat.) occurring in the Holarctic, Palearctic, Nearctic and Orien tal–Madagascan–Afrotropical zones. Our study demonstrated that the Palearctic clade of Pons et al. (2004) actually includes species from the Oriental region (*Cosmodela* and part of *Calochroa*) and Africa (*Calomera fimbriata* from Zambia). These clades are sister to the Oriental/Madagascan/Afrotropical clade. Thus, major groups of clade I include both Oriental and Afrotropical/Madagascan species, whereas New World species (*Cicindelidia* and *Cicindela* excluding *Sophiodela*) are restricted to one clade. Therefore, the primary range of clade I is likely to be the Oriental and Afrotropical/Madagascan regions, although our sampling is insufficient for the latter region.

We estimated the divergence time of clade I to be 33 mya (early Oligocene) based on the mitochondrial COI clock rate (0.0177 per my; Papadopoulou et al., 2010), which is older than the estimate of 10.9–8.4 mya (mid Miocene) in Pons et al. (2004). Although both divergence time estimations contain high uncertainty, we consider the estimation based on the COI clock rate more plausible because the clock rate was estimated based on sequence divergence during a longer time interval than the latter. If our estimation based on the COI clock rate is correct, the divergence of clade I followed the collision of the Indian subcontinent into Asia and the Himalayan orogeny, which may have promoted diversification of Cicindelina (Pearson and Vogler, 2001). The Indian subcontinent might bring species of Gondwanan origin that were new to Asia. *Calomera* is distributed widely in the Old World, and it is of interest how this group has spread after the mid Miocene. Further molecular phylogenetic study including more Afrotropical/Madagascan taxa, as well as those in regions between Africa and India, is needed to reveal the role of the Indian subcontinent as a carrier and the dispersal between Africa and Asia after the collision of the Indian subcontinent into Eurasia. A divergence of Palaearctic and Nearctic species occurred during the Miocene, and global climatic cooling and expansion of grasslands and savannas during the mid-Miocene may have promoted the dispersal and radiation of *Cicindela* s. str. and *Cicindelidia* species as Pons et al. (2004) suggested. In contrast, the *Sophiodela* group has been confined to the Oriental and Sino-Japanese regions and has not diversified, as *Cicindela* s. str. *Sophiodela* may have remained in its traditional niche in forest areas, while *Cicindela* s. str. radiated by adaptation to dry, open habitats, which prevailed in the northern hemisphere during the mid-Miocene.

Of the two species of the subgenus *Sophiodela* evaluated, *C. chinensis* occurs on the East Asian mainland (China and Korea) and in two separate island regions of Japan (Okinawa and Japanese main islands). The two Japanese subspecies form a monophyletic clade, which diverged from the mainland population 1 mya (mid Pleistocene; 95% HPDI, 0.6–1.6 mya) according to the estimation based on the COI evolutionary rate. However, another *Sophiodela* species, C. *ferriei*, occurs in the Amami Islands between the two Japanese subspecies of *C. chinensis*. *Cicindela ferriei* diverged from *C. chinensis* 2.5 mya (early Pleistocene; 95% HPDI, 1.5–3.9 mya). These estimated divergence times imply that *C. ferriei* colonized Japan first, and *C. chinensis* later, in two separate regions. Geologically, the Ryukyu Islands, including Amami and Okinawa, separated from the main islands of Japan around 1.7 mya; subsequently, land bridges between the East Asian mainland and each of the Ryukyu Islands and Japanese main islands may have formed, but no land bridge occurred between Amami and the Japanese main islands (Machida et al., 2001; Watanabe et al., 2006; Osozawa et al., 2011). Therefore, a lineage of *C. chinensis* from the East Asian mainland may have dispersed separately to the Japanese main islands and to Okinawa via land bridges during glacial periods. *Cicindela chinensis* might not have reached Amami due to the lack of available land bridges, or it might have reached Amami but did not colonize successfully due to the presence of *C. ferriei*. The Amami Islands harbor endemic mammals, such as *Tokudaia osimensis* (Muridae) and *Pentalagus furnessi* (Leporidae), which do not have sister species in other regions of Japan and might have been derived directly from East Asian mainland species during the mid-Miocene (Suzuki et al., 2000; Yamada et al., 2002). These endemic species indicate the existence of specific land connections between the East Asian mainland and Amami allowing colonization by terrestrial animals.

4.3. Elytral color pattern

Most species of the *Sophiodela* group share colorful elytral patterns with vivid red, green, and/or blue colors among the species evaluated (Fig. 2). We found that the elytral coloration of the tiger beetles studied had phylogenetic signals, suggesting that the color pattern of the *Sophiodela* group originated from their common ancestor. The species of the *Sophiodela* group inhabit three types of habitat (streamside, open forest floor and sandy water edge), and a color component was associated with one of these habitats (PC4 on open forest floor). Therefore, both phylogenetic constraints and adaptation to specific habitats may have affected the coloration of the *Sophiodela* group. Note that analyzing the complex elytral color pattern of tiger beetles is difficult and our analysis is

only a preliminary one. Further studies need to examine the relationships between color pattern and habitat conditions, considering the combination of patches with different coloration and metallic colors, as well as background colorations in the habitats.

Vogler and Kelley (1998) argue that the color pattern is associated with defensive tactics in *Cicindela*: specifically, they found that bright iridescent coloration, which is effective in predator evasion during flight, is not tightly correlated with chemical defense. The typical coloration in the *Sophiodela* group comprises partly metallic red, blue and green with white maculations. This pattern may be cryptic on a ground surface of complex texture and dappled sunlight (Hori, 1982), and the metallic coloration may be effective in predator evasion during flight. Defensive chemicals of the *Sophiodela* group species are largely unknown, although Pearson et al. (1988) detected some benzaldehyde (the major defensive chemical in tiger beetles) in three *Cosmodela* species. Further study of defensive chemicals is needed, as well as studies of the function of color pattern in the *Sophiodela* group species to reveal their defensive tactics.

In conclusion, our molecular phylogenetic analysis demonstrated that a specific color pattern indicates the common ancestry of diverse species previously classified into three different groups in *Cicindela* s. lat., which were together called the *Sophiodela* group in our study. To understand the origin and adaptive significance of the colorful pattern, further studies need to examine the function of color patterns, such as in predation avoidance.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.11.006.

References

Acciavatti, E., Pearson, D.L., 1989. The tiger beetles genus *Cicindela* (Coleoptea, Insecta) from the Indian subcontinent. Ann. Carnegie Mus. 58, 77–353.
Baele, G., Lemey, P., Bedford, T., Rambaut, A., Suchard, M.A., Alekseyenko, A.V., 2012. Improving the accuracy of demographic and molecular clock model comparison while accommodating phylogenetic uncertainty. Mol. Biol. Evol. 29, 2157–2167.
Blomberg, S.P., Garland, T., Ives, A.R., 2003. Testing for phylogenetic signal in comparative data: behavioral traits are more labile. Evolution 57, 717–745.
Brower, A.V.Z., DeSalle, R., 1998. Patterns of mitochondrial versus nuclear DNA sequence divergence among nymphalid butterflies: the utility of *wingless* as a source of characters for phylogenetic inference. Insect Mol. Biol. 7, 1–10.
Cassola, F., Pearson, D.L., 2000. Global patterns of tiger beetle species richness (Coleoptera: Cicindelidae): their use in conservation planning. Biol. Conserv. 95, 197–208.
Drummond, A.J., Suchard, M.A., Xie, D., Rambaut, A., 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol. Biol. Evol. 29, 1969–1973.
Eberhard, W.G., 1985. Sexual Selection and Animal Genitalia. Harvard University Press, Cambridge, MA.
Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797.
Fowler, M.A., 1912. The fauna of British India, including Ceylon and Burma. In: Coleoptera. General Introduction and Cicindelidae and Paussidae. Today & Tomorrow's Printers & Publishers, New Delhi.
Holt, B.G., Lessard, J.-P., Borregaard, M.K., Fritz, S.A., Araujo, M.B., et al., 2013. An update of Wallace's zoogeographic regions of the world. Science 339, 76–78.
Hori, M., 1982. The biology and population dynamics of the tiger beetle, *Cicindela japonica* (Thunberg). Physiol. Ecol. Jpn. 19, 77–212.
Hosken, D.J., Stockley, P., 2004. Sexual selection and genital evolution. Trends Ecol. Evol. 19, 87–93.
Horn, W., 1915. Coleoptera Adephaga (family Carabidae, subfamily Cicindelinae). In: Wytsman, P. (Ed.), Genera Insectorum. Desmet-Vereneuil, Brussels.
Kim, C.G., Zhou, H.Z., Imura, Y., Tominaga, O., Su, Z.H., Osawa, S., 2000. Pattern of morphological diversification in the *Leptocarabus* ground beetles (Coleoptera, Carabidae) as deduced from mitochondrial ND5 gene and nuclear 28S rDNA sequences. Mol. Biol. Evol. 17, 137–145.
Lanfear, R., Calcott, B., Ho, S.Y.W., Guindon, S., 2012. PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. Mol. Biol. Evol. 29, 1695–1701.
Machida, H., Ota, Y., Moriwaki, H., Nagaoka, S., 2001. Regional geomorphology of the Japanese Islands. Geomorphology of Kyushu and the Ryukyus, vol. 7. University of Tokyo Press, Tokyo.
Nakane, T., 1955. New or little-known Coleoptera from Japan and its adjacent region. XII. Sci. Rep. Saikyo Univ. (Nat. Sci. Liv. Sci.) 2, 24–42.
Osozawa, S., Shinjo, R., Armid, A., Watanabe, Y., Horiguchi, T., Wakabayashi, J., 2011. Palaeogeographic reconstruction of the 1.55 Ma synchronous isolation of the Ryukyu Islands, Japan, and Taiwan and inflow of the Kuroshio warm current. Int. Geol. Rev. 54, 1369–1388.
Papadopoulou, A., Anastasiou, I., Vogler, A.P., 2010. Revisiting the insect mitochondrial molecular clock: the mid-Aegean trench calibration. Mol. Biol. Evol. 27, 1659–1672.
Paradis, E., Claude, J., 2002. Analysis of comparative data using generalized estimating equations. J. Theor. Biol. 218, 175–185.
Paradis, E., Claude, J., Strimmer, K., 2004. APE: analyses of phylogenetics and evolution in R language. Bioinformatics 20, 289–290.
Pearson, D.L., Vogler, A.P., 2001. Tiger Beetles: the Evolution, Ecology, and Diversity of the Cicindelids. Cornell University Press, Ithaca, New York.
Pearson, D.L., Blum, M.S., Jones, T.H., Fales, H.M., Gonda, E., Witte, B.R., 1988. Historical perspective and the interpretation of ecological patterns: defensive compounds of tiger beetles (Coleoptera: Cicindelidae). Am. Nat. 132, 404–416.
Pons, J., Vogler, A.P., 2005. Complex pattern of coalescence and fast evolution of a mitochondrial rRNA pseudogene in a recent radiation of tiger beetles. Mol. Biol. Evol. 22, 991–1000.
Pons, J., Brarraclough, T.G., Throdorides, K., Cardoso, A., Vogler, A.P., 2004. Using Exon and Intron sequences of the gene Mp20 to resolve basal relationships in *Cicindela* (Coleoptera: Cicindelidae). Syst. Biol. 53, 554–570.
Rambaut, A., Suchard, M.A., Xie, D., Drummond, A.J., 2014. Tracer v1.6. Available: <http://beast.bio.ed.ac.uk/Tracer>.
Rivalier, E., 1961. Démembrement du genre *Cicindela* L. (Suite) (1). IV. Faune indomalaise. Rev. Franc. Entomol. 28, 121–149.
Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Hohna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61, 539–542.
Schultz, T.D., 1986. Role of structural colors in predator avoidance by tiger beetles of the genus *Cicindela* (Coleoptera: Cicindelidae). Bull. Entomol. Soc. Am. 32, 142–146.
Schultz, T.D., 1991. Tiger hunt. Nat. History, 38–44.
Shelford, V.E., 1917. Color and color-pattern mechanism of tiger beetles. Illinois Biol. Monogr. 3, 395–592.
Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., Flook, P., 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved PCR primers. Ann. Entomol. Soc. Am. 87, 651–701.
Sota, T., Hayashi, M., 2004. Molecular phylogenetic analysis of the genus *Donacia* (Coleoptera, Chrysomelidae) in Japan based on mitochondrial gene sequences. In: Jolivet, P., Santiago-Blay, J.A., Schmitt, M. (Eds.), New Developments in the Biology of Chrysomelidae. SPB Academic Publishing, The Hague, pp. 105–116.
Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. http://dx.doi.org/10.1093/bioinformatics.
Suzuki, H., Tsuchiya, K., Takezaki, N., 2000. A molecular phylogenetic framework for the Ryukyu endemic rodents *Tokudaia osimensis* and *Diplothrix legata*. Mol. Phylogenet. Evol. 15, 15–24.
Vogler, A.P., Barraclough, T.G., 1998. Reconstructing shifts in diversification rate during the radiation of Cicindelidae (Coleoptera). In: Ball, G.E., Casale, A., Taglianti, A.G. (Eds.), Phylogeny and Classification of Caraboidea (Coleoptera: Adephaga). Museo Regionale di Scienze Naturali, Trino, pp. 251–260.
Vogler, A.P., Goldstein, P.Z., 1997. Adaptation, cladogenesis, and the evolution of habitat association in North American tiger beetles. In: Givnish, T.J., Systsma, K.

J. (Eds.), 2002, Molecular Evolution and Adaptive Radiation. Cambridge University Press, New York, pp. 353–373.
Vogler, A.P., Kelley, K.C., 1998. Covariation of defensive traits in tiger beetles (genus *Cicindela*): a phylogenetic approach using mtDNA. Evolution 52, 529–538.
Watanabe, K., Takahashi, H., Kitamura, A., Yokoyama, R., Kitagawa, T., Takeshima, H., Sato, S., Yamamoto, S., Takehana, Y., Mukai, T., Ohara, K., Iguchi, K., 2006. Biogeographical history of Japanese freshwater fishes: phylogeographic approaches and perspectives. Jpn. J. Ichthyol. 53, 1–38.
Wickler, W., 1968. Mimicry in Plants and Animals (R.D. Martin, Trans.). McGraw-Hill, New York.
Wiesner, J., 1992. Checklist of the Tiger Beetles of the World. Verlag Erna Bauer, Keltern.
Yamada, F., Takaki, M., Suzuki, H., 2002. Molecular phylogeny of Japanese Leporidae, the Amami rabbit *Pentalagus furnessi*, the Japanese hare *Lepus brachyurus*, and the mountain hare *Lepus timidus*, inferred from mitochondrial DNA sequences. Genes Genet. Syst. 77, 107–116.