

# Comparative Phylogeography of Three *Leptocarabus* Ground Beetle Species in South Korea, Based on the Mitochondrial COI and Nuclear 28S rRNA Genes

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We analyzed the intraspecific gene genealogies of three *Leptocarabus* ground beetle species (*L. seishinensis*, *L. semiopacus*, *L. koreanus*) in South Korea using sequence data from the mitochondrial cytochrome oxidase subunit I (COI) and nuclear 28S rRNA (28S) genes, and compared phylogeographical patterns among the species. The COI data detected significant genetic differentiation among local populations of all three species, whereas the 28S data showed genetic differentiation only for *L. seishinensis*. The clearest differentiation of *L. seishinensis* among local populations was between the northern and southern regions in the COI clades, whereas the 28S clade, which likely indicates relatively ancient events, revealed a range expansion across the northern and southern regions. *Leptocarabus semiopacus* had the most shallow differentiation of the COI haplotypes, and some clades occurred across the northern and southern regions. In *L. koreanus*, four diverged COI clades occurred in different regions, with partial overlaps. We discuss the difference in phylogeographical patterns among these *Leptocarabus* species, as well as between these and other groups of carabid beetles in South Korea.

**Key words:** carabid beetles, phylogeography, nested clade analysis, statistical parsimony, molecular phylogeny

## INTRODUCTION

Since the emergence of phylogeography (Avice *et al.*, 1987; Avice, 2000), comparative analyses of phylogeographical patterns across different taxa and regions have provided a powerful technique for revealing the evolutionary history and current status of biodiversity (Bermingham and Moritz, 1998; Arbogast and Kenagy, 2001). Comparative phylogeography, with extensive geographic sampling, can provide substantial insight into the evolutionary process of closely related taxa across a continuum from population divergence to species divergence (Avice, 2000, 2004; Templeton, 2001; Barraclough and Nee, 2001).

Ground beetle species belonging to the flightless subtribe Carabina (=genus *Carabus sensu lato*; family Carabidae) are restricted in their dispersal ability and serve as important organisms for analyzing the process of geographical differentiation. Here, we examined the molecular phy-

logeny and phylogeography of three closely related *Leptocarabus* ground beetles in South Korea, *L. seishinensis* (Lapouge), *L. semiopacus* (Reitter), and *L. koreanus* (Reitter), to explore differences in the current and historical biogeography of closely related species in the same region. *Leptocarabus* is adapted to cool climatic conditions and occurs mainly in mountainous regions up to alpine zones (Sota, 1996; Sota *et al.*, 2000; Sota and Ishikawa, 2004). The three species in South Korea have similar life histories, although *L. seishinensis* and *L. semiopacus* inhabit higher elevations than *L. koreanus* (Kim and Lee, 1992). *Leptocarabus* consists of 20 species in East Asia, including the Korean peninsula and the Japanese islands (Imura and Mizusawa, 1996). Kim *et al.* (2000a) studied the phylogeny of 14 *Leptocarabus* species, including the above three Korean species. However, their study focused on the species phylogeny and did not analyze the phylogeography of individual species.

Comparative phylogeographic analyses of different groups of organisms within one region can provide inferences about the processes of genetic differentiation, such as common vicariance events and the various dispersal and colonization histories of different species. Recently, carabid

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beetles have been studied for this purpose in different regions of the world (e.g., Emerson *et al.*, 1999, 2000; Clarke *et al.*, 2001; Moya *et al.*, 2004). In our previous study of *Coptolabrus*, another Carabina group in South Korea (Zhang *et al.*, 2005), two closely related species exhibited a similar geographic differentiation pattern in the mitochondrial gene genealogy, which likely reflects their recent dispersal and colonization history. The phylogeographic pattern of *Leptocarabus* in South Korea may differ from that of *Coptolabrus* because of habitat differences: *Leptocarabus* is confined to mountain zones, whereas *Coptolabrus* inhabits both mountains and lowlands down to coastal areas.

In addition, comparative phylogeographic analyses of one group of organisms in different geographic regions provide inferences about the differences in historical and geographical factors that determine the group's differentiation (speciation) processes. Kim *et al.* (2000b) studied the phylogeny of five Japanese *Leptocarabus* species using the mitochondrial ND5 gene. This study revealed frequent sharing of the same ND5 haplotype, or lineage, between species. The discrepancy between this molecular phylogeny and morphological taxonomy revealed a serious problem in the phylogeny of closely related *Leptocarabus* species. In particular, three species in the central to northern part of Honshu, Japan, including two widely sympatric species pairs, shared the same or closely related haplotypes. Although trans-species polymorphism of mitochondrial haplotypes is a well-known phenomenon in other carabid beetles, inferred to be an effect of interspecific hybridization (e.g., Sota and Vogler, 2001; Sota *et al.*, 2001; Sota, 2002; Takami and Suzuki, 2005; Zhang *et al.*, 2005), the case of Japanese *Leptocarabus* is extraordinary and requires further assessment and comparative studies of congeneric species in other areas.

In this study, we sought to resolve the species relationships and explore differences in phylogeographic patterns among the three species, as well as compare them to other species inhabiting South Korea and Japan. We used sequence data from the mitochondrial cytochrome oxidase subunit I (COI) and nuclear 28S rRNA (28S) genes to construct phylogenetic trees and to investigate population genetics and phylogeographic patterns using nested clade analysis.

## MATERIALS AND METHODS

### Sampling, DNA extraction, PCR, and sequencing

A total of 162 adult beetles of the three species was obtained from nine locations in South Korea (Table 1; see also Fig. 1). Upon collection, beetles were preserved in 99% ethanol. *Leptocarabus arboreus*, collected from Japan, was used as the outgroup. Muscle tissue of the beetles was digested with proteinase K in CTAB buffer, and total DNA was extracted using the standard phenol–chloroform method. Primers COS1751C (5'-GGA GCT CCT GAT ATA GCT TTY CC-3'; Sota and Hayashi, 2004) and COA2703 (5'-CT CCA ATR AAT ACA ATA AAT TG-3'; Zhang *et al.*, 2005) were used to PCR-amplify 894 bp of the COI gene region. Primer pair 28S-01/28SR-01 (Kim *et al.*, 2000a) was used to amplify 832- to 852-bp fragments of the nuclear 28S gene region. For direct sequencing of the PCR products, dye terminator cycle-sequencing reactions were performed using the ABI PRISM BigDye Terminator Cycle Sequencing FS Ready Reaction Kit, followed by electrophoresis on an ABI 377 sequencer (Applied Biosystems, Foster City, CA, USA).

**Table 1.** Sample localities and the number of samples sequenced.

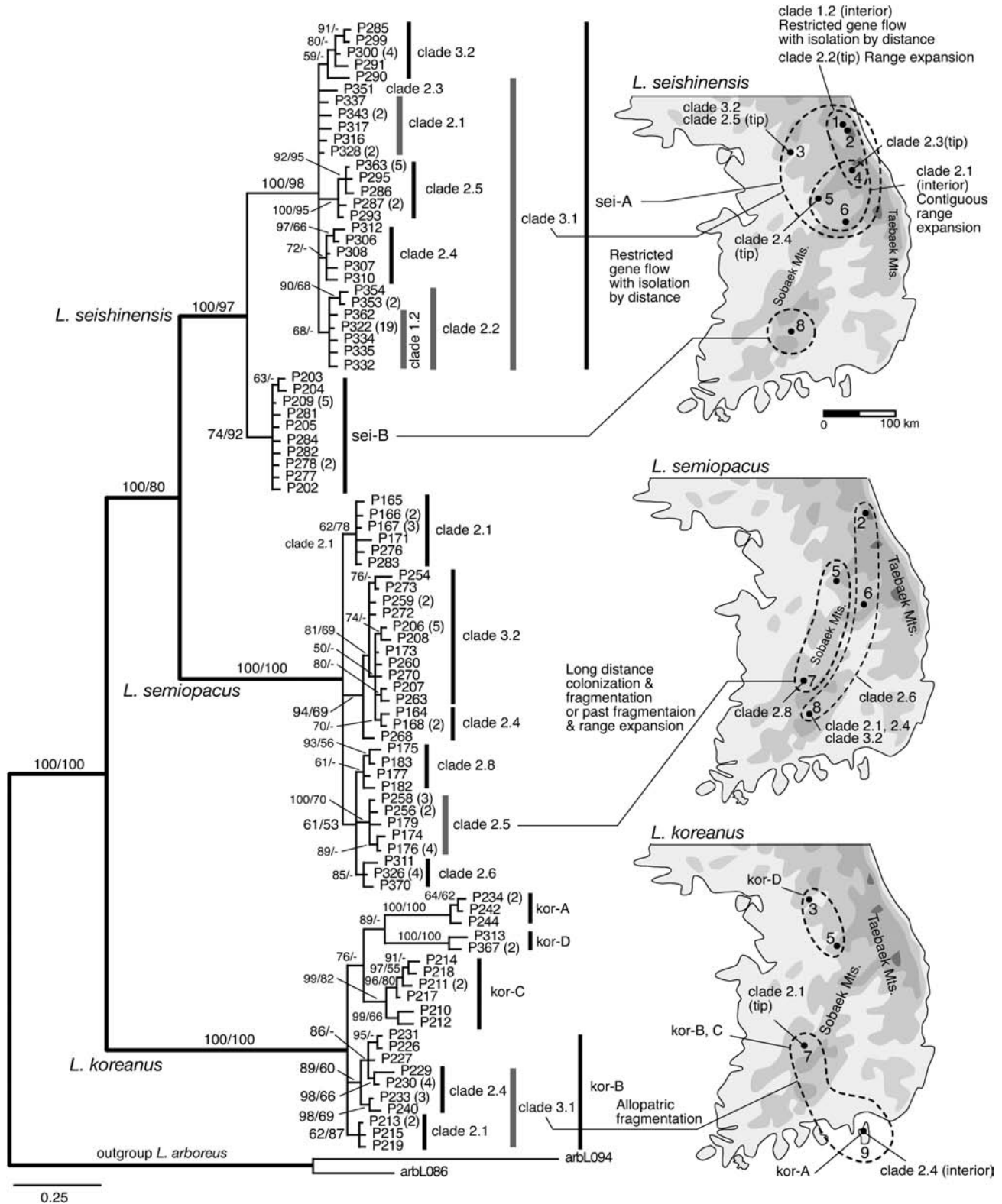
Species	Sample locality (No./code)	No. of		
		Coordinates		
		N°	E°	COI/ 28S
<i>L. seishinensis</i>				
	Baekdamsa (1/PAE)	38.17	128.38	3/3
	Mt. Seoraksan (2/SOR)	38.14	128.41	6/7
	Chuncheon (3/CHU)	37.88	127.73	18/14
	Mt. Odaesan (4/ODA)	37.75	128.48	21/22
	Mt. Chiaksan (5/CHI)	37.36	128.06	7/6
	Mt. Sobaeksan (6/SOB)	36.96	128.41	3/2
	Jeungsan-ri, Mt. Jirisan (8/CHN)	35.32	127.76	15/6
<i>L. semiopacus</i>				
	Mt. Seoraksan (2/SOR)	38.14	128.41	1/1
	Mt. Odaesan (4/ODA)	37.75	128.48	0/1
	Mt. Chiaksan (5/CHI)	37.36	128.06	1/1
	Mt. Sobaeksan (6/SOB)	36.96	128.41	4/6
	Mt. Deogyusan (7/TOG)	35.82	127.72	10/6
	Jeungsan-ri, Mt. Jirisan (8/CHN)	35.32	127.76	34/11
<i>L. koreanus</i>				
	Chuncheon (3/CHU)	37.88	127.73	2/2
	Mt. Chiaksan (5/CHI)	37.36	128.06	1/1
	Mt. Deogyusan (7/TOG)	35.82	127.72	12/9
	Mt. Beugbyeongsan, Geojedo I. (9/KOJ)	34.88	128.67	17/11
Total				155/111
Outgroup:				
<i>L. arboreus</i>				
	Tumagoi-mura, Gunma, Japan	35.56	138.14	1/1
	Ohshika-mura, Nagano, Japan	36.46	138.44	1/1

The number of sequences obtained for each species and gene is presented in Table 1. The sequence data have been deposited in GenBank (accession numbers for 28S, DQ184516–DQ184540; for COI, DQ184541–DQ184632).

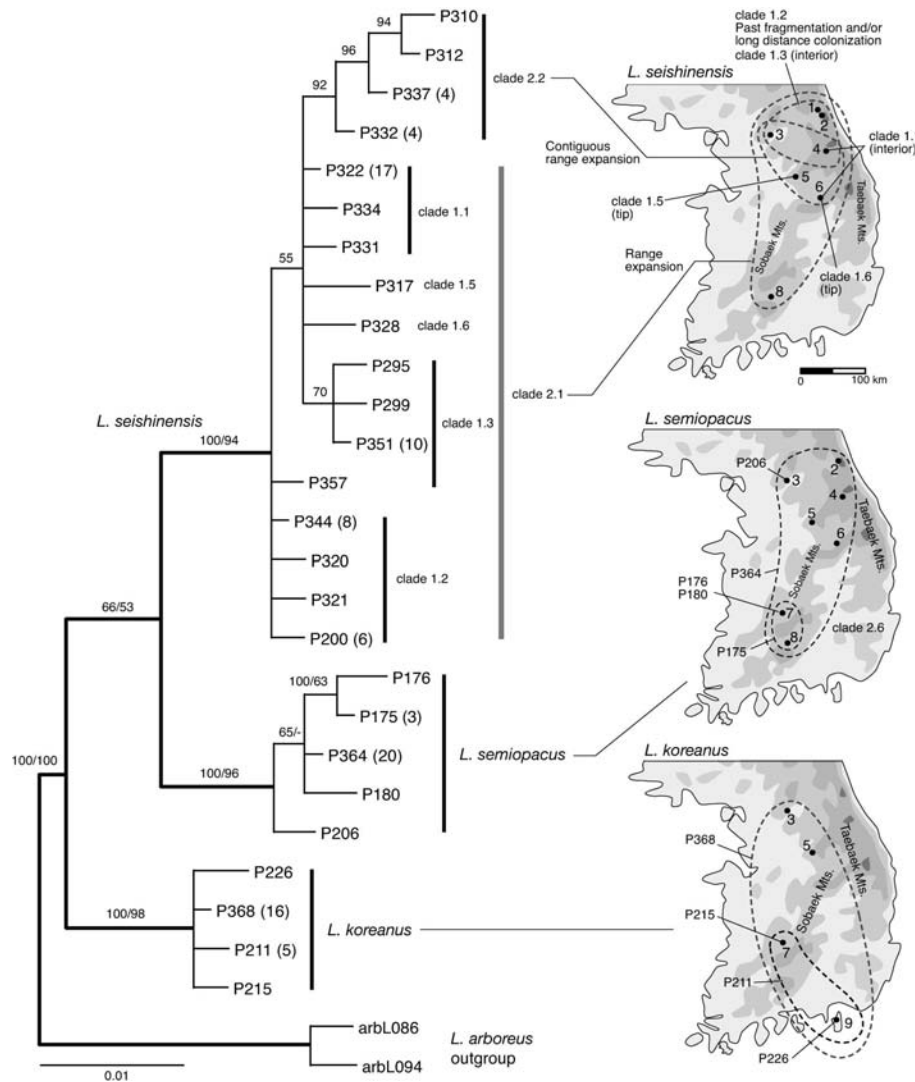
### Phylogenetic analysis

DNA sequences were aligned using the multiple-sequence alignment program Clustal X version 1.83 (Chenna *et al.*, 2003) and later refined manually. Since selection on mutations and intragenic recombinations within the studied sequences could result in misleading inferences in the phylogenetic and phylogeographic analysis, we tested for the neutrality of mutations following the method of Fu and Li (1993) and used DnaSP version 4.0 (Rozas *et al.*, 2003) to compute  $D^*$  and  $F^*$  test statistics. In addition, we confirmed the absence of intragenic recombination using TOPALi version 0.23 (Milne *et al.*, 2004) for both the 28S and COI sequences, as widespread recombination has been suggested to occur in animal mtDNA sequences (Tsaousis *et al.*, 2005). Putative recombination breakpoints were sought using a 10-bp sliding window and 10-bp increment along the alignment and  $DSS$  (difference in sum of squares) statistics for the sliding windows. Along a sequence alignment, each significant peak in  $DSS$  corresponds to a recombination breakpoint. The statistical significance of  $DSS$  peaks (recombination breakpoints) was tested at  $P=0.05$  using 100 parametric bootstraps.

To find the best-fit substitution model for each sequence data set, a likelihood ratio test (LRT) was performed using Modeltest version 3.5 (Posada and Crandall, 1998). For phylogenetic reconstruction with each sequence data set, we performed a Bayesian Markov-Chain Monte-Carlo (MCMC) analysis using MrBayes version 3.1 (Huelsenbeck and Ronquist, 2001). Substitution models selected by Modeltest were used in this analysis. The MCMC analysis was run for one million generations, with four chains, and every hundredth tree was sampled. After visually examining the likelihood scores, data for the first 200,000 generations were discarded as burn-in.



**Fig. 1.** Fifty percent majority consensus of 8,000 trees resulting from Bayesian analysis of mitochondrial COI haplotypes (left), and maps of the Korean Peninsula showing geographic distribution of haplotypes (clades) of the three *Leptocarabus* species (right). Branch lengths are proportional to mean branch lengths of the 8,000 trees. Numerals above the branches are Bayesian posterior probabilities followed by bootstrap percentages obtained from parsimony analysis (when >50%). Terminal nodes (P-numbers) are sample codes and represent unique sequences (haplotypes). When the same haplotype appeared two or more times in the sample, the frequency is indicated in parentheses after the P-number. Vertical grey bars indicate clades that showed significant geographical association (see Table 4). In the maps, black dots with numbers are sampling sites (see Table 1). Inference from NCA is indicated when available for a clade (see Table 4).



**Fig. 2.** Fifty-percent majority consensus of 8,000 trees resulting from Bayesian analysis of nuclear 28S sequences (left), and maps of the Korean Peninsula showing phylogeographic patterns of the three *Leptocarabus* species (right). See the legend for Fig. 1 for further explanation.

We also performed a parsimony analysis using PAUP\* version 4.0b10 (Swofford, 2002). Heuristic searches were conducted using 100 random-addition replicates with tree bisection–reconnection branch swapping (MulTree option in effect). The confidence level of each node was assessed by 1,000 bootstrap replications.

#### Analysis of molecular variance

To examine the extent of genetic variation among local populations, we used analysis of molecular variance (AMOVA) implemented in Arlequin version 2000 (Schneider *et al.*, 2000). The significance of the fixation index ( $F_{ST}$ ) was tested using a nonparametric analysis with 1,000 permutations, as described by Excoffier *et al.* (1992). In addition, to examine whether the differences in  $F_{ST}$  among the three species were statistically significant, we created 100 data sets for each gene by bootstrap resampling of sequences within each local population and performed AMOVA for each of the bootstrap data sets. With 100  $F_{ST}$  values for each species, we determined 95% confidence intervals. We considered that  $F_{ST}$  values to differ between species if the 95% confidence intervals did not overlap.

#### Nested clade analyses for intraspecific phylogeography

The nested clade analysis has widely been used to infer the

complex historical processes involved in intraspecific phylogeography (Templeton 2001, 2002, 2004), although its statistical reliability has been questioned (Knowles and Maddison, 2002). To apply the nested clade analysis to the COI and 28S data, statistical parsimony networks (Templeton *et al.*, 1995) were constructed using TCS program version 1.8 (Clement *et al.*, 2000) with a 95% connection limit. For the 28S gene, intraspecific networks could be estimated without gap sites, although the interspecific sequence alignment required gaps. Loops that appeared in the networks were resolved, whenever possible, following the criteria of Crandall and Templeton (1993). Nested clades were determined at one-step and higher levels for use in a subsequent nested clade analysis of each species, after construction of the network. Cladogram nesting was made with the nesting algorithm described by Templeton *et al.* (1987) and Templeton and Sing (1993).

With nested cladograms obtained as above, we assessed the intraspecific phylogeography of sequences by nested clade analyses using GeoDis version 2.2 (Posada *et al.*, 2000). The geographical association of haplotypes or clades was first tested using the permutational contingency test (Templeton and Sing, 1993). If the test identified significant nonrandom patterns, the pattern of geographic structure was further analyzed using two distance mea-

sures. One was the clade distance ( $D_c$ ), which measures the geographical range of a particular clade. The other was the nested clade distance ( $D_n$ ), which measures how a particular clade is geographically distributed relative to its closest evolutionary sister clades (*i.e.*, clades in the same higher-level nesting category). The distributions of these two distance measures were tested against the null hypothesis of no geographical associations within the nesting clade by means of 1,000 permutational analyses. The subsequent inference of phylogeographical events was performed using the program AUTOINFERR 1.0 (Zhang *et al.*, 2006), which is based on Templeton's (2004) inference key.

## RESULTS

### Sequence variation and phylogenetic analysis

The alignment of the COI data set required no gaps. For the 28S sequences, the intraspecific alignment required no gaps, and only eight gaps were needed for the alignment of all species. In total, 91 unique COI haplotypes were found for all three species: 38 for *L. seishinensis*, 32 haplotypes for *L. semiopacus*, and 21 for *L. koreanus* (Fig. 1). Only 26 haplotypes were identified among all three species for the 28S gene: 17 for *L. seishinensis*, 5 for *L. semiopacus*, and 4 for *L. koreanus* (Fig. 2).

COI exhibited higher sequence variation, with 142 parsimony-informative characters (excluding the outgroup) over the total length of 894 bp, than 28S, which had 28 parsimony-informative characters (excluding gaps) over the total alignment of 855 bp. The level of sequence variation differed among the three species (Table 2). The largest substitution rate for COI was found for *L. koreanus* (2.2%), followed by *L. seishinensis* (1.3%) and *L. semiopacus* (0.9%; Table 2). For 28S, *L. seishinensis* had the largest substitution rate (0.22%), while *L. semiopacus* and *L. koreanus* had much lower rates (0.09% and 0.07%, respectively; Table 2).

Selective neutrality was confirmed for the two genes ( $P > 0.1$  for all  $D^*$  and  $F^*$  test statistics). In addition, no evidence of recombination events (recombination breakpoints across sequences) was detected ( $P > 0.05$  for all test statistics with DSS). Thus, the phylogenetic and phylogeographical analyses presented here have not suffered from the effects of recombination and selection.

The Tamura–Nei model (Tamura and Nei, 1993) with a proportion of invariable sites and the gamma shape param-

eter (TrN+I+G) was the best-fit model for the COI gene, while the Hasagawa–Kishino–Yano model (Hasegawa *et al.*, 1985) with a proportion of invariable sites (HKY+I) was the best fit for the 28S gene. However, due to the available settings in MrBayes, the GTR (general time reversible) +I+G model instead of TrN+I+G was used for COI. The COI and 28S gene trees resulting from Bayesian analyses presented the same basic relationships for the three species, (*L. arboreus*, (*L. koreanus*, (*L. semiopacus*, *L. seishinensis*)); Figs. 1 and 2). A parsimony analysis of the COI data resulted in 451,700 shortest trees of 410 steps (consistency index [CI] excluding uninformative sites=0.595; retention index [RI]=0.945; the number of trees saved was restricted by the available computer memory). The 28S data resulted in 24 shortest trees of 115 steps (CI=0.826, RI=0.936; gaps were treated as missing data). The strict consensus of the above trees did not contradict trees resulting from the Bayesian analysis; hence, only the latter trees are reported in Figs 1 and 2.

### Geographical differentiation revealed by AMOVA

An analysis of molecular variance (AMOVA) revealed significant genetic differentiation among local populations (fixation indices  $F_{ST} > 0$ ) within the COI lineages of each species and within the 28S lineages of *L. seishinensis* (Table 2). The COI gene indicated that the three species had significantly different fixation indices ( $F_{ST}$ ) with respect to haplotypes within populations, as shown by the nonoverlapping 95% confidence intervals obtained by the bootstrap resampling procedure (Table 2). A significant difference existed for the  $F_{ST}$  of the 28S gene between *L. seishinensis* and the other two species, but not between *L. semiopacus* and *L. koreanus*, for which the  $F_{ST}$  was not significantly greater than zero (Table 2).

### Nested clade analysis of intraspecific phylogeography

*Leptocarabus seishinensis* had two major COI clades (sei-A and sei-B), which were not connected within a 95% connection limit (Figs. 1 and 3, top). For *L. semiopacus*, all the COI haplotypes were connected in one network at the three-step level (Fig. 3, middle). The COI haplotypes of *L. koreanus* were distributed in four separate networks that

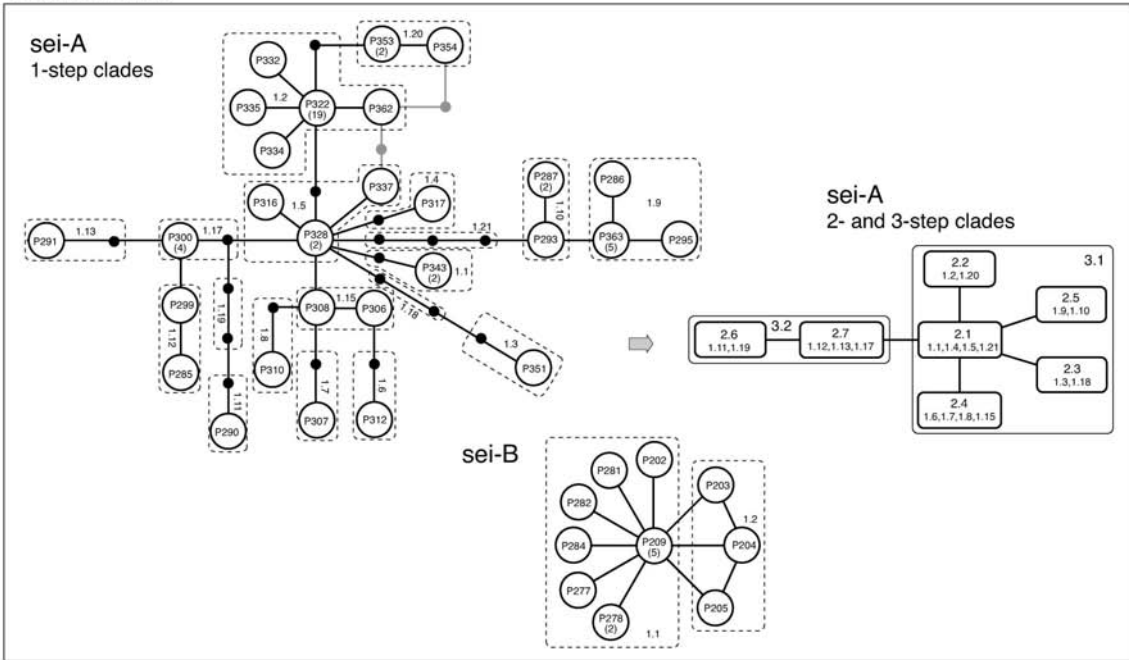
**Table 2.** Substitution rate and analysis of molecular variance for population differentiation in three species that show polymorphism in mitochondrial COI and nuclear 28S gene sequences.

Gene	Pairwise sequence divergence (Mean±SD)	Molecular variance				$F_{ST}^a$	95% C.I. of $F_{ST}^b$ lower–upper
		among populations		within population			
Species		d.f.	Variance (%)	d.f.	Variance (%)		
<b>COI</b>							
<i>L. seishinensis</i>	1.31±1.23	6	4.621 ( 79.16)	73	1.217 ( 20.84)	0.792**	0.758–0.813
<i>L. semiopacus</i>	0.93±0.47	4	1.941 ( 37.73)	44	3.204 ( 62.27)	0.377**	0.353–0.381
<i>L. koreanus</i>	2.24±1.77	3	5.014 ( 47.64)	27	5.510 ( 52.36)	0.476**	0.454–0.673
<b>28S</b>							
<i>L. seishinensis</i>	0.22±0.14	6	0.428 ( 49.05)	53	0.445 ( 50.95)	0.491**	0.522–0.717
<i>L. semiopacus</i>	0.09±0.13	5	−0.054 (−15.37)	20	0.407 (115.37)	−0.154	−0.185–0.360
<i>L. koreanus</i>	0.07±0.08	3	0.002 ( 0.51)	19	0.308 ( 99.49)	0.005	−0.161–0.338

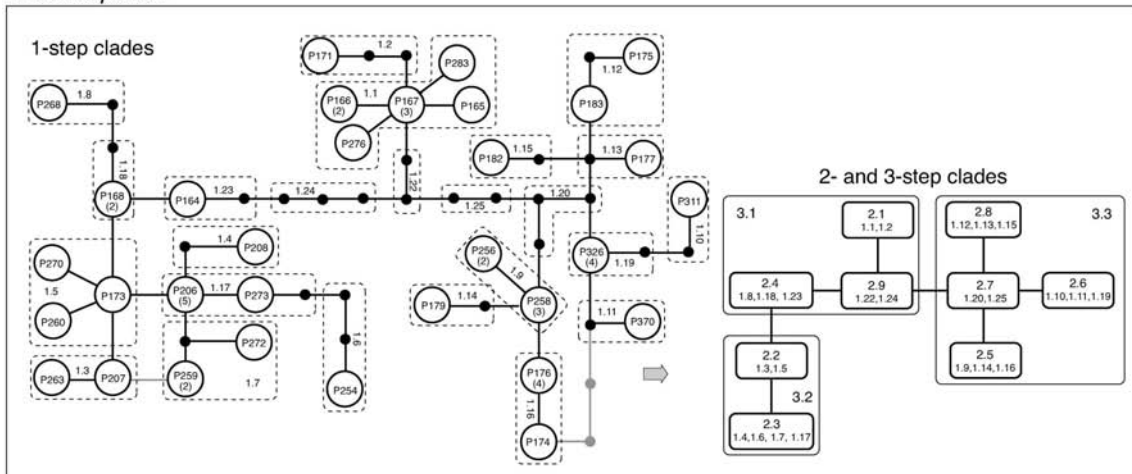
<sup>a</sup>  $F_{ST}$ , fixation index. \*\* Significantly greater than zero at  $P=0.01$ .

<sup>b</sup> 95% confidence interval obtained from 100 data sets derived by bootstrap resampling of haplotypes within populations

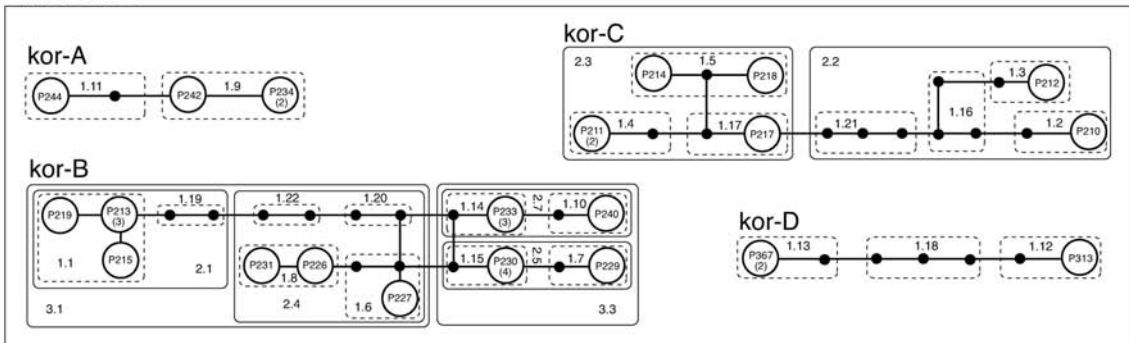
*L. seishinensis*



*L. semiopacus*



*L. koreanus*



**Fig. 3.** Parsimony networks and nested clade pattern for mitochondrial COI haplotypes. The codes in large circles indicate unique haplotypes. Each solid line represents one mutational step, and each black dot indicates an undetected intermediate haplotype. When a haplotype (P-number) occurred two or more times in the sample, the frequency is indicated in parentheses below the P-number. Loops that could be resolved prior to the cladogram nesting are shown by grey lines and circles. Clades are indicated by rectangles with rounded corners (broken line for 1-step clades; solid line for 2-step or higher clades), with the clade numbers shown.

**Table 3.** Results of the nested geographical distance analyses of the mitochondrial COI and nuclear 28S genes for three *Leptocarabus* ground beetles.

Gene		Permutational <sup>a</sup>		Haplotype/ clade	Status	Geographic distance <sup>b</sup>	
Species	Nested clade	$\chi^2$	<i>P</i>			<i>D<sub>c</sub></i>	<i>D<sub>n</sub></i>
Mitochondrial COI							
<i>L. seishinensis</i>							
	1.2	17.39	0.006	P334	tip	0.00	8.40 S
				P322	interior	15.86	13.96
				P335	tip	0.00	39.09
				P332	tip	0.00	39.09
				P362	tip	0.00	4.85
				I-T		<b>15.86 L</b>	-2.70
	2.1	38.61	0.001	1.1	tip	0.00	73.52
				1.4	tip	0.00	38.41
				1.5	interior	11.85	19.14
				I-T		11.85	<b>-42.68 S</b>
	2.2	18.65	0.003	1.2	interior	<b>1.11 S</b>	<b>2.67 S</b>
				1.20	tip	0.00	<b>41.59 L</b>
				I-T		1.11	<b>-38.92 S</b>
	3.1	82.72	0.000	2.1	interior	24.88	<b>80.01 L</b>
				2.2	tip	<b>4.11 S</b>	<b>37.90 S</b>
				2.3	tip	0.00	16.51
				2.4	tip	<b>0.00 S</b>	53.25
				2.5	tip	<b>0.00 S</b>	51.03
				I-T		22.34	<b>37.67 L</b>
	Total cladogram	20.16	0.003	3.1	tip	49.69	49.94
				3.2	tip	<b>0.00 S</b>	48.99
<i>L. semiopacus</i>							
	2.5	11.00	0.002	1.9	interior	<b>0.00 S</b>	<b>44.44 L</b>
				1.14	tip	0.00	11.22
				1.16	tip	<b>0.00 S</b>	<b>11.22 S</b>
				I-T		0.00	<b>23.21 L</b>
	2.6	12.00	0.062	1.19	interior	0.00	59.41
				1.10	tip	0.00	24.94
				1.11	tip	0.00	73.29
				I-T		0.00	10.29
	Total cladogram	24.81	0.000	2.5	tip	<b>17.92 S</b>	<b>147.68 L</b>
				2.6	tip	52.55	64.33
				2.8	tip	<b>0.00 S</b>	136.91
<i>L. koreanus</i>							
	3.1	8	0.020	2.1	tip	<b>0.00 S</b>	<b>40.20 S</b>
				2.4	interior	<b>0.00</b>	<b>95.13 L</b>
				I-T		0.00	<b>54.94 L</b>
Nuclear 28S							
<i>L. seishinensis</i>							
	1.2	25.33	0.000	P344	interior	<b>3.40 S</b>	<b>108.69 S</b>
				P320	tip	0.00	112.22
				P321	tip	0.00	112.22
				P200	tip	<b>0.00 S</b>	<b>209.17 L</b>
				I-T		3.40	<b>-76.24 S</b>
	2.1	80.02	0.000	1.2	tip	138.54	<b>144.94 L</b>
				1.3	tip	131.52	<b>140.02 L</b>
				1.1	interior	<b>42.36 S</b>	<b>59.29 S</b>
				1.6	tip	0.00	<b>23.60 S</b>
				1.5	tip	0.00	40.52
				I-T		<b>-85.19 S</b>	<b>-77.12 S</b>
	2.2	10	0.023	1.7	interior	<b>0.02 S</b>	<b>27.23 S</b>
				1.4	tip	0.00	<b>29.74 L</b>
				I-T		<b>0.00 S</b>	<b>-2.51 S</b>
	Total cladogram	13.12	0.029	2.1	tip	<b>41.69 S</b>	<b>46.31 S</b>
				2.2	tip	<b>113.07 L</b>	<b>110.74 L</b>
<i>L. semiopacus</i>							
	1.4	2.28	0.706	P364	interior	90.44	93.56
				P175	interior	27.78	162.85
	Total cladogram	8.5	0.534	1.1	tip	0.00	124.84
				1.2	tip	0.00	124.84
				1.3	tip	0.00	177.90
				1.4	interior	97.89	101.02
				I-T		97.89	-41.50
<i>L. koreanus</i>							
	1.1	6.41	0.303	P368	interior	130.46	134.74
				P211	tip	38.13	101.27
				P215	tip	0.00	82.84
				I-T		<b>98.69 L</b>	36.55
	Total cladogram	0.48	1.000	1.1	tip	25.65	27.43
				1.2	tip	32.33	32.33

<sup>a</sup> Permutation test for the null hypothesis of no association between nested clades and geographical location within a nested clade.

<sup>b</sup> *D<sub>c</sub>*, clade distance; *D<sub>n</sub>*, nested clade distance. Distances in bold letters with S or L indicate significantly shorter (S) or longer (L) than expected from no association between nested clades and geographic location (at *P*=0.05).

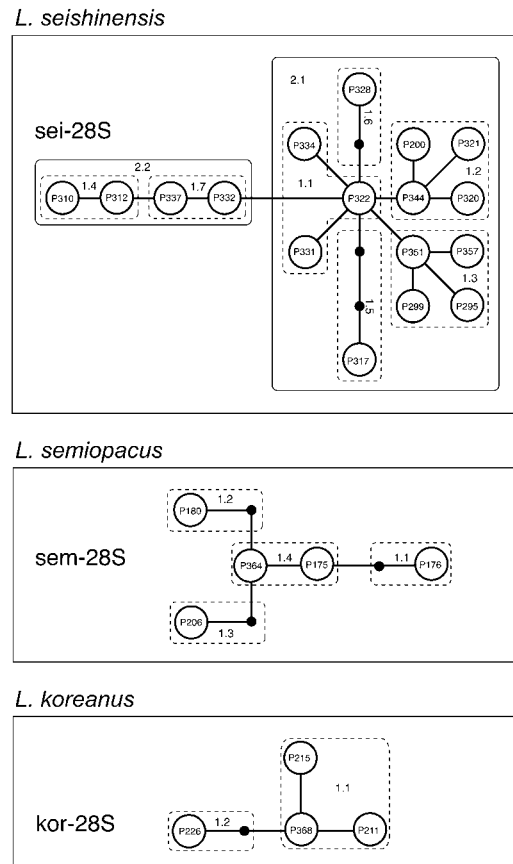
were not connected within a 95% connection limit (Fig. 3, bottom). For the 28S gene, the haplotypes of each species were connected in one network (Fig. 6). The network for *L. seishinensis* consisted of two 2-step clades, but that for each of the other two species was spun into one 2-step clade.

Geographical distance analysis revealed significant associations between clades and geographical locations within each species for COI. However, for 28S, only clades of *L. seishinensis* showed significant geographic association (Table 3). The two major clades of COI haplotypes for *L. seishinensis* (sei-A and sei-B) were distributed in the northern and southern regions, respectively (Fig. 1). Within the sei-A clade, restricted gene flow with isolation by distance was inferred for clade 1.2, and contiguous range expansion for clade 2.1. In addition, range expansion was inferred for clade 2.2, and restricted gene flow with isolation by distance for clade 3.1 (Table 4; Fig. 1). In *L. semiopacus*, only clade 2.5 revealed a significant geographical association, except for the total cladogram; long distance colonization or past fragmentation was inferred for clade 2.5 (Table 4; Fig. 1). In *L. koreanus*, allopatric fragmentation between the peninsula and Geojedo Island was inferred within clade 3.1 (Fig. 1). This inference was enhanced by the large number of mutation steps between local haplotypes (Fig. 3).

For the 28S gene, past fragmentation and/or long-distance colonization was inferred for clade 1.2 in the northern region (Table 4; Fig. 2). Because the branch lengths were short in this clade (Fig. 4), a colonization event likely occurred (Templeton, 2004). Range expansion was inferred for clade 2.2 in the northern region and for clade 2.1 occurring across the study area (Table 4; Fig. 2).

**Table 4.** Inferences from nested clade phylogeographical analyses for clades in Table 3 showing a significant association between the nested clade and geographic location.

Gene		
Species Clade	Chain of inference	Inference
Mitochondrial COI		
<i>L. seishinensis</i>		
1.2	1-2-3-4 No	Restricted gene flow with isolation by distance
2.1	1-2-11-12 No	Contiguous range expansion
2.2	1-2-11 Yes	Range expansion
3.1	1-2-3-4 No	Restricted gene flow with isolation by distance
<i>L. semiopacus</i>		
2.5	1-2-3-5-6-13 Yes	Long distance colonization, possibly coupled with subsequent fragmentation, or past fragmentation followed by range expansion
<i>L. koreanus</i>		
3.1	1-19 No	Allopatric fragmentation
Nuclear 28S		
<i>L. seishinensis</i>		
1.2	1-2-3-5-15 No	Past fragmentation and/or long-distance colonization
2.1	1-2-11 Yes	Range expansion
2.2	1-19-20-2-11-12 No	Contiguous range expansion



**Fig. 4.** Parsimony networks and nesting pattern for nuclear 28S alleles for three *Leptocarabus* species. See the legend for Fig. 3 for further explanation.

## DISCUSSION

In 155 samples sequenced for the mitochondrial COI gene, we found no case of shared sequences between species. This result differs from that found in Japanese *Leptocarabus*, which showed extensive sharing of identical mitochondrial haplotypes among species (Kim *et al.*, 2000b). The nuclear 28S also showed no trans-species polymorphism, despite the fairly low level of sequence variation across species (maximum sequence difference, 2.65% for 28S vs. 12.75% for COI). These findings indicate that reproductive isolation among the Korean species has been strong enough to prevent introgressive hybridization, despite their similar morphologies and life cycles (Kim and Lee, 1992). In contrast, the reproductive isolation of Japanese *Leptocarabus* species either is incomplete (Kubota, 1991), or gene flow between species occurred until recently, probably owing to their relatively short history of divergence.

In this study, we used both mitochondrial and nuclear genes to reveal biogeographical events of different time spans based on the genes' different coalescent times (Templeton, 2002). In our previous phylogeographic study of South Korean *Coptolabrus*, we used two nuclear gene sequences, phosphoenolpyruvate carboxykinase and wingless, in addition to mitochondrial COI (Zhang *et al.*, 2005). Although these nuclear gene sequences exhibited significant geographic variation, they showed complex allelic net-



works, with many loops and high frequencies of heterozygosity, demonstrating the difficulties in using nuclear allelic data in nested clade analyses (Zhang *et al.*, 2005). In this study, we used 28S as a nuclear DNA marker and detected no putative heterozygotes in the data. The resulting networks were relatively simple, owing to less extensive sequence variation. The absence of variation within individuals, however, contradicts our detection of allelic diversity within populations; this may have resulted from the unequal amplification of a numerically dominant allele type belonging to the highly repetitive ribosomal gene.

Since the 28S data exhibited a low level of variation, we could make biological inferences only for *L. seishinensis* from the 28S nested clade analysis. Therefore, most of the biogeographical inferences here rely on the mitochondrial COI results. AMOVA indicated that the extent of genetic differentiation among local populations within species was greater in *L. seishinensis* than in *L. semiopacus* and *L. koreanus* for both the COI and 28S sequences. This finding should be interpreted with caution, as sampling was unintentionally more intensive in the number of localities and specimens for *L. seishinensis*. The sample sizes of *L. semiopacus* and *L. koreanus* from northern sites were small, and thus the molecular variance among local populations may have been underestimated. However, the level of nucleotide diversity for each local population appears to differ among the species. For populations with 10 or more samples for COI, the within-population nucleotide diversity (average pairwise difference) was 0.16–0.56% for *L. seishinensis*, but 0.52–0.84% for *L. semiopacus* and 1.21–1.33% for *L. koreanus*. Therefore, geographic differentiation with the fixation of haplotypes is likely more advanced in *L. seishinensis* than in *L. semiopacus*, and especially, *L. koreanus*.

The distribution pattern of locally unique haplotype clades of COI differed among the species. In *L. seishinensis*, two separate clades, sei-A and sei-B, occurred in the north and south, respectively. However, the nested clade analysis for nuclear 28S revealed a range expansion over the south-north region in this species. Diploid nuclear genes have longer coalescent times than haploid mitochondrial genes and can indicate relatively ancient events (Templeton 2002). Therefore, the differentiation in COI may have followed the range expansion detected by 28S. *Leptocarabus koreanus* also showed north-south differentiation of COI clades. Among the four separate clades of COI, kor-D occurred in the north and kor-A, B, and C occurred in the south. Although allopatric fragmentation was detected in southern clade 3.1, diverse haplotypes co-occurred in the south. In contrast with the other two species, *L. semiopacus* exhibited a shallow coalescence of COI haplotypes (Fig. 1), with all the COI haplotypes united in a single network (Fig. 3), and geographical segregation was absent for the highest clades (Fig. 1). The nested clade analysis detected a wide northward range expansion of a mitochondrial clade (2.5) in this species. Thus, the timing of recent range expansion and local differentiation appears to be different among the three species.

*Leptocarabus seishinensis* and *L. semiopacus* inhabit middle and upper elevations of mountainous areas and are adapted to very cool habitats (Kim and Lee, 1992), whereas *L. koreanus* does not appear to be cold-adapted, and instead colonizes lower elevations. *Leptocarabus koreanus*,

which occurs on Geojedo, a coastal island, is an exceptional case of an island distribution of *Leptocarabus* (Kwon and Lee, 1984). *Leptocarabus koreanus* apparently expanded its range southward during a glacial period, when the current coastal islands were part of the continental peninsula. However, the fact that no *Leptocarabus* species occurs on Jeju, the largest island of South Korea, 100 km off the Korean peninsula, suggests a restricted range expansion. Jeju Island was colonized by two *Coptolabrus* species by the late Pleistocene (the estimated time of differentiation between the Jeju and peninsular populations is 110,000–40,000 years ago; Zhang *et al.*, 2005). Since these beetles inhabit coastal areas, their range expansion during a regression (low sea-level) would have been more extensive than that of *Leptocarabus*. In *Coptolabrus*, genetic differentiation between the northern and southern regions of the Korean peninsula was not clear, and COI clades revealed gene flow along the Sobaek Mountains. This result is similar to that for *L. semiopacus*, but contrasts with those for *L. seishinensis* and *L. koreanus*, which showed differentiation between the northern and southern regions. However, *Coptolabrus* species exhibited differentiation between the eastern and western regions of the peninsula. This difference between the two groups may stem from disparities in habitat preference and colonization history. *Coptolabrus* inhabits lowlands and lower mountain zones and disperses mainly via lowland habitats, while *Leptocarabus* disperses along mountain zones, such as the Sobaek Mountains, which run longitudinally along the peninsula. Thus, the comparative phylogeography of closely related species, as well as of related groups, can reveal variation in the historical biogeography of related organisms in the same region.

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