

Mitochondrial DNA Sequence-Based Population Genetic Structure of the Firefly, *Pyrocoelia rufa* (Coleoptera: Lampyridae)

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*The genetic divergence, population genetic structure, and possible speciation of the Korean firefly, *Pyrocoelia rufa*, were investigated on the midsouthern Korean mainland, coastal islets, a remote offshore island, Jedu-do, and Tsushima Island in Japan. Analysis of DNA sequences from the mitochondrial COI protein-coding gene revealed 20 mtDNA-sequence-based haplotypes with a maximum divergence of 5.5%. Phylogenetic analyses using PAUP, PHYLIP, and networks subdivided the *P. rufa* into two clades (termed clade A and B) and the minimum nucleotide divergence between them was 3.7%. Clade A occurred throughout the Korean mainland and the coastal islets and Tsushima Island in Japan, whereas clade B was exclusively found on Jeju-do Island. In the analysis of the population genetic structure, clade B formed an independent phylogeographic group, but clade A was further subdivided into three groups: two covering western and eastern parts of the Korean peninsula, respectively, and the other occupying one eastern coastal islet and Japanese Tsushima Island. Considering both phylogeny and population structure of *P. rufa*, the Jeju-do Island population is obviously differentiated from other *P. rufa* populations, but the Tsushima Island population was a subset of the Korean coastal islet, Geoje. We interpreted the isolation of the Jeju-do population and the grouping of Tsushima Island with Korean coastal islets in terms*

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of Late Pleistocene–Holocene events. The eastern–western subdivision on the Korean mainland was interpreted partially by the presence of a large major mountain range, which bisects the midpart of the Korean peninsula into western and eastern parts.

KEY WORDS: firefly; *Pyrocoelia rufa*; mitochondrial DNA; COI gene; population genetic structure; genetic diversity; speciation.

INTRODUCTION

Fireflies are often regarded as a heart touching, indicative organism representing environmental cleanliness in Korea, but information on many ecological aspects and even taxonomy is largely unclear (Kim and Nam, 1981). The Korean firefly, *Pyrocoelia rufa*, has been recorded to occur in China, in Japan, on Tsushima Island, and mostly in the Korean mainland (Kim and Nam, 1981; Suzuki, 1997). However, it was only recently that an extensive investigation of firefly habitats was made and a new updated distribution chart of the species is now available (Kim *et al.*, 2003).

In Korea, since the first report of the firefly on Jeju-do Island by Okamoto (1924), eight species have been reported (Kim and Nam, 1981), but a recent extensive research unfortunately resulted in the discovery of only three species: *Hotaria unimunsana* and *Luciola lateralis* belonging to Luciolinae and *Pyrocoelia rufa* belonging to Lampyrinae (Kim *et al.*, 2003). Among them *P. rufa* is rarest in Korea and has the largest body size. In fall (August–September), females oviposit about 40–120 eggs under the rocks and roots of grasses where enough moisture is available, and the eggs are hatched in May–June (Kim *et al.*, 2003). Larval fireflies are subjected to ecdysis four times, and thereafter are subjected to metamorphosis approximately in August. Ten- to twelve-days after metamorphosis, pupal fireflies become adult fireflies, and their lifespan continues for 15–20 days until they end their lives (Kim *et al.*, 2003). Fireflies emit species-specific light for the purpose of mating. A flying male *P. rufa* emits light in response to the light emitted by a brachypterous female sitting on grass (J. Kim *et al.*, 2003).

I. Kim *et al.* (2000c) previously suggested the possibility that the Korean fireflies from the remote Jeju-do Island population are phylogenetically divergent from others in a molecular study. However, the statement was largely based on a comparison between only a few individuals from the Korean mainland and Jeju-do Island. In fact, the southern Korean coastline is characterized by numerous small islands (called “islets”) that lie within 1–3 km of the mainland (some are difficult to access). The only large island is Jeju-do, which is about 100 km offshore. Also, a remote Japanese island named Tsushima is located closer to the Korean mainland (about 50 km to the nearest Korean mainland). However, most of these localities have never been investigated. Furthermore, there is a tendency to lump most islet

populations together with the neighboring Korean mainland populations under the false assumption that the mainland is the contemporary source of the island populations.

In the taxonomic perspective, Matsumura (1928) long ago classified the Jeju-do population as *P. saishutonis*, the Tsushima Island population as *P. tsushimana*, and the Korean mainland one as *P. coreana* on the basis of morphological data. However, no molecular approach has dealt with this hypothesis until now.

In this study, we investigate population genetic structure and genetic divergence of the Korean firefly on the midsouthern Korean mainland, a few coastal islets, a remote offshore island, Jedu-do, and Tsushima Island in Japan and also provide information on the taxonomic status of the Jeju-do Island population. For the purpose of this study, we sequenced a portion of mitochondrial DNA (mtDNA) of *P. rufa* collected from 14 localities in Korea and two localities on Tsushima Island (a total of 107 specimens). We selected a portion of the cytochrome oxidase subunit I (COI) gene, which includes the membrane-spanning helices M3, M4, and M5, external loops E2 and E3, and internal loops I2 (Lunt *et al.*, 1996). This portion of the COI gene has proved useful for the study of intraspecific genetic variation in insects (Bae *et al.*, 2001; Kim *et al.*, 2000a,b; Simon *et al.*, 1994).

MATERIALS AND METHODS

Insects

Adult fireflies (*Pyrocoelia rufa*) were caught from 14 localities in Korea from July 1999 to October 2000, and the alcohol-preserved samples from two Tsushima Island locales were collected in October 1995 (Fig. 1). Korean samples were frozen at -70°C until molecular analyses were performed after a brief external morphological examination.

Mitochondrial DNA (mtDNA)

Total DNA was extracted following the standard Proteinase K method (Kocher *et al.*, 1989). A part of the COI gene was amplified by PCR using primers *CI-J-1751* (5'-GGAGCTCCTGACATAGCATTC-3') and *CI-N-2191* (5'-CCCGGTAA-ATTAATAATAACTTC-3') (Simon *et al.*, 1994). PCR conditions were as follows: after an initial denaturation step at 94°C for 5 min, 40 cycles of 94°C for 30 s, 50°C for 40 s, and 72°C for 45 s, and a final extension step at 72°C for 7 min were performed. To confirm the successful DNA amplification, electrophoresis was carried out using $0.5 \times$ TAE buffer in 1% agarose gel. The PCR product was then purified using a PCR purification Kit (Qiagen, Germany). DNA sequencing was performed using an ABI 310 Genetic Analyzer (PE Applied Biosystems, USA). Each strand was sequenced twice for accuracy. Sequence alignment was performed using IBI MacVector (ver. 6.5). When homologous sequences from two

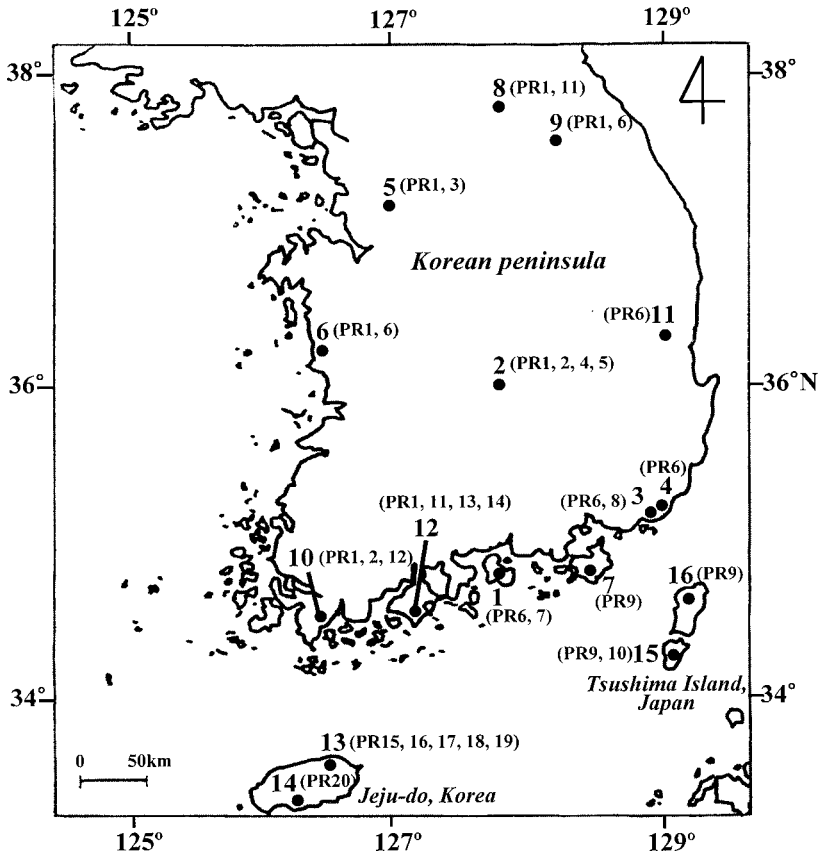


Fig. 1. Detailed map of midsouthern part of Korean peninsula and Japanese island Tsushima showing collecting localities (bold numbers correspond to location numbers in Table I) and haplotypes (in parentheses) obtained at each.

individuals differed by \geq one nucleotide base, the sequences were considered as different haplotypes. Haplotype designations were applied to new sequences as they were discovered (PR1–PR20).

Phylogenetic Analysis Using PAUP, Networks, and PHYLIP

PAUP (Phylogenetic Analysis using Parsimony) ver. 3.1 (Swofford, 1993) was used to infer possible phylogenetic relationships among the matrilineal sequences of *P. rufa*. The homologous mtDNA sequences from one individual of *Hotaria unmunisana* collected in Busan were used as an outgroup. The analysis was performed using an equal weighting of transitions and transversions by heuristic search as well as several ratios up to and including 1:20. The reliability of the trees was tested by 1000 iterations of bootstrapping (Felsenstein, 1985). With intraspecific mtDNA

sequence data it often happens that parsimony analyses provide limited resolution because of polytomies, possibly caused by back mutations and parallel mutations. One solution, which we employed, is to prepare one-step median networks, which provide insight into probable relationships among closely related lineages (Bandelt *et al.*, 1995).

As an alternative to the parsimony analysis, we used the Neighbor-Joining (NJ) method and the maximum likelihood (ML) method incorporated in PHYLIP (Phylogeny Inference Package) ver. 3.5c (Felsenstein, 1993). To obtain a phylogenetic tree, the data set was first iterated 1000 times using the subprogram SEQBOOT. Next, in the case of the NJ method, the iterated data set was run using the subprogram DNADIST to obtain a distance matrix between pairs of haplotypes with the option of Kimura's 2-parameter method (Kimura, 1980), which attempted to correct observed dissimilarities for multiple substitution in sequences evolving with a transition bias. Individual trees from each distance matrix were obtained using the subprogram NEIGHBOR. In the case of the ML method, individual trees were directly obtained using the subprogram DNAML. In both cases, the *H. unmunsana* sequence was included in the analysis to root the trees. Finally, a consensus tree representing reliability at each branch in the trees was obtained using the subprogram CONSENSE.

Genetic Distance, Migration Estimate, and Population Structuring

Genetic distance, coefficient of coancestry, and migration rate were estimated from mtDNA sequences and subroutines in the Arlequin version 2.0 (Schneider *et al.*, 2000). Population pairwise genetic distance (F_{ST}) and a permutation test of the significant differentiation of the pairs of localities (1000 bootstraps) were obtained following the approach described in Excoffier *et al.* (1992) and the distances between DNA sequences were calculated by the Kimura 2-parameters method (Kimura, 1980). Although F_{ST} values do not increase linearly with divergence time, they can be linearized (assuming that mutation rate is low and divergence time is relatively short) as: $D = -\log(1 - F_{ST})$, where D is the coancestry coefficient that is approximately linear with divergence time (Reynolds *et al.*, 1983). Pairwise F_{ST} values were used to estimate per generation migration rate, Nm (the product of the effective population size, N_e , and migration rate, m) based upon the equilibrium relationship: $F_{ST} = 1/(2Nm + 1)$. Genetic relationships among populations and sets of populations were assessed by the Holsinger and Mason-Gamer (H-MG) method (Holsinger and Mason-Gamer, 1996). A detailed rationale of this method is described in the original study of Holsinger and Mason-Gamer (1996) and other reports, which utilized this method (Kim *et al.*, 1998). Unlike other variance analyses, this approach generated the hierarchical relationships of the groups without specifying the hierarchical structure of the populations before the analysis (Holsinger and Mason-Gamer, 1996). Therefore, any structure present in the data set emerged naturally. In addition to the hierarchical structure among

populations, these statistics enabled us to test the significance of the genetic differentiation detected at each hierarchical level (10,000 bootstraps). To raise the reliance of these statistics, localities from which more than four individuals were collected were used, so localities 4 (Yongho-dong, Busan) and 16 (Sasuna, Tsushima Island) were excluded from these analyses.

RESULTS

Mt DNA Sequence Analysis

A total of 20 haplotypes (PR1–PR20) was obtained from the 403 bp of the COI gene from 107 individuals of the fireflies, *Pyrocoelia rufa*, collected from 14 localities in Korea and two locales from Japan. Individual haplotype and GenBank accession numbers are listed in Table I. Sequence alignment revealed 28 variable nucleotides: nine were T \leftrightarrow C transitions, 11 G \leftrightarrow A transitions, two each were C \leftrightarrow A and T \leftrightarrow G transversions, and four were A \leftrightarrow T transversions (Fig. 2). Twenty-six among 28 variable sites were in the third place of codons, but two were in the second place (nucleotide positions 81 and 231). These nonthird place sites caused substitutions of arginine to histidine in PR5 and of alanine to valine in PR14, respectively.

Sequence divergence among 20 haplotypes by pairwise comparisons ranged from 0.2 to 5.5% (1–22 bp), and the largest sequence divergence was observed when PR4 found only in locality 2 (Muju-gun) was compared with PR16 found in two individuals from locality 13 (Bugjeju-gun) (Table II).

PAUP, PHYLIP, and Networks Analysis

Phylogenetic relationships among haplotypes are depicted in Fig. 3. Because analyses run with transition: transversion weightings of 1:0, 1:1, 1:5, 1:10, and 1:20 did not affect the topology of the tree, only the result obtained by unordered analysis is presented (Fig. 3). Twenty haplotypes obtained in this study were subdivided into two independent groups (termed clade A and clade B), although haplotype relationships within each clade were mostly not resolved, possibly by small nucleotide difference (e.g., 1 or 2 bp). Each clade was somewhat different in haplotype composition. For example, clade A consists of a large number of haplotypes (14 haplotypes among 20) and is larger in maximum sequence divergence (1.5%) than clade B (Fig. 3 and Table II). On the other hand, clade B contained six haplotypes with a maximum sequence divergence of 0.7% (Fig. 3 and Table II). Figure 4 represents the result of probability-based phylogenetic analyses using the neighbor-joining (NJ) method. This method generated more branches in clade A than PAUP, but most of them were weakly supported. Nevertheless, each monophyletic group designated in the PAUP analysis included identical haplotypes, suggesting that *P. rufa* can be subdivided into two distinct genetic groups. We also performed the

Table I. Trapping Localities, Animal Numbers, Mitochondrial COI Haplotypes, and GenBank Accession Numbers

Collecting locality (no. of individuals)	Collection date	Animal number	Sex	COI haplotype	GenBank accession number
1. Bonghwa-ri, Samdong-myon, Namhae-gun, Gyeongsangnam-do (8)	1999. 7. 29	F50	NA	PR6	AF277807
		F51	NA	PR6	AF277808
		F52	NA	PR6	AF277809
		F53	NA	PR6	AF277810
		F54	NA	PR6	AF277811
		F55	NA	PR6	AF277812
		F56	NA	PR6	AF277813
		F57	NA	PR7	AF277814
2. Suan-ri, Seolcheon-myon, Muju-gun, Jeonlabug-do (9)	1999. 7. 20	F58	NA	PR1	AF277815
		F59	NA	PR1	AF277816
		F60	NA	PR1	AF277817
		F61	NA	PR2	AF277818
		F62	NA	PR4	AF277819
		F63	NA	PR2	AF272702
		F64	NA	PR2	AF277820
		F65	NA	PR5	AF277821
3. Goejong-dong, Saha-gu, Busan-si (8)	1999. 8. 12	F66	NA	PR1	AF277822
		F67	NA	PR6	AF277823
		F68	NA	PR6	AF277824
		F69	NA	PR6	AF277825
		F70	NA	PR6	AF277826
		F90	M	PR6	AF445559
		F91	M	PR6	AF445560
		F92	M	PR8	AF445561
4. Yongho-dong, Nam-gu, Busan-si (1)	1999. 8. 26	F94	M	PR6	AF445562
		F71	M	PR6	AF277827
5. Muk-ri, Leedong-myon, Yongin-si, Gyeonggi-do (4)	1999. 9. 2	F72	M	PR3	AF277828
		F73	M	PR1	AF277829
		F74	M	PR1	AF277830
		F75	M	PR1	AF277831
6. Bolyeong-si, Chungcheongnam-do (6)	1999. 9. 18	F78	M	PR1	AF445563
		F79	M	PR1	AF272697
		F80	M	PR1	AF445564
		F81	M	PR6	AF445565
		F82	M	PR1	AF445566
		F84	M	PR1	AF445567
7. Nambu-myon, Geoje-si Gyeongsangnam-do (8)	2000. 7. 16	F261	NA	PR9	AF445568
		F262	NA	PR9	AF445569
		F263	NA	PR9	AF445570
		F264	NA	PR9	AF445571
		F265	NA	PR9	AF445572
		F267	NA	PR9	AF445573
		F268	NA	PR9	AF445574
		F269	NA	PR9	AF445575

Table I. (Continued.)

Collecting locality (no. of individuals)	Collection date	Animal number	Sex	COI haplotype	GenBank accession number
8. Chuncheon-si, Gangwon-do (7)	2000. 8. 18	F272	NA	PR11	AF445576
		F273	NA	PR11	AF445577
		F274	NA	PR11	AF445578
		F275	NA	PR11	AF445579
		F344	NA	PR1	AF445580
		F345	NA	PR11	AF445581
		F346	NA	PR1	AF445582
9. Heungjeong-ri, Bongpyeong-myon, Pyeongchang-gun, Gangwon-do (8)	2000. 8. 8	F282	M	PR6	AF445583
		F283	M	PR6	AF445584
		F284	M	PR1	AF445585
		F285	M	PR6	AF445586
		F286	M	PR6	AF445587
		F287	M	PR6	AF445588
		F288	M	PR6	AF445589
10. Haenam-gun, Jeonlanam-do (8)	2000. 8. 10	F293	NA	PR2	AF445591
		F294	NA	PR2	AF445592
		F295	NA	PR2	AF445593
		F296	NA	PR2	AF445594
		F298	F	PR1	AF445595
		F299	F	PR1	AF445596
		F300	F	PR12	AF445597
11. Cheongsong-gun, Gyeongsangbug-do (7)	2000. 9. 9	F301	F	PR1	AF445598
		F321	M	PR6	AF445599
		F322	NA	PR6	AF445600
		F323	NA	PR6	AF445601
		F324	NA	PR6	AF445602
		F325	NA	PR6	AF445603
		F326	NA	PR6	AF445604
12. Goheung-si, Jeonlanam-do (5)	2000. 10. 7	F327	NA	PR6	AF445605
		F331	F	PR13	AF445606
		F332	F	PR13	AF445607
		F333	F	PR1	AF445608
		F334	F	PR14	AF445609
		F335	F	PR11	AF445610
		13. Pyeongdae-ri, Gujwa-eub, Bugjeju-gun, Jeju-do (8)	1999. 9. 16	F87	M
F88	M			PR16	AF445612
F89	M			PR15	AF272698
F256	NA			PR19	AF445613
F257	NA			PR17	AF445614
F258	NA			PR15	AF445615
F259	NA			PR15	AF445616
14. Yelae1-dong, Seogwipo-si, Jeju-do (6)	2000. 8. 30	F260	NA	PR18	AF445617
		F302	NA	PR20	AF445618
		F303	NA	PR20	AF445619
		F304	NA	PR20	AF445620
		F305	NA	PR20	AF445621
		F306	NA	PR20	AF445622
		F307	M	PR20	AF445623

Table I. (Continued.)

Collecting locality (no. of individuals)	Collection date	Animal number	Sex	COI haplotype	GenBank accession number
15. Kyozyuka, Izuhara-machi, Tsushima Island, Kyshu, Japan (11)	1995. 10. 15	F100	M	PR9	AF445624
		F101	M	PR9	AF445625
		F102	M	PR9	AF445626
		F103	M	PR9	AF445627
		F104	M	PR9	AF445628
		F105	NA	PR9	AF445629
		F106	NA	PR10	AF445630
		F107	NA	PR9	AF445631
		F108	NA	PR9	AF445632
		F109	NA	PR9	AF445633
		F110	NA	PR9	AF445634
16. Sasuna, Kamiagata-machi, Tsushima Island, Kyushu, Japan (3)	1995. 10. 15	F111	NA	PR9	AF445635
		F112	NA	PR9	AF445636
		F113	NA	PR9	AF445637

Note. NA, not identifiable.

maximum-likelihood (ML) based phylogenetic analysis, but the topology obtained by this method was almost identical with that of NJ method (data not shown).

To illustrate further the genetic relationships among *P. rufa* haplotypes, we used an unrooted one-step median network, which visualizes a possible evolutionary pathway among closely related haplotypes (Fig. 5). Although we expected more resolution among the closely related haplotypes, it provided limited information. For example, the network suggested that haplotypes belonging to clade A could have been derived from a single founder, haplotype PR1 (labeled as 1), showing star phylogeny, but relationships among others were not resolved. Nevertheless, the network confirmed the result obtained from PAUP and PHYLIP analyses that haplotypes were subdivided into two clades, separated by 3.7% (15 mutational steps) of minimum nucleotide divergence.

Regional Distribution and Frequency of Haplotype

The distribution and frequency of haplotypes are listed in Tables I and III, respectively. Among 20 haplotypes all but PR1, PR2, PR6, PR9, and PR11 were found only in one locality, and 11 of them were found only as single individuals, indicating regional restriction and rarity of most haplotypes. The most frequent haplotype was PR6 (27 among 107 individuals) and next was PR1 (19 among 107 individuals). In terms of distribution, these two haplotypes were found in five and six localities, respectively (Tables I and III).

When members of haplotypes in each clade were related to their geographic location, they were perfectly divided into two groups without overlap (Figs. 1, 3,

			30		60	
PR1	TCGAATAAAT	AATATAAGAT	TTTGACTTCT	TCCACCATCA	TTATCATTAC	TTTAAATAAG
PR2
PR3
PR4
PP5
PR6
PR7
PR8
PR9
PR10
PR11
PR12
PR13
PR14
PR15C.....
PR16C.....	.G.....
PR17C.....
PR18C.....
PR19C.....
PR20C.....
			90		120	
PR1	AAGATTAATT	GAAAGTGGGG	CAGGTACGGG	ATGAACAGTT	TATCCGCCCT	TATCAGCAAA
PR2
PR3
PR4
PP5
PR6A.....
PR7A.....A.....
PR8A.....A.....
PR9A.....A.....
PR10A.....A.....
PR11A.....A.....
PR12A.....A.....
PR13
PR14	T.....
PR15A.....	.A.....A..T.
PR16A.....	.A.....A..T.
PR17A.....	.A.....A..T.
PR18A.....	.A.....A..T.
PR19A.....	.A.....A..T.
PR20A.....	.A.....A..T.
			150		180	
PR1	TATTGCACAT	AGAGGTC CCT	CTGTAGATT	AGCAATTTT	AGACTTCATT	TAGCAGGTAT
PR2
PR3
PR4	G.....
PP5
PR6	G.....
PR7

Fig. 2. Sequence alignment of 20 mitochondrial haplotypes (designated as PR1–PR20) obtained from 403-bp COI sequences of *P. rufa*. GenBank accession numbers of individual fireflies are listed in Table I.

4, and 5). Clade B (haplotypes PR15–PR 20) occurs in Jeju-do Island (localities 13 and 14) exclusively and clade A (haplotypes PR1–PR14) occurs in all localities but localities 13 and 14. Thus, phylogenetic analysis and haplotype distribution collectively suggest that *P. rufa* is subdivided into two geographic groups.

PR8	G.....	
PR9	
PR10	
PR11	
PR12	
PR13	C.....	
PR14	
PR15	G.....	G.....G..	
PR16	G.....	G..A..G..	
PR17	G..A..G..	
PR18	G.....	G..A..G..	
PR19	G.....	G..A..G..	
PR20	G.....	G..A..G..	
				210			
PR1	TTCCTCAATT	CTTGGAGCAG	TGAATTTTAT	CTCAACTATT	ATTAATATAC	GCCCTAATAG	
PR2	
PR3	
PR4	
PP5	A.....	
PR6	
PR7	
PR8	
PR9	
PR10	
PR11	
PR12	
PR13	
PR14	
PR15	C.....	A.....	
PR16	C.....	A.....	
PR17	C.....	A.....	
PR18	C.....	A.....	
PR19	C.....	A.....	
PR20	C.....	A.....	
				270			
PR1	AATAATATTT	GATCAAATAC	CTTTATTTGT	ATGAGCAGTT	CTAATTACTG	CCATCTTATT	
PR2	
PR3	
PR4	
PP5	
PR6	
PR7	A.....	
PR8	
PR9	
PR10	G.....	
PR11	
PR12	
PR13	
PR14	
PR15	A.....A..T.....	

Fig. 2. (Continued.)

Gene Flow and Population Genetic Structure

Genetic distance (F_{ST}), coancestry coefficients (D), and per generation migration rates (Nm) are shown in Table IV. Among 82 pairwise comparisons, 67 showed significant genetic differentiation ($p < 0.05$). The maximum value of genetic

PR16A.....A..T....
PR17A.....A..T....
PR18A.....A..T....
PR19A.....A..T....
PR20A.....A..T....
			330		360
PR1	GCTTTTATCT	CTACCTGTTT	TAGCAGGAGC	TATCACAATA	TTATTAACAG ATCGAAATTT
PR2
PR3	A.....
PR4	A.....
PP5
PR6	A.....
PR7
PR8	A.....
PR9
PR10
PR11
PR12T.....
PR13
PR14
PR15	..C.....
PR16	..C.....
PR17	..C.....
PR18	..C.....
PR19	..C.....
PR20	..C.....
			390	403	
PR1	AAATTCCTCA	TTTTTTGACC	CAGCGGGAGG	AGGAGATCCT	GTA
PR2A.....
PR3
PR4
PP5A.....
PR6
PR7
PR8
PR9A.....
PR10A.....
PR11
PR12
PR13
PR14
PR15T.....	..A.....C..A
PR16T.....	..A.....C..A
PR17T.....	..A.....C..A
PR18A.....C..A
PR19T.....C..A
PR20T.....C..A

Fig. 2. (Continued.)

differentiation ($F_{ST} = 1$) was observed in four pairwise comparisons as in localities 7 (Geoge-si) and 10 (Henam-gun), 7 and 14 (Seogwipo-si), 11 (Cheongsong-gun) and 14, and 12 (Goheong-si) and 14. All these pairs include either coastal islet (Geoje-si) or offshore island (Jeju-do), but no between-islet (or Island) and no within-mainland localities are involved (Table IV). Coancestry coefficients ($D = 0$ is identical, shared ancestry) ranged from 0 to 4.95, and the highest value was observed in a comparison between localities 3 (Busan-si) and 14 (Seogwipo-si).

Table II. Pairwise Comparisons Among 20 Haplotypes Obtained From the Partial Sequences of Mitochondrial COI

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. PR1	—	0.002	0.002	0.005	0.005	0.007	0.007	0.010	0.007	0.010	0.002	0.005	0.002	0.002	0.045	0.050	0.045	0.045	0.042	0.047
2. PR2	1	—	0.005	0.007	0.002	0.010	0.010	0.012	0.005	0.007	0.005	0.007	0.005	0.005	0.042	0.047	0.042	0.042	0.045	0.045
3. PR3	1	2	—	0.002	0.007	0.005	0.010	0.007	0.010	0.012	0.005	0.007	0.005	0.047	0.052	0.047	0.047	0.045	0.045	0.050
4. PR4	2	3	1	—	0.010	0.002	0.012	0.005	0.012	0.015	0.007	0.010	0.007	0.007	0.050	0.055	0.050	0.050	0.047	0.052
5. PR5	2	1	3	4	—	0.012	0.012	0.015	0.007	0.010	0.007	0.010	0.007	0.045	0.050	0.045	0.045	0.047	0.047	0.047
6. PR6	3	4	2	1	5	—	0.010	0.002	0.010	0.012	0.005	0.012	0.010	0.047	0.052	0.047	0.047	0.045	0.045	0.050
7. PR7	3	4	4	5	5	4	—	0.007	0.005	0.007	0.005	0.012	0.010	0.042	0.047	0.042	0.042	0.040	0.045	0.045
8. PR8	4	5	3	2	6	1	3	—	0.007	0.010	0.007	0.015	0.012	0.045	0.050	0.045	0.045	0.042	0.042	0.047
9. PR9	3	2	4	5	3	4	2	3	—	0.002	0.005	0.012	0.010	0.037	0.042	0.037	0.037	0.040	0.040	0.040
10. PR10	4	3	5	6	4	5	3	4	1	—	0.007	0.015	0.012	0.040	0.045	0.040	0.040	0.042	0.042	0.042
11. PR11	1	2	2	3	3	2	2	3	2	3	—	0.007	0.005	0.005	0.042	0.047	0.042	0.040	0.040	0.045
12. PR12	2	3	3	4	4	5	5	6	5	6	3	—	0.007	0.007	0.045	0.050	0.045	0.042	0.042	0.047
13. PR13	1	2	2	3	3	4	4	5	4	5	2	3	—	0.005	0.047	0.052	0.047	0.047	0.045	0.050
14. PR14	1	2	2	3	3	4	4	5	4	5	2	3	2	—	0.047	0.052	0.047	0.047	0.045	0.050
15. PR15	18	17	19	20	18	19	17	18	15	16	17	18	19	19	—	0.005	0.005	0.005	0.002	0.002
16. PR16	20	19	21	22	20	21	19	20	17	18	19	20	21	21	2	—	0.005	0.005	0.007	0.002
17. PR17	18	17	19	20	18	19	17	18	15	16	17	18	19	19	2	2	—	0.005	0.007	0.002
18. PR18	18	17	19	20	18	19	17	18	15	16	17	18	19	19	2	2	2	—	0.005	0.007
19. PR19	17	18	18	19	18	19	18	16	17	16	17	16	17	18	1	1	3	3	3	—
20. PR20	19	18	20	21	19	20	18	19	16	17	18	19	20	20	1	1	3	3	1	2

Note. Numbers above the diagonal are mean distance values; numbers below the diagonal are absolute distance values.

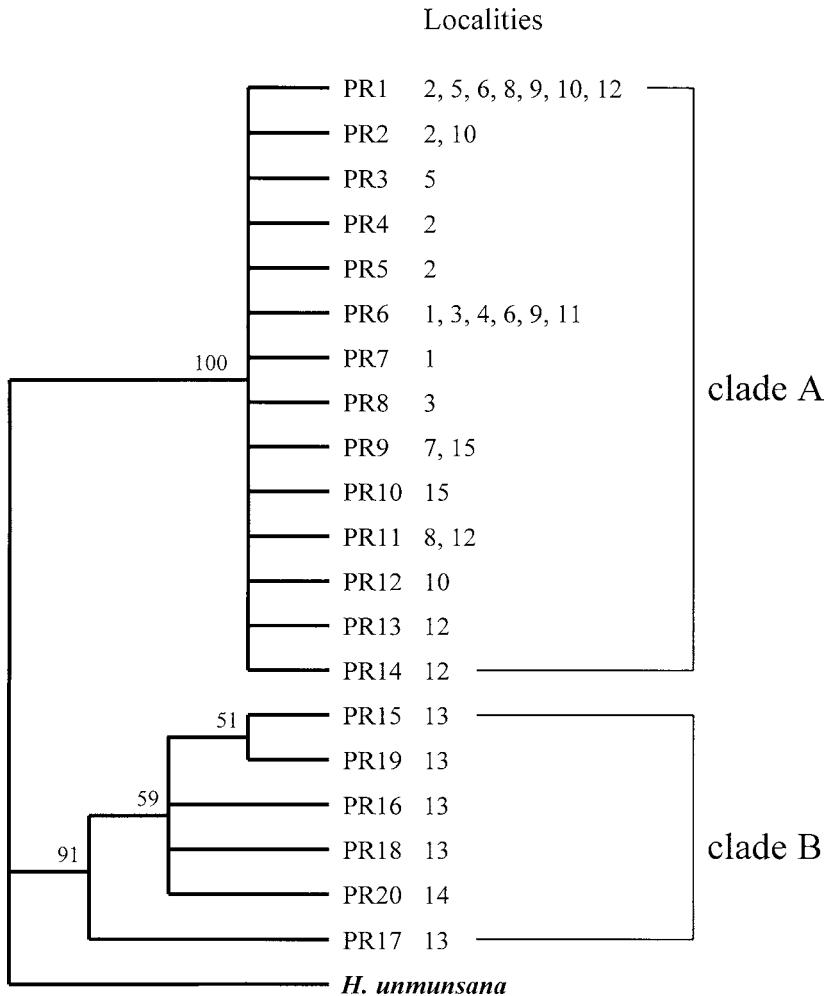


Fig. 3. A PAUP analysis of mitochondrial COI gene sequences of *P. rufa* using homologous sequence of another firefly, *Hotaria unmunšana*, as an outgroup. The tree shown is an unordered tree obtained with the option of “retain groups with frequency >50%” by majority-rule consensus of three equally parsimonious trees from the heuristic search. The numbers shown on the branches represent bootstrap values for 1000 replicates. Tree length is 120 steps long, Consistency Index is 0.800, and Retention Index is 0.975.

The remaining 15 comparisons among 82 showed no significant genetic differentiation (Table IV). Interestingly, these localities did not show any “genetic isolation by distance” including localities on the mainland (Fig. 6). For example, although localities 8 (Chuncheon-si) and 9 (Pyeongchang-gun) are neighboring

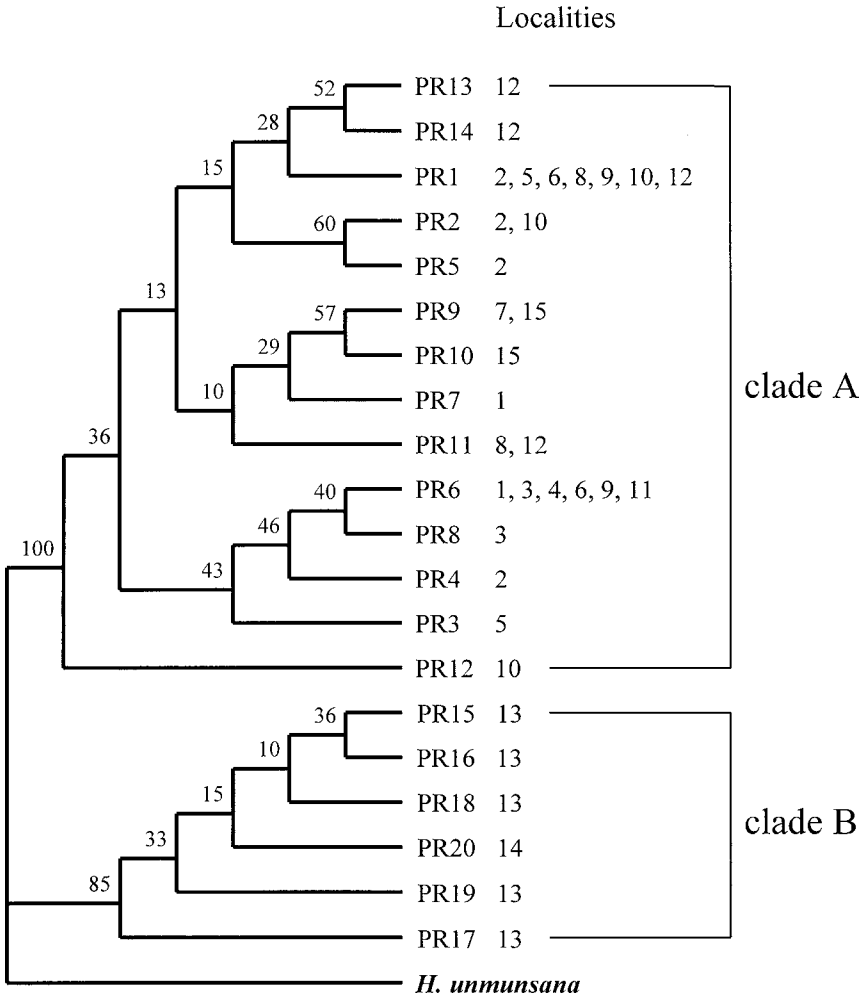


Fig. 4. PHYLIP analysis of mitochondrial COI gene sequences of *P. rufa*. The tree was obtained using the subprogram NEIGHBOR incorporated in PHYLIP with the option of Kimura's 2-parameter method (1980). The tree was rooted using *H. unmunšana*. The numbers shown on the branches, which represent bootstrap values for 1,000 replications, were obtained using the subprogram CONSENSUS.

(only about 50 km apart), substantial genetic differentiation between them was detected ($F_{ST} = 0.70$) and the value was statistically significant ($p < 0.01$). On the other hand, localities 5 (Yongin-si) and 10 (Henam-gun), separated by at least 400 km are not differentiated ($F_{ST} = 0.18$) enough to show a statistically significant genetic distance and, instead, per generation female migration rate was substantial ($Nm = 2.57$; Fig. 6).

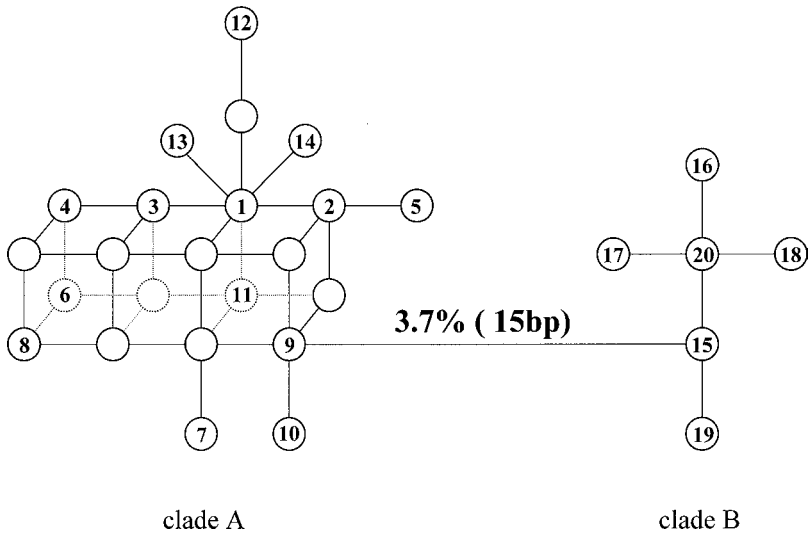


Fig. 5. Parsimonious one-step median networks analysis among 20 *P. rufa* haplotypes. Each bar indicates one nucleotide difference from the neighboring haplotype and the empty circles indicate the hypothetical haplotypes, which were not found in this study. Numbers on each circle denote haplotype name, omitting the antecedent letters, PR. Note that PR9 in clade A and PR15 in clade B require 15 mutational steps (3.7%) to connect between clade A and clade B.

A comparison of pairwise estimates of F_{ST} and/or gene flow showed that mainland populations of *P. rufa* were best explained by an east–west subdivision. The localities on the western part of the Korean peninsula (localities 2, 5, 6, 8, 10, and 12) are mostly interrelated, but none of these has genetic affinity to the localities on the eastern part of the Korean peninsula (localities 1, 3, 4, 9, and 11) (Fig. 6). At the same time, although most of the eastern localities are interrelated, none of these has genetic affinity to the localities on the western site of the Korean peninsula (Fig. 6). Along with this subdivision, the coastal island-locality, Geoje-si (7), was uniquely interrelated with one locality on Tsushima Island (locality 15, $F_{ST} = 0$, $Nm = \text{infinite}$). The two localities on the remote Jeju-do Island were genetically connected exclusively ($Nm = 2.45$). Thus, gene flow data collectively suggest the presence of four genetic groups in the *P. rufa* populations surveyed.

Hierarchical Population Genetic Structure

To test further genetic relationships among firefly populations, a hierarchical analysis among localities was performed by the H-MG method (Holsinger and

Table III. Relative Frequencies of mtDNA COI Haplotypes Through the Populations

Haplotype	1 (8)	2 (9)	3 (8)	4 (1)	5 (4)	6 (6)	7 (8)	8 (7)	9 (8)	10 (8)	11 (7)	12 (5)	13 (8)	14 (6)	15 (11)	16 (3)
PR1	0	0.444	0	0	0.75	0.833	0	0.286	0.125	0.375	0	0.2	0	0	0	0
PR2	0	0.333	0	0	0	0	0	0	0	0.5	0	0	0	0	0	0
PR3	0	0	0	0	0.25	0	0	0	0	0	0	0	0	0	0	0
PR4	0	0.111	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PR5	0	0.111	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PR6	0.875	0	0.875	1	0	0.167	0	0	0.875	0	1	0	0	0	0	0
PR7	0.125	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PR8	0	0	0.125	0	0	0	0	0	0	0	0	0	0	0	0	0
PR9	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0.909	1
PR10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.091	0
PR11	0	0	0	0	0	0	0	0.714	0	0	0	0.2	0	0	0	0
PR12	0	0	0	0	0	0	0	0	0	0.125	0	0	0	0	0	0
PR13	0	0	0	0	0	0	0	0	0	0	0	0.4	0	0	0	0
PR14	0	0	0	0	0	0	0	0	0	0	0	0.2	0	0	0	0
PR15	0	0	0	0	0	0	0	0	0	0	0	0	0.375	0	0	0
PR16	0	0	0	0	0	0	0	0	0	0	0	0	0.25	0	0	0
PR17	0	0	0	0	0	0	0	0	0	0	0	0	0.125	0	0	0
PR18	0	0	0	0	0	0	0	0	0	0	0	0	0.125	0	0	0
PR19	0	0	0	0	0	0	0	0	0	0	0	0	0.125	0	0	0
PR20	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0

Note. Numbers in parentheses indicate sample size at each population.

Table IV. Mitochondrial COI Sequence Data of Coancestry Coefficients, Genetic Distance, and Per-Generation Female Migration Rate Between Pairs of Localities

	1	2	3	5	6	7	8	9	10	11	12	13	14	15
1. Nambhe-gun	0													
	0													
	0													
2. Muju-gun	1.11	0												
	0.67***	0												
	0.25	0												
3. Busan-si	0	1.52	0											
	-0.05	0.78***	0											
	Inf	0.141	0											
5. Yongin-si	1.22	0.05	2.17	0										
	0.71**	0.05	0.89**	0										
	0.21	9.141	0.06	0										
6. Bolyeong-si	0.95	0.07	1.53	0	0									
	0.61*	0.07	0.78**	-0.15	0									
	0.32	6.59	0.14	Inf	0									
7. Geoje-si	2.02	1.50	3.44	3.06	2.01	0								
	0.87***	0.78***	0.97***	0.95**	0.87**	0								
	0.08	0.14	0.02	0.03	0.08	0								
8. Chuncheon-si	1.11	0.53	1.92	0.69	0.29	2.34	0							
	0.67***	0.41**	0.85***	0.50	0.25*	0.90***	0							
	0.25	0.72	0.09	0.51	1.47	0.05	0							
9. Pyeongchang-gun	0	1.11	0.00	1.30	0.96	2.34	1.21	0						
	-0.08	0.67**	0.00	0.73**	0.62*	0.90**	0.70***	0						
	Inf	0.25	1061	0.19	0.31	0.05	0.21	0						

10. Henam-gun	1.29	0	1.78	0.18	0.19	1.64	0.62	1.31	0	0.62	1.31	0
	0.73***	-0.06	0.83***	0.16	0.17*	0.81***	0.46**	0.73***	0	0.46**	0.73***	0
11. Cheongsong-gun	0.19	Inf	0.10	2.57	2.44	0.12	0.58	0.18	0	0.58	0.18	0
	0	1.59	0	2.79	1.69	Inf	2.27	0	1.89	0	2.27	0
12. Gohyeung-si	-0.02	0.80***	-0.02	0.94**	0.82**	1.00***	0.90***	-0.02	0.85***	0	0.90***	0
	Inf	0.13	Inf	0.03	0.11	0.00	0.06	Inf	0.09	0	0.06	0
13. Bugjeju-gun	1.08	0.20	1.64	0.07	0.03	1.86	0.32	1.12	0.24	1.78	0	1.78
	0.66***	0.18*	0.81***	0.7	0.03*	0.85***	0.28*	0.67**	0.22*	0.83***	0	0.83***
14. Seogwipo-si	0.26	2.32	0.12	6.87	14.5	0.09	1.32	0.24	1.81	0.10	0	0
	2.67	2.56	2.99	2.65	2.59	2.91	2.75	2.77	2.58	3.06	2.48	0
15. Kyozeuka	0.93***	0.92***	0.95***	0.93**	0.93**	0.95***	0.94***	0.94***	0.92***	0.95***	0.92***	0
	0.04	0.04	0.03	0.04	0.04	0.03	0.03	0.03	0.04	0.03	0.05	0
16. Seogwipo-si	3.56	3.26	4.95	4.67	3.68	Inf	4.29	3.85	3.42	Inf	3.47	0
	0.97***	0.96***	0.99**	0.99***	0.98***	1.00***	0.99**	0.98***	0.97***	1.00**	1.00**	0
17. Kyozeuka	0.02	0.02	0.00	0.01	0.01	0.00	0.01	0.01	0.02	0.00	0.02	2.45
	1.99	1.52	2.95	2.56	1.94	0	2.09	2.25	1.63	3.60	1.84	2.96
18. Kyozeuka	0.86***	0.78***	0.95***	0.92***	0.86**	-0.03	0.88***	0.90**	0.80**	0.97**	0.84**	0.95***
	0.08	0.14	0.03	0.04	0.08	Inf	0.07	0.06	0.12	0.01	0.10	0.03

Note. Distance method was utilized for that of Kimura's 2-parameter method (Kimura, 1980). Value at the first line of each column is the estimate of coancestry coefficients (D), at the second line is genetic distance (F_{ST}), and third is per-generation female migration rate (Nm).

* $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. Inf means infinite.

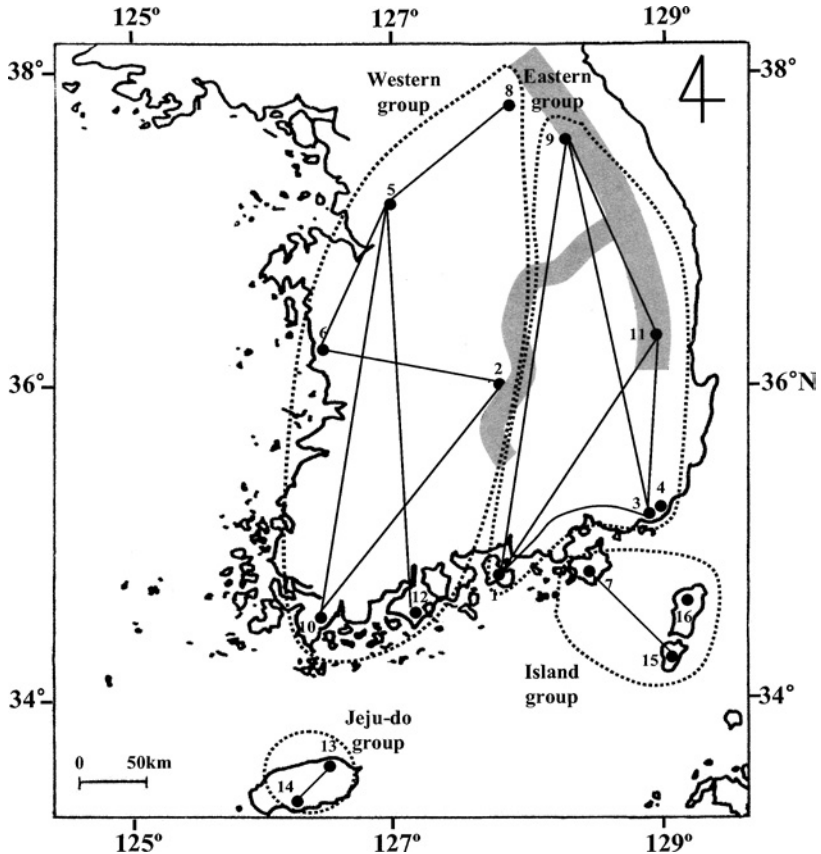


Fig. 6. A map showing a summary of gene flow and population genetic structure of *P. rufa* populations. The lines connecting pairs of localities denote nonsignificant F_{ST} values at the level of $p > 0.05$ (summarized from Table IV). The dotted lines denote population structure generated by the H-MG method (1996; summarized from Fig. 7). The gray bars indicate an approximate stretch of the major mountain range called “Bekdudegan” in Korea.

Mason-Gamer, 1996) as presented in Fig. 7. *P. rufa* populations in Korea and Japan were subdivided into several statistically significant groups. Four groups were notable (termed western, eastern, island, and Jeju-do groups). The genetic distances separating these groups (maximum $\hat{g}_{st} = 1$) ranged from 0.612 ($p = 0.000$) to 0.905 ($p = 0.000$) and these estimates were substantially high among the distance values represented at other nodes (-0.289 – 0.134). These four groupings were concordant with the result obtained in the population differentiation and/or gene flow analyses (Table IV), confirming the presence of four genetic groups in *P. rufa* populations.

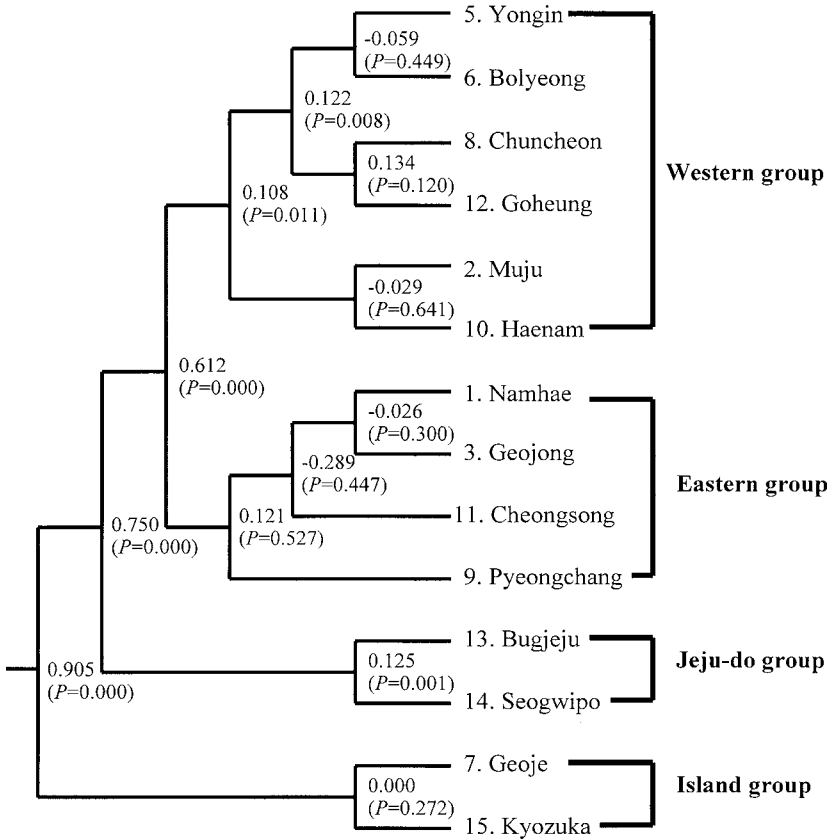


Fig. 7. Dendrogram resulting from the Holsinger and Mason-Gamer (1996) analysis of localities, from which the sample size was ≥ 5 . The number at each node is the distance between its two daughter nodes and the p value is the significance of differentiation (based on 10,000 random resamplings).

DISCUSSION

Populations of nearly all species tend to exhibit at least some level of genetic differentiation unless they are panmictic (Ehrlich and Ravin, 1969). Additional structure might be found for several reasons such as dispersal ability, habitat specificity, mating system, and so on. Along with these behavioral causes, probably other complications would be generated and it makes prediction difficult when numerous physically isolated and/or semi-isolated islands/islets are involved in the study area and the possibility of geographic isolation is also involved.

Phylogenetic analysis of *Pyrocoelia rufa* showed that the Korean fireflies are subdivided largely into two clades and distribution of each clade is concordant

with geographic separations: one exclusively from Jeju-do Island (clade B) and the other from all localities but Jeju-do Island (clade A) (Table II; Fig. 5). Further, within clade A, fireflies in the Korean mainland, coastal islets, and Tsushima Island were structured in some unexpected forms as revealed in the analyses of gene flow and population genetic structure (Table IV and Figs. 6 and 7). In the following discussion, we separate our study into genetic differentiation of the Jeju-do population and population genetic structure in the Korean peninsula. Although, for convenience, we have treated these as separate items, they are interrelated and interdependent at some level.

Genetic Differentiation of the Jeju-do Population

Island biogeography has been an important part of evolutionary theory since the days of Darwin and Wallace. To a large extent our continuing interest derives from the expectation that geographic isolation is an obvious potential source of reproductive isolation. Both phylogenetic and population genetic analyses consistently suggest that the Jeju-do population is significantly differentiated from all others (Figs. 3, 4, 5, and 7). Because Jeju-do Island is a remote offshore island (>100 km from the closest mainland), it would be reasonable to speculate that the geographic isolation of the island might be a source of phylogenetic differentiation. In fact, differentiation of the Jeju-do population was suggested long ago by Matsumura (1928). He proposed, on the basis of morphological data, that the Jeju-do population should be considered an independent species, *P. saishutonis*, separated from the Korean mainland population, *P. coreana*. He also placed the Tsushima Island population into the *P. tsushimana* species. In fact, the minimum sequence divergence of the haplotypes found in Jeju-do population is larger than the within-species level (3.7%; Fig. 6). For example, other studies which utilized the same mitochondrial gene showed that the sequence divergence was 0.2% for domestic silkworm (Kim *et al.*, 2000b), 0.2% and 1.2% for two species of mushroom flies (Bae *et al.*, 2001), about 0.23% and 0.12% for two species of the rice planthoppers (Mun *et al.*, 1999), 0.4% for the spruce budworm species (Sperling and Hickey, 1994), 0.5% for *Heliconius* butterflies (Brower, 1994), 1.4% for the diamondback moth (Kim *et al.*, 2000a), and 4.0% for another firefly in Korea (*L. lateralis*; Kim *et al.*, 2001). Excluding *L. lateralis*, which also has a taxonomic implication, the divergence was $\leq 1.4\%$ in the mitochondrial COI gene. These data show that the degree of genetic divergence between the two clades of *P. rufa* is far greater than the within-species level of other insect species. Thus, the old hypothesis on the Jeju-do population appears to be supported by our mtDNA data, although more detailed morphological, nuclear, and breeding (light-signaling pattern) data are required to confirm the present genetic data. However, our mtDNA data do not support the idea that the Tsushima Island population is *P. tsushimana*.

Although we presently have no means of ascertaining the evolutionary rate in the COI protein-coding gene in the *P. rufa* genome, it nevertheless seems likely that our data reflect relatively well documented Late Pleistocene–Holocene events. According to Ohshima (1990), the last glacial advance (the Haupt Würm age) was about 20,000 years ago (BP). At this time, the lowered sea level (estimated at 80 ± 5 m) meant that the Korean peninsula and the present Jeju-do Island were joined (Ohshima, 1990; Park, 1988). Later Jeju-do Island became such about 12,000 BP and the near-shore islets (Namhe and Geoje) formed about 7,000 BP when the sea level rose to its present state (Ohshima, 1990). Thus, for about 12,000 BP, *P. rufa* on the Jeju-do Island may have started to accumulate an independent genetic variation separated from other populations, although further evidence may be required to test this hypothesis (*e.g.*, other organisms and other geological data).

Population Genetic Structure in the Korean Mainland

The analyses of gene flow and hierarchical population genetic structure show that the Korean firefly populations in the Korean mainland, coastal islets, and Tsushima Island form three separate groups (the western, eastern, and island groups; Figs. 6 and 7), although they contain phylogenetically more or less continuous haplotypes. Two mainland groups were generated, bisecting the Korean mainland into western and eastern parts (the western and eastern groups). The remaining island group includes both one eastern coastal islet in the Korean peninsula and the Japanese Tsushima Island.

Avise *et al.* (1987) proposed five different distribution patterns of mtDNA clones. Among them category III is one that possesses phylogenetically adjacent clones, each of which is nonetheless confined to a subset of the geographic range of the species. Although phylogenetic analysis did not reveal further subdivision within clade A (Figs. 3 and 4), haplotypes in clade A are mostly confined to the given locality: nine among 14 are confined to a single locality in clade A (Table III). This may be reflected in the data of F_{ST} (Table IV) and population genetic structure, forming western, eastern, and island groups (Fig. 7). Thus, our mtDNA data represents category III of the phylogeographic pattern *sensu* Avise (Avise 1989; Avise *et al.*, 1987). Avise *et al.* (1987) suggested that the most likely explanation for the occurrence of category III involves historically limited gene flow between populations in the species not subdivided by firm long-term zoogeographic barriers to dispersal.

Considering that the adult female of *P. rufa* possesses degenerative back wings, which render flight almost impossible, mitochondrial DNA-based gene flow would be highly hampered, although passive dispersal and crawling are possible. Thus, newly arisen mutations would be confined to a subset of the species range and long-term accumulation of the closely related haplotypes in a population will result in a population structure termed “island” (Avise *et al.*, 1987). In fact, our pairwise

F_{ST} data show that 67 pairs of localities among 82 comparisons have significant genetic differentiation ($P < 0.05$), although some of these include island localities (Table III).

The two mainland groups (groups A and B), being bisected into western and eastern parts of the Korean peninsula, are interesting in that some far distant localities within the groups are well interrelated, whereas spatially close groups are not interrelated. Thus, our *P. rufa* data appear not to reflect “isolation by distance.” Instead, our data appear to reflect some other aspect of the population structure. Kim *et al.* (2001) observed similar pattern from another firefly species, *Luciola lateralis*, which undergoes aquatic larval stages and occurs in extremely limited habitats (*e.g.*, terraced rice field and wet fields). They claimed that the “east–west” division of *L. lateralis* could be explained by the presence of a major mountain range, called “Bekdudegan,” which divides the midnorthern Korean peninsula into western and eastern parts (Fig. 6). They explained that the presence of this mountain range may have provided different habitat types largely due to altitude, and therefore different types of streams, flow rates of the streams, and so on. In the case of *P. rufa*, the collection localities of the western part of the Korean mainland are mostly located away from the mountain ranges and are at a low altitude. On the other hand, the collection localities of the eastern part are relatively close to “Bekdudegan” and are located at a high altitude. It seems, therefore, that difference in altitude on each side may have served at least as one cause, generating different selection pressure. Along with this geomorphological feature in the Korean peninsula, a limited dispersal ability of the Korean firefly may have initiated the difference in the genetic structure of the western and eastern divisions. Subsequently, these two groups independently may have influenced the populations of the southern parts and those of neighboring islets in the southern part of the Korean peninsula, respectively, resulting in the current population genetic structure. Although we suggest a possible explanation for the observed genetic subdivision in terms of geomorphological feature in the Korean mainland, detailed evidence can be obtained by further research with *P. rufa* populations from the boundaries of “Bekdudegan.”

Although our *P. rufa* data appear to be similar to those of *L. lateralis* in the mainland, a more complex structure might be found in the coastal islets including Tsushima Island in Japan. Surprisingly, *P. rufa* captured in the two localities of Tsushima Island exclusively share one haplotype (PR9) with the ones captured on one Korean coastal islet, Geoje (locality 7; Table I), and this resultantly groups these localities into another independent group (island group; Figs. 6 and 7). The result is unexpected because Matsumura (1928) long ago placed the Tsushima Island population into the *P. tsushimana* group independently of all other populations, and many Japanese fireflies found in islands including Tsushima Island, possess their own taxonomic status (Suzuki, 1997). Currently we do not have an obvious answer for this observation, but one possible explanation includes the

Late Pleistocene–Holocene events as explained previously. During the last glacial advance (the Haupt Würm age) in about 20,000 BP, a lowered sea level (estimated at 80 ± 5 m; Ohshima, 1990) extended the Korean peninsula to the present Jeju-do Island including also Tsushima Island (Ohshima, 1990; Park, 1988). At this time, slow but steady gene flow from the Korean mainland to Tsushima Island may have mingled the pre-existing gene pool of the Tsushima Island population. Later, when the island arose, *P. rufa* was fixed with the most frequent haplotype by losing rare haplotypes as seen in isolated small population (Neigel and Avise, 1993). Although the present study on the population genetic structure of *P. rufa* provides limited information, an extended study (*e.g.*, using more samples from different localities, time period, and molecular markers) will provide a better understanding of the process and mode of population divergence in *P. rufa*.

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