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Genomic Structure of the Luciferase Gene and Phylogenetic Analysis of the Firefly, *Pyrocoelia rufa*

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We describe here the complete nucleotide sequence and the exon-intron structure of the luciferase gene of the firefly, *Pyrocoelia rufa*. The luciferase gene of the *P. rufa* firefly consisted of six introns and seven exons coding for 548 amino acid residues. From the translational start site to the end of last exon, however, the genomic DNA length of the *P. rufa* luciferase gene from the Korean and Chinese samples spans 1,968 bp and 1983 bp, respectively, and 3 amino acid residues were different to each other. Additionally, we also analyzed mitochondrial cytochrome oxidase I (COI) gene of the Chinese *P. rufa* fireflies. Analysis of DNA sequences from the mitochondrial COI protein-coding gene revealed 4 mitochondrial DNA sequence-based haplotypes with a maximum divergence of 0.7%. With the 20 *P. rufa* haplotypes found in Korea, phylogenetic analyses using PAUP and PHYLIP subdivided the *P. rufa* into three clades, termed clades A and B for the Korean sample, and clade C for the Chinese sample.

Key words: Firefly, *Pyrocoelia rufa*, Luciferase gene, Mitochondrial DNA, Cytochrome oxidase I gene, Phylogenetic analysis

Introduction

The Korean firefly, *Pyrocoelia rufa*, is one of the abundant firefly species in Korea and is also found in China and only at Tsushima in case of Japan (Kim and Nam, 1981; Suzuki, 1997, 2001). The *Pyrocoelia*-group in the firefly is divided into two lineages. The first one consists of *P. rufa*, *P. miyako* and *P. atripennis*, and the second of *P. fumosa*, *P. oshimana*, *P. matsumurai matsumurai*, *P. m. kumejimensis*, *P. discicollis* and *P. abdominalis* (Suzuki, 1997, 2001). The body sizes of the former group are larger than those of the other Lampyrine species, and members of the group are characterized by the continuous broadcast of strong light. *P. rufa* has the largest luminescent organs among the former group (Suzuki, 1997, 2001). In fall (August – September), *P. rufa* females oviposit about 40 ~ 120 eggs under the rocks and roots of glasses where enough moisture is available, and the eggs are hatched on May ~ June (Kim *et al.*, 2003). Larval fireflies are subjected to ecdysis four times, and thereafter are subjected to metamorphoisis approximately on August. Ten- to twelve-days after metamorphosis, pupal fireflies become adult fireflies, and their lifespan continues for 15 – 20 days until they finish their lives (Kim *et al.*, 2003).

The firefly luciferase gene is widely used as a genetic marker or as a reporter gene in a variety of organisms including bacteria, plants and animals (De Wet *et al.*, 1987; DiLella *et al.*, 1988; Howard *et al.*, 1988; Kondo *et al.*, 1992; Miller *et al.*, 1992; Jacobs *et al.*, 1993; Bailey *et al.*, 1994; Vikas *et al.*, 1995). Today, more than 10 luciferase genes have been isolated from various firefly species (Tatsumi *et al.*, 1992; Cho *et al.*, 1999; Masuda *et al.*, 1989; Devine *et al.*, 1993; Ohmiya *et al.*, 1995; Choi *et al.*, 2002). The nucleotide sequences of a cDNA encoding the luciferase of *P. rufa* have been reported (Lee *et al.*, 2001).

Choi *et al.* (2003) cloned and sequenced the genomic

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structure of the luciferase from the *Hotaria*-group fireflies and elucidated their phylogenetic relationships using luciferase and mitochondrial cytochrome oxidase I (COI) genes. Kim *et al.* (2004) also elucidated the genomic structure of the luciferase from *Luciola lateralis* and their phylogenetic relationships using luciferase and mitochondrial COI genes. The genetic divergence, population genetic structure, and possible speciation of *P. rufa* were investigated on the midsouthern Korean mainland, coastal islets, a remote offshore, Jeju - do, and Tsushima Island in Japan (Lee *et al.*, 2003).

In this study, we analyzed the genomic structure of the luciferase from *P. rufa* collected in Korea and China, and investigated the phylogenetic analysis from the mitochondrial COI gene of *P. rufa* between the Korean and Chinese samples.

Materials and Methods

Insects

The firefly, *Pyrocoelia rufa* used in this study was collected in Korea and China. The firefly samples in Korea were previously described (Lee *et al.*, 2003). The firefly samples in China were caught at Wuhan, Hubei, China from June to July 2003.

PCR of *P. rufa* luciferase genomic DNA

Genomic DNA was extracted from the larvae of *P. rufa* by Wizard™ Genomic DNA Purification Kit, according to the manufacturers instructions (Promega). The primers used for amplification of the genomic DNA of the luciferase from the *P. rufa* were 5'-ATGGAAGATGATAGTAAACATATTATGCAT-3' for the translational start sequence region and 5'-TTACAATTTGGATTTTTGTCCCATTGTAAGG-3' for the 3' coding region, based on the luciferase cDNA of *P. rufa* (Lee *et al.*, 2001). After a 35-cycles amplification (94°C for 1 min; 55°C for 1 min; 72°C for 1 min), PCR products were ethanol precipitated, centrifugated at 10,000 × g for 15 min, and rinsed with 70% ethanol. These DNAs were analyzed 1.0% agarose gel electrophoresis. The PCR products for sequencing were cloned into pGem-T vector (Promega, Madison, WI).

Amplification of *P. rufa* mitochondrial COI gene

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'-GGAGCTCCTGACATAGCATTCCC-3') and CI-N-2191 (5'-CCCGGTAAAATTAAAATATAAACTTC-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 for 40 s, and 72 for 45 s, and a final extension step at 72°C for 7 min. To confirm the successful DNA amplification, electrophoresis was carried out using 0.5 × TAE buffer in 1% agarose gel. The PCR product was then purified using a PCR purification Kit (QIAGEN, Germany) following manufacturers instruction.

DNA sequencing and data analysis

DNA sequencing was performed using an automatic sequencer (model 310 Genetic Analyzer; Perkin-Elmer Applied Biosystems, Foster City, CA). Sequence alignment was performed using IBI MacVector (ver. 6.5). PAUP (Phylogenetic Analysis using Parsimony) ver. 4.0b8 (Swofford, 2000) was used to infer possible phylogenetic relationships among the matralines of *P. rufa*. The homologous mtDNA sequences from one individual of *Hotaria unmunsana*, collected in Busan was used as an outgroup (Choi *et al.*, 2003). 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 1,000 iterations of bootstrapping (Felsenstein, 1985). As an alternative to the parsimony analysis, we used Neighbor-Joining (NJ) method and 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 1,000 times using the subprogram SEQBOOT. Next, 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. The *H. unmunsana* sequence was included in the analysis to root the tree. Finally, a consensus tree representing reliability at each branch in the trees was obtained using the subprogram CONSENSE.

Results and Discussion

Genomic structure of the luciferase gene of *P. rufa* firefly

To identify the genomic DNA of the luciferase gene of the firefly, *P. rufa*, we have designed the PCR primer set based on the sequences of the luciferase cDNA of *P. rufa* already known in our laboratory (Lee *et al.*, 2001). The genomic DNA of the luciferase gene from the Korean and Chinese samples of the *P. rufa* firefly was cloned and sequenced (Fig. 1).

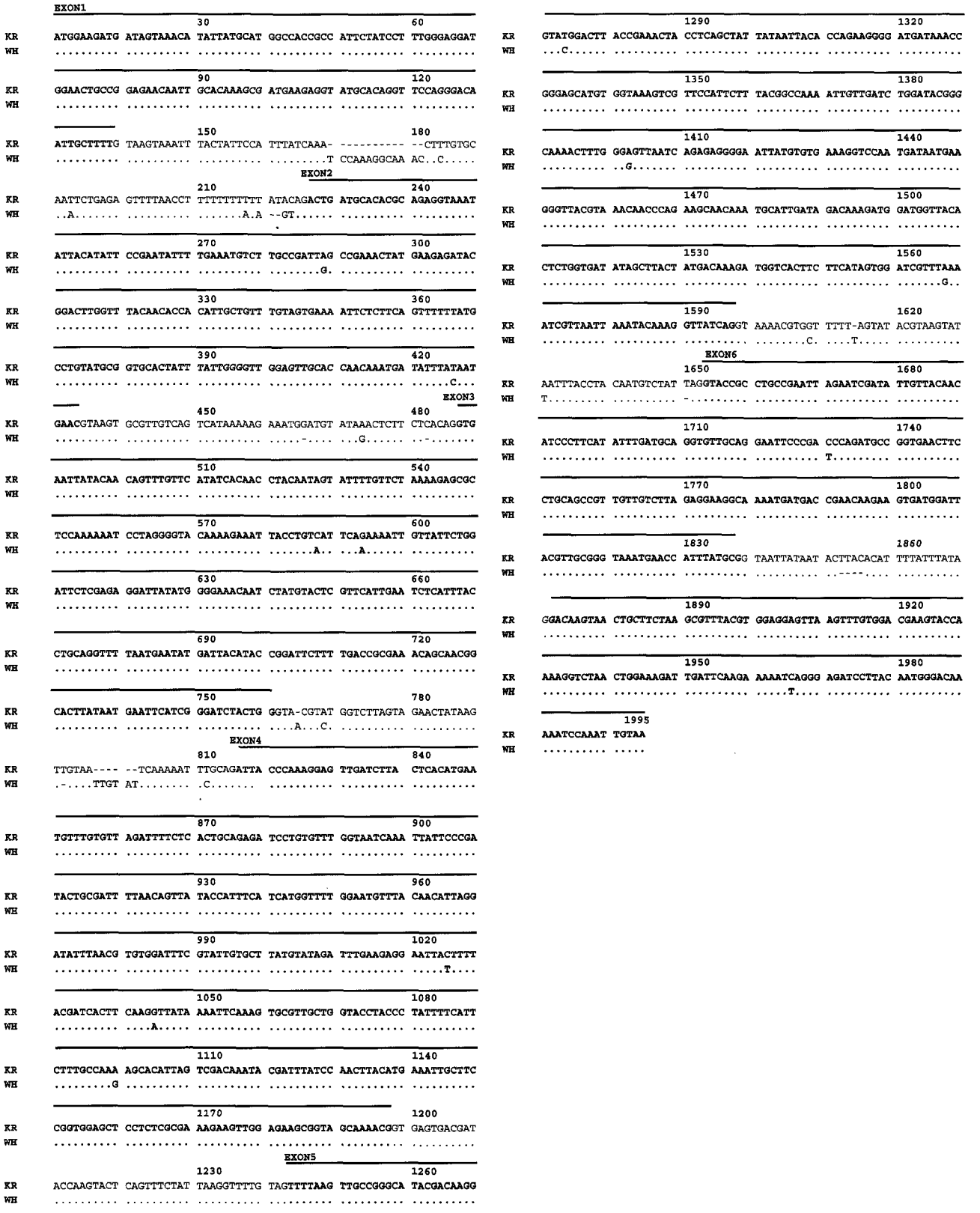
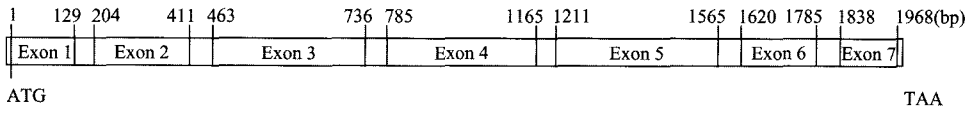


Fig. 1. The nucleotide sequence and genomic organization of *P. rufa* luciferase gene. The firefly used in this study was collected in the Korea (KR) and China (WH). Nucleotide numbers are presented on the upper, and the first base of initiation codon of the ORF is defined as +1. Exons are labeled with bold-lines.

A

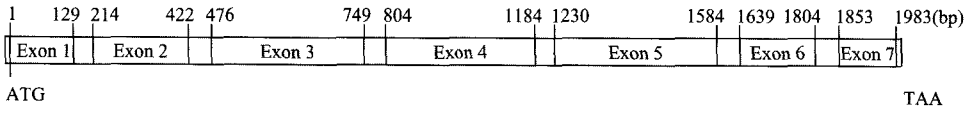


B

Exon	Length of exon(bp)	Position in gene close	Sequence at exon-intron junction		
			M E D D		I A F
1	129	1-129	ATGGAAGATGAT	.....	ATTGCTTTTgtaagtaaatt
			T D A H		Y N E
2	208	204-411	tacagACTGATGCACAC	.....	TATAATGAACgtaagtg
			E L Y N		G S TG
3	274	463-736	ctacagGTGAATTATACA	.....	GGATCTACTGGgtacgt
			L P K		V A K R
4	381	785-1165	aattcgcagATTACCCAAA	.....	GTAGCAAAACGgtgagt
			F K L		K G Y Q
5	355	1211-1565	ggttttgtagTTTTAAGTTG	.....	AAAGGTTATCAGgtaaa
			V P P A		Y V A
6	166	1620-1785	ctattagGTACCGCCTGCC	.....	TACGTTGCGGgtaaatgaa
			G Q V T		K L Stop
7	131	1838-1968	atttatagGACAAGTAACT	.....	AAATTGtaa

Fig. 2. Genomic organization of the luciferase gene of the Korean *P. rufa* firefly. (A) Exon/intron structures. Numbers indicate the length (bp) of exons and introns. (B) Lengths of exons and exon/intron boundaries.

A



B

Exon	Length of exon(bp)	Position in gene close	Sequence at exon-intron junction		
			M E D D		I A F
1	129	1-129	ATGGAAGATGAT	.....	ATTGCTTTTgtaagtaaatt
			T D A H		Y N E
2	208	215-422	agtagACTGATGCACAC	.....	TACAATGAACgtaagtg
			E L Y N		G S TG
3	274	476-749	tcacagGTGAATTATACA	.....	GGATCTACTGGgtaacg
			L P K		V A K R
4	381	804-1184	aatttgcagATTACCCAAA	.....	GTAGCAAAACGgtgagt
			F K L		K G Y Q
5	355	1230-1584	ggttttgtagATTTAAGTTG	.....	AAAGGTTATCAGgtaaa
			V P P A		Y V A
6	166	1639-1804	tctatagGTACCGCCTGCC	.....	TACGTTGCGGgtaaatgaa
			G Q V T		K L Stop
7	131	1853-1983	atttatagGACAAGTAACT	.....	AAATTGtaa

Fig. 3. Genomic organization of the luciferase gene of the Chinese *P. rufa* firefly. (A) Exon/intron structures. Numbers indicate the length (bp) of exons and introns. (B) Lengths of exons and exon/intron boundaries.

Comparison of the genomic sequence with the sequence of cloned luciferase cDNA in the *P. rufa* revealed the presence of seven exons (Fig. 1 and 2). From the known cDNA 5 site to the end of exon 7, the gene is 1,968 bp

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                                30                                  60
KR    MEDDSKHIMH  MEDDSKHIMH  GTAGEQLHKA  MKRYAQVPGT  IAFTDAHAEV  NITYSEYFEM
WH    ..........  ..........  ..........  ..........  ..........  ..........
                                90                                 120
KR    SCRLAETMKR  YGLGLQHHIA  VCSENSLQFF  MPVCGALFIG  VGVAPTNDIY  NERELYNSLF
WH    ..........  ..........  ..........  ..........  ..........  ..........
                               150                                 180
KR    ISQPTIVFCS  KRALQKILGV  HKKLPVIQKI  VILDSREDYM  GKQSMYSFIE  SHLPAGFNEY
WH    ..........  ..........  Q.........  ..........  ..........  ..........
                               210                                 240
KR    DYIPDSFDRE  TATALIMNSS  GSTGLPKGVD  LTHMNVCVRF  SHCRDPVFGN  QIIPDTAILT
WH    ..........  ..........  ..........  ..........  ..........  ..........
                               270                                 300
KR    VIPFHHGFGM  FTTLGYLTCG  FRIVLMYRFE  EELFLRSLQD  YKIQSALLVP  TLFSFFAKST
WH    ..........  ..........  ..........  ..........  ..........  ..........
                               330                                 360
KR    LVDKYDLSNL  HEIASGGAPL  AKEVGEAVAK  RFKLPGIRQG  DGLTETTSAI  IITPEGDDKP
WH    ..........  ..........  ..........  ..........  Y.........  ..........
                               390                                 420
KR    GACGKVVPFF  AAKIVDLDTG  KTLGVNQRGE  LCVKGPMIMK  GYVNNPEATN  ALIDKDGWLH
WH    ..........  T.........  ..........  ..........  ..........  ..........
                               450                                 480
KR    SGDIAYYDKD  GHFFIVDRLK  SLIKYKGYQV  PPAELESILL  QHPFIFDAGV  AGIPDPDAGE
WH    ..........  ..........  ..........  ..........  ..........  ..........
                               510                                 540
KR    LPAAVVVLEH  GKMMTEQEVM  DYVAGQVTAS  KRLRGGVKFV  DEVPKGLTGK  IDSRKIREIL
WH    ..........  ..........  ..........  ..........  ..........  ..........
          548
KR    TMGQKSKL
WH    ........
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Fig. 4. The deduced amino acid sequence of the luciferase gene of *P. rufa* firefly. The firefly used in this study was collected in the Korea (KR) and China (WH). Residues are numbered according to the *P. rufa* luciferase cDNA sequences previously known (Lee *et al.*, 2001), and identical residues are dotted.

long and consisted of 548 amino acid residues. On the other hand, genomic structure of luciferase gene from the Chinese *P. rufa* sample was identical in their exon number and coding sequence size (548 amino acid residues), but the genomic DNA length differed from the Korean *P. rufa* sample (Fig. 1 and 3). Furthermore, the amino acid sequence of the luciferase gene of the Chinese *P. rufa* sample differed from the Korean sample by three amino acid residues (Fig. 4). The intron boundaries are listed in Fig. 2B and 3B. The consensus sequences, including an invariant GT at the intron 5 boundary and an invariant AG at its 3 boundary were very well conserved in two samples.

When compared with the Korean *P. rufa* firefly sample, the Chinese *P. rufa* luciferase gene differed by three amino acid residues in the coding region. Also, the length of introns differed between two samples, which resulted in genomic DNA length difference. However, the genomic structure of the luciferase gene in both samples consisted of six introns and seven exons coding for 548 amino acid residues. This is the same as that of luciferases from other fireflies, such as *L. lateralis* (Kim *et al.*, 2004) and *Hotaria*-group fireflies (Choi *et al.*, 2003).

Phylogenetic analysis using the mitochondrial COI gene

Fig. 5 shows the nucleotide sequences of the 403 bp region of the COI gene from the Chinese samples of the *P. rufa* firefly. The nucleotide sequences of the COI gene from the Chinese *P. rufa* samples were compared with those of the Korean *P. rufa* firefly already known in our laboratory (Lee *et al.*, 2003). A total of 4 haplotypes (PR-C1 – PR-C4) was obtained from the 403 bp of the COI gene from 10 individuals of the *P. rufa* firefly collected from Wuhan in China.

Table 1 shows the nucleotide sequence divergence among the *P. rufa* haplotypes. Sequence divergence among 4 haplotypes from the Chinese samples by pairwise comparisons ranged from 0.2 to 0.7% (one bp three bp). Sequence divergence among 24 haplotypes, including 20 haplotypes found in Korean *P. rufa* firefly, by pairwise comparisons ranged from 0.2 to 8.4% (one bp 34 bp), and the largest sequence divergence was observed when PR4 found in Korean sample was compared with PR-C4 found in three individuals from the Chinese sample.

Phylogenetic relationships among haplotypes are depicted in Figure 6. 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. 6). Twenty-four haplotypes used in this study were subdivided into three groups (termed clades A and B for Korean sample, and clade C for Chinese sample), although haplotype relationships within each clade were mostly not resolved, possibly by small nucleotide difference (*e.g.*, one or two bp). Each clade was somewhat different in haplotype composition. For example, clade A consists of a large number of haplotypes (14 haplotypes among 24) and is larger in maximum sequence divergence (1.5%) than clade B (Lee *et al.*, 2003). Clade B contained six haplotypes with a maximum sequence divergence of 0.7% (Lee *et al.*, 2003). Also, clade C for Chinese sample contained four haplotypes with a maximum sequence divergence of 0.7% (Fig. 6 and Table 1).

Fig. 7 represents the result of probability-based phylogenetic analyses using the neighbor-joining (NJ) method. Each monophyletic group designated in the PAUP analysis included identical haplotypes, suggesting that *P. rufa* can be subdivided into three distinct genetic groups.

Phylogenetic analysis of *P. rufa* showed that the fireflies are subdivided largely into three clades and distribution of each clade is concordant with geographic separations: clade B exclusively from Jeju-do Island, clade A from all localities but Jeju-do Island (Lee *et al.*, 2003), and clade C from Wuhan, China.

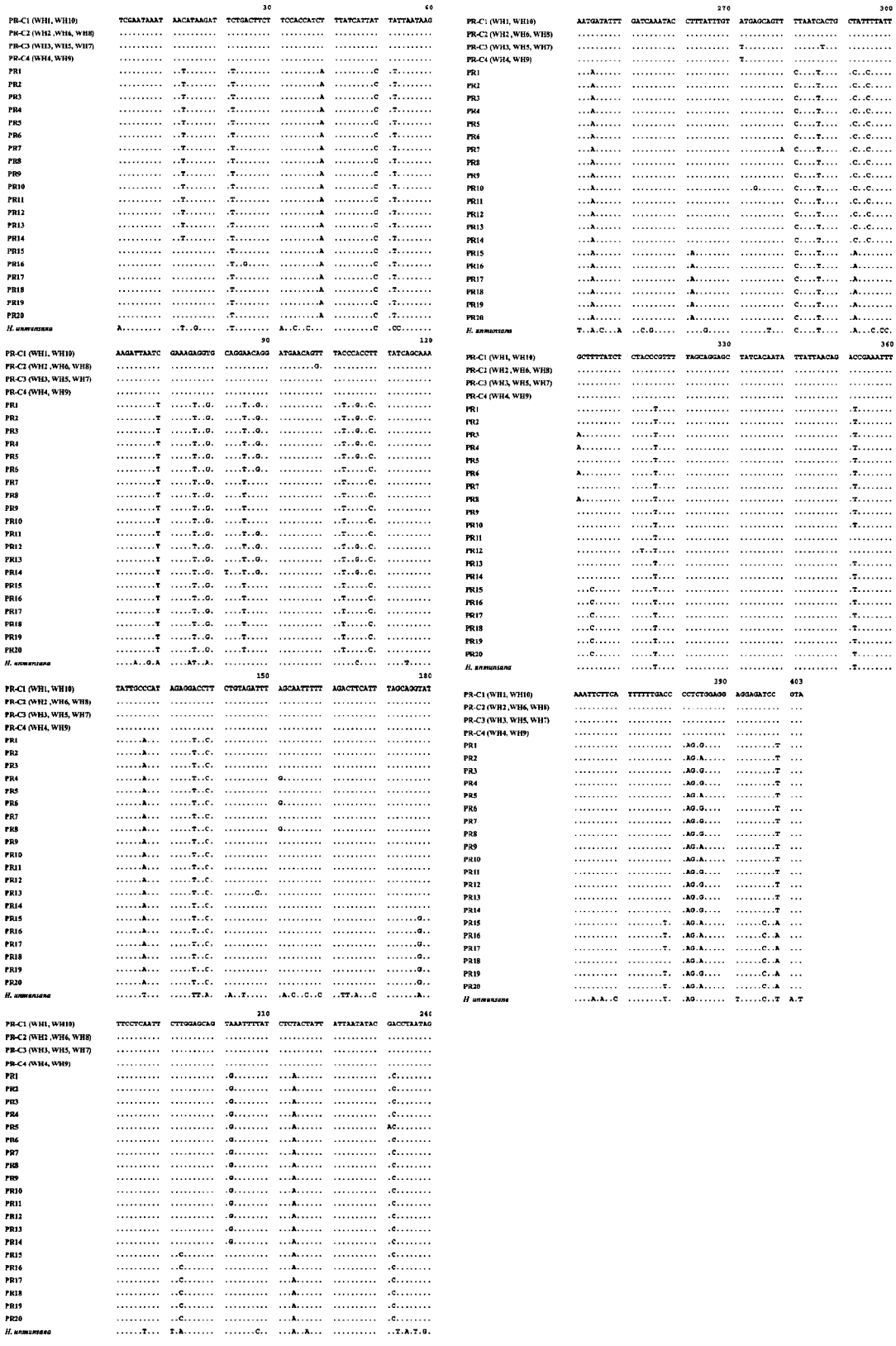

Fig. 5. Sequence alignment of 4 mitochondrial haplotypes (designated as PR-C1 PR-C4) obtained from 403 bp COI sequences of *P. rufa* fireflies collected in Wuhan, China. Sequences of 20 mitochondrial haplotypes (PR1 PR20) are taken from the Korean *P. rufa* firefly (Lee *et al.*, 2003).

Table 1. Pairwise comparisons among nucleotide sequences of COI genes obtained from this study and known *P. rufa* COI genes obtained through GenBank search

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. PR-C1 (WH1,WH10)	–	0.002	0.002	0.005	0.069	0.072	0.072	0.072	0.072	0.074	0.074	0.074	0.074
2. PR-C2 (WH2, WH6, WH8)	1	–	0.005	0.007	0.072	0.074	0.074	0.074	0.074	0.077	0.077	0.077	0.077
3. PR-C3 (WH4, WH9)	1	2	–	0.002	0.072	0.074	0.074	0.074	0.074	0.077	0.077	0.077	0.077
4. PR-C4 (WH3, WH5, WH7)	2	3	1	–	0.074	0.077	0.077	0.077	0.077	0.079	0.079	0.079	0.079
5. PR9	28	29	29	30	–	0.005	0.002	0.005	0.037	0.007	0.005	0.007	0.037
6. PR7	29	30	30	31	2	–	0.007	0.005	0.042	0.007	0.010	0.007	0.042
7. PR10	29	30	30	31	1	3	–	0.007	0.040	0.010	0.007	0.010	0.040
8. PR11	29	30	30	31	2	2	3	–	0.042	0.002	0.005	0.007	0.042
9. PR18	29	30	30	31	15	17	16	17	–	0.045	0.042	0.045	0.005
10. PR1	30	31	31	32	3	3	4	1	18	–	0.002	0.010	0.045
11. PR2	30	31	31	32	2	4	3	2	17	1	–	0.012	0.042
12. PR8	30	31	31	32	3	3	4	3	18	4	5	–	0.045
13. PR15	30	31	31	32	15	17	16	17	2	18	17	18	–
14. PR17	30	31	31	32	15	17	16	17	2	18	17	18	2
15. PR19	30	31	31	32	16	16	17	16	3	17	18	17	1
16. PR20	30	31	31	32	16	18	17	18	1	19	18	19	1
17. PR3	31	32	32	33	4	4	5	2	19	1	2	3	19
18. PR5	31	32	32	33	3	5	4	3	18	2	1	6	18
19. PR6	31	32	32	33	4	4	5	2	19	3	4	1	19
20. PR12	31	32	32	33	5	5	6	3	18	2	3	6	18
21. PR13	31	32	32	33	4	4	5	2	19	1	2	5	19
22. PR14	31	32	32	33	4	4	5	2	19	1	2	5	19
23. PR16	31	32	32	33	17	19	18	19	2	20	19	20	2
24. PR4	32	33	33	34	5	5	6	3	20	2	3	2	20
25. *Hotaria unmunsana*	85	86	86	87	80	81	81	81	76	81	81	80	76

Table 1. continud

	14	15	16	17	18	19	20	21	22	23	24	25
1. PR-C1 (WH1,WH10)	0.074	0.074	0.074	0.077	0.077	0.077	0.077	0.077	0.077	0.077	0.079	0.211
2. PR-C2 (WH2, WH6, WH8)	0.077	0.077	0.077	0.079	0.079	0.079	0.079	0.079	0.079	0.079	0.082	0.213
3. PR-C3 (WH4, WH9)	0.077	0.077	0.077	0.079	0.079	0.079	0.079	0.079	0.079	0.079	0.082	0.213
4. PR-C4 (WH3, WH5, WH7)	0.079	0.079	0.079	0.082	0.082	0.082	0.082	0.082	0.082	0.082	0.084	0.216
5. PR9	0.037	0.040	0.040	0.010	0.007	0.010	0.012	0.010	0.010	0.042	0.012	0.199
6. PR7	0.042	0.040	0.045	0.010	0.012	0.010	0.012	0.010	0.010	0.047	0.012	0.201
7. PR10	0.040	0.042	0.042	0.012	0.010	0.012	0.015	0.012	0.012	0.045	0.015	0.201
8. PR11	0.042	0.040	0.045	0.005	0.007	0.005	0.007	0.005	0.005	0.047	0.007	0.201
9. PR18	0.005	0.007	0.002	0.047	0.045	0.047	0.045	0.047	0.047	0.005	0.050	0.189
10. PR1	0.045	0.042	0.047	0.002	0.005	0.007	0.005	0.002	0.002	0.050	0.005	0.201
11. PR2	0.042	0.045	0.045	0.005	0.002	0.010	0.007	0.005	0.005	0.047	0.007	0.201
12. PR8	0.045	0.042	0.047	0.007	0.015	0.002	0.015	0.012	0.012	0.050	0.005	0.199
13. PR15	0.005	0.002	0.002	0.047	0.045	0.047	0.045	0.047	0.047	0.005	0.050	0.189
14. PR17	–	0.007	0.002	0.047	0.045	0.047	0.045	0.047	0.047	0.005	0.050	0.186
15. PR19		–	0.005	0.045	0.047	0.045	0.042	0.045	0.045	0.007	0.047	0.189
16. PR20	1		–	0.05	0.047	0.050	0.047	0.05	0.050	0.002	0.052	0.186
17. PR3	19	18		–	0.007	0.005	0.007	0.005	0.005	0.052	0.002	0.199
18. PR5	18	19	19		–	0.012	0.010	0.007	0.007	0.050	0.010	0.203
19. PR6	19	18	20	2		–	0.012	0.010	0.010	0.052	0.002	0.201
20. PR12	18	17	19	3	4		–	0.007	0.007	0.050	0.010	0.196
21. PR13	19	18	20	2	3	4		–	0.005	0.052	0.007	0.203
22. PR14	19	18	20	2	3	4	3		–	0.052	0.007	0.203
23. PR16	2	3	1	21	20	21	20	21		–	0.055	0.189
24. PR4	20	19	21	1	4	1	4	3	3	22	–	0.201
25. *Hotaria unmunsana*	75	76	75	80	82	81	79	82	82	76	81	–

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

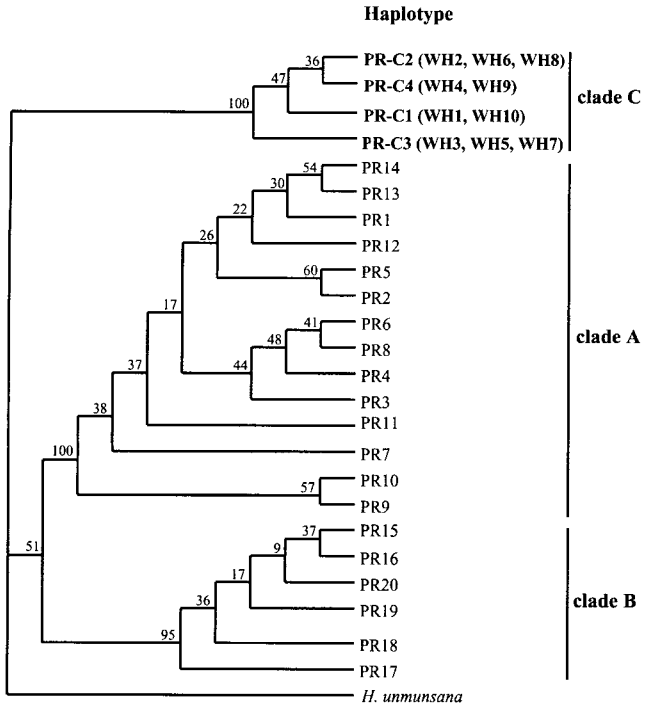

Fig. 6. A PAUP analysis of mitochondrial COI gene sequences of *P. rufa* using homologous sequence of another firefly, *Hotaria unmunsana*, as an outgroup. The tree shown is an unordered tree obtained with the option of "retain groups with frequency >50%" by majority-rule consensus of three equally parsimonious trees from the heuristic search. The numbers shown on the branches represent bootstrap values for 1,000 replicates. Tree length is 120 steps long, Consistency Index is 0.800, and retention index is 0.975.

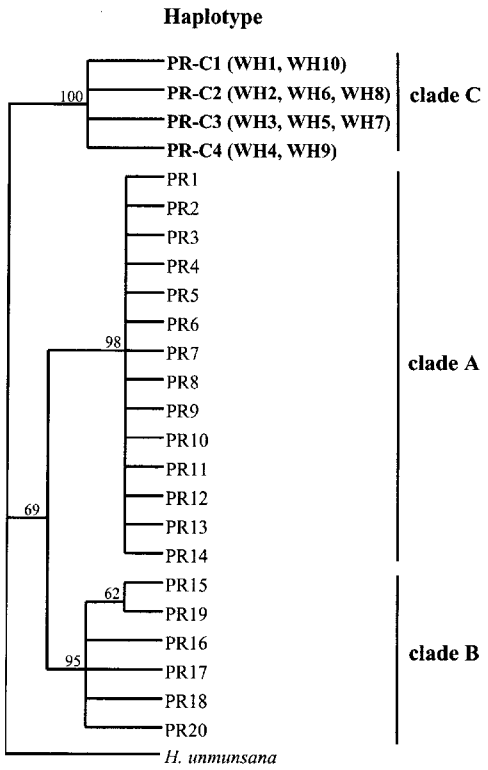

Fig. 7. PHYLIP analysis of mitochondrial COI gene sequences of *P. rufa*. The tree was obtained using the subprogram NEIGHBOR incorporated in PHYLIP with the option of Kimura's 2-parameter method (1980). The tree was rooted using *H. unmunsana*. The numbers shown on the branches, which represent bootstrap values for 1,000 replications, were obtained using the subprogram CONSENSUS.

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