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Mitochondrial DNA Sequence Variation of the Tiny Dragonfly, *Nannophya pygmaea* (Odonata: Libellulidae)

Ki-Gyoung Kim, Sang Kyun Jang[1], Dong Woo Park[1], Mee Yeon Hong[1], Kyoung-Hee Oh, Kee Young Kim[2], Jae Sam Hwang[2], Yeon Soo Han[1] and Iksoo Kim[1,*]

Biological Resources Research Department, National Institute of Biological Resources, Incheon, Korea
[1]College of Agriculture & Life Sciences, Chonnam National University, Gwangju 500-757, Korea
[2]Department of Agricultural Biology, National Institute of Agricultural Science &Technology, Rural Development Administration, Suwon 441-100, Korea

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The tiny dragonfly, *Nannophya pygmaea* (Odonata: Libellulidae) is one the smallest dragonflies in the world and listed as a second-degree endangered wild animal and plant in Korea. For the long-term conservation of such endangered species, an investigation on nation-wide genetic magnitude and nature of genetic diversity is required as a part of conservation strategy. We, thus, sequenced a portion of mitochondrial COI gene, corresponding to "DNA Barcode" region (658 bp) from 68 *N. pygmaea* individuals collected over six habitats in Korea. The sequence data were used to investigate genetic diversity within populations and species, geographic variation within species, phylogeographic relationship among populations, and phylogenetic relationship among haplotypes. Phylogenetic analysis and uncorrected pairwise distance estimate showed overall low genetic diversity within species. Regionally, populations in southern localities such as Gangjin and Gokseong in Jeollanamdo Province showed somewhat higher genetic diversity estimates than those of remaining regions in Korean peninsula. Although geographic populations of *N. pygmaea* were subdivided into two groups, distance- or region-based geographic partition was not observed.

Key words: Mitochondrial DNA, COI gene, *Nannophya pygmaea*, Tiny dragonfly, Endangered species, Population genetic structure, Conservation genetics.

*To whom the correspondence addressed
College of Agriculture and Life Sciences, Chonnam National University, Gwangju 500 - 757, Korea. Tel: +82-62-530-2073; Fax: +82-62-530-2079; E-mail: ikkim81@chonnam.ac.kr

Introduction

Nannophya pygmaea is a dragonfly of the family Libellulidae in the insect order Ordonata, which often belongs to the Paleoptera together with another insect order Ephemeroptera. The species known variously as the scarlet dwarf, northern pygmyfly, or tiny dragonfly, is distributed from Southeast Asia to China, Korea and Japan, and occasionally found south to Australia (Ishida *et al.*, 1988). This species is one of the smallest modern ordonatan species recorded, with a wingspan of ~20 mm, or about 3/4 of an inch.

Biodiversity is threatened with a massive destruction of the habitat by increase of human population, urbanization, greenhouse phenomenon, contamination and so on. Due by these factors, substantial number of species have been exterminated and are under the pressure of extinction. These threats not only influence to species diversity but also threaten various levels of diversity, such as subspecies, regional population, and genetic diversity (Soulé, 1986; Wilson, 1992).

In this regard, Korea is not exception. For over the last 50 years many species were exterminated by various damages. With the concern on this issue, more than 200 species distributed in Korea was designated as endangered species, and this chart include 20 insect species. Among these the tiny dragonfly, *Nannophya pygmaea* (Odonata: Libellulidae) is listed as a second-degree endangered wild animal and plant in Korea. There have been some studies on habitat, distribution, and morphology of the species (Bae *et al.*, 1999; Kim, 1997), but no study has been made regarding genetic aspects of the species yet. In this study, we, thus, investigated genetic diversity, geographic variation within

Table 1. A list of trapping localities, animal numbers, mitochondrial COI haplotypes, and GenBank accession numbers of *N. pygmaea*

Collecting locality (no. of individuals)	Collection date	Animal number	Sex	COI haplotype	GenBank accession number
1. Munkyeong ,	2006. 08. 14	NP0073	F	BARNP10	EU048699
Kyeongsangbuk Province (19)	〃	NP0075	F	BARNP10	EU048726
	〃	NP0077	F	BARNP10	EU048700
	〃	NP0078	F	BARNP06	EU048701
	2007. 08. 02	NP0079	F	BARNP10	EU048702
	〃	NP0080	M	BARNP02	EU048703
	2006. 08. 14	NP0081	M	BARNP10	EU048704
	〃	NP0082	M	BARNP04	EU048705
	〃	NP0083	M	BARNP06	EU048706
	〃	NP0084	M	BARNP06	EU048707
	〃	NP0085	M	BARNP10	EU048708
	〃	NP0086	M	BARNP10	EU048709
	2006. 08. 14	NP0087	M	BARNP06	EU048710
	〃	NP0088	M	BARNP06	EU048711
	〃	NP0089	M	BARNP10	EU048712
	〃	NP0090	M	BARNP10	EU048713
	〃	NP0091	M	BARNP10	EU048714
	〃	NP0092	M	BARNP10	EU048715
	〃	NP0093	M	BARNP10	EU048716
2. Suwon,	2006. 08. 14	NP0094	F	BARNP02	EU048717
Kyeonggi Province (9)	〃	NP0095	F	BARNP06	EU048718
	〃	NP0096	F	BARNP04	EU048719
	〃	NP0097	F	BARNP04	EU048720
	〃	NP0098	F	BARNP10	EU048721
	〃	NP0099	F	BARNP02	EU048722
	〃	NP0100	M	BARNP06	EU048723
	〃	NP0101	M	BARNP02	EU048724
	〃	NP0102	M	BARNP10	EU048725
3. Muuido,	2006. 08. 14	NP0103	F	BARNP10	EU048727
Incheon (10)	〃	NP0104	F	BARNP10	EU048728
	〃	NP0105	F	BARNP10	EU048729
	〃	NP0106	F	BARNP10	EU048730
	〃	NP0107	M	BARNP10	EU048731
	〃	NP0108	M	BARNP10	EU048732
	〃	NP0109	M	BARNP10	EU048733
	〃	NP0110	M	BARNP10	EU048734
	〃	NP0111	M	BARNP10	EU048735
	〃	NP0112	M	BARNP10	EU048736

M, male; and F, female.

Table 1. Continue

Collecting locality (no. of individuals)	Collection date	Animal number	Sex	COI haplotype	GenBank accession number
4. Ganggin,	2006. 08. 22	NP0120	F	BARNP03	EU048737
Jeollanamdo Province (10)	〃	NP0121	F	BARNP03	EU048738
	〃	NP0122	F	BARNP01	EU048739
	〃	NP0123	F	BARNP07	EU048740
	〃	NP0124	F	BARNP09	EU048741
	〃	NP0125	M	BARNP03	EU048742
	〃	NP0126	M	BARNP03	EU048743
	〃	NP0127	M	BARNP09	EU048744
	〃	NP0128	M	BARNP10	EU048745
	〃	NP0129	M	BARNP05	EU048746
5. Gokseong A,	2007. 08. 04	NP0130	F	BARNP10	EU048757
Jeollanamdo Province (10)	〃	NP0131	F	BARNP08	EU048758
	〃	NP0132	F	BARNP06	EU048759
	〃	NP0133	F	BARNP10	EU048760
	〃	NP0134	F	BARNP06	EU048761
	〃	NP0135	M	BARNP05	EU048762
	〃	NP0136	M	BARNP06	EU048763
	〃	NP0137	M	BARNP06	EU048764
	〃	NP0138	M	BARNP05	EU048765
	〃	NP0139	M	BARNP04	EU048766
6. Gokseong B,	2006. 08. 22	NP0140	F	BARNP10	EU048747
Jeollanamdo Province (10)	〃	NP0141	F	BARNP05	EU048748
	〃	NP0142	F	BARNP08	EU048749
	〃	NP0143	F	BARNP05	EU048750
	〃	NP0144	F	BARNP04	EU048751
	〃	NP0145	M	BARNP03	EU048752
	〃	NP0146	M	BARNP10	EU048753
	〃	NP0147	M	BARNP02	EU048754
	〃	NP0148	M	BARNP10	EU048755
	〃	NP0149	M	BARNP10	EU048756

M, male; and F, female.

species, and phylogeographic relationship of the species by sequencing a portion of mitochondrial COI gene.

Materials and Methods

Sampling

Adult *N. pygmaea* were sampled from six localities in August, 2006 and 2007 after a proper permission was obtained. In detail, collection permission for Suwon and Muuido populations was obtained from the Han River Basin Environmental Office (permission number 2006-10), for Munkyeong from the Daegu Regional Environmental Office (permission numbers 2006-2and 2007-5), and for Ganggin and Gokseong from the Yeongsan River Basin Environmental Office (permission numbers 2006-7 and 2007-5). Sampling locality, date of collection, and individual sex are listed in Table 1 and locality map is shown in Fig 1. Detailed administrative district for each sampling location was omitted for the protection of the endangered species.

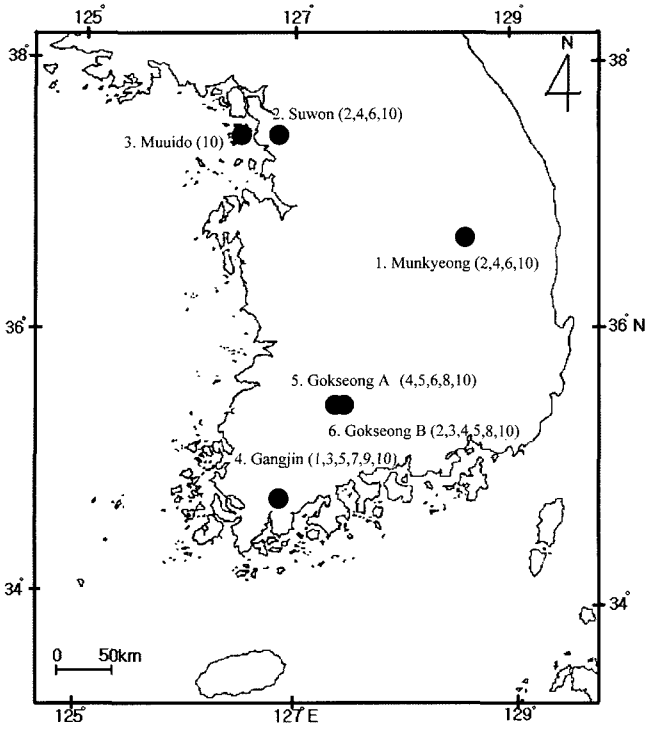

Fig. 1. Sampling location of *N. pygmaea* Ramber in Korea. General locality names are as follows: 1, Munkyung, Kyeongsangbukdo Province; 2, Suwon, Gyeonggi Province; 3, Muuido, Incheon; 4, Gangjin, Jeollanamdo Province; 5, Gokseong A, Jeollanamdo Province; and 6, Gokseong B, Jeollanamdo Province. Within parenthesis denotes haplotype name, omitting the antecedent alphabets, BARNP.

DNA extraction, Primer, PCR, and Sequencing

The total DNA was extracted using the Wizard Genomic DNA Purification Kit, in accordance with the manufacturer's instructions (Promega, USA). For the amplification of a portion of mt COI gene, corresponding to "DNA Barcode" region being utilized for global animal identification (Hebert *et al.*, 2003) a pair of primer was designed based on Folmer *et al.* (1994): LCO1490, 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198, 5'-TAAACTTCAGGGTGACCAAAAAATAC-3'. After an initial denaturation step at 94°C for 7 min, a 35-cycle amplification (94°C for 1 min, 50~55°C for 1 min, and 72°C for 1 min) was conducted. The final extension step was continued for 7 min and 45 s at 72°C. To confirm the successful DNA amplification, electrophoresis was carried out using 0.5 × TAE buffer on 0.5% agarose gel. The PCR product was then purified using PCR purification Kit (QIAGEN, Germany). The COI gene fragments were directly sequenced from PCR products. DNA sequencing was performed using the ABI PRISM® BigDye® Terminator v1.1 Cycle Sequencing Kit under the ABI 377 or ABI 310 Genetic Analyzer (PE Applied Biosystems, USA). All products were sequenced from both strands. Sequence alignment was performed using CLUSTAL X programs (ver. 1.8; Thompson *et al.*, 1997). When homologous sequences from two individuals differed by ≥ one nucleotide base, the sequences were considered as different haplotypes. Haplotype designations were applied to new sequences as they were discovered (*i.e.*, BARNP01, BARNP02, PBARN03 and so forth).

Phylogenetic analysis using PAUP and networks

Phylogenetic analysis was performed by maximum-parsimony (MP) method (Fitch, 1971) using PAUP* (Phylogenetic Analysis Using Parsimony and Other Method*) ver. 4.0b10 (Swofford, 2002). To root trees, the nearly complete *Orthetrum triangulare melania* mitogenome sequence was utilized (Yamauchi *et al.*, 2004) as an outgroup. The analysis was performed using an equal weighting of transitions and transversions by heuristic search. The reliability of the trees was tested by 1,000 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).

Genetic diversity indices

Genetic diversity estimates, such as haplotype diversity and nucleotide diversity within each locality were obtained using Arlequin ver. 3.0 (Excoffier *et al.*, 2005). On the other hand, maximum sequence divergence within population was obtained by extracting the estimate of unrooted pairwise distance within population from PAUP (Swofford, 2002).

Genetic distance and migration estimate

Genetic distance and migration rate were estimated from mtDNA sequences and subroutines in the Arlequin version 3.0 (Excoffier et al., 2005). Population pairwise genetic distance (F_{ST}) and a permutation test of the significant differentiation of the pairs of localities (1,000 bootstraps) were obtained following the approach described in Excoffier *et al.* (1992) and the distance between DNA sequences were calculated by the Kimura 2-parameters method (Kimura, 1980). Pairwise F_{ST} values were used to estimate per generation migration rate, Nm (the product of the effective population size Ne and migration rate, m) based upon the equilibrium relationship: $F_{ST} = 1/(2Nm+1)$.

```
                                                  30                                                  60
                    T  L  Y   L  I  F  G    A  W  A   G  M  V   G  T  A  L   S  V  L
BARNP01(NP0122) AACCCTATAC CTAATTTTTG GAGCATGAGC AGGAATAGTA GGAACTGCAT TAAGTGTATT
BARNP02(NP0080) .......... ........C. .......G.. .......... .......... ..........
BARNP03(NP0120) .......... ........C. .......... .......... .......... ..........
BARNP04(NP0082) .......... ........C. .......... .......... .......... ..........
BARNP05(NP0129) .......... ........C. .......... .......... .......... ..........
BARNP06(NP0078) .......... ........C. .......... .......... .......... ..........
BARNP07(NP0123) .......... ........C. .......... .......... .......... ..........
BARNP08(NP0131) .......... ........C. .......... .......... .......... ..........
BARNP09(NP0124) .......... ........C. .......... .......... .......... ..........
BARNP10(NP0067) .......... ........C. .......... .......... .......... ..........
                                                  90                                                 120
                    I  R  I   E  L  G  Q    P  G  L   L  I  G   D  D  Q  I   Y  N  V
BARNP01(NP0122) AATTCGAATT GAATTGGGAC AGCCGGGATC TTTAATTGGA GATGATCAAA TTTATAACGT
BARNP02(NP0080) .......... .......... .......... .......... .......... ..........
BARNP03(NP0120) .......... .......... .......... .......... ........G. ..........
BARNP04(NP0082) .......... .......... .......... .......... .......... ..........
BARNP05(NP0129) .......... .......... .......... .......... .......... ..........
BARNP06(NP0078) .......... .......... .......... .......... .......... ..........
BARNP07(NP0123) .......... .......... .......... .......... .......... ..........
BARNP08(NP0131) .......... .......... .......... .......... .......... ..........
BARNP09(NP0124) .......... .......... .......... .......... .......... ..........
BARNP10(NP0067) .......... .......... .......... .......... .......... ..........
                                                 150                                                 180
                    I  V  T   A  H  A  F    V  M  I   F  F  M   V  M  P  I   M  I  G
BARNP01(NP0122) TATCGTTACT GCTCATGCTT TTGTAATAAT TTTCTTTATG GTTATACCTA TTATAATTGG
BARNP02(NP0080) .......... .......... .......... .......... .......... ..........
BARNP03(NP0120) .......... .......... .......... .......... .......... ..........
BARNP04(NP0082) .......... .......... .......... .......... .......... ..........
BARNP05(NP0129) .......... .......... .......... .......... .......... ..........
BARNP06(NP0078) .......... .......... .......... .......... .......... ..........
BARNP07(NP0123) .......... .......... .......... .......... .......... ..........
BARNP08(NP0131) .......... .......... .......... .......... .......... ..........
BARNP09(NP0124) .......... .......... .......... .......... .......... ..........
BARNP10(NP0067) .......... .......... .......... .......... .......... ..........
                                                 210                                                 240
                    G  F  G   N  W  L  V    P  L  M   L  G  A   P  D  M  A   F  P  R
BARNP01(NP0122) AGGATTTGGA AACTGATTAG TACCATTAAT ACTGGGTGCT CCAGATATAG CATTCCCACG
BARNP02(NP0080) .......... .......... .......... .......... .......... ..........
BARNP03(NP0120) .......... .......... .......... .......... .......... ..........
BARNP04(NP0082) .......... .......... .......... ...A...... .......... ..........
BARNP05(NP0129) .......... .......... .......... .......... .......... ..........
BARNP06(NP0078) .......... .......... .......... .......... .......... ..........
BARNP07(NP0123) .......... .......... .......... .......... .......... ..........
BARNP08(NP0131) .......... .......... .......... .......... .......... ..........
BARNP09(NP0124) .......... .......... .......... .......... .......... ..........
BARNP10(NP0067) .......... .......... .......... .......... .......... ..........
                                                 270                                                 300
                    L  N  N   M  S  F  W    L  L  P   P  S  F   T  L  L  L   A  A  S
BARNP01(NP0122) GCTAAATAAT ATAAGATTTT GATTACTACC ACCATCTTTT ACATTACTTC TTGCCAGAAG
BARNP02(NP0080) .......... .......... .......... .......... .......... ..........
BARNP03(NP0120) .......... .......... .......... .......... .......... ..........
BARNP04(NP0082) .......... .......... .......... .......... .......... ..........
BARNP05(NP0129) .......... .......... .......... .......... .......... ..........
BARNP06(NP0078) .......... .......... .......... .......... .......... ..........
BARNP07(NP0123) .......... .......... .......... .......... .......... ..........
BARNP08(NP0131) .......... .......... .......... .......... .......... ..........
BARNP09(NP0124) .......... .......... .......... .......... .......... ..........
BARNP10(NP0067) .......... .......... .......... .......... .......... ..........
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Fig. 2. Sequence alignment of ten mitochondrial haplotypes (designated as BARNP01 - BARNP10) obtained from 658-bp CO gene sequences of *N. pygmaea*. Only nucleotide positions that differ from haplotype BARNP01 are indicated. Within parenthesis is the animal numbers corresponding to its haplotype. Amino acid sequences corresponding to the nucleotides are presented at the top of the nucleotide sequences.

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                                                330                                 360
                    M  V  E   S  G  A  G    T  G  W   T  V  Y   P  P  L  A   G  A  I
BARNP01(NP0122) TATAGTTGAA AGAGGAGCTG GAACTGGATG AACGGTTTAT CCTCCATTAG CAGGGGCAAT
BARNP02(NP0080) .......... .......... .......... .......... .......... ..........
BARNP03(NP0120) .......... .......... .......... .......... .......... ..........
BARNP04(NP0082) .......... .......... .......... .......... .......... ..........
BARNP05(NP0129) .......... .......... .......... .......... .......... ..........
BARNP06(NP0078) .......... .......... .......... .......... .......... ..........
BARNP07(NP0123) .......... .......... .......... .......... .......... ..........
BARNP08(NP0131) .......... .......... .......... .......... .......... ..........
BARNP09(NP0124) .......... .......... .......... .......... .......... ..........
BARNP10(NP0067) .......... .......... .......... .......... .......... ..........
                                                390                                 420
                    A  H  A   G  A  S  V    D  L  T   I  F  S   L  H  L  A   G  V  S
BARNP01(NP0122) TGCCCATGCT GGTGCTTCAG TAGATTTAAC AATTTTCTCT TTACACCTAG CAGGTGTCTC
BARNP02(NP0080) .......... .......... .......... .......... .......... ..........
BARNP03(NP0120) .......... .......... .......... .......... .......... ..........
BARNP04(NP0082) .......... .......... .......... .......... .......... ..........
BARNP05(NP0129) .......... .......... .......... .......... .......... ..........
BARNP06(NP0078) .......... .......... .......... .......... .......... ..........
BARNP07(NP0123) .......... .......... .......... .......... .......... ..........
BARNP08(NP0131) .......... .......... .......... .......... .......... ..........
BARNP09(NP0124) .......... .......... .......... .......... .......... ..........
BARNP10(NP0067) .......... .......... .......... .......... .......... ..........
                                                450                                 480
                    S  I  L   G  A  I  N    F  I  T   T  V  I   N  M  K  S   P  G  M
BARNP01(NP0122) ATCTATTTTA GGAGCTATTA ATTTTATTAC TACTGTAATC AATATAAAAT CTCCTGGCAT
BARNP02(NP0080) .......... .......... .......... .......... .......... ..........
BARNP03(NP0120) .......... .......... .......... .......... .......... ..........
BARNP04(NP0082) .......... .......... .......... .......... .......... ..........
BARNP05(NP0129) .......... .......... .......... .......... .......... ..........
BARNP06(NP0078) .......... .......... .......... .......... .......... ..........
BARNP07(NP0123) .......... .......... .......... .......... .......... ..........
BARNP08(NP0131) .......... .......... .......... .......... .......... ..........
BARNP09(NP0124) .......... .......... .......... .......... .......... ..........
BARNP10(NP0067) .......... .......... .......... .......... .......... ..........
                                                510                                 540
                    K  L  D   Q  M  P  L    F  V  W   A  V  V   I  T  A  V   L  L  L
BARNP01(NP0122) AAAATTAGAT CAAATACCAT TATTTGTATG AGCAGTAGTA ATTACTGCTG TATTACTTTT
BARNP02(NP0080) .......... .......... .......... .......... .......... ..........
BARNP03(NP0120) .......... .......... .......... .......... .......... ..........
BARNP04(NP0082) .......... .......... .......... .......... .......... ..........
BARNP05(NP0129) .......... .......... .......... .......... .......... ..........
BARNP06(NP0078) .......... .......... .......... .......... .......... ..........
BARNP07(NP0123) .......... .......... .......... .......... .......... ..........
BARNP08(NP0131) .......... .......... .......... .......... .......... ..........
BARNP09(NP0124) .......... .......... .......... .......... .......... ..........
BARNP10(NP0067) .......... .......... .......... .......... .......... ..........
                                                570                                 600
                    L  S  L   P  V  L  A    G  A  I   T  M  L   L  T  D  R   N  I  N
BARNP01(NP0122) ATTATCTTTA CCAGTACTAG CAGGGGCCAT TACTATGTTA TTAACGGATC GAAATATTAA
BARNP02(NP0080) .......... .......... .......... .......... .......... ..........
BARNP03(NP0120) .......... .......... .......... .......... .......... ..........
BARNP04(NP0082) .......... .......... .......... .......... .......... ..........
BARNP05(NP0129) .......... .......... .......... .......... .....A.... ..........
BARNP06(NP0078) .......... .......... .......... .......... .........C. ..........
BARNP07(NP0123) .......... .......... .......... .......... .......... ..........
BARNP08(NP0131) .......... .......... .......... .......... .......... ..........
BARNP09(NP0124) .......... .......... .......... .......... .......... ..........
BARNP10(NP0067) .......... .......... .......... .......... .......... ..........
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Fig. 2. Continue.

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                                                    630                                 658
                    T  S  F  F  D  P  A   G  G  G  D  P  I  L  Y  Q  H   L  F
BARNP01(NP0122) TACATCATTT TTTGATCCTG CAGGGGGAGG AGATCCTATT TTATATCAAC ACCTCTTT
BARNP02(NP0080) .......... .......... .......... .......... .......... ........
BARNP03(NP0120) .......... .......... .......... .......... .......... ........
BARNP04(NP0082) .......... .......... .......... .......... .......... ........
BARNP05(NP0129) .......... .......... .......... .......... .......... ........
BARNP06(NP0078) .......... .......... .......... .......... .......... ........
BARNP07(NP0123) .......... .....C.... .......... .......... .......... ........
BARNP08(NP0131) .......... ........C. .......... .......... .......... ........
BARNP09(NP0124) .......... .......... ....A..... .......... .......... ........
BARNP10(NP0067) .......... .......... .......... ...C...... .......... ........
```

Fig. 2. Continue.

Hierarchical genetic structure

Genetic relationships among populations and sets of populations were assessed by the Holsinger and Mason-Gamer (H-MG) method (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).

Results and Discussion

COI gene sequence analysis

A total of ten haplotypes (BARNP01 - BARNP10) was obtained by sequencing 658-bp of COI gene from 68 adult *N. pygmaea* (Table 1; Fig. 2). Sequence alignment revealed ten variable nucleotide positions, which are all transitions, and these all did not replace amino acids (Fig. 2).

Sequence divergence among haplotypes

Pairwise comparison between pairs of haplotypes was performed to know about the divergence and relationships among haplotypes (Table 2). The divergence among ten haplotypes ranged from 0.152% to 0.450% (1~3 bp). Comparison of BARNP05 to other haplotypes always provided the highest estimate, 0.450% (3 bp), except for the comparison to BARNP10, wherein the distance was 0.304% (2 bp). Considering other similar studies, which utilized homologous region of mitochondrial genome, the maximum sequence divergence was moderate to low in *N. pygmaea*. For example, the estimate was 0.2% for domestic silkworm (Kim *et al.*, 2000), 0.2% and 1.2% for two species of mushroom flies (Bae *et al.*, 2001), ~0.23% and 0.12% for two species of the rice planthoppers (Mun *et al.*, 1999), 0.4% for spruce budworm species (Sperling and Hickey, 1994), 0.5% for *Heliconius* butterflies (Brower, 1994), 0.9% for the diamondback moth (Li *et al.*, 2006), 4.0% for the firefly, *Luciola lateralis* (Kim *et al.*, 2001), 5.0% for another firefly *Pyrocoelia rufa* (Lee *et al.*, 2003). Excluding two firefly species, which have been reported to have a taxonomic implication (Kim *et al.*, 2001; Lee *et al.*, 2003) it was approximately ≤ 1.2% in the insect mitochondrial COI gene, although exact comparison may not be possible, at least because of the areas covered in each study. Thus, the magnitude of sequence divergence of the *N. pygmaea* is moderate to low compared with other insect species. On the other hand, another

Table 2. Pairwise comparisons among ten *N. pygmaea* haplotypes obtained from the partial sequences of mitochondrial COI gene

Haplotype	1	2	3	4	5	6	7	8	9	10
1	-	0.00304	0.00304	0.00304	0.00456	0.00304	0.00304	0.00304	0.00304	0.00152
2	2	-	0.00304	0.00304	0.00456	0.00304	0.00304	0.00304	0.00304	0.00152
3	2	2	-	0.00304	0.00456	0.00304	0.00304	0.00304	0.00304	0.00152
4	2	2	2	-	0.00456	0.00304	0.00304	0.00304	0.00304	0.00152
5	3	3	3	3	-	0.00456	0.00456	0.00456	0.00456	0.00304
6	2	2	2	2	3	-	0.00304	0.00304	0.00304	0.00152
7	2	2	2	2	3	2	-	0.00304	0.00304	0.00152
8	2	2	2	2	3	2	2	-	0.00304	0.00152
9	2	2	2	2	3	2	2	2	-	0.00152
10	1	1	1	1	2	1	1	1	1	-

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

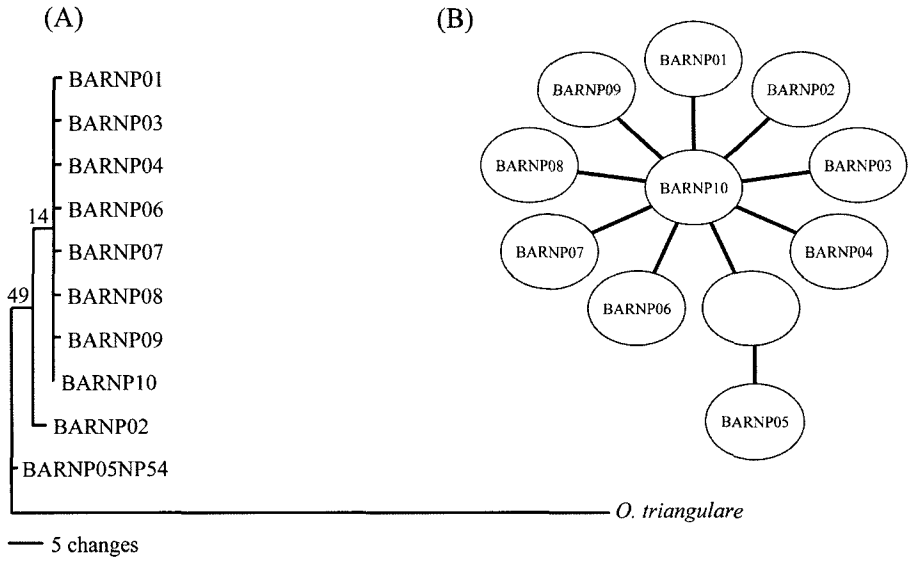

Fig. 3. Relationships among ten haplotypes of *N. pygmaea*. (A) Phylogenetic analysis of ten haplotypes of *N. pygmaea*. The tree was acquired via the MP method incorporated in the PAUP* (Phylogenetic Analysis Using Parsimony and Other Method*) ver. 4.0b10 software (Swofford, 2002). The tree length is 113 steps, Consistency Index is 0.973, Retention Index is 0.4000, and Homoplasy Index is 0.027. *Orthetrum triangulare melania* mitogenome sequence was utilized (Yamauchi et al., 2004) was incorporated in the analysis in order to root the tree. The numbers on the branches represent bootstrap values of 1,000 replications. (B) Parsimonious one-step median networks analysis among ten haplotypes of *N. pygmaea*. Each bar indicates one nucleotide difference from the neighboring haplotype, and the empty circle indicates the hypothetical haplotype, which was not found in this study.

dragonfly, *Libellula quadrimacular*, showed <1% within Europe, 0.2~2.3% within North America, and >0.8% within Japan when limited number of individuals from a few localities were analyzed for mitochondrial COI gene. Thus, the maximum sequence divergence of *N. pygmaea* is much lower than that of *L. quadrimacular*.

Phylogenetic analysis

Phylogenetic relationships among haplotypes were investigated to know they are related to each other (Fig. 3). PAUP analysis by maximum parsimony method showed that all haplotypes were weakly associated or unresolved, although BARNP05 was slightly divergent from all other haplotypes (Fig. 3A). Lack of resolution among most haplotypes is may be due to very low genetic divergence among them. To further illustrate the genetic relationships among *N. pygmaea* haplotypes, we used an unrooted one-step median network, which visualizes a possible evolutionary pathway among closely related haplotypes (Fig. 3B). Although we expected more resolution in the closely

Table 3. Relative frequencies of mitochondrial COI haplotypes through the populations

Haplotype	Locality					
	1. Munkyeong (19)	2. Suwon (9)	3. Muuido (10)	4. Gangjin (10)	5. Gokseong A (10)	6. Gokseong B (10)
BARNP01 (NP0122)				0.1 (1)		
BARNP02 (NP0080)	0.0526 (1)	0.333 (3)				0.1 (1)
BARNP03 (NP0120)				0.4 (4)		0.1 (1)
BARNP04 (NP0082)	0.0526 (1)	0.222 (2)			0.1 (1)	0.1 (1)
BARNP05 (NP0129)				0.1 (1)	0.2 (2)	0.2 (2)
BARNP06 (NP0078)	0.263 (5)	0.222 (2)			0.4 (4)	
BARNP07 (NP0123)				0.1 (1)		
BARNP08 (NP0131)					0.1 (1)	0.2 (1)
BARNP09 (NP0124)				0.2 (2)		
BARNP10 (NP0067)	0.632 (12)	0.222 (2)	1 (10)	0.1 (1)	0.2 (2)	0.4 (4)

Numbers in parentheses indicate sample size at each population.

Table 4. Within-locality diversity estimates

Locality	SS[a]	NH[b]	H[c]	NP[d]	MSD[e] (%)	MPD[f]	p[g]
1. Munkyeong	19	4	0.5556	3	0.304	0.619883	0.000942
2. Suwon	9	4	0.8333	3	0.304	1.277778	0.001942
3. Muuido	10	1	0	0	0	0	0
4. Gangjin	10	6	0.8444	6	0.456	1.688889	0.002567
5. Gokseong A	10	5	0.8222	5	0.456	1.644444	0.002499
6. Gokseong B	10	6	0.8444	6	0.456	1.511111	0.002297

[a]Sample size
[b]Number of haplotypes
[c]Haplotype diversity
[d]Number of polymorphic sites
[e]Maximum sequence divergence
[f]Mean number of pairwise differences
[g]Nucleotide diversity

Table 5. Fixation indices (F_{ST}) and migration rate (Nm) between pairs of populations

	2. Suwon	3. Muuido	4. Gangjin	5. Gokseong A	6. Gokseong B
1. Munkyeong	F_{ST}=0.06560 Nm=9.81130	F_{ST}=0.14305 Nm=4.77704	F_{ST}=0.00000* Nm=2.03132	F_{ST}=0.04365 Nm=11.90759	F_{ST}=0.02705 Nm=4.41860
2. Suwon		F_{ST}=0.00015* Nm=2.05357	F_{ST}=0.00520 Nm=2.56043	F_{ST}=0.29595 Nm=7.97450	F_{ST}=0.30040 Nm=9.88930
3. Muuido			F_{ST}=0.00000* Nm=2.71429	F_{ST}=0.00030* Nm=2.31250	F_{ST}=0.01250 Nm=8.50000
4. Gangjin				F_{ST}=0.01295 Nm=3.28947	F_{ST}=0.15220 Nm=20.00000
5. Gokseong A					F_{ST}=0.31400 Nm=35.50000
6. Gokseong B					

*$p < 0.05$.

related haplotypes, it provided us limited information. All haplotypes, except for BARNP05 were very closely related to BARNP10, with a minimum sequence divergence. In the case of BARNP05, it is somewhat divergent from the most common one and others, but overall *N. pygmaea* haplotypes found in Korea were all highly close to each other.

Genetic diversity indices

Geographic distribution and frequency of haplotypes are listed in Tables 3. Among the ten haplotypes found in this study three haplotypes, BARNP01, BARNP07, and BARNP09, were found only in one locality, but others were found in more than two localities. BARNP10 was found in all localities with a high frequency.

With this information in part, the within-locality diversity was estimated in terms of haplotype diversity (H), maximum sequence divergence (MSD), mean number of pairwise differences (MPD), and nucleotide diversity (π) (Table 4). In a range of 0~1 in H, Suwon (locality 2), Gangjin (locality 4), Gokseong A (locality 5), and Gokseong B (locality 6) were comparatively high (H=0.8222~0.8444), whereas the samples collected from Muuido (locality 3) showed zero diversity, providing a single haplotype (BARNP10; Table 4). In terms of π, Gangjin (locality 4), Gokseong A (locality 5), and Gokseong B (locality 6) showed comparatively high estimate (π= 0.002297~0.002567). Although Suwon (locality 2) showed comparatively high H, the estimate of π was not high as others. Taking these diversity estimates into consideration, the populations in southern localities such as Gangjin (locality 4), Gokseong A (locality 5), and Gokseong B (locality 6) in Jellanamdo Province are relatively higher in genetic diversity than that of remaining localities. Although limited, this result may reflect that the southern localities in Korean peninsula provide better habitats for *N. pygmaea* and may have sustained larger populations than remaining regions. For the long term

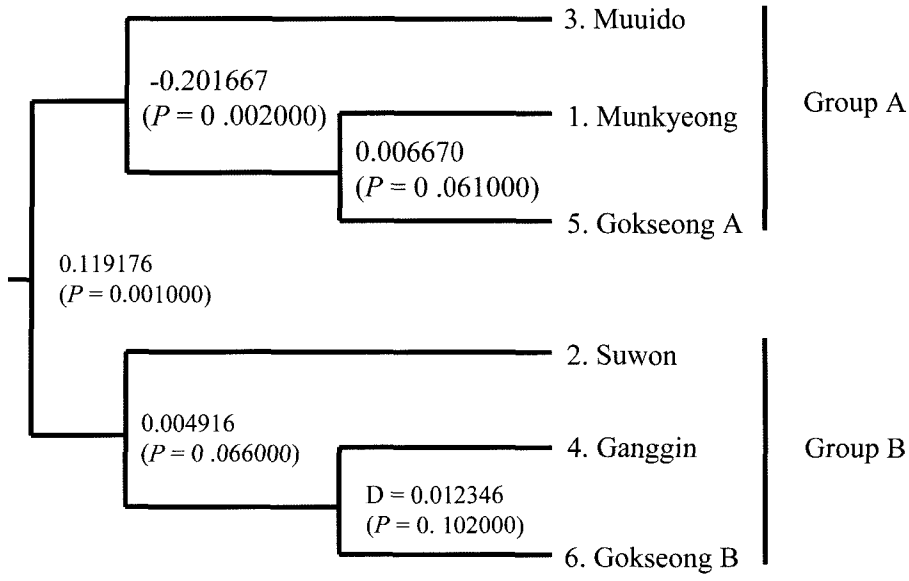

Fig. 4. Hierarchical relationships among localities analyzed using Holsinger and Mason-Gamer method (1996). The value at each node is the distance between its two daughter nodes and the *p* value is the significance of differentiation (based on 10,000 random resamplings). Two statistically significant groups are denoted as groups A and group B.

conservation of the species over a nation-wise scale, thus, southern region of Korean peninsula may require more effort to preserve species-level genetic diversity. However, for further decisive conclusion, more scrutinized sampling and analysis of rapidly evolving gene of *N. pygmaea* may be required.

Gene flow

Genetic distance (F_{ST}) and per-generation migration rates (*Nm*) between pairs of populations are shown in Table 5. Test of statistical significance of pairwise F_{ST} estimates were found in four cases: between Munkyeong (locality 1) and Gangjin (locality 4), between Suwon (locality 2) and Muuido (locality 3), between Muuido (locality 3) and Gangin (locality 4), and between Muuido (locality 3) and Gokseong A (locality 5). Thus, Muuido has three among four cases, which shows implication in statistical significance of pairwise F_{ST}. This probably indicates the nature of isolation of this locality as island, unlikely other populations. Except for Muuido, a significant genetic differentiation was found only in the comparison between Munkyeong (locality 1) and Gangjin (locality 4), showing that populations of *N. pygmaea* in Korean peninsula are very well interconnected to each other. Consistent result was obtained from gene flow estimate (*Nm*), providing the least gene flow estimate in a comparison between Munkyeong (locality 1) and Gangjin (locality 4) (*Nm*= 2.03132), except for the implication of Muuido (locality 3) (Table 5).

Hierarchical population genetic structure

To better understand the nature of genetic diversity of *N. pygmaea* in Korea the hierarchical relationships among localities were analyzed (Fig. 4). The six Korean localities were structured into two groups (p=0.001): Muuido+Munkyeong+Gokseong A group (termed group A) and Suwon+Ganggin+Gokseong B (termed group B). Although group B does not have any subgroup partitioned with statistically significant estimate, group A was further subdivided into Muuido and (Munkyeong+Gokseong A). Overall, the data represent no immediate relationship between geographic distance and genetic distance. The clearest example can be Gokseong A and Wolbokgri, which are located only ~10 km distant to each other, but they belong to different group. On the other hand, Gangjin and Suwon were grouped together as like a single panmictic population, although the distance between them is more than 300 km.

Species of Ordonata, composed of three suborders, have capability to fly. In particular, species of the suborder, Anisoptera, wherein *N. pygmaea* is belonged, contains many active fliers. Recent molecular analysis of another anisopteran dragonfly, *L. quadrimaculata*, also showed a high interconnection among samples collected within continent (Artiss, 2004). However, the reasoning for the interconnection among local samples would be different between *L. quadrimaculata* and *N. pygmaea*. This is because some continental populations of *L. quadrimaculata* are facultative migrants, undergoing occasional periodic mass migrations (Russell *et al.*, 1988), whereas *N.*

pygmaea is smallest dragonfly with a wingspan of ~20 mm and unlikely to have such behavior, although it has not been documented. Instead, it seems that the result of geographic interconnection may have been observed by population reduction and consequent low genetic diversity in the species.

Summarized, the sequence analysis of the tiny dragonfly, *N. pygmaea* provided overall low genetic diversity in this species, in particular, compared to within-continental estimate of another anisopteran dragonfly, *L. quadrimacular*. Geographically, southern localities such as Gangjin and Gokseong in Jeollanamdo Province showed somewhat higher diversity estimates than remaining regions. Genetic isolation by distance was not observed considering the F_{ST}, *Nm*, and hierarchical population genetic structure. For further decisive conclusion, more scrutinized sampling and analysis of rapidly evolving gene of *N. pygmaea* may be required.

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