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[Note]

Additional mitochondrial DNA sequences from the dragonfly, *Nannophya pygmaea* (Odonata: Libellulidae), which is endangered in South Korea

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Abstract

The tiny dragonfly, *Nannophya pygmaea* (Odonata: Libellulidae), is an endangered insect in South Korea. Previously, a partial mitochondrial DNA sequence that corresponded to a DNA barcoding region has been used to infer genetic diversity and gene flow. In this study, we additionally sequenced the barcoding region from *N. pygmaea* that had been collected from three previously sampled populations (40 individuals) and these sequences were combined with the preexisting data. We also selected and sequenced an additional mitochondrial gene (ND5) to find further variable gene regions in the mitochondrial genome. DNA barcoding sequences of 108 individuals from five South Korean localities showed that genetic diversity was highest in Gangjin, Jeollanam-do Province. Muuido, which was previously occupied by a single haplotype, was also found to have an identical haplotype, which confirmed the low genetic diversity on this islet. Gene flow among populations is highly limited, and no clear distance- or region-based geographic partitioning was observed. Phylogenetic relationships among haplotypes showed that there were no discernable haplotypes in South Korea. ND5 provided slightly more haplotypes compared to the barcoding region in 40 individuals (14 vs. 10 haplotypes in the COI gene). It also had a slightly higher within-locality diversity estimate, which suggested that ND5 had potential as mitochondrial DNA-based marker for population genetic analysis.

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Introduction

Nannophya pygmaea (Odonata: Libellulidae) is often called the "tiny" or "Scarlet Dwarf" dragonfly. It has a wingspan of ~20 mm and is one of the smallest recorded modern odonate species (Won *et al.*, 2009). The species ranges across the Indian Peninsula to Australia, including Korea (Ishida *et al.*, 1988; Karube, 2009; Won *et al.*, 2009). In Korea, the species is listed as a second-degree endangered wild animal and plant (Korean Ministry of Environment, 2006). Therefore, a population genetics analysis of *N. pygmaea* has been performed using a portion

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of mitochondrial DNA (mtDNA) corresponding to a DNA barcoding region (the 658 bp region of the COI gene). The main finding was that genetic diversity was low in Korean *N. pygmaea* populations. However, the results also showed that diversity was slightly higher in southern localities, such as Gangjin and Gokseong in Jeollanam-do Province, than in other areas (Kim *et al.*, 2007).

In this study, we additionally sampled 40 *N. pygmaea* from three previously collected localities in 2016, and these data were combined with the pre-existing DNA barcoding sequence data to expand the mtDNA-based population genetic analysis data on *N. pygmaea*

Materials and Methods

DNA sequencing and analysis

The mitochondrial (mt) COI gene sequences, corresponding to a DNA barcoding region (658 bp), were amplified under the following conditions: an initial denaturation step at 94°C for 5 min, a 35-cycle amplification (94°C for 1 min, 48–52°C for 1 min, and 72°C for 1 min), and a final extension step of 7 min at 72°C. The primers for the COI sequences were designed using the complete mt genome sequences from *N. pygmaea* (Jeong *et al.*, Submitted). These were NP-LCOF (5′-TTTCTACTAAT CATAAGGATATTGG-3′), and NP-HCOR (5′-TAAACTTC CGGATGACCAAAGAATCA-3′). The variability of several mt ND genes were also considered (e.g., Wan *et al.*, 2013), and eventually, ND5 was chosen based on amplification efficiency, sequence divergence, and the number of variable sites after several individual *N. pygmaea* were sequenced. The primers for the amplification of a 730 bp partial ND5 gene were designed using the complete mt genome sequences from *N. pygmaea* (Jeong *et al*., Submitted). These were forward, 5′-TAATAGTATATACTCCCGTG-3′, and reverse, 5′-GCTCATGTTGAAGCTCCTG-3′. After an initial denaturation step at 94°C for 5 min, a 30-cycle amplification (94°C for 1 min, 48–52°C for 1 min, and 72°C for 1 min) was conducted. The final extension step was 7 min at 72°C. The PCR product was then purified using a PCR Purification Kit (Qiagen, Germany). Electrophoresis was carried out in 0.5 × TAE buffer on 0.5% agarose gels to confirm successful DNA amplification. The DNA sequencing was conducted using the ABI PRISM® BigDye® Terminator ver. 3.1 Cycle Sequencing Kit with an ABI 3100 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA). All products were sequenced from both strands.

The sequences of both strands from each individual were aligned using the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/; Sievers *et al.*, 2011) and finalized for each individual sample. When the sequences from each individual differed by ≥1 nucleotide or insertion/deletion (indel) after alignment using PAUP ver. 4.0b (Swofford, 1999), they were considered to be different haplotypes. Haplotype designations for ND5 were applied to new sequences as they were discovered (i.e., NPND501, NPND502, NPND503, etc.), whereas haplotypes for the DNA barcoding region were designated by following Kim *et al.* (2007).

Genetic diversity indices

Within-population haplotype diversity (h) and nucleotide diversity (π) were estimated according to Nei (1987) using Arlequin ver. 3.5 (Excoffier and Lischer, 2010). Maximum sequence divergence within each locality was obtained by extracting the within-locality estimates of the unrooted pairwise distances from PAUP (Swofford, 1999). The genetic distance and per-generation female migration rate were estimated from subroutines in Arlequin ver. 3.5 (Excoffier and Lischer, 2010). Pairwise F_{ST} was used to estimate the per-generation female migration rate, and Nm (the product of the effective population size, Ne, and the migration rate, m) was obtained according to Excoffier *et al.* (1992) using the equilibrium relationship: $F_{ST} = 1/(2Nm + 1)$. Any significant differences between the pairs of localities (1,000 bootstraps) were analyzed using a permutation test, which followed the approach described by Excoffier *et al.* (1992). The distances between DNA sequences were calculated by the Kimura 2-parameters method (Kimura, 1980). In this analysis, only populations with more than two haplotypes were considered. This meant that four of the five populations were subjected to analysis.

Phylogenetic analysis

During the phylogenetic analysis, the GTR + GAMMA

+ I model, which was selected by comparing the Akaike Information Criterion scores (Akaike, 1974) using Modeltest ver. 3.7 (Posada and Crandall, 1998), was used for the maximum-likelihood (ML) method. The ML method was conducted using RAxML-HPC2 on XSEDE ver. 8.0.24 (Stamatakis, 2006), which is found on the CIPRES Portal ver. 3.1 (Miller *et al.*, 2010). To root the phylogenetic trees, six *N. pygmaea* haplotypes, originally from Malaysia, were downloaded from GenBank (GenBank accession numbers KT991525, KT991529, KT991527, KT991526, KT991528, and KT991531; Low *et al.*, 2016). The generated tree was viewed with FigTree software ver. 1.4.2 (tree.bio.ed.ac.uk/software/figtree).

Results and Discussion

Sequence analyses

DNA sequencing of the COI DNA barcoding region and ND5 from 40 individuals identified ten haplotypes (BARNP01, BARNP02, BARNP03, BARNP05, BARNP07, BARNP09, BARNP10, BARNP11, BARNP13, and BARNP14) and 15 haplotypes (NPND501–NPND515) (Table 1), respectively. They had maximum sequence divergences of 0.680% (4 bp) and 0.822% (6 bp), respectively (data not shown). Therefore, the newly selected ND5 provided slightly greater variability than the DNA barcoding region analysis. When COI and ND5 were concatenated (1,388 bp), a total of 20 haplotypes (NPCOND01–NPCOND20) were generated, with the maximum sequence divergence ranging from one (0.072%) to 10-bp (0.72%; data not shown). Thus, concatenated sequences provided more variable sites compared to each individual gene sequence. Furthermore, genetic diversity estimates were higher for the concatenated sequences than for each individual gene sequence, which confirmed that an additional gene sequence with equivalent or higher viability would help detect genetic diversity in *N. pygmaea*.

When previously published COI data (68 individuals from five localities; Kim *et al.*, 2007) were combined together with the current data (40 individuals from three localities), a total of 108 individuals from five localities resulted in 14 haplotypes, with a maximum sequence divergence of 0.680% (4 bp; data not shown). Therefore, the combined data increased the number of haplotypes, but not the maximum sequence divergence.

Genetic diversity indices

Among the 14 haplotypes, eight haplotypes were only found in Gangjin (locality 4) and nine haplotypes were found in Gokseong (locality 5). Munkyeing (locality 1) and Suwon (locality 2) were found to contain four haplotypes, and Muuido (locality 3) was found to contain one haplotype. BARNP10 was found in all localities at a relatively high frequency (data not shown). 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 2). Suwon, Gangjin, and Gokseong had comparatively high *H* values (H = 0.8154~0.8737). However, the samples collected from Muuido showed zero diversity and contained only one haplotype (BARNP10; Table 2). In terms of π, Gangjin and Gokseong had a comparatively high estimate (π = 0.002465~0.002640). Although Suwon had the second highest *H*, its π estimate was third behind Gangjin and Gokseong. These diversity estimates show that, the populations in the southern localities, such as Gangjin and Gokseong in Jellanamdo Province, have a relatively higher genetic diversity compared to the other localities. This result, even after extended sampling, is consistent with previous results obtained by Kim *et al.* (2007). Therefore, the previous rationale that the southern localities on the Korean peninsula may have sustained larger populations than the other sampling regions is further supported by these results (Kim *et al.*, 2007).

Gene flow

The genetic distance (F_{ST}) and per-generation migration rates (*Nm*) results between pairs of populations indicated that the pairwise F_{ST} estimates were statistically different for nearly all population pairs, except for the Gangjin and Gokseong pair (Table 3). These results are consistent with the previous finding that the two southern localities do not show any genetic differentiation (Kim *et al.*, 2007). This may be due to the geographic closeness of the two localities and agreed with the gene flow estimate

Table 1. A list of trapping localities, insect numbers, and mitochondrial COI and ND5 haplotypes for *Nannophya pygmaea*

Collection locality (nos. of individuals)	Insect number	COI haplotype	GenBank accession number	ND5 haplotype	GenBank accession number	COI+ND5 haplotype
1. Muuido Island,	NP6318	BARNP10	MF491643	NPND501	MF491683	NPCOND01
Incheon (10)	NP6319	BARNP10	MF491644	NPND501	MF491684	NPCOND01
	NP6320	BARNP10	MF491645	NPND501	MF491685	NPCOND01
	NP6321	BARNP10	MF491646	NPND501	MF491686	NPCOND01
	NP6322	BARNP10	MF491647	NPND501	MF491687	NPCOND01
	NP6323	BARNP10	MF491648	NPND501	MF491688	NPCOND01
	NP6324	BARNP10	MF491649	NPND502	MF491689	NPCOND02
	NP6325	BARNP10	MF491650	NPND501	MF491690	NPCOND01
	NP6326	BARNP10	MF491651	NPND501	MF491691	NPCOND01
	NP6327	BARNP10	MF491652	NPND501	MF491692	NPCOND01
2. Gokseong-gun,	NP6669	BARNP10	MF491653	NPND513	MF491693	NPCOND03
Jeollanam-do	NP6670	BARNP10	MF491654	NPND512	MF491694	NPCOND04
Province (20)	NP6671	BARNP11	MF491655	NPND512	MF491695	NPCOND05
	NP6672	BARNP05	MF491656	NPND504	MF491696	NPCOND06
	NP6673	BARNP02	MF491657	NPND512	MF491697	NPCOND07
	NP6674	BARNP05	MF491658	NPND512	MF491698	NPCOND06
	NP6675	BARNP05	MF491659	NPND505	MF491699	NPCOND06
	NP6676	BARNP10	MF491660	NPND506	MF491700	NPCOND08
	NP6677	BARNP10	MF491661	NPND512	MF491701	NPCOND09
	NP6678	BARNP05	MF491662	NPND504	MF491702	NPCOND06
	NP6679	BARNP02	MF491663	NPND504	MF491703	NPCOND07
	NP6680	BARNP02	MF491664	NPND507	MF491704	NPCOND07
	NP6681	BARNP12	MF491665	NPND508	MF491705	NPCOND10
	NP6682	BARNP05	MF491666	NPND503	MF491706	NPCOND11
	NP6683	BARNP10	MF491667	NPND509	MF491707	NPCOND03
	NP6684	BARNP05	MF491668	NPND509	MF491708	NPCOND12
	NP6685	BARNP02	MF491669	NPND510	MF491709	NPCOND13
	NP6686	BARNP02	MF491670	NPND512	MF491710	NPCOND14
	NP6687	BARNP05	MF491671	NPND508	MF491711	NPCOND06
	NP6688	BARNP05	MF491672	NPND513	MF491712	NPCOND11
3.Gangjin-gun,	NP6689	BARNP09	MF491673	NPND512	MF491713	NPCOND15
Jeollanam-do	NP6690	BARNP13	MF491674	NPND511	MF491714	NPCOND16
Province (10)	NP6691	BARNP03	MF491675	NPND512	MF491715	NPCOND17
	NP6692	BARNP07	MF491676	NPND513	MF491716	NPCOND18
	NP6693	BARNP10	MF491677	NPND513	MF491717	NPCOND19
	NP6694	BARNP03	MF491678	NPND514	MF491718	NPCOND17
	NP6695	BARNP14	MF491679	NPND513	MF491719	NPCOND20
	NP6696	BARNP13	MF491680	NPND515	MF491720	NPCOND16
	NP6697	BARNP01	MF491681	NPND512	MF491721	NPCOND19
	NP6698	BARNP01	MF491682	NPND514	MF491722	NPCOND04

Table 2. Within-locality diversity estimates for COI haplotypes from *Nannophya pygmaea*

Locality	SS[a]	NH[b]	*H*[c]	NP[d]	MSD[e] (%)	MPD[f]	π[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	20	1	0.0000	0	0.000	0.000000	0.000000
4. Gangjin	20	8	0.8737	8	0.608	1.736842	0.002640
5. Gokseong	40	9	0.8154	8	0.456	1.621795	0.002465

[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 3. Fixation indices (F_{ST}) and the migration rate (*Nm*) between pairs of populations based on the COI gene from *Nannophya pygmaea*

	1	2	4	5
1. Munkyeong				
2. Suwon	F_{ST} = 0.10739* *Nm* = 4.15610			
4. Gangjin	F_{ST} = 0.11967* *Nm* = 3.67821	F_{ST} = 0.12402* *Nm* = 3.53169		
5. Gokseong	F_{ST} = 0.12739* *Nm* = 3.42497	F_{ST} = 0.09871* *Nm* = 4.56559	F_{ST} = 0.04849 *Nm* = 9.81130	

*, $P < 0.05$

(*Nm*) results where the highest gene flow estimate was for a comparison between Gangjin and Gokseong (*Nm* = 9.81130 vs. 3.42497–4.56559; Table 3).

Phylogenetic analysis

A phylogenetic analysis, which investigated the relationships and divergence among the *N. pygmaea* haplotypes, strongly supported the monophyly of the *N. pygmaea*, which was originated in Korea, and had separated from the *N. pygmaea* haplotypes that had originated in Malaysia. This was confirmed in both the BI and ML analyses (Fig. 1). No haplotype formed a distinguishable subgroup in both analyses, which indicated that *N. pygmaea* haplotypes found in Korea are all very close to each other.

In summary, even though the new sampling was undertaken about 10 years after the previous study, the previous finding by Kim *et al.* (2007) was supported by these results. The sequence analysis of *N. pygmaea* showed that overall genetic diversity was low, which confirmed previously reported results (Kim *et al.*, 2007). Southern localities, such as Gangjin and Gokseong in Jeollanamdo Province, still showed somewhat higher diversity estimates than the remaining regions. These results suggest that the *N. pygmaea* populations in Korea are genetically stable, even though the genetic diversity rate is low.

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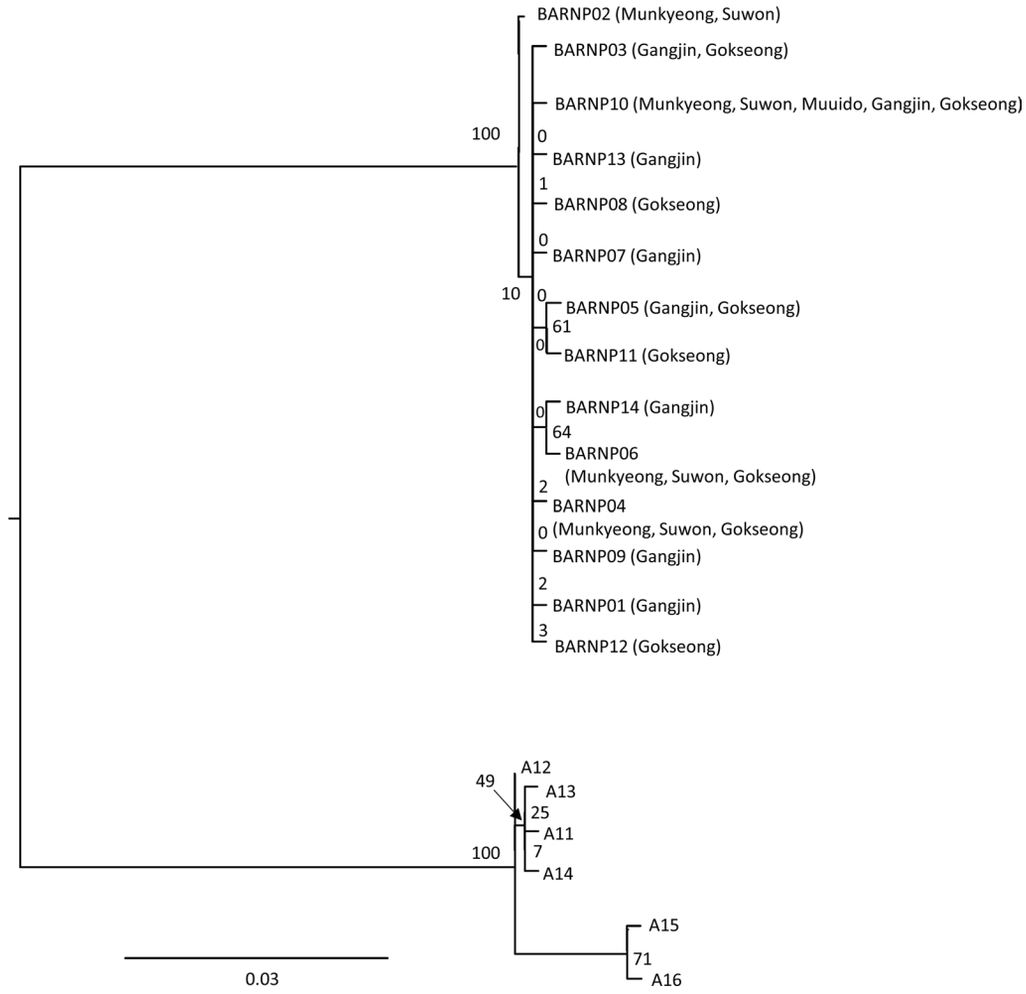

Fig. 1. Phylogenetic analysis of haplotypes from the DNA barcoding region in *Nannophya pygmaea.* The tree was acquired via the ML method. The numbers at each node specify the bootstrap percentages of 1,000 pseudoreplicates. Locality names corresponding to each haplotype are provided in parentheses. *Nannophya pygmaea* haplotypes from A11 to A16 originated in Malaysia (Low et al., 2016) and were used as outgroups in order to root the tree.

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