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Report

Molecular phylogenetic analysis of *Nannophya pygmaea* from the Tono area of Gifu Prefecture, Japan, and South Korea

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Abstract

To investigate the phylogeny of the Tiny dragonfly, *Nannophya pygmaea* (Odonata: Libellulidae), 41 individuals were collected from 14 sites in the Tono area of Gifu Prefecture, Japan (TGJ group). We amplified a partial sequence of the mitochondrial cytochrome c oxidase subunit I (COI) gene (658 bp.) from the TGJ group and compared it with the same sequence of 68 *N. pygmaea* specimens were collected from 6 sites, South Korea (SK group), to analyze the genetic diversity. The TGJ group comprised four haplotypes (TGJ01–04), all of which differed from those in the SK group. Neighbor-joining phylogenetic tree generated based on the COI sequences revealed divergence between the two groups. The distribution of the 10 haplotypes in the SK group was random and did not vary with distance or among the geographic regions in South Korea, but this pattern of genetic distribution was not observed in our study. Rather, although the distribution of the TGJ01 haplotype was ubiquitously in the Tono area, the remaining haplotypes (TGJ02, TGJ03 and TGJ04) exhibited localized distributions. Although our study was limited by the number of individuals and the size of the area surveyed, we were able to clarify the phylogeny and genetic variation in *N. pygmaea* in the Tono area of Gifu Prefecture, Japan.

Key words: *Nannophya pygmaea*, mitochondrial COI gene, genetic diversity,

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Introduction

Nannophya pygmaea (Odonata: Libellulidae), one of the smallest dragonflies in the world, is distributed from China to Korea and Japan, through Southeast Asia, and Australia (Ishida *et al*., 1988). In Japan, this species is widely distributed throughout the Japanese mainland and is listed as an endangered species in some prefectures (Ishida *et al*., 1988). The habitats of *N. pygmaea* include specific marshes that are too shallow to support fish species that prey on dragonfly larvae. Since *N. pygmaea* adults remain at the same marsh after emergence (Ishida *et al*., 1988), the species is well suited for use as an environmental indicator species. However, populations of *N. pygmaea* are threatened by extensive habitat destruction, mainly due to factors such as urbanization, and water pollution. These threats have a negative influence of the populations of this species and can act to decrease overall genetic diversity.

For example, in South Korea, *N. pygmaea* is listed as a second-degree endangered wild animal, generally due to the same environmental problems that exist in Japan (Kim *et al*., 2007). Given this background, Kim *et al*. (2007) sequenced a region of the mitochondrial cytochrome c oxidase subunit I (COI) gene from 68 specimens collected in 6 sites in South Korea. Their study showed that the populations in the southern part of South Korea exhibited higher levels of genetic diversity compared to regions elsewhere in the country. Moreover, phylogenetic analysis and uncorrected pairwise distance estimates showed that genetic diversity estimates were relatively low in this species (Kim *et al*., 2007). For the long-term conservation of the species at a national scale, Kim *et al*. (2007) proposed that

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Table 1. Frequencies of 4 haplotypes of *Nannophya pygmaea* in 14 sampling sites on the Tono area of Gifu Prefecture, Japan.

Locality	Number of haplotype			
	TGJ01	TGJ02	TGJ03	TGJ04
Kitaogi-cho, Tajimi (four different sampling sites) (35° 20', 137° 02')	1	-	-	1
	1	-	-	-
	1	-	-	-
	3	-	-	-
Kita-machi, Tajimi (35° 20', 137° 06')	4	-	-	-
Hoshigadai, Tajimi (35° 19', 137° 08')	3	-	-	-
Izumikitayama-cho, Toki (35° 23', 137° 11')	3	1	-	-
Tsurusato-cho, Toki (35° 16', 137° 13')	3	-	-	-
Kamado-cho, Mizunami (35° 25', 137° 17')	4	-	-	-
Takenami-cho, Ena (four different sampling sites) (35° 25', 137° 21')	4	-	2	-
	2	-	-	-
	3	-	1	-
	1	-	1	-
Naegi, Nakatsugawa (35° 32', 137° 28')	2	-	-	-
Total	35	1	4	1

the populations in the southern part of the country required more efforts to preserve species-level genetic diversity.

While some studies have been conducted on the habitat preference (Yoshida *et al*., 2004) and distribution (Ishida *et al*., 1988) of this species in Japan, no studies have examined the genetic diversity of *N. pygmaea* to date. In order to clarify the genetic diversity of *N. pygmaea* in Japan, we therefore investigated the partial sequences of the mitochondrial COI gene region of the populations of this species in the Tono area of Gifu Prefecture of Japan. Moreover, the sequence data were then used to estimate the genetic diversity within a target population and to perform a molecular phylogenetic analysis using the specimens from the Japanese populations (this study) and South Korean populations (Kim *et al*., 2007).

Materials and Methods

We collected 41 *N. pygmaea* specimens from 14 sites in the Tono area of Gifu Prefecture (Table 1). The left middle legs of *N. pygmaea* specimens were removed for DNA analysis, where after the specimens were released at the collection sites. The tissues thus collected were stored at −20°C until

Table 2. The haplotypes defined by polymorphic nucleotide sites in a partial sequence of the mitochondrial COI genes of *Nannophya pygmaea* on the Tono area of Gifu Prefecture, Japan, and South Korea.

Haplotype	Nucleotide potition [1]														
	19	28	109	214	271	352	367	478	484	586	589	616	619	625	634
Tono area [2]															
TGJ01	C	A	A	G	A	A	T	C	A	G	T	T	T	G	C
TGJ02	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*
TGJ03	*	*	*	*	C	*	*	*	T	*	*	*	*	*	*
TGJ04	*	*	*	*	*	G	C	T	*	*	*	*	*	*	*
South Korea [3]															
BARNP01	T	*	*	*	*	*	*	*	*	*	*	*	*	*	T
BARNP02	*	G	*	*	*	*	*	*	*	*	*	*	*	*	T
BARNP03	*	*	G	*	*	*	*	*	*	*	*	*	*	*	T
BARNP04	*	*	*	A	*	*	*	*	*	*	*	*	*	*	T
BARNP05	*	*	*	*	*	*	*	*	*	A	*	*	*	*	.
BARNP06	*	*	*	*	*	*	*	*	*	*	C	*	*	*	T
BARNP07	*	*	*	*	*	*	*	*	*	*	*	C	*	*	T
BARNP08	*	*	*	*	*	*	*	*	*	*	*	*	C	*	T
BARNP09	*	*	*	*	*	*	*	*	*	*	*	*	*	A	T
BARNP10	*	*	*	*	*	*	*	*	*	*	*	*	*	*	T

Asterisk indicates the identical nucleotide to which of TGJ01.
1) Variation site are shown as nucleotide positions from 5' end of PCR amplified region, except forward primer sequence.
2) TGJ01 - 04 were confirmed on Tono area of Gifu Prefecture in Japan
3) BARNP01 - 10 were confirmed in South Korea (Kim *et al*., 2007)

total genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Germany) and purified using a GeneClean Spin Kit (MP Biomedicals, CA).

PCR was performed in 50 µl reaction mixtures with 0.5 units of KOD-Plus-DNA Polymerase (Toyobo, Japan) and 0.2 µM of each primer, according to the manufacturer's instructions. The primer sequences for the regions flanking the COI locus were (forward) 5′-GG TCAACAAATCATAAAGATATTGG-3′, and (reverse) 5′-TAAACTTCAGGGTGACCA AAAAATCA-3′ (Muraji *et al*., 2001). PCR reactions were performed using a DNA Thermal Cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA) using an initial denaturation step of 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 47°C for 30 s, extension at 68°C for 90 s, and a final extension step of 68°C for 90 s. All PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) and subjected to dye-terminator cycle sequencing using a DTCS Quick Start Mix (Beckman Coulter, CA) with an automatic sequencer (CEQ 2000XL, Beckman Coulter, Inc.).

The COI sequences were aligned with the published sequences from South Korean species (Kim *et al*., 2007) using MEGA5.05 software (Tamura *et al*., 2011), which was also used to conduct phylogenetic analyses. Specifically, neighbor-joining (NJ) tree were generated from the COI sequences using the Maximum Composite Likelihood algorithm in MEGA5.05. Bootstrap analysis was performed to estimate the robustness of each node in the phylogeny (10,000 replicates). The partial sequence of *Orthetrum triangulare melania* COI (Accession No. AB126005) was used as an outgroup.

Results and Discussion

The alignment of a continuous stretch of 658 bp of the COI sequences revealed nucleotide variability at six positions. A total of four haplotypes (TGJ01–04) was obtained for the *N. pygmaea* specimens from 14 sites in the Tono area of Gifu Prefecture (Table 2). The TGJ01 haplotype was identified in 85% (35/41) of all of the specimens in the Tono area of Gifu Prefecture (TGJ) group, and is distributed throughout the Tono area of Gifu Prefecture. The distribution of the TGJ01 haplotype appeared to be ubiquitously and independent of both distance and geographic location in the Tono area. However, the remaining three haplotypes (TGJ02, TGJ03 and TGJ04) showed evidence of local adaptation. In particular, the TGJ02 and TGJ04 haplotypes were both only observed in one individual each in Izumikitayama-cho in Toki and Kitaogi-cho in Tajimi, respectively

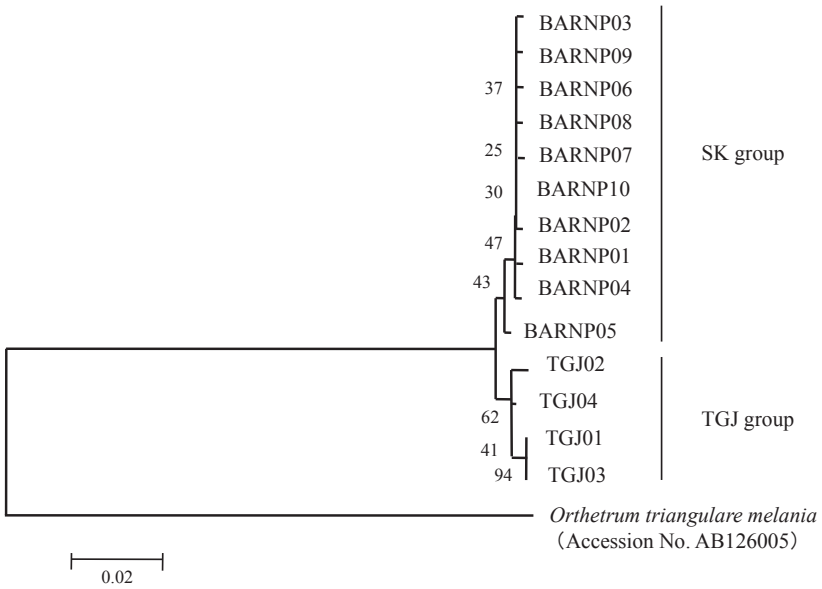

Fig.1. Neighbor-joining tree based on mitochondrial COI gene partial sequences of *Nannophya pygmaea* on Tono area of Gifu Prefecture, Japan (TGJ group) and South Korea (SK group). *Orthetrum triangulare melania* (Accession No. AB126005) was used as an outgroup. Numbers beside the branches indicate bootstrapvalues in 10,000 replicates. Branch lengths represent nucleotide substitutions. The alphabet and number were shown each haplotype name (shown in Table 1 and 2). Sequence data of SK group was quoted from the report of Kim *et al*. (2007).

(Table 1). The TGJ03 haplotype was confirmed in four individuals in Takenami-cho in Ena (Table 1). Although the different geographic populations of *N. pygmaea* in South Korea contained 10 haplotypes, the distribution of these haplotypes did not show any variation with distance or among geographic regions (Kim *et al*., 2007). This genetic pattern was not comparable to that observed in our study, as the regional genetic variation observed in *N. pygmaea* specimens from the Tono area was less than that observed among specimens from South Korea.

Sequence alignment of the four *N. pygmaea* haplotypes (TGJ01–04) identified in this study and the 10 *N. pygmaea* haplotypes (BARNP01–10) identified in South Korea (Kim *et al*., 2007) revealed 15 variable nucleotide positions. In addition, NJ analysis revealed that the *N. pygmaea* sequences formed two distinct clades (TGJ and SK groups) (Fig. 1). We identified the intraspecific COI variants in both groups and found that the four endemic haplotypes comprising the TGJ group were responsible for the separation of the clades.

Although somewhat limited in geographical scale, we conducted this preliminary case study to clarify the genetic diversity and regional genetic variation of *N. pygmaea* populations in the Tono area of Gifu Prefecture. Future studies will therefore involve genetic surveys of *N. pygmaea* populations in other regions so that we can compare such findings with those of this study.

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摘　要

岐阜県東濃地方と韓国に生息するハッチョウトンボ（*Nannophya pygmaea*）の遺伝的多様性についての比較

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ハッチョウトンボのミトコンドリア COI 遺伝子部分領域（658塩基）について，岐阜県東濃地方14地点 41個体（東濃グループ）の DNA 配列を決定した．決定された東濃グループの COI と，韓国で報告された 6地点 68個体（韓国グループ）の COI について分子系統学的解析を行った．東濃グループからは，韓国グループからは確認されていない新たな 4つのハプロタイプ（TGJ01-04）が確認された．そして，近隣接合法による分子系統樹を構築した結果，東濃グループと韓国グループは異なる 2つのクレードに分かれた．韓国グループの10のハプロタイプは，韓国国内にランダムに分布し，地理的局在性は認められないと報告されている．一方，東濃グループでは最も多く確認された TGJ01（85%）については東濃全体にランダムに分布していたが，残りの 3つのハプロタイプ（TGJ02, TGJ03 and TGJ04）については地域局在性が認められた．本報告では，調査域が東濃全域に及んでいないが，東濃のハッチョウトンボは韓国のものと分子系統的に異なり，遺伝的変異に地理的局在性があることが明らかとなった．

キーワード：ハッチョウトンボ, ミトコンドリア COI 遺伝子, 遺伝的多様性

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