Molecular Phylogenetic Relationships in four *Oxya* species (Orthoptera : Catantopidae)

Yasuyoshi AIKAWA[1], Yoshikazu ANDO[1] and Yasuyuki SHIROTA[2]

[1] *Laboratory of Entomology*
[2] *Laboratory of Evolutionary Biology*

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Introduction

Four species of rice grasshoppers, *Oxya yezoensis*, *O. japonica japonica*, *O. chinensis formosana and O. hyla intricata*, occur primarily in paddy fields and grasslands in Japan. They are well known as pests of rice. Although these species are similar in many morphological traits, they can be separated by the characters of the male genitalia and female subgenital plate (4, 5).

ZHU and ANDO (18) studied parthenogenesis in the first three species and demonstrated that *O. yezoensis* had the lowest ability of parthenogenesis followed by *O. chinensis formosana*, whereas *O. japonica japonica* had the highest ability. Reciprocal interspecific crosses between *O. yezoensis* and *O. chinensis formosana* produced fertile hybrids as did intraspecific crosses (ANDO and ZHU, unpublished data). These observations suggest that *O. yezoensis* and *O. chinensis formosana* are closely related to each other. Although some ecological features of Japanese *Oxya* species are known, their phylogenetic relationships have not been well understood (19). In this study, we investigated the phylogenetic relationships of four Japanese *Oxya* species by comparing a part of the mitochondrial cytochrome oxidase I (COI) gene. In addition, we applied the second internal transcribed spacer (ITS2) of the rDNA gene for analysing the closely related *Oxya* species of *O. yezoensis* and *O. chinensis formosana*.

Materials and Methods

Insects

We analysed four *Oxya* species including one subspecies : *O. yezoensis*, *O. japonica japonica* and *O. j. vitticollis*, *O. chinensis formosana*, and *O. hyla intricata*. *O. yezoensis* is distributed predominantly in northeastern Honshu, and found in limited areas in Hokkaido, southeastern Honshu, and Kyushu (4, 5, 1). *O. japonica japonica* is primarily distributed in Taiwan, the southern part of the Korea peninsula, the southern part of China, India, Southeast Asia, Hawaii and Japan. In Japan, it occurs in Honshu, Shikoku, Kyushu and the southwestern island chain. *O. chinensis formosana* occurs in Taiwan, China and the southwestern island chain of Japan. *O. hyla intricata* occurs in the middle and south part of China and tropical Asia (the east of Myanmar). In Japan, it is distributed in the southwestern island chain, and the northern limit of its distribution is Okinawa Island (7, 4, 5). *O. japonica* is divided into two subspecies : *O. japonica japonica* and *O. japonica vitticollis*. The latter occurs in Melanesia, the east part of Republic of Indonesia, and the east part of Australia (7). As mentioned above, *O. japonica japonica* is distributed not only in Japan but also in many other countries. We thus analysed these two subspecies derived from foreign countries to compare with Japanese *O. japonica japonica* population. The collecting sites of the insects used are shown in Figs. 1 and 2. Three individuals were sequenced from each

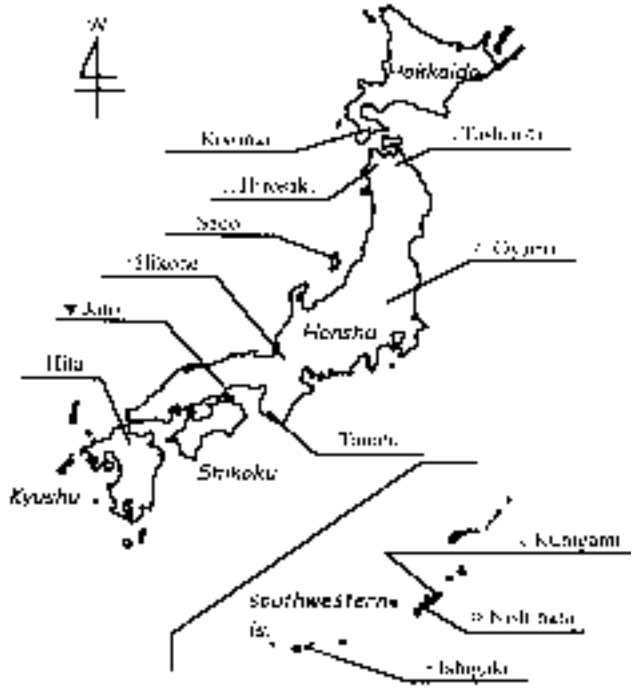

Fig. 1. Collection sites for four *Oxya* species in Japan. ○ : *O. yezoensis*, ▼ : *O. japonica japonica*, ◎ : *O. chinensis formosana*, ※ : *O. hyla intricata*. Three specimens were sequenced from each collection site.

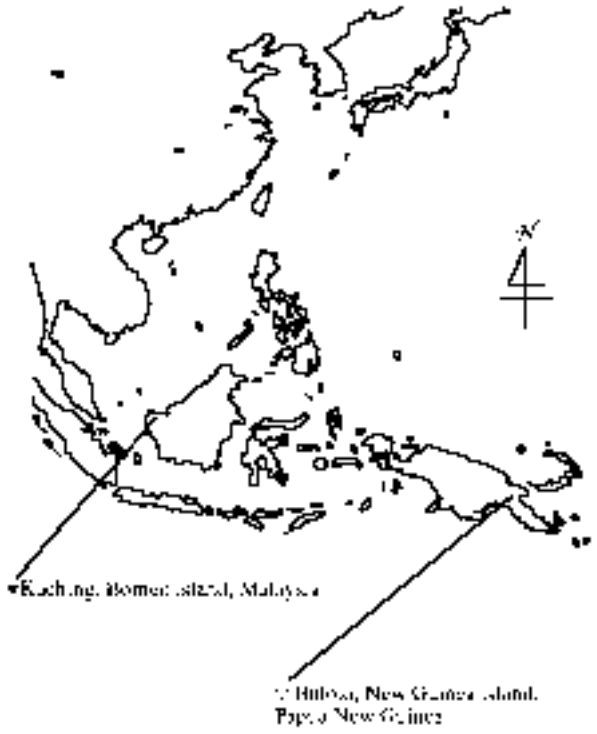

Fig. 2. Collection sites for two *O. japonica* subspecies in Malaysia and Papua New Guinea. ▼ : *O. japonica japonica*, ▽ : *O. j. vitticollis*. One specimen was sequenced from each collection site.

site, but only one individual in Kuching (Borneo island : Malaysia) and Bulolo (Papua New Guinea). A total of 38 specimens that had been preserved in ethanol at room temperature were sequenced.

DNA extraction, amplification and sequencing

DNA was extracted from a hind leg of an adult or a nymph, or a whole first instar nymph. DNA extraction, amplification, and sequencing were performed according to the method of OZAKI and OHBAYASHI (11). We collected and analyzed a region of sequence data from COI in all individuals, and from ITS2 in *O. yezoensis* and *O. chinensis formosana*. Fragments of COI and ITS2 were amplified by the polymerase chain reaction (14). We amplified the COI fragment with sense strand primer (COIS ; 5 'GGATCACCTGATATAGCATTCCC 3 ') and antisense strand primer (COIA ; 5 'CCCGGTAAAATTAAAATATAAACTTC 3 ')(6). Likewise, the ITS2 fragment was amplified with primers ITS2-5.8S (5 'TGTGAACTGCAGGACA CATG 3 ') and ITS2-28S (5 ' ATGCTTAAA-TTTAGGGGGTAGTC 3 ') (12).

Analysis of sequence

Sequences were aligned by CLUSTAL W (17) and analysed by maximum parsimony (MP), neighbor joining (NJ), and maximum likelihood (ML). MP analysis was performed with a PAUP computer software package (PAUP version 3.1.1) (16). Equal weighting and a weighting scheme of 1-3 for transitions (TI) and transversions (TV) were used in this analysis. The shortest tree was found with the heuristic algorithm. NJ analysis was conducted with KIMURA's two-parameter model to correct for multiple substitutions (8). NJ tree was constructed with NJ procedure in Phylogeny Inference Package (PHYLIP version 3.573c ; 3). ML analysis was performed with DNAML in PHYLIP. Bootstrap support was assessed for MP and NJ analyses based on 1,000 replication, but only 100 replications for ML analysis because of computational limitations.

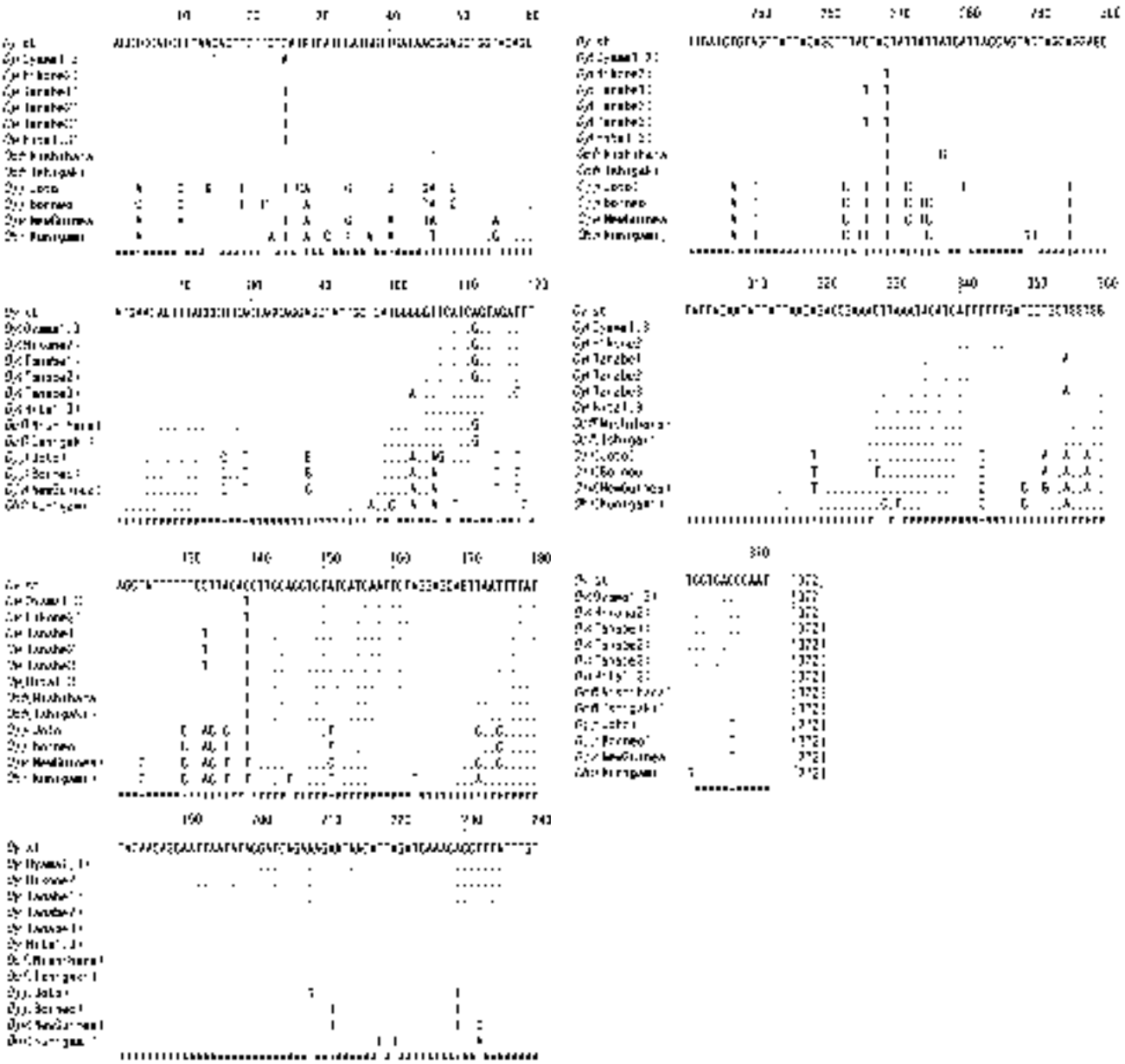

Fig. 3. Nucleotide sequences of a 372-bp region of the COI gene. Dotes indicate the bases identical with those of the first sequence (*Oy*-st). Asterisk indicates bases identical to all the species ; cross indicates parsimony informative sites. *Oy* : *O. yezoensis*, *Ocf* : *O. chinensis formosana*, *Ojj* : *O. japonica japonica*, *Ojv* : *O. japonica vitticollis*, *Ohi* : *O. hyla intricata*. The sequence of *Oy*-st was observed in all populations except Oyama.

Results

COI sequences and variation

We collected 372bp of sequence data from COI in all individuals. Figure 3 shows the aligned nucleotide sequences. There were no insertions or deletions in this region. Considering all the species, 73 sites were variable in the 372-bp region, of which 44 were parsimony-informative. The sequence divergences are shown in Table 1. Intraspecific variation ranged from 0.00 to 4.75% and interspecific variation from 0.00 to 14.53%.

In *O. yezoensis*, the same sequence was obtained from all but the Tanabe population, and there were no substitutions in the four northern populations including the Kikonai, Hirosaki, Tashirotai and Sado populations.

Table 1. KIMURA (1980)'s two-parameter distance for COI gene

species[a] (strains)	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.
1. *Oy*-St	1.08	0.81	1.91	1.35	2.18	0.81	1.35	0.81	13.13	11.81	13.21	13.55
2. *Oy* (Oyama 1, 3)		0.81	1.63	1.08	2.46	1.08	1.36	0.81	13.48	12.47	13.55	13.91
3. *Oy* (Hikone 2)			1.08	0.54	1.91	0.54	0.54	0.00	12.81	11.50	12.88	13.23
4. *Oy* (Tanabe 1)				0.54	1.81	1.08	1.63	1.08	11.83	11.16	11.90	12.24
5. *Oy* (Tanabe 2)					1.36	0.54	1.08	0.54	12.49	11.81	12.56	12.91
6. *Oy* (Tanabe 3)						1.36	2.46	1.91	10.89	10.23	10.95	11.92
7. *Oy* (Hita 1, 3)							1.08	0.54	12.17	11.50	12.24	12.59
8. *Ocf* (Nishihara)								0.54	13.13	11.81	13.21	13.23
9. *Ocf* (Ishigaki)									12.81	11.50	12.88	13.23
10. *Ojj* (Joto)										4.75	5.90	14.53
11. *Ojj* (Borneo)											4.73	14.26
12. *Ojv* (New Guinea)												11.97
13. *Ohi* (Kunigami)												

[a] *Oy* : *O. yezoensis*, *Ocf* : *O. chinensis formosana*, *Ojj* : *O. japonica japonica*, *Ojv* : *O. japonica vitticollis*, *Ohi* : *O. hyla intricata*. The sequence of *Oy*-st was observed in all populations except Oyama.

Table 2. Frequency of nucleotide substitution (%) among 13 sequences

codon position	Transition		Transversion				Substitution rate
	T/C	A/G	A/T	A/C	T/G	G/G	
1st position	85.28	4.94	0.00	4.84	4.94	0.00	17.81
2nd position	43.94	19.45	36.38	0.00	0.23	0.00	4.11
3rd position	30.98	12.89	45.04	5.42	4.14	1.53	78.08
Overall	39.64	11.95	37.89	5.23	4.08	1.21	100.00

Table 3. Average base composition (%) of the COI fragment

codon position	A		C		G		T	
	Mean	Min. −Max.	Mean	Min. −Max.	Mean	Min. −Max.	Mean	Min. −Max.
1st position	24.19	23.39 −25.00	18.32	16.94 −19.35	32.49	33.87 −32.26	25.00	23.39 −26.61
2nd position	12.56	12.10 −12.90	35.60	35.48 −36.29	13.82	13.71 −14.52	38.02	37.10 −38.71
3rd position	51.50	50.00 −52.42	8.29	5.65 −10.48	3.23	1.61 −4.84	36.98	33.06 −39.52
Overall	29.42	28.76 −30.11	20.74	19.35 −21.77	16.51	15.86 −17.74	33.33	31.99 −34.68

Modes of substitution

Most substitutions in the region of COI were found at the third codon (Fig. 3). Considering all the species, the position variance at the third codon was 78.08%. On the other hand, the corresponding values for the first and second positions were 17.81 and 4.11%, respectively (Table 2). As in mtDNA of many other insects (2, 9, 13), a high A+T content was observed in COI fragments of the *Oxya* species. This tendency was obvious in the third position of codons in which 88.48% of nucleotides were A or T. The first and second positions showed a relatively low A+T content of 49.19 and 50.58%, respectively (Table 3). The frequency of different types of nucleotide substitutions in the first codon position was quite different : a strong T-C transition bias was observed, even though nucleotides in this position did not show highly biased contents (Table 2). Among the nucleotide substitutions detected at this position, 76.92% were silent substitutions.

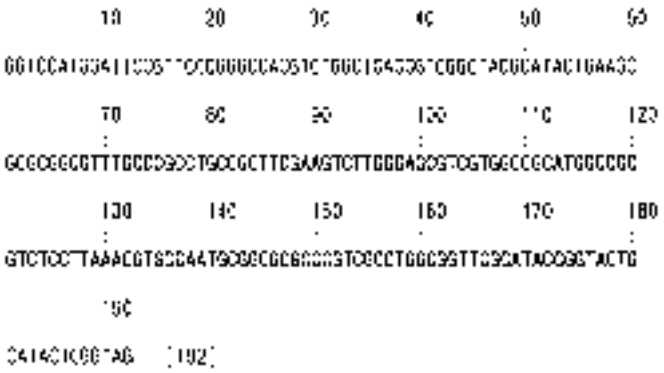

Fig. 4. Nucleotide sequences of ITS2 and flanking 5.8S and 28S regions in *O. yezoensis* and *O. chinensis formosana*. The sequences were identical in the two species.

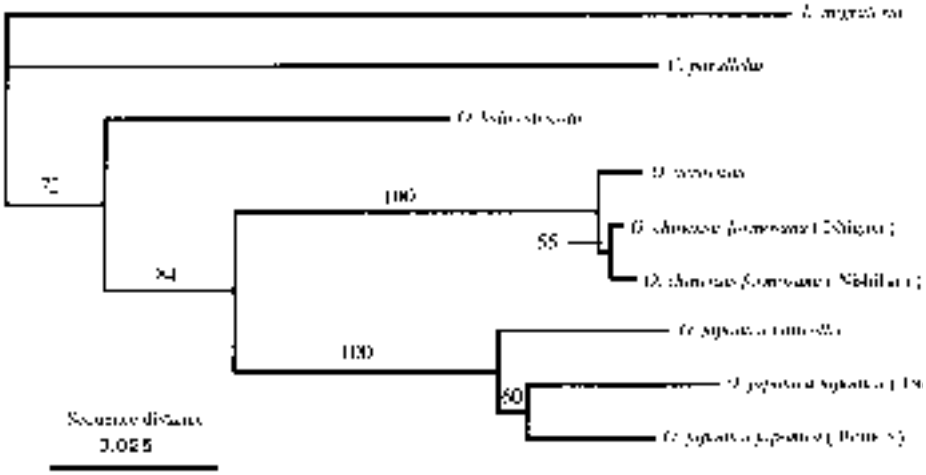

Fig. 5. Phylogenetic relationships estimated with neighbor joining (NJ) analysis method. Bootstrap values are shown above the branches (1000 replicates).

ITS2 sequences

We collected 192bp of sequence data from ITS2 in seven groups of *O. yezoensis* and two groups of *O. chinensis formosana* ; the population of *O. yezoensis* in Tashirotai was excluded because the data were not clear. All individuals had the same sequence ; there were no insertions, deletions or substitutions (Fig. 4).

Phylogenetic analysis

The molecular phylogenetic trees were constructed by the MP, NJ and ML methods. The phylogenetic relationships were estimated without considering the geographic variation of *O. yezoensis* ; the sequences of *O. yezoensis* had seven patterns but only differed in up to nine nucleotides. The results showed that *O. yezoensis* and *O. chinensis formosana* were monophyletic (Fig. 5). Their relationships were supported by bootstrap analyses (the bootstrap value was 100 in all the methods). The *O. japonica* groups belonged to the same linage, and *O. hyla intricata* was the most distantly related to the others. The phylogenetic trees estimated with three methods were basically the same.

Discussion

In the 372-bp region of COI sequence, there were 0.00—2.46% of sequence divergence between *O. yezoensis* and *O. chinensis formosana*, whereas *O. yezoensis* had up to 2.46% of intraspecific sequence divergence ; interspecific divergence of the two species was as match as intraspecific variation of *O. yezoensis*. Although some morphological differences are noticed, these species can not be separated by COI sequence data. We presume that these two species have diverged from a common ancestor so recently that the nucleotide substitutions did not reflect the morphological differences. The molecular data from COI sequences suggest that *O. yezoensis* and *O. chinensis formosana* are genetically close to each other. These two species are isolated geographically (4), but not reproductively (ANDO and ZHU, unpublished data). The phylogenetic trees estimated in this article agreed with the results of interspecific crosses (ANDO and ZHU, unpublished data).

By the sequence of ITS2, separation of *O. yezoensis* from *O. chinensis formosana*, or local populations of *O. yezoensis* is not possible. The length of ITS2 sequence in these *Oxya* species is about 200bp. This is nearly half the length of that in *Drosophila* (15). It has been suggested that ITS2 sequences in some dipterans and hymenopterans have changed relatively rapidly and may be useful for phylogenetic studies (10). ITS2 sequence for closely related *Oxya* species is well conserved, and is not useful to separate these species or their local populations in Japan. Although this region may prove useful in comparisons at the genus or subfamily level, but its usefulness in *Oxya* remains to be questioned.

The *O. japonica* groups belong to the same linage. There were 17-21bp of nucleotide substitutions (4.73—5.90%) among these groups. Considering that the sequence distances among the *O. japonica* groups did not range so widely (Table 1), these groups may have dispersed at a certain period. More detail studies are necessary to solve the problem of the dispersal and differentiation of *O. japonica*.

The male genitalia of *O. hyla intricata* is short, stubby and weakly sclerotised (7, 5), whereas that of the other species is long and strongly sclerotised ; especially in *O. yezoensis*, it is thick and robust. This character in *O. hyla intricata* is unique among genus *Oxya*. The molecular phylogenetic trees agree with a hypothesis based on this morphological observation : the evolutionary direction in the genitalia is to sclerotise harder and to increase in size. Following this hypothesis, it appears that *O. hyla intricata* is the most ancestral species and *O. yezoensis* is the most recently derived species.

There remain some questions about the distribution of *O. japonica* and the relationships among closely related taxa and local populations. More suitable molecular markers than those used in the present study will be necessary to answer to these questions. Further studies along this line will help clarify the phylogenetic relationships within the genus *Oxya* and may facilitate the ecological studies of *Oxya* species and local populations in Japan.

Summary

Four rice grasshopper species belonging to the genus *Oxya* are common in Japan : *O. yezoensis*, *O. chinensis formosana*, *O. japonica japonica*, and *O. hyla intricata*. In this study, nucleotide sequences of a 372-bp region of the cytochrome oxidase subunit I (COI) gene in mtDNA were used for constructing the molecular phylogenetic trees of four Japanese *Oxya* species and foreign *O. japonica* species. The second internal transcribed spacer (ITS2) in nuclear rDNA was also sequenced to separate two closely related species *O. yezoensis* and *O. chinensis formosana*. The results indicated that these two species were closely related to each other, and *O. japonica* and *O. hyla intricata* were distantly related to them. ITS2 sequences in *O. yezoensis* and *O. chinensis formosana* were identical. These two species are related so closely that it was not possible to separate them using the sequences of COI and ITS2. The molecular phylogenetic trees constructed were consistent with information on the morphology and reproductive compativility in those species.

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イナゴ属4種の分子系統関係

相川　康慶・安藤　喜一・城田　安幸

環境生物学講座

日本に生息するイナゴは主にハネナガイナゴ，タイワンハネナガイナゴ，コバネイナゴ及びコイナゴの4種がある。本研究では主として日本産イナゴ属4種の，ミトコンドリアDNAにあるCOI遺伝子の一部分372塩基の配列をもとに系統解析を行った。また核のrDNA中に存在するスペーサー領域ITS2の塩基配列データをもとに，特に近縁だと考えられるコバネイナゴとタイワンハネナガイナゴの種間及び地方個体群間の比較を試みた。得られた系統樹からタイワンハネナガイナゴとコバネイナゴは遺伝的に近縁であり，ハネナガイナゴ及びコイナゴは遠いことが明らかになった。ITS2の塩基配列は192塩基を比較したところ，コバネイナゴとタイワンハネナガイナゴの全個体で完全に一致した。

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