Eur. J. Entomol. **105**: 545–552, 2008
http://www.eje.cz/scripts/viewabstract.php?abstract=1367
ISSN 1210-5759 (print), 1802-8829 (online)

Limited variation in mitochondrial DNA of maize-associated *Ostrinia nubilalis* (Lepidoptera: Crambidae) in Russia, Turkey and Slovenia

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Key words. *Ostrinia*, mtDNA, COII, geographical variation, phylogeography

Abstract. A sequence analysis of the mitochondrial cytochrome oxidase subunit II (COII) gene in Russian and Turkish maize-associated populations of *Ostrinia nubilalis* and a Slovenian population of *O. nubilalis* probably infesting maize revealed little diversity. This lack of diversity may have resulted from bottleneck event(s) when the maize-associated population of *O. nubilalis* expanded from small population(s) in association with the cultivation of maize in Europe ca. 500 years ago. In the genealogy of COII genes obtained in the present and previous studies, Eurasian samples were substantially differentiated from North American samples. Since the North American population of *O. nubilalis* came from Europe, our finding suggests that there is geographical differentiation in European maize-associated *O. nubilalis*, and that maize-associated populations of *O. nubilalis* expanded multiple times in Europe. Finally, a phylogenetic analysis of the COII gene did not support that *O. nubilalis* and *O. furnacalis* are the closest relatives within the *O. furnacalis* species group.

INTRODUCTION

The European corn borer, *Ostrinia nubilalis* (Hübner), is one of the most serious pests of maize plants in the northern hemisphere (Showers, 1993). The native habitat of *O. nubilalis* is Europe, North Africa, and Western and Central Asia, but *O. nubilalis* was accidentally introduced into North America at the turn of the 20th century (Showers, 1993).

O. nubilalis is treated, in most cases, as a single extremely polyphagous species: > 200 plant species have been recorded as larval hosts (Ponsard et al., 2004, and references therein). However, it is suspected that the nominal species *O. nubilalis* includes different populations adapted to particular host plants (Frolov, 1998). Recent studies have revealed that, at least in France, *O. nubilalis* comprises two sympatric host-associated species: a maize- and mugwort-associated species. The mugwort-associated species infests mostly mugwort and hop, while the maize-associated species infests mostly maize, and occasionally other plants such as sunflower, cocklebur, bird pepper, and sorghum (Leniaud et al., 2006). These two host-differentiated species are genetically differentiated from each other (Bourguet et al., 2000; Martel et al., 2003; Leniaud et al., 2006), and show assortative mating in the field and in cages (Malausa et al., 2005; Bethenod et al., 2005). The mechanisms resulting in reproductive isolation include differences in the female sex pheromone blend (Thomas et al., 2003; Pélozuelo et al., 2004; Bontemps et al., 2004; Leniaud et al., 2006), an unidentified factor that works at close-range in mate discrimination (Pélozuelo et al., 2007), time of year of adult emergence (Thomas et al., 2003; but see also Malausa et al., 2005) and choice of host by ovipositing females (Bethenod et al., 2005). Also, in the European part of the former Soviet Union, the mating of maize- and mugwort/hemp-associated populations of *O. nubilalis* is generally less successful than the mating success within each population (Frolov, 1998). Moreover, the host-associated populations show several ecological distinctions (Frolov, 1998; Frolov et al., 2007). Thus, the maize-associated *O. nubilalis* is likely to have species status in much of Europe. Interestingly, the *Ostrinia* populations associated with plants other than maize in France and the European part of the former Soviet Union are not always the same (Frolov, 1998; Frolov et al., 2007). The mugwort-associated species in France is morphologically indistinguishable from the maize-associated species of *O. nubilalis*. On the other hand, in several regions of the former Soviet Union, borers collected from hop/hemp are morphologically identified as *O. scapulalis* or *O. narynensis*. Frolov et al. (2007) argue that the mugwort/hop/hemp-associated *O. nubilalis*, *O. narynensis* and *O. scapulalis* in Europe, and *O. orientalis* in

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the Far East represent a biological species, but this argument is not yet unanimously accepted.

The maize-associated *O. nubilalis* would have colonized maize plants after the introduction of the crop into Europe ca. 500 years ago (for a review, Rebourg et al., 2003). Damage to maize by *O. nubilalis* in Europe was first recorded in Russia and France in 1879, namely >350 years after the introduction of maize into Europe (Keppen, 1883; Robin & Laboulbène, 1884). The maize-associated *O. nubilalis* may have already been present prior to the introduction of maize, utilizing plants other than maize as the larval host. Alternatively, it may have evolved from a preexisting *O. nubilalis* population after maize cultivation began in Europe. Malausa et al. (2007) suggested that the latter hypothesis is not very likely based on a population genetic analysis of the variation in four nuclear genes. In either case, it is likely that the population of maize-associated *O. nubilalis* increased rapidly in size and geographical range as maize cultivation spread in Europe. It is not clear whether this expansion started in a single or multiple geographical region(s).

When a population expands from a small size, it may develop several population genetic characteristics, particularly in animal mitochondrial DNA (mtDNA), such as little molecular diversity, deviations from the neutral equilibrium state of molecular evolution, a star-like pattern in gene genealogy, and an excess of rare sequence types (Tajima, 1989; Slatkin & Hudson, 1991; Fu, 1997; Avise, 2000). Such patterns are evidence of a population expansion, and except for the limited molecular diversity, can be clearly detected only when a population accumulates enough mutations in mtDNA after the onset of population expansion. Therefore, the maize-associated *O. nubilalis* may show a low level of mtDNA diversity, but not clear evidence of population expansion, such as a star-like genealogy and excess of rare sequence types, since the supposed expansion of the maize-associated *O. nubilalis* population occurred < 500 years ago, which is probably too recent for a significant accumulation of mutations in mtDNA (cf. Vilà et al., 1999).

There are few population genetic studies on mtDNA of the European populations of *O. nubilalis*. Martel et al. (2003) examined mtDNA variation within and between the maize- and mugwort-associated species in France by conducting a restriction fragment length polymorphism (RFLP) analysis. They found that one haplotype was predominant at all localities sampled. Keszthelyi & Ács (2005) examined mtDNA variation in Hungarian and Egyptian samples of maize-associated *O. nubilalis*, by performing a single-strand conformation polymorphism analysis and did not find any mtDNA polymorphisms. This lack of diversity in mtDNA may be because the population of maize-associated *O. nubilalis* expanded from a small size, which would be supported if the limited diversity of mtDNA in the maize-associated *O. nubilalis* also applies to a wider region of Europe. It is not clear whether there is geographical differentiation in the maize-associated species of *O. nubilalis* in Europe. For the North American population of *O. nubilalis*, Coates et al. (2004) examined the cytochrome oxidase subunit I (COI) and COII genes of mtDNA in 15 individuals and found 15 haplotypes. They also examined the COI-COII region of mtDNA in 1414 individuals using PCR-RFLP, and found significant differences in the haplotype frequency between the Atlantic coast and Midwestern regions as well as between sympatric univoltine and bivoltine populations in Minnesota.

O. nubilalis is a member of the *O. furnacalis* species group in which Mutuura & Munroe (1970) list ten species. The ten species are morphologically very similar to each other, and their phylogeny is uncertain. In this species group, larvae of *O. nubilalis* and *O. furnacalis* use maize as a major host plant. These two species are often compared in physiological/biochemical studies (e.g. Roelofs et al., 2002; Linn et al., 2007). However, it is unknown whether these two species are the closest relatives in this species group. Kim et al. (1999) examined single sequences of the COII gene in each of six species of the *O. furnacalis* species group. They found that *O. nubilalis* is more closely related to *O. orientalis* and *O. scapulalis* than to *O. furnacalis*, but it is necessary to examine a larger number of sequences per species to be certain.

In the present study, the mitochondrial COII gene of Russian and Turkish feral samples of *O. nubilalis* infesting maize, Slovenian feral samples for which the host plant was not specified and samples from two laboratory populations of maize-associated *O. nubilalis* (France and Slovenia) was sequenced. Using these COII gene sequences and those of previous studies, the geographical distribution and genealogy of COII gene haplotypes in *O. nubilalis* were examined. Phylogenetic relationships of *O. nubilalis* with other *Ostrinia* species were preliminarily examined using the COII gene, with particular emphasis on the relationship between *O. nubilalis* and *O. furnacalis*.

MATERIAL AND METHODS

Natural populations of maize-associated *O. nubilalis* were sampled at Krasnodar, southwestern Russia and Adana, southern Turkey. Larvae were collected from stems of maize. The larvae from Russia were reared to adulthood in the laboratory and DNA extracted from ovaries (n = 22). The ovaries were used because they were also used for surveying infections with *Wolbachia,* which is found in male testes and female ovaries (see below). The larvae from Turkey were preserved in ethanol until used for DNA extraction (n = 7). Moths of French and Slovenian laboratory populations of maize-associated *O. nubilalis* were obtained (n = 2 for Bordeaux, France; n = 9 for Bjlje, Primorska region, Slovenia) and preserved in ethanol until used for DNA extraction.

Feral moths of *O. nubilalis* were caught by sweep netting or using a light trap at Adana, Turkey (n = 3) and four localities in Slovenia (n = 6 at Dobrovnik, Prekmurje region; n = 11 at Crni log, Prekmurje region; n = 1 at Tosko Celo, central Slovenia; and n = 3 at Ozeljan, Primorska region), and preserved in ethanol until used for DNA extraction. At Adana, Dobrovnik and Ozeljan, the moths were collected in a maize field. At Tosko Celo and Crni log, the moths were collected in a forest. The larval host plants of these field-collected moths were not specified.

TABLE 1. COII sequences used in the study. n – number of DNA sequences. * – these sequences are shorter than the others.

Species	Locality	n	Accession No.	Reference
O. nubilalis	Russia/Turkey/Slovenia/France	64	AB121300-11, AB126394-445	Present study
	France	1	EU219734	—
	China	1	EU070917	—
	USA	1	AB029540	Kim et al. (1999)
	USA	1	AF321880	—
	USA	1	AF442957	Coates et al. (2005)
	USA	14	not deposited	Coates et al. (2004)
O. furnacalis	Japan/China/Philippines	165	AB121251-72, AB127196-285, AB127287-339	Hoshizaki et al. (2008)
	Japan	1	AB029538	Kim et al. (1999)
	China	4	EF626670-3	—
	Vietnam	5	AJ560785-9*	—
O. orientalis	Japan	27	AB121273-99	Present study
	Japan	1	AB029539	Kim et al. (1999)
	China	1	EF622418	—
O. scapulalis	Japan	1	AB029541	Kim et al. (1999)
	China	1	EF622419	—
O. kurentzovi	China	1	EF622420	—
O. narynensis	China	1	EU070916	—
O. zaguliaevi	Japan	1	AB029542	Kim et al. (1999)
O. zealis	Japan	1	AB029543	Kim et al. (1999)
	China	1	EU070915	—
O. palustralis	Japan	1	AB029545	Kim et al. (1999)
	Japan	1	AB185108	Ohno et al. (2006)
O. latipennis	Japan	1	AB029544	Kim et al. (1999), Ohno et al. (2006)
	China	1	EU070914	—
O. ovalipennis	Japan	2	AB185106-7	Ohno et al. (2006)

O. orientalis inhabits Eastern Asia. This species is very closely related to *O. nubilalis* (Mutuura & Munroe, 1970; Ishikawa et al., 1999; Kim et al., 1999; Fu et al., 2004), but does not use maize as the larval host (Ishikawa et al., 1999). We used *O. orientalis* samples (n = 28, including one sample used in Kim et al., 1999) collected at seven localities in Japan (Hokkaido, Niigata, Chiba, Nagano, Kyoto, Wakayama and Tokushima). Frolov et al. (2007) revised the taxonomy of *O. nubilalis* and its relatives: *O. orientalis*, *O. narynensis*, and the mugwort-associated *O. nubilalis* are synonymized with *O. scapulalis*. However, in the present paper "*O. nubilalis*" and "*O. orientalis*" are used in the classical sense for ease of description.

Genomic DNA was extracted either from whole insects, or the legs or female ovaries of single insects, following the method of Milligan (1992) with slight modifications. Single whole bodies were ground with a mixture of 300 ml of 2 × CTAB buffer [100 mM Tris–HCl, pH 8.0; 1.4 M NaCl; 20 mM EDTA; 2% hexadecyltrimethylammonium bromide (CTAB)], 1 µl of 2-mercaptoethanol, 0.75 µl of RNase A (10 mg/ml) and 1.5 µl of proteinase K (20 mg/ml). When DNA was extracted from legs or ovaries, volumes of the solutions were reduced to 1/3 or 1/6, respectively. The homogenates were incubated at 55°C for two hours, and extracted sequentially with chloroform, phenol/chloroform (1 : 1), and chloroform. The DNA pellet obtained by isopropanol precipitation was dissolved in 200 µl of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA).

An mtDNA region spanning the COII gene was amplified using the polymerase chain reaction (PCR) and *Taq* DNA polymerase (Sawady Technology, Tokyo, Japan). The thermal profile of the PCR was 94°C for 5 min, 32 cycles of 94°C for 36 s, 47°C for 1 min, and 70°C for 2 min, and finally 70°C for 7 min. For the amplification, a forward primer O-tLEU (Kim et al., 1999; 5'-TAGTGCAATGGATTTAAACC-3') and an external reverse primer B-tLYS (Liu & Beckenbach, 1992; 5'-GTTTAAGAGACCAGTACTTG-3') were used. The PCR products were purified from agarose gels using a Gene Clean III kit (BIO101, La Jolla, CA, USA) and directly sequenced using a Dye Terminator Sequencing FS Ready Reaction kit or BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Biosystems, Foster City, CA, USA). The nucleotide sequences were determined in both directions, using four primers: O-tLEU, B-tLYS, an internal forward primer COII-L3 (5'-GATATTGAAGTTACGAATATTCAG-3') and an internal reverse primer COII-R3 (5'-CATTTATAGGGGTTATATAAG AATC-3'). The sequence products were analyzed on an automated sequencer (ABI prism 377, PE Biosystems).

Nucleotide sequences of the COII gene of *O. nubilalis* and the other 10 *Ostrinia* species were obtained from the literature and DDBJ/GENBANK/EMBL (Table 1). Sequences were aligned using Clustal W (Thompson et al., 1994). Aligned sequences were compiled into haplotypes using MacClade 3.05 (Maddison & Maddison, 1992). A parsimony network (Templeton et al., 1992) of COII haplotypes in *O. nubilalis* was constructed using TCS 1.13 (Clement et al., 2000). In the network construction, parsimonious connections with 95% confidence were statistically verified for haplotypes that differed by up to 11 mutations. We estimated the phylogenetic relationship of haplotypes within and among *Ostrinia* species in two ways. First, pairwise sequence divergences between haplotypes were estimated with Kimura's two-parameter model, and then the neighbour-joining

Haplotype	3333333333333333 0012224444455555 56 5620342567724455 92 0437739324784645 71	Reference
A	GCATATAAAAAAGGTATG	Present study
B	.............A...A	Present study
C	.............A....	Present study Kim et al. (1999) EU070917
D	.......G.....A....	Present study
E	.T.............C.	EU219734
F	...ATA......AA....	Coates et al. (2004)
G	C..ATA.......AG...	AF442957 AF321880
H	...ATAC.C....A....	Coates et al. (2004)
I	...ATA.....TAA....	Coates et al. (2004)
J	C..ATA......AA....	Coates et al. (2004)
K	...ATA......AAG...	Coates et al. (2004)
L	...ATA....T..A....	Coates et al. (2004)
M	...ATA...T...A.T..	Coates et al. (2004)
N	..GATA.TTTGTAA.T..	Coates et al. (2004)

Fig. 1. Variable sites of the mitochondrial COII gene in *O. nubilalis*. Identity with the top sequence is denoted by a dot. The nucleotide position number refers to the COII gene in *Drosophila yakuba* (Clary & Wolstenholme, 1985).

tree was constructed using PHYLIP 3.67 (Felsenstein, 2004). A bootstrap test was performed with 1000 resamplings for the evaluation of branch support. Second, we used MODELTEST 3.7 (Posada & Crandall, 1998) to determine the model of the molecular evolution. The Akaike information criterion was chosen, and the data were best represented by the HKY + I + G model (freqA = 0.3899, freqC = 0.1213, freqG = 0.0922, freqT = 0.3965; Ti/tv ratio = 3.0521; Proportion of invariable sites = 0.7036; Gamma distribution shape parameter = 0.7008). With these parameter values, we used TREEFINDER (Jobb et al., 2004) to construct a maximum likelihood (ML) phylogeny of haplotypes. A bootstrap test was performed with 100 resamplings for the evaluation of branch support.

The distribution of COII haplotypes among groups of *O. nubilalis* samples was examined by using an exact test of population differentiation (Raymond & Rousset, 1995), which was implemented by Arlequin 3.1 (Excoffier et al., 2005), with 200,000 steps in the Markov chain. Tajima's *D* statistic was used to test the neutrality of evolution of the COII sequence (Tajima, 1989). Fu's F_s statistic was calculated: large negative values of F_s indicate an excess of recent mutations and reject a historical stasis in population size (Fu, 1997). These population genetic calculations were performed using DnaSP ver. 3.53 (Rozas & Rozas, 1999).

Parasitic bacteria of the genus *Wolbachia* modify the reproduction of arthropod hosts in various ways, and consequently a *Wolbachia* infection may affect the mtDNA polymorphism of a host (Johnstone & Hurst, 1996; Ballard, 2000). *Wolbachia* has not been found to infect *O. nubilalis* (Kageyama et al., 2004), but does infect two closely related species, *O. furnacalis* and *O. scapulalis*, causing the production of all-female progeny by male-killing (Sakamoto et al., 2007; see also Kageyama & Traut, 2004). In the present study, to avoid confusion caused by the effects of *Wolbachia* on mtDNA, we confirmed using a diagnostic PCR assay that all the samples used were uninfected with *Wolbachia* (Kageyama et al., 2004; S. Hoshizaki, pers. observ.). As a positive control in the PCR assay, we used DNA samples of *O. furnacalis* and *O. scapulalis* that were infected with *Wolbachia* from our previous studies (Kageyama et al., 2002, 2003).

TABLE 2. Distribution of COII haplotypes from *O. nubilalis* in Russia (RUS), Turkey (TUR), Slovenia (SVN), and France (FRA). For samples whose host plants are unspecified, m and f indicate moths collected in maize fields and forests, respectively. SV-m includes the Dobrovnik and Ozeljan samples, and SV-f includes Tosko Celo and Crni log samples.

Haplotype	Host: maize				Host: unspecified		
	RUS	TUR	SVN	FRA	TUR-m	SVN-m	SVN-f
A	4	–	–	–	–	1	1
B	1	2	–	–	–	1	–
C	17	5	9	2	3	6	11
D	–	–	–	–	–	1	–

RESULTS

There were only four haplotypes (haps. A–D; Fig. 1) of COII genes (682 bp) from 64 individuals of *O. nubilalis* from Russia, Turkey and Slovenia (DDBJ/GENBANK/EMBL accession numbers AB121300–AB121311, AB126394–AB126445). These haplotypes were compared with other *O. nubilalis* COII sequences available in the literature and DDBJ/GENBANK/EMBL database (Table 1). A total of 15 COII haplotypes were found in *O. nubilalis* (Fig. 1).

The frequency distributions of haplotypes in Russian and Turkish natural populations of *O. nubilalis* infesting maize were similar: one particular haplotype, hap. C, was predominant (Table 2). The haplotype frequencies in these populations were not significantly different ($p = 0.113 \pm 0.001$). Only hap. C was found in the two laboratory populations (France and Slovenia) of maize-associated *O. nubilalis.* The absence of polymorphisms may be due to the small sample sizes and/or laboratory maintenance of populations. No significant difference in haplotype frequency was found between the two groups of moths collected in different habitats (maize field and forest) in Slovenia ($p = 0.567 \pm 0.002$).

Phylogenetic relationships among the 15 haplotypes of *O. nubilalis* COII sequences were estimated using TCS analysis (Fig. 2). In this network, the COII sequences from Eurasian samples and those from North American samples were substantially differentiated. The Eurasian and North American samples did not share haplotypes other than hap. C.

Population genetic evidence for a population expansion was looked for in *O. nubilalis*. The TCS network of haplotypes in *O. nubilalis* (Fig. 2) as a whole, or its European part, did not show a star-like pattern, which is characteristic of a population expansion event in the past. Tajima's *D* value for the natural population of maize-associated insects (Russian and Turkish samples were pooled; $n = 29$, $D = -0.288$, $p > 0.10$) did not indicate a significant departure from neutral molecular evolution. Values of F_s also did not provide evidence for an excess of rare haplotypes in Russian and Turkish *O. nubilalis* infesting maize ($n = 29$, $F_s = -0.214$, $p > 0.10$). Thus, there was no evidence for a rapid population expansion in the maize-associated *O. nubilalis*.

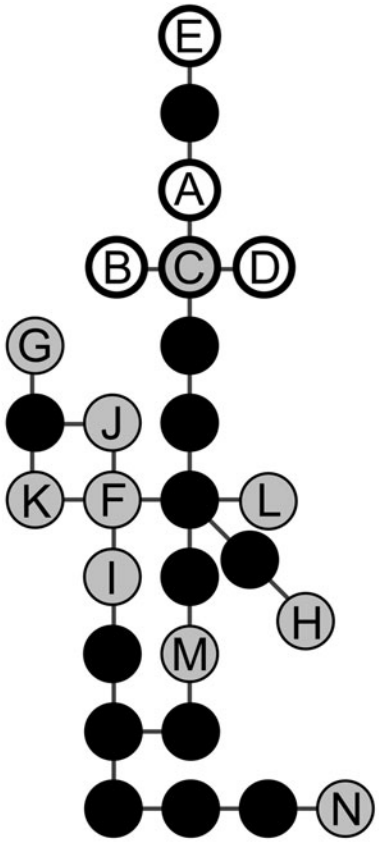

Fig. 2. A parsimonious network illustrating the phylogenetic relationships among COII gene haplotypes in *O. nubilalis*. Black circles indicate hypothetical haplotypes that were not found in the present study. Circles with a thick line indicate haplotypes found in Eurasian samples and grey circles indicate those found in samples from North America.

The COII sequences representing 11 *Ostrinia* species were compiled into 69 haplotypes. In this comparison, 667 nucleotides portion shared by all the sequences was used because five sequences (AJ560785-9) were shorter (667 bases) than the others (682 bases). Their phylogenetic relationships were estimated using NJ (not shown) and ML (Fig. 3) trees. In both trees, the *O. furnacalis* species group (*O. furnacalis*, *O. nubilalis*, *O. orientalis*, *O. scapulalis*, *O. narynensis*, *O. kurentzovi*, *O. zaguliaevi*, and *O. zealis*) formed a well-supported clade. The two trees showed generally similar patterns of topology and supports, and their major difference was in the position of *O. zaguliaevi*, which was positioned at the tip of a basal branch in the *O. furnacalis* group on the NJ tree, but positioned within the *O. furnacalis* clade on the ML tree. Within *O. nubilalis*, the North American haplotypes other than hap. C of *O. nubilalis* exclusively formed a well-supported clade on both trees. The Eurasian haplotypes of *O. nubilalis* were scattered together with those of other species outside the North American clade. Thus, the parsimony network and ML/NJ tree showed the tendency for phylogenetic separation of Eurasian and North American samples of *O. nubilalis.* In both trees, *O. nubilalis*, *O. orientalis*, *O. scapulalis*, *O. narynensis*, and *O. kurentzovi* formed a large clade, which was not strongly supported by the bootstrap test. Two maize-pest species, *O. furnacalis* and *O. nubilalis*, did not share clades. Haps. A and C of *O. nubilalis* were shared by *O. orientalis*.

DISCUSSION

In terms of the mitochondrial COII gene, the Russian and Turkish populations of maize-associated *O. nubilalis* similarly showed a low level of polymorphism. This is consistent with previous findings on mtDNA of maize-associated *O. nubilalis* in France, Hungary, and Egypt: little polymorphism was found (Martel et al., 2003; Keszthelyi & Ács 2005). Such small variation in mtDNA may be a consequence of population bottleneck event(s) when the maize-associated *O. nubilalis* population expanded in association with the cultivation of maize in Europe. In Russian and Turkish populations of *O. nubilalis* infesting maize, there was no population genetic evidence of the population expansion, as predicted from the very recent occurrence of population expansion event(s) in the maize-associated *O. nubilalis* (see Introduction).

The poor mtDNA diversity found in the maize-associated *O. nubilalis* in Turkey and Russia might be explained by an alternative hypothesis. In general, diversity can become drastically reduced by strong natural selection operating on the mtDNA or other maternally inherited genetic factors (referred to as the selective sweep; Johnstone & Hurst, 1996; Ballard, 2000). In particular, the rapid spread of a maternally-inherited factor in a host population can lead to the fixation of a single mtDNA variant, and a representative of such a genetic factor in insects is the parasitic bacterium, *Wolbachia* (Werren, 1997; Stouthamer et al., 1999). However, such a scenario mediated by *Wolbachia* infection can be disregarded since none of the *O. nubilalis* samples used in the present study were infected with *Wolbachia*.

A more interesting finding of the present study is the substantial divergence between North American and Eurasian COII sequences. This suggests geographical differentiation among some European populations of maize-associated *O. nubilalis* because the North American population of *O. nubilalis* was introduced from Europe (Showers, 1993). It also tempts us to speculate that the maize-associated *O. nubilalis* population expanded in multiple localities in its natural distribution range in association with the cultivation of maize. However, the present findings give little information on the geographical pattern of differentiation among local populations of European *O. nubilalis* associated with maize, due to the geographically limited range of the samples in the present study. The mtDNA polymorphism in the maize-associated *O. nubilalis* should be examined over a wider geographical area of its natural range.

In the maize-associated *O. nubilalis* in Europe, there are two races based on the female sex pheromone blend (Z and E races). The Z race occurs throughout Europe, with the two races apparently coexisting in Italy and Switzerland (Anglade et al., 1984). It would be interesting to compare the mtDNA variation between populations in southern Europe and other regions.

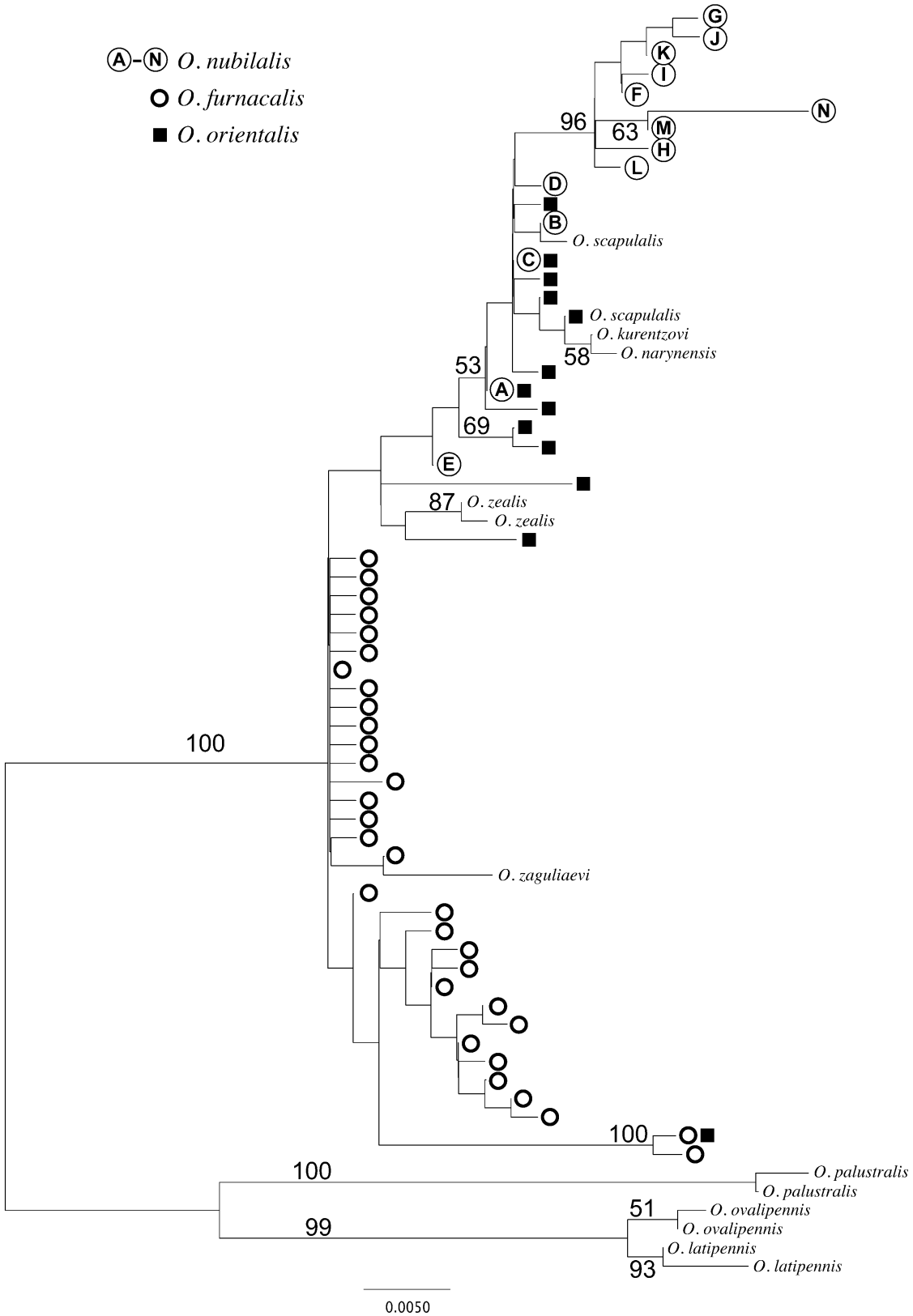

Fig. 3. A maximum likelihood tree of COII gene haplotypes in 11 species of *Ostrinia*. The bar indicates the genetic distance. Bootstrap support values greater than 50 (100 resamplings) are shown on branches.

In the phylogenetic tree of COII genes in 11 species of *Ostrinia*, *O. furnacalis* is monophyletic, while *O. nubilalis* formed a clade together with *O. orientalis*, *O. scapulalis*, *O. narynensis*, and *O. kurentzovi*. This does not support that *O. nubilalis* and *O. furnacalis* are the closest relatives in the *O. furnacalis* species group. These two species favour maize and other Poaceae plants as host for their larvae. However, if the two species are not the

closest relatives, it does not necessarily support that the similar host-preferences between the two species share the evolutionary origin. The closer relationship among *O. nubilalis*, *O. orientalis*, and *O. scapulalis* is consistent with the diversity of sex pheromone components among *Ostrinia* species. The sex pheromone of females of *O. nubilalis*, *O. orientalis*, and *O. scapulalis* has the same major components [(*E*)- and (*Z*)-11-tetradecenyl acetates], while that of *O. furnacalis* females has components [(*E*)- and (*Z*)-12-tetradecenyl acetates] unique in this genus (reviewed by Ishikawa et al., 1999). Indeed, in terms of its external morphology, *O. nubilalis* is indistinguishable from *O. orientalis* (Mutuura & Munroe, 1970).

However, the present results do not allow us to draw a conclusion about the species phylogeny in the genus *Ostrinia*, including which species is closest to *O. nubilalis*. First, neither *O. nubilalis*, *O. orientalis*, *O. scapulalis* nor *O. furnacalis* are monophyletic in the COII tree. Second, sampling density in the present analysis was biased to *O. nubilalis*, *O. furnacalis* and *O. orientalis*. More rigorous sampling of other species is required. Finally, the statistical support was weak for most internal branches of the COII tree. To obtain a more reliable phylogenetic estimate, it would be necessary to examine not only longer mitochondrial but also nuclear sequences.

Finally, we address the importance of a phylogeographic / phylogenetic perspective in ecological studies on *O. nubilalis*. In the present paper, we suggest that there may be a geographical differentiation in European maize-associated *O. nubilalis*. If so, it is relevant to the development of a management strategy for *O. nubilalis* in Europe. It may also be relevant to the study of speciation between the maize-associated and mugwort/hop-associated *O. nubilalis*. A better knowledge of the geographical pattern of differentiation in maize-associated *O. nubilalis* in Europe would help to extend the *O. nubilalis* speciation study done in France, which is reviewed in the Introduction.

ACKNOWLEDGEMENTS. This work was supported in part by Grants-in-Aid (Nos 12640675 and 13460021) from the Ministry of Education, Science, Sports and Culture, Japan. We thank T. Takanashi for help with rearing the insects. We also thank F. Marion-Poll for the French samples of *O. nubilalis* and for comments on the manuscript.

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Received October 23, 2007; revised and accepted July 11, 2008