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Origins and genetic diversity of the ragweed beetles, *Ophraella communa* (Coleoptera: Chrysomelidae), that were introduced into Italy and Japan based on an analysis of mitochondrial DNA sequence data

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Abstract. *Ophraella communa* (Coleoptera: Chrysomelidae) is an oligophagous herbivorous beetle that feeds on *Ambrosia artemisiifolia*. It is native to North America, but was accidentally introduced into Japan in 1995 and Europe in 2013. We analyzed partial DNA sequences of the mitochondrial cytochrome oxidase II gene for *O. communa* collected from 29 locations in the United States, Japan and Italy. Overall, the results of our analyses indicate that the introduced Japanese populations have lower genetic variation than the native populations. The sequences for the Italian specimens did not share haplotypes with Japanese specimens. These results indicate that the introduced Japanese populations originated from a single introduction, and that the Italian and Japanese populations have different origins.

INTRODUCTION

Inferring the introduction history from genetic markers is important in elucidating the patterns of colonization and expansion of introduced species and biocontrol agents (Estoup & Guillemaud, 2010). In some cases, the introduced populations have experienced bottlenecks and founding events. Consequently, they have a reduced genetic variation in their introduced ranges (Hufbauer et al., 2004; Sax et al., 2005). In contrast, if the introduced populations are derived from multiple native populations, then the genetic diversity can be higher than that of native populations (Allendorf & Lundquist, 2003; Kolbe et al., 2004; Sakai et al., 2001). High genetic diversity caused by multiple introductions presumably facilitates the adaptation of the introduced species to the exotic environment because the level of genetic variation strongly influences the degree and speed of evolutionary change (Barrett & Schluter, 2008). The resulting evolutionary change in the introduced species could amplify its effect on native ecosystems (Lavergne & Molofsky, 2007).

Ragweed leaf beetle, *Ophraella communa* LeSage, is an oligophagous species whose preferred host is ragweed, *Ambrosia artemisiifolia* L., on which it prefers to feed on the leaves. *Ambrosia artemisiifolia* is native to North America, but was recently (and accidentally) introduced into many countries in eastern Asia (Japan and Taiwan in 1996, Korea in 2000, mainland China in 2001; Shiyake & Moriya, 2005) and Europe (Boriani et al., 2013; Bosio et al., 2014; Müller-Schärer et al., 2014). In both its native and exotic ranges, *A. artemisiifolia* is a problematic weed (Ding et al., 2006), because of its high fecundity and production of large amounts of highly allergenic pollen (Bassett & Crompton 1975, 1982). *Ophraella communa* is known to be an effective biocontrol agent of introduced populations of *A. artemisiifolia* (Cao et al., 2011, Guo et al., 2011; Zhou et al., 2014).

O. slobodkini is a sibling species to *O. communa* and also feeds on the leaves of ragweed. *O. slobodkini* occurs in the southern United States (Florida), and these two species have allopatric distributions (Futuyma, 1991) and can be distinguished from one another genetically and morphologically, but the morphological differences are slight (Futuyma, 1991). Because introduced *O. communa* have not been investigated using genetic markers, it is possible that the sibling species, *O. slobodkini* has been introduced (cryptically) into Japan and Italy.

The main purpose of this study is to characterize the genetic structure of native *O. communa* populations in North American and introduced populations of *O. communa* and *O. slobodkini* in Japan and Italy, in order to infer the invasion history in the aforementioned introduced ranges, using genetic markers. We analyzed partial mitochondrial cytochrome oxidase II (COII) DNA sequences, rather than the more traditional COI sequences, because a previous study had shown that COI haplotypes of native *O. communa* showed no regional clustering pattern (Knowles et

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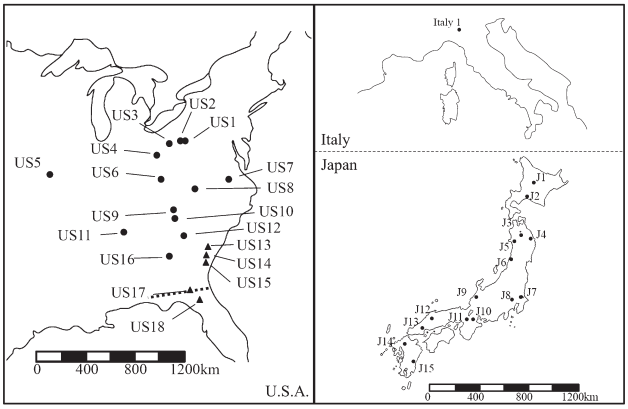

Native range Introduced range

Fig. 1. Maps showing locations in the native and introduced ranges at which samples were collected. Black circles denote sites where *Ophraella communa* were sampled. Black triangles denote sites where *Ophraella slobodkini* were sampled. Dashed line represents the Florida-Georgia border.

al., 1999). Specifically, we asked three questions: (1) How much genetic variation exists in Japanese, Italian and native *O. communa* populations? (2) Where are the possible source populations of introduced populations? (3) Has there been a cryptic introduction of the aforementioned regions by *Ophraella slobodkini*?

MATERIAL AND METHODS

Collection of samples

During 2008–2013, a total of 89 individuals of *Ophraella* were collected from 18 locations in the United States, 15 locations in Japan and the location of a recent introduction in Italy (Table 1, Fig. 1). The individuals were collected by sweeping and hand picking from *A. artemisiifolia* and *A. trifida* in the introduced range and from *A. artemisiifolia* in the native range (Fukano & Doi, 2013). We collected 1–6 individuals per population except for the Italian population (n = 13). These specimens were preserved in ethanol (99.5%) until analyzed in the laboratory at Tokyo University of Agriculture and Technology, Japan.

Table 1. Localities, year collected and host plants of *Ophraella communa* (US1–US12, US16, J1–J15 and Italy1) and *Ophraella slobodkini* (US13–US15, US17 and US18) that were examined using mitochondrial CO II DNA sequencing.

No.	Locality	Latitude	Longitude	Year collected	n	Host plant
	Native Area (USA)					
US1	Trafford, PA	N40.388	W79.775	2010	1	*Ambrosia artemisiifolia*
US2	Donegal, PA	N40.112	W79.398	2012	2	*Ambrosia artemisiifolia*
US3	Valley Grove, WV	N40.091	W80.536	2012	2	*Ambrosia artemisiifolia*
US4	Williamstown, WV	N39.390	W81.443	2012	1	*Ambrosia artemisiifolia*
US5	O'Fallon, MO	N38.791	W90.699	2010	1	*Ambrosia artemisiifolia*
US6	Cabin Creek, WV	N38.197	W81.507	2012	1	*Ambrosia artemisiifolia*
US7	Innsbrook, VA	N37.624	W77.598	2010	4	*Ambrosia artemisiifolia*
US8	Blue Redge, VA	N37.410	W79.798	2008	5	*Ambrosia artemisiifolia*
US9	Statesville, NC	N35.799	W80.828	2012	1	*Ambrosia artemisiifolia*
US10	Robbin Park, NC	N35.454	W80.885	2012	1	*Ambrosia artemisiifolia*
US11	Acworth, GA	N34.069	W84.676	2012	2	*Ambrosia artemisiifolia*
US12	Blue Ridge, VA	N34.307	W81.004	2012	1	*Ambrosia artemisiifolia*
US13	St. George, SC	N33.201	W80.578	2012	2	*Ambrosia artemisiifolia*
US14	Yamassee, SC	N32.631	W80.836	2012	1	*Ambrosia artemisiifolia*
US15	Savannah, GA	N32.072	W81.199	2012	3	*Ambrosia artemisiifolia*
US16	Forsyth, GA	N33.040	W83.943	2012	3	*Ambrosia artemisiifolia*
US17	Live Oak, FL	N30.323	W82.962	2010	1	*Ambrosia artemisiifolia*
US18	Millpond, FL	N29.681	W82.448	2010	5	*Ambrosia artemisiifolia*
	Introduced Area (Japan)					
J1	Asahikawa, Hokkaido	N43.769	E142.330	2009	1	*Ambrosia artemisiifolia*
J2	Tomakomai, Hokkaido	N42.665	E141.662	2009	2	*Ambrosia artemisiifolia*
J3	Hirosaki, Aomori	N40.599	E140.436	2009	1	*Ambrosia artemisiifolia*
J4	Hachinohe, Aomori	N40.516	E141.529	2009	1	*Ambrosia trifida*
J5	Minamiakita, Akita	N39.947	E140.159	2009	2	*Ambrosia artemisiifolia*
J6	Higashitagawa, Yamagata	N38.633	E140.012	2009	3	*Ambrosia artemisiifolia*
J7	Tsukuba, Ibaragi	N36.024	E140.106	2009	1	*Ambrosia artemisiifolia*
J8	Tachikawa, Tokyo	N35.689	E139.387	2009	2	*Ambrosia trifida*
J9	Kanazawa, Ishikawa	N36.569	E136.634	2011	3	*Ambrosia trifida*
J10	Tsuchiyama, Mie	N34.934	E136.288	2011	5	*Ambrosia artemisiifolia*
J11	Yawata, Kyoto	N34.887	E135.692	2011	2	*Ambrosia trifida*
J12	Izumo, Shimane	N35.335	E132.886	2011	2	*Ambrosia trifida*
J13	Yamagichi, Yamaguchi	N34.309	E131.584	2011	6	*Ambrosia artemisiifolia*
J14	Kasuya, Fukuoka	N33.623	E130.495	2011	5	*Ambrosia trifida*
J15	Miyakonojo, Miyazaki	N31.705	E131.094	2011	2	*Ambrosia trifida*
	Introduced Area (Italy)					
Italy1	Broni, Lombardia	N45.075	E9.275	2013	13	*Ambrosia artemisiifolia*

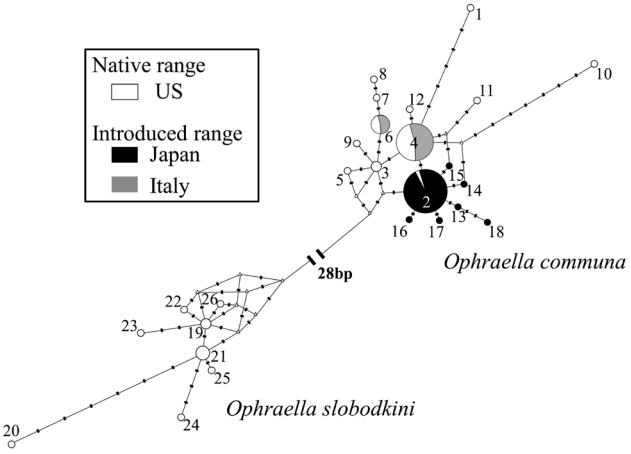

Fig. 2. Haplotype network for cytochrome c oxidase subunit (CO II) DNA sequences of *Ophraella communa* (right) and *Ophraella slobodkini* (left). The circle size is proportional to the number of individuals (1–33). Each line represents a single mutation. Empty triangles represent inferred non-sampled or extinct haplotypes.

DNA Sequencing

DNA was extracted from whole bodies of *O. communa* and *O. slobodkini* using a DNeasy blood & tissue kit (Qiagen Inc., Hilden, Germany). The COII region of the extracted DNA was amplified using polymerase chain reaction (PCR) with primer modified-A-tLeu (Simon et al., 1993; AATTTGGCAGATTATGCA) and C2-N-3661 (Simon et al., 1994; CCACAAATTTCTGAACATTGACCA). PCR was done using Takara Ex-Taq DNA polymerase (Takara Bio, Shiga, Japan) and a temperature profile of 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 2 min. In a previous study, COI sequences were obtained for *O. communa* and *O. slobodkini* (Funk et al., 1995). To identify the two species, we sequenced COI from the specimens collected in southern areas of the United States within the area where the ranges of *O. communa* and *O. slobodkini* overlap. The PCR conditions (e.g., temperature profile and primers) used for COI were the same as those used by Funk et al. (1995). DNA sequencing was performed using an ABI 3130 DNA sequencer (Applied Biosystems, Foster City, CA). The resulting DNA sequences were deposited in GenBank with accession numbers KJ683911–KJ683936.

Data analysis

DNA sequences were edited visually and manually using MEGA 5.0 (Tamura et al., 2011). Multiple alignment of COII sequences was performed using ClustalW (Thompson et al., 1994) and MEGA default parameters. The 369 base pairs of partial sequences from the COII region were used for further analyses. To examine relationships among haplotypes of individuals of *O. communa* from the United States, Japan and Italy, we constructed a haplotype network (using Network 4.6.0; Fluxus Technology Ltd.), followed by median joining (Bandelt et al., 1999). The median joining algorithm estimates genetic networks from the minimum number of nucleotide differences. To estimate the genetic

Table 2. List of haplotypes and GenBank Accession numbers (KJ683911–KJ683936) for all haplotypes sampled in this study. A total of 18 haplotypes were recorded for *Ophraella communa* (Hap1–18) and 8 (Hap18–26) for *O. slobodkini*. Population names in bold type were collected from *Ambrosia trifida* and those in normal type collected from *Ambrosia artemisiifolia.* Numbers in parentheses are sample sizes.

Haplotype no.	Sample size	Populations	Accession no.
Hap1	1	US2(1)	KJ683911
Hap2	33	US1 (1), J1 (1), J2 (2), J5 (1), J6 (2), J7 (1), **J8** (2), **J9** (2), J10 (4), **J11** (2), **J12** (2), J13 (6), J14 (5), J15 (2)	KJ683912
Hap3	2	US8 (1), US4(1)	KJ683913
Hap4	23	US2 (1), US3 (2), US7 (2), US8 (2), US9 (1), US10 (1), US16 (3), Italy1 (11)	KJ683914
Hap5	1	US6 (1)	KJ683915
Hap6	5	US5 (1), US8 (1), US12 (1), Italy1 (2)	KJ683916
Hap7	1	US11 (1)	KJ683917
Hap8	1	US11 (1)	KJ683918
Hap9	1	US8 (1)	KJ683919
Hap10	1	US7 (1)	KJ683920
Hap11	1	US7 (1)	KJ683921
Hap12	1	US8 (1)	KJ683922
Hap13	1	J10 (1)	KJ683923
Hap14	1	**J9** (1)	KJ683924
Hap15	1	**J4** (1)	KJ683925
Hap16	1	J3 (1)	KJ683926
Hap17	1	J6 (1)	KJ683927
Hap18	1	J5 (1)	KJ683928
Hap19	2	US13 (1), US18 (1)	KJ683929
Hap20	1	US13 (1)	KJ683930
Hap21	4	US14 (1), US15 (2), US18 (1)	KJ683931
Hap22	1	US15 (1)	KJ683932
Hap23	1	US17 (1)	KJ683933
Hap24	1	US18 (1)	KJ683934
Hap25	1	US18 (1)	KJ683935
Hap26	1	US18 (1)	KJ683936

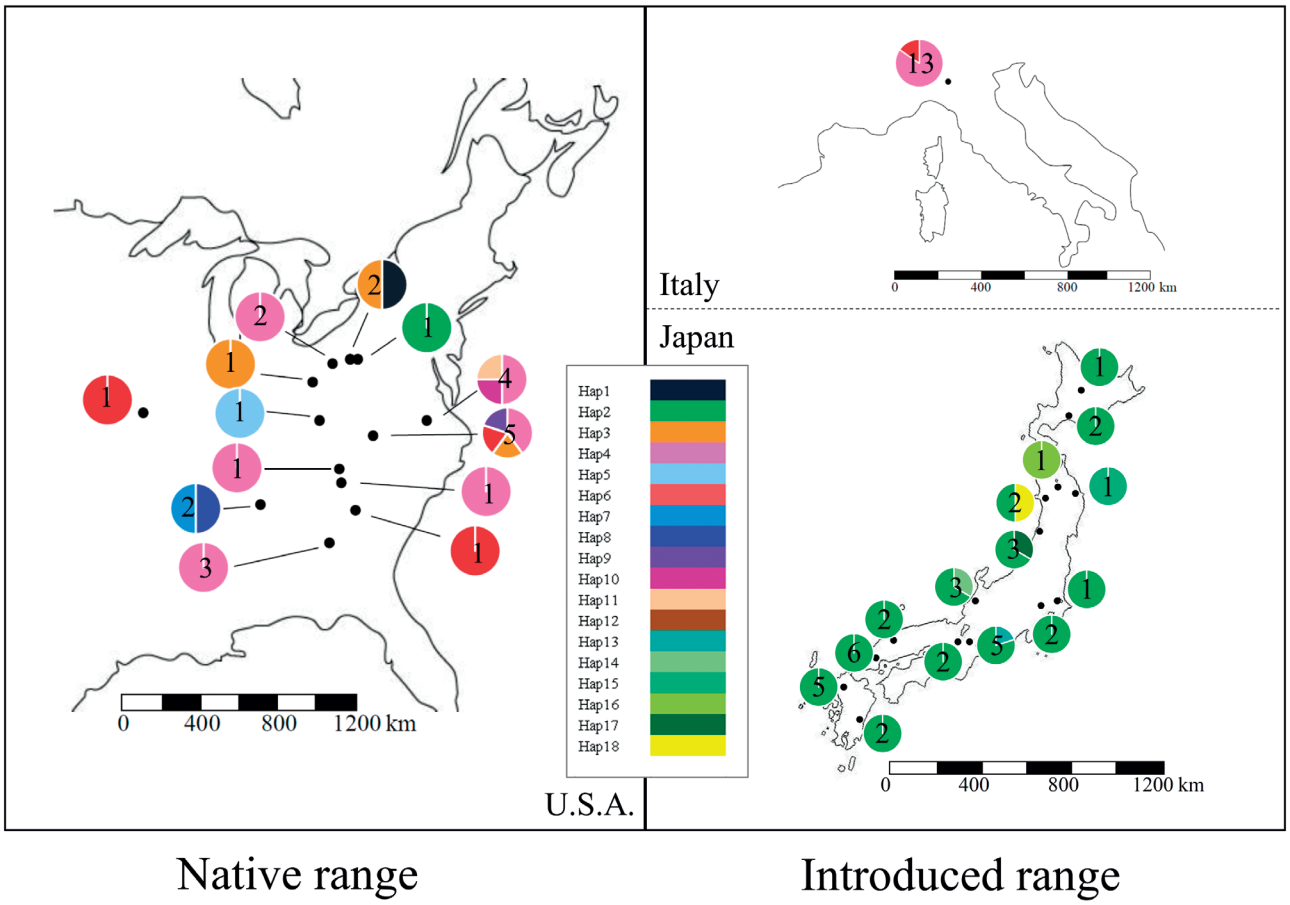

Fig. 3. The geographical distribution of haplotypes of *Ophraella communa* in USA, Japan and Italy. The numbers in the pie charts indicate the number of samples.

diversity of US, Japanese and Italian *O. communa* individuals, two standard diversity indices, haplotype diversity and nucleotide diversity (Nei, 1987), were calculated using Arlequin 3.5.1.2 (Excoffier & Lischer, 2010). When estimating the genetic diversity in different countries, data from within each country were pooled. We sampled too few samples per population to examine population level differences. To determine the demographic history of the native and introduced populations of *O. communa*, we calculated Tajima's D, which determines departure from neutrality by measuring the disparity between the number of segregating sites and pairwise genetic distance (Tajima, 1989). Fu and Li's F* and D*, which are variations of Tajima's test (Fu & Li, 1993), were calculated using the DnaSP program. These neutrality indices can be used to estimate past bottlenecks in population size or population expansions. We also estimated the nucleotide diversity in each country (average number of nucleotide differences per site within countries) and haplotype diversity (the likelihood that two individuals randomly chosen from a country have different haplotypes).

RESULTS

Based on the COII sequences, there were 26 haplotypes in the total sample of 89 individuals (Table 2). The haplotypes cluster into two distinct groups separated by at least 33 mutational steps (Fig. 2). Individuals from the two clusters were assigned respectively to *O. communa* and *O. slobodkini* based on COI sequences (Funk et al., 1995). Whereas 12 haplotypes of *O. communa* were found in the native populations, seven and two haplotypes were found, respectively, in the populations introduced into Japan and Italy (Table 1, Fig. 2). Eight haplotypes of *O. slobodkini* were found only in the southern populations in the native range, but not in the introduced populations (Fig. 1). No regional clustering of haplotypes was found among the native individuals. For example, haplotype 4 was found in widely separated native populations. Most of the Japanese individuals (84.2%) have one haplotype (Hap. 2), which

Table 3. Genetic diversity and neutrality tests of native and introduced *Ophraella communa*.

	N	H	π	Tajima's D	Fu and Li's D*	Fu and Li's F*
Total	77	0.729 ± 0.038	0.00528 ± 0.00088	–2.141*	–3.950*	–3.904*
Native range (US)	25	0.785 ± 0.081	0.00802 ± 0.00192	–2.011*	–3.221*	–3.334*
Introduced range (Japan)	38	0.294 ± 0.097	0.00141 ± 0.00061	–2.095*	–2.717*	–2.955*
Introduced range (Italy)	13	0.282 ± 0.142	0.00230 ± 0.00115	–0.394	1.086	0.803

N – number of samples; H – haplotype diversity; π – nucleotide diversity; * $p < 0.05$; ** $p < 0.01$.

they share with an individual from the native population (Trafford, PA). The Japanese individuals showed a star-like pattern of six haplotypes arising from the most common haplotype (Fig. 2). Two haplotypes (Hap. 4 and Hap. 6) found in Italian individuals were also found in native individuals but not Japanese individuals (Fig. 2; Table 2).

Overall, there were lower levels of nucleotide and haplotype diversity in *O. communa* in its introduced ranges than in its native range (Table 3). Tajima's D, Fu and Li's D*, and Fu and Li's F* statistics showed significant negative values for Japanese individuals and native individuals, but not for Italian individuals (Table 3).

DISCUSSION

In this study, we analyzed partial sequences of mitochondrial DNA to infer the invasion history of *O. communa* and compare the genetic diversity in native and introduced ranges. Although our dataset is preliminary and limited in terms of the numbers of individuals and populations sampled, our results suggest that introduced Japanese populations are less genetically diverse than the native North American population (Figs 2, 3; Table 3). Most Japanese individuals (33/38) shared one of the haplotypes found in the native range. As a result, the nucleotide and haplotype diversities of Japanese populations was much lower than that of populations in the United States (Table 2). In some of the Japanese populations there were none of the haplotypes recorded in the United States (Haplotype13–18) and these haplotypes differed by only a few nucleotides from the main haplotype in Japan (Haplotype 2). The unique haplotypes in Japan may result from sampling error because we collected only a few individuals per population. The low genetic variation recorded in the introduced populations indicate that the Japanese populations of *O. communa* resulted from a single major introduction rather than multiple introductions. For Japanese populations, we collected *O. communa* individuals feeding on both *A. artemisiifolia* and *A. trifida*. However, we recorded no genetic differentiation between these host-associated *O. communa* populations (Table 2). We also could not find a clear relationship between these haplotypes and the year in which the *Ophraella* beetles were collected.

Although our results suggest that the introduced Japanese populations were derived from a single major introduction, we were unable to identify the exact source populations of the introduced Japanese and Italian populations. This is because no regional clustering of haplotypes occurs among the native populations (Fig. 3). The absence of a regional clustering pattern in the native populations is consistent with the results of a previous study on *O. communa* based on mitochondrial COI sequences (Knowles et al., 1999). They suggest that this pattern reflects historical expansion associated with Pleistocene alternation of glacial and interglacial episodes. The significant negative values of the neutrality indices recorded for the native individuals (Table 3) are also consistent with the demographic history of past bottleneck events and/or recent population growth. Two haplotypes of Italian individuals were shared by native individuals collected from several US populations. Although Italian individuals were collected from only one population, Italian haplotypes were not shared by Japanese individuals, suggesting that the introduction into Europe was independent of that into Japan. In the populations tested there was no evidence of a cryptic introduction of *O. slobodkini* into Japan and Italy. Futuyma (1991) reports a species boundary between *O. communa* and *O. slobodkini* along the Georgia-Florida border (Fig. 1). However, we found that the northernmost sampling site for *O. slobodkini* was near St. George, South Carolina in the native range (US13, US14 and US 15 in Fig. 1). During recent decades, data indicate that the boundary between *O. communa* and *O. slobodkini* might be shifting northwards.

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