Appl. Entomol. Zool. 43 (1): 19–23 (2008)
http://odokon.org/

Estimating colony number of *Bombus terrestris* (Hymenoptera, Apidae) queens foraging in Biratori, Hokkaido, Japan

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(Received 19 January 2007; Accepted 17 July 2007)

Abstract
Commercial colonies of European bumble bee, *Bombus terrestris*, have been widely used for pollinating tomato plants in greenhouses in Japan; however, a wild colony of the species was found in Hokkaido in 1996, after which several studies were conducted to investigate the ecological impact of the naturalization of *B. terrestris* to Japanese ecosystems. To quantify the status of naturalization, it is necessary to establish a method for estimating the population size of the alien bees established in the field. Locating bumble bee colonies in the field is, however, very difficult because they usually nest underground. In this study, we estimated the number of colonies using genetic data from *B. terrestris* queens foraging near greenhouses in Biratori, Hokkaido, Japan, applying the Shared Loci Correspondence Analysis (SLCA). Sampled 39 queens were assigned to 30 different families, which indicates that most queens came from different colonies. Based on the findings, we discuss the importance of urgent measures to prohibit *B. terrestris* from escaping from greenhouses and becoming naturalized in Hokkaido.

Key words: Bumble bee; microsatellites; invasive alien species; Shared Loci Correspondence Analysis; SLCA

INTRODUCTION

Bombus terrestris is a widespread European species of bumble bees. Colonies of this species are relatively larger than other bumble bees, and workers visit a wide range of flowers. This efficient pollinator species has been commercialized in Europe, and commercial colonies of *B. terrestris* have been imported into many countries for pollination of agricultural crops in greenhouses. Since 1991, this species has also been imported into Japan for the pollination of tomato plants in greenhouses (Ono, 1998). More than 70,000 colonies of *B. terrestris* are imported from Europe each year, because they help to produce less labor intensive and pesticide-free tomatoes (Kunitake and Goka, 2006).

Japanese ecologists, however, have warned of the ecological risks caused by the biological invasion of this species (Washitani, 1998). This exotic bumble bee has the potential to affect native bumble bees or the native pollination system in three ways (Goka et al., 2001): transmission of exotic parasites and pathogens (Goka et al., 2001), genetic disturbance caused by hybridyzation with native bees (Goka, 1998), and competition for ecological niches, such as food or nesting sites (Hingston and Mcquillan, 1999). In 1996, a feral colony of *B. terrestris* was found in the vicinity of a greenhouse in Monbetsu in the Hidaka region of Hokkaido (Washitani and Matsumura, 1998). Since then, several studies have investigated the state of invasion and its ecological impact on native species, mainly in Hokkaido (Matsumura et al., 2004; Inari et al., 2005; Goka et al., 2006).

Estimation of the population size of naturalized *B. terrestris* is needed as basic data for assessing ecological risks and eradicating the naturalized alien bees. Each bumble bee colony is usually founded by a single queen, and queens of *B. terrestris* are monoandrous (Estoup et al., 1995; Schmid-Hempel and Schmid-Hempel, 2000). The

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DOI: 10.1303/aez.2008.19

number of reproductive individuals released varies greatly, from zero to tens of bees, among colonies (Goulson, 2003); therefore, the number of colonies, rather than the number of individuals, represents the effective population size. The number of colonies also represents propagule pressure of the species, because bumble bees are social and colonial insects. To prevent more expansion and establishment in the field, we should decrease propagule pressure on native ecosystems; however, locating bumble bee colonies in the field is often difficult because *B. terrestris* queens usually nest underground using pre-existing holes made by mice and voles (Goulson, 2003).

Using genotypes of foraging worker bees, we can estimate the number of *B. terrestris* colonies with so-called full-sib reconstruction methods (Konovalov et al., 2005) because workers within a colony are full siblings. Several surveys have been conducted to estimate the number of bumble bee colonies using genetic data obtained by microsatellite DNA (Chapman et al., 2003; Darvill et al., 2004; Knight et al., 2005). Chapman et al. (2003) used a maximum likelihood approach, which maximizes the likelihood of overall full-sib configurations of sample individuals (Thomas and Hill, 2000; Wang, 2004). Maximum likelihood approaches require a large number of individuals because they need an accurate estimation of population allele frequencies. Most of these methods are computationally intensive and, in many cases, do not work successfully on large challenging data sets (Butler et al., 2004). Darvill et al. (2004) and Knight et al. (2005) used a pairwise likelihood approach, which infers the potential relationship of a pair of individuals, and then clusters them into sibling families. To study the initial phase of intrusion, the pairwise approach would be a suitable method for estimating the number of foraging bumble bees with DNA data.

Recently, Kokuvo et al. (2007) proposed a simple pairwise scoring method, Shared Loci Correspondence Analysis (SLCA), to reconstruct nestmate patterns of haplodiploid species. SLCA does not require knowledge of population allele frequencies. Using haplodiploid genetics and the Mendelian exclusion principal, SLCA enables the estimation of full siblings of haplodiploid species, even with a small data set (Kokuvo et al., 2007).

In this study, we conducted a brief survey for investigating the initial phase of intrusion by *B. terrestris* in the field. In May 2005, we sampled 39 *B. terrestris* queens foraging in Biratori, Hokkaido, Japan, and estimated the population size of this alien bees using the SLCA algorithm (Kokuvo et al., 2007) and a pairwise method using software, KINSHIP (Darvill et al., 2004; Knight et al., 2005). The impact of the intrusion of *B. terrestris* on natural habitats will be discussed.

MATERIALS AND METHODS

Sample collection. On four different days in May 2005, 39 *Bombus terrestris* queens foraging on *Taraxacum* spp. and *Corydalis ambigua* were captured with a net over about two hours a day at six sites in Biratori, Hokkaido, Japan (Fig. 1).

In general, *Bombus* species have an annual life cycle (Goulson, 2003). Queens emerge from hibernation in late winter or spring, and at this time of year they can often be seen searching for suitable nest sites. In 2005, commercial colonies were also used from March to August in the Biratori region (Yoneda et al., 2007); thus, our sample was possibly from commercial colonies in greenhouses or wild colonies.

In these regions, the use of commercial colonies of *B. terrestris* has rapidly increased since 1997 (Inari et al., 2005), and about 1,000 colonies were shipped into Biratori in 2005 (Yoneda et al., 2007). Thus, this area is suitable for studying the initial phase of the intrusion process of *B. terrestris*. Each sample was kept in a plastic tube, chilled immediately, and later frozen for DNA extraction at

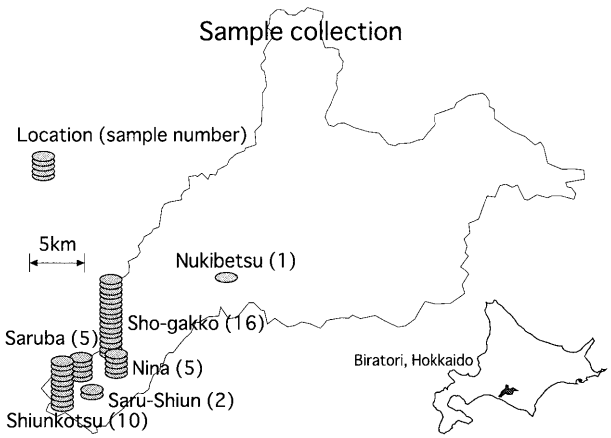

Fig. 1. A map of Biratori indicating the location and sample sizes at each site.

Table 1. Number of alleles (n), allele frequencies, and observed heterozygosities (H_o) of six microsatellite loci for *Bombus terrestris*

Locus	n	H_o	Allele frequency									
			a	b	c	d	e	f	g	h	i	j
B11	10	0.615	0.282	0.256	0.218	0.115	0.038	0.026	0.026	0.013	0.013	0.013
B96	4	0.462	0.513	0.385	0.077	0.026						
B100	8	0.744	0.398	0.180	0.167	0.103	0.103	0.026	0.013	0.013		
B118	5	0.641	0.385	0.372	0.103	0.077	0.026					
B121	4	0.359	0.795	0.103	0.090	0.013						
B132	10	0.872	0.321	0.179	0.128	0.115	0.077	0.051	0.051	0.038	0.026	0.013

−80°C.

DNA extraction and microsatellite genotyping. Bees were genotyped by six microsatellite DNA loci. DNA was extracted from an entire middle leg of each sample. The leg was homogenized in a tube containing 30 μl of lysis buffer (1 mg/ml proteinase K, 0.01 M NaCl, 0.1 M EDTA, 0.01 M Tris-HCl (pH 8.0), 0.5% Nonidet P-40). The homogenate was incubated at 50°C for 60 min and then at 94°C for 10 min. After incubation, the homogenate was diluted with 270 μl of TE buffer (0.001 M EDTA, 0.01 M Tris-HCl (pH 8.0)) and used as a DNA template in polymerase chain reaction (PCR). Six microsatellite loci (B11, B96, B100, B118, B121, and B132) were amplified by PCR using locus-specific primer pairs developed by Estoup et al. (1996). PCRs were performed and polymorphic patterns of each microsatellite locus was analyzed, following the protocols described by Kokuvo et al. (2007). The mean number of alleles per locus and heterozygosity was 6.83 (range 4–10) and 61.5%, respectively (Table 1).

Estimating the number of colonies. We used EstNest 1.0 to estimate the number of colonies in a sample. As sample queens might be from commercial or wild colonies, the estimated number of colonies is the number of commercial colonies in 2005 and feral or commercial colonies in the previous year.

EstNest 1.0 is a program for reconstructing nestmate patterns among haplodiploid females using Shared-Loci Correspondence Analysis (SLCA) algorithms (Kokuvo et al., 2007). The confidence intervals and standard errors of the estimate were calculated by Jackknife resampling (Efron, 1982).

We also used another pairwise method to estimate the number of colonies following the algorithm described in Knight et al. (2005). Sister relationships among individuals were established using the likelihood function of KINSHIP 1.3.1 (Goodnight and Queller, 1999) where R_m=0.5 and Rp=1.0. Confidence in the sister pair assignment was calculated from 1,000,000 simulations. To minimize type I errors, only sisters designated at $p \leq 0.001$ (the most stringent value that KINSHIP will return) were used for further analysis. The total number of colonies was calculated as the total number of sister groups found by KINSHIP. We did not estimate the number of colonies that were not sampled because we were interested in the number of colonies from which the sampled bees came.

RESULTS AND DISCUSSION

We conducted genetic analysis to estimate the number of colonies of 39 *Bombus terrestris* queens foraging in the field in Biratori, Hokkaido, Japan. We found that foraging queens were from many colonies, suggesting that most came from different colonies. A total of 28.82±1.62 families (mean± t·SD, range 22–32, n=39) were detected by Jackknife resampling with SLCA. Estimated sister pairs for 39 individuals by KINSHIP and SLCA are shown in Fig. 2. Using whole individuals, 30 colonies were estimated by SLCA. Twenty-three were assigned to one individuals, 8 to two individuals, and 6 to three individuals. On the other hand, 31 colonies were estimated by KINSHIP. Twenty-three were assigned to one individuals, and the other 8 colonies were assigned to two individuals.

Accumulation of knowledge and quantitative data is necessary for better understanding of intrusion and effective management of this efficient pol-

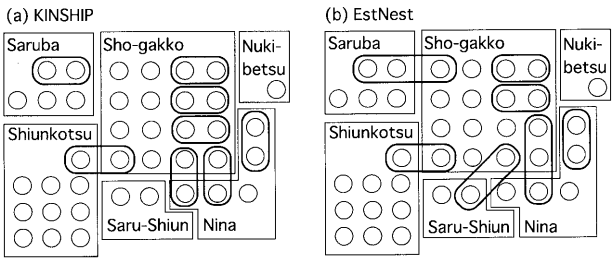

Fig. 2. Estimated sister groups (thick ellipse) for (a) KINSHIP and (b) EstNest 1.0. Each circle and its position represent 39 individuals at each sample site (thin rectangle), that is, two circles at the same position in (a) and (b) show the same individual.

linator. Unfortunately, we cannot know the actual number of colonies in the wild, and we should be careful when evaluating this estimate. Although Kokuvo et al. (2007) showed that SLCA can accurately reconstruct nest-mate patterns among native Japanese bumble bees collected from three wild colonies of *B. ardens*, further research is required to evaluate the accuracy of this algorithm. An evaluation of SLCA using simulated data is now being conducted by the authors.

Propagule pressure is a key factor for alien species to expand their distribution in new conditions (Williamson, 1996). In order to calculate the propagule pressure of commercial *B. terrestris*, we estimated the effective population size, i.e. the number of colonies, of the species. Our results indicate that a large number of colonies release bees into the wild, suggesting that the propagule pressure of the species is high in our study area. Our estimate and the method used here would provide valuable information to discuss the impact of commercial bee populations on native bee species. This method could also be used to estimate the number of colonies of foraging workers in the field.

In 2006, *B. terrestris* was listed as an invasive alien species by the Japanese government, but their importation and rearing is permitted on the condition that the greenhouse where they will be used is fully netted and colonies are burned or otherwise destroyed at the end of their useful life (Goka, 2006a, b; Kunitake and Goka, 2006). Proper usage of this efficient pollinator is essential both for Japanese agriculture and Japanese ecosystems. Our present study shows, however, that a large number of colonies may be releasing bees into the wild. In order to allow the sustainable and continuous use of this efficient pollinator, steps should be taken as soon as possible to prevent additional commercial bees from being released into the wild, and we should develop more efficient methods to control and eradicate escaped alien bumble bees. Long-term monitoring studies and activities for the extermination of *B. terrestris* in the field have been conducted by the Japanese Society of Conservation Biology, and the results of annual "sighting" surveys are published on their web site (http://www003.upp.so-net.ne.jp/consecol/). Very valuable whole body samples and census data have been accumulated by the research team, and the collaboration of scientists in various fields is required to provide a more detailed understanding of this introduced species of bees.

ACKNOWLEDGEMENTS

We are grateful to Dr. Y. Kunitake and Dr. H. Mano for valuable comments and discussions on earlier versions of the manuscript. We also thank Dr. J. Yokoyama and Mr. Y. Saito for their continuous help with sample collections, and Shinya Yoshimura for computer skills. This work was supported in part by Grants-in-Aid for Scientific Researches (No. 17405004 to YT and KG) from the JSPS and by Research Project for Utilizing Advanced Technologies in Agriculture, Forestry and Fisheries (No. 1701 to KG and YT).

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APPENDIX

Genotypes of each individuals. Capital letter represents each sample sites: NI: Nina, SS: Saru-Shiun, NU: Nukibetsu, SI: Shiunkotsu, SA: Saruba, SH: Sho-gakko.

ID	B11	B118	B96	B100	B121	B132
NI01	fi	ad	cc	bf	cc	de
NI02	bf	cd	ac	bg	cc	di
NI03	ff	ad	cc	bd	cc	de
SS04	bd	bb	cc	ag	bc	cj
SS05	be	de	aa	bh	cc	bf
NI06	ad	cc	cc	bf	cc	cf
NI07	dd	ad	aa	ad	cc	bf
SI08	ff	cc	ad	bf	bc	bd
NU09	bb	ce	aa	bb	cc	ef
SI10	ee	bb	ab	bf	cc	bd
SI11	ff	dd	aa	aa	bc	bb
SI12	be	cd	ac	ae	cc	bd
SA13	ab	dd	ac	bb	bc	fh
SA14	dg	cd	aa	bf	cd	de
SA15	bb	cd	aa	bb	cc	bd
SA16	dg	dd	aa	bf	cd	de
SI17	fi	ce	bb	bf	bc	ch
SI18	eg	ce	ac	bb	cc	bd
SI19	df	cd	ab	bg	cd	ce
SI20	bb	cc	aa	dg	cc	ej
SI21	ee	bd	aa	gg	cd	eg
SI22	ee	bd	ac	bb	cd	bg
SH23	bf	cd	ac	bg	bc	bi
SH24	dh	cd	cc	bd	cc	ba
SH25	bd	ac	ab	af	cc	ff
SH26	bd	cc	cc	bb	cc	bd
SH27	bf	dd	ac	bd	cc	dj
SH28	bf	ac	ad	bf	bc	bb
SH29	bb	cd	ac	af	cc	bh
SH30	df	ac	cc	bf	cc	cd
SH31	dd	cd	cc	bd	cc	bb
SH32	bd	ad	cc	ag	cc	bi
SH33	bb	cd	ac	aa	ac	bi
SH34	df	de	ac	bf	dd	be
SH35	df	cc	aa	ac	cc	bf
SH36	bf	dd	ac	bd	cc	dj
SH37	dd	ac	ac	ac	cc	bb
SH38	dd	dd	ab	ad	cc	bf
SA39	cj	cc	aa	ff	bc	cf