Genetic Characterization of Russian Honey Bee Stock Selected for Improved Resistance to *Varroa destructor*

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ABSTRACT   Maintenance of genetic diversity among breeding lines is important in selective breeding and stock management. The Russian Honey Bee Breeding Program has strived to maintain high levels of heterozygosity among its breeding lines since its inception in 1997. After numerous rounds of selection for resistance to tracheal and varroa mites and improved honey production, 18 lines were selected as the core of the program. These lines were grouped into three breeding blocks that were crossbred to improve overall heterozygosity levels of the population. Microsatellite DNA data demonstrated that the program has been successful. Heterozygosity and allelic richness values are high and there are no indications of inbreeding among the three blocks. There were significant levels of genetic structure measured among the three blocks. Block C was genetically distinct from both blocks A and B (F_{ST} = 0.0238), whereas blocks A and B did not differ from each other (F_{ST} = 0.0074). The same pattern was seen for genic (based on numbers of alleles) differentiation. Genetic distance, as measured by chord distance, indicates that all of the 18 lines are equally distant, with minimal clustering. The data indicate that the overall design of the breeding program has been successful in maintaining high levels of diversity and avoiding problems associated with inbreeding.

KEY WORDS   *Apis*, breeding program, Russian honey bees, genetic differentiation

Genetic diversity of managed stocks of honey bees, *Apis mellifera* L., has been measured throughout the United States and Italy (Sheppard and Berlocher 1989, Schiff and Sheppard 1995, Schiff and Sheppard 1996, Bourgeois et al. 2008). Maintenance of genetic diversity among breeding lines is an important factor in selective breeding and stock management. Reductions in diversity promote susceptibility to disease outbreaks and other negative conditions associated with inbreeding (Sherman et al. 1988, Sherman et al. 1998, Baer and Schmid-Hempel 1999, Baer and Schmid-Hempel 2001, Tarpy 2003, Tarpy and Seeley 2006, Seeley and Tarpy 2007).

To foster diversity among breeding lines, some breeding programs maintain separate breeding lines that are then crossbred to promote diversity (Willham and Pollack 1985). This is common in livestock breeding programs and has allowed for genetic improvement of traits without sacrificing genetic diversity. Line breeding and outcrossing to produce hybrid production queens have been implemented in Western Australian honey bees for the purpose of maintaining genetic diversity (Chapman et al.

2008). Line breeding and hybrid formation were used by G. H. Cale, Jr., to produce the renowned Starline hybrid and later the Midnite hybrid for Dadant and Sons, Inc. (Witherell 1976). These hybrids were produced by developing inbred lines, testing the quality of various hybrids of the inbred lines with other lines, and then rejecting poor-quality crosses. The inbred lines were difficult to maintain because of decreased fitness and were consequently replaced regularly with new inbred lines that also proved successful in tests of hybrids.

Closed population breeding is an alternative to line-hybrid breeding (Page et al. 1982). This system has numerous advantages. It can be done with natural matings, has much greater flexibility in choice of matings, and permits improvement through selective breeding while simultaneously permitting the maintenance of genetic variability, most importantly at the sex allele locus *csd* (Laidlaw and Page 1986).

Russian honey bee queens were first brought into the United States in 1997 for the purpose of genetic improvement of varroa mite resistance in managed honey bee stocks (Rinderer et al. 1997, Rinderer et al. 2005). Yearly importations continued through 2002 to include a total of 362 queens. From tests of individual queens and numerous field trials of groups of siblings, 18 lines in total were established
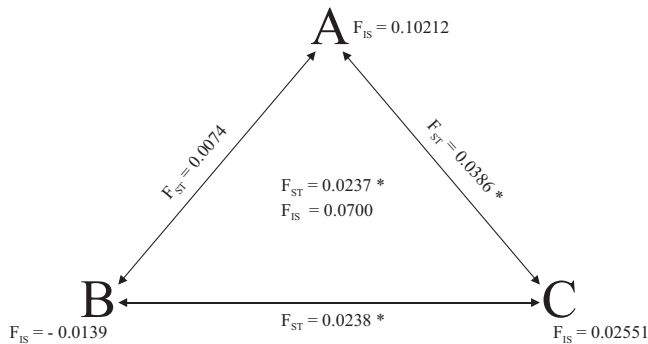
Fig. 1. Graphical representation of three-block breeding design for Russian honey bees. Genetic diversity estimates are listed along each axis. Values inside of figure represent overall diversity estimates. *$P < 0.05$.

that had resistance to tracheal and varroa mites and good honey production (Rinderer et al. 1997). The lines were divided into three groups of six lines each, to form a closed breeding population having a three-block design. Lines within each block were deliberately crossed with lines from the other two blocks (Fig. 1) under controlled conditions to promote maintenance of genetically diverse lines. Queens were allowed to free-mate in flight in isolated apiaries on coastal islands containing drones from predetermined sources (from the remaining two blocks). Initial breeding efforts resulted in a release of three lines of improved stock to commercial beekeepers in 2000 (Rinderer et al. 2000). Controlled releases of numerous lines to select beekeepers have continued through 2008.

Establishment of the Russian Honey bee Breeders Association (RHBA) occurred in 2007. Members of this organization will be the recipients of the final release of Russian stock. The breeding plan that incorporates the three-block design will be adopted by the RHBA. The 18 individual members will each produce sibling groups of two of the 18 lines and selectively breed for further improvements. They will use the appropriate drone sources for their lines that they will receive from other members. Hence, although the breeding population remains closed, the breeding will be done in many locations. Members do not have the potential for island matings but all have some degree of isolation in mating yards. For the most part, "drone flooding" produces a very high proportion ($\approx$95%) of desired matings (Hellmich and Waller 1990, Hellmich 1991). Because Russian hybrid colonies have intermediate resistance to *Varroa destructor* Anderson & Trueman (Harris and Rinderer 2004) colonies in selection apiaries that have queens with some degree of mismating should perform less well on colony evaluations for *V. destructor* population growth and would be culled from the program.

The success of the breeding program relies on maintenance of the diversity among the 18 lines constituting the three genetic blocks. The purpose of this study was to determine the current level of genetic diversity among three breeding groups of Russian honey bees represented in the final stock release.

Materials and Methods

Sampling. Emerging worker bees per colony were sampled from colonies representing each of the 18 selected lines of Russian honey bees maintained at apiaries managed by the USDA–ARS Honey Bee Breeding, Genetics, and Physiology Laboratory and apiaries of a beekeeper, Charlie Harper, collaborating with our research and breeding program. Samples were immediately placed on ice and stored at $-70°C$ until processed.

DNA Extraction. DNA was extracted from the thorax of individual bees (two bees per colony). Samples were first homogenized in 600 μl of lysis buffer (100 mM Tris, pH 8.0, 10 mM EDTA, pH 8.0, and 1% SDS) and 100 mg of 1-mm glass beads for 3 min at speed 8 in the Bullet Blender (Next Advance, Inc., Averill Park, NY) and then treated with proteinase K (20 mg/ml) at 55°C for 1 h. Two hundred microliters of 7.5 M NH_4OAc was then added for protein precipitation, followed by ethanol precipitation and lyophilization. Pure genomic DNA was rehydrated in 50 μl of Millipore filtered and deionized distilled H_2O (Millipore, Billerica, MA) and stored at $-20°C$.

Genotyping. In total, 49 microsatellite markers (Solignac et al. 2007) were screened for polymorphism among the DNA samples. Thirty-four were polymorphic, of which nine were used for subsequent analyses. GenBank accession numbers and amplification conditions are listed in Table 1. The 5′ end of the forward primer was modified by the addition of the complementary sequence to the M13–Cy5-labeled primer (Univ) of sequence GAGTTTTCCCAGTCACGAC. Amplification conditions were optimized on a PTC-200 thermal cycler (MJ Research, Watertown, MA) and were used for all subsequent reactions. The optimized amplification profile was 1 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C, and ended with 10 min at 72°C. Each 6.5-μl reaction included 1.5 pmol of each primer (MF, R, and Univ); 2 mM of each dNTP; 1$\times$ polymerase chain reaction (PCR) buffer II [10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 0.01% (wt:vol) gelatin; Applied Biosystems, Foster City, CA]; 1.5 mM $MgCl_2$; 0.35 U of

Table 1. Locus information for microsatellite DNA used for population genetic analyses for Russian honey bee stocks

Locus	Accession no.	Block A			Block B		Block C	
		Repeat unit	Gene diversity	Allelic richness	Gene diversity	Allelic richness	Gene diversity	Allelic richness
K0405	AADG02002083	(CT)9	0.745	5.631	0.667	3.895	0.668	3.000
SV185	BI507391	(AAC)12	0.309	2.000	0.655	3.891	0.235	2.696
UN393	AADG05008245	(TC)12	0.841	6.608	0.871	8.271	0.782	5.633
K0820	AADG02004220	(TG)10	0.709	4.922	0.632	3.792	0.786	5.634
BI109	BI507109	(TC)13	0.788	6.424	0.633	4.700	0.848	8.436
K1168	AADG05004246	(GA)11	0.791	4.969	0.805	5.631	0.795	6.558
SV167	BI506173	(AAT)9	0.617	4.250	0.659	2.999	0.611	4.000
SV131	BI506633	(TC)9	0.677	5.584	0.772	5.889	0.735	4.694
AT082	AJ509542	(GGT)10	0.542	2.946	0.411	2.900	0.489	2.750

Primer sequences were published previously (Solignac et al. 2007).

Amplitaq Gold polymerase (Applied Biosystems); and template DNA (50–100 ng).

Amplification products were analyzed with a CEQ8000 genetic analyzer (Beckman Coulter, Inc., Fullerton, CA) and CEQ8000 software (Beckman Coulter, Inc.). Four microliters of PCR product was mixed with Frag-400 size standards (Beckman Coulter, Inc.) and deionized formamide. Samples were run according to the manufacturer's recommendations.

Data Analysis. The software MICROCHECKER (Van Oosterhout et al. 2004) was used to assess the presence of null alleles at each locus within each population. Measures of allelic richness (mean number of alleles per locus) and gene diversity (heterozygosity) were generated in FSTAT (Goudet 2001). Assessment of population genetic structure was completed using Weir and Cockerham (1984) estimators. Additionally, global and pairwise values of θ (Wright 1951) were estimated in Genetix v 4.05 (Belkhir et al. 1996). Significance was determined with tests based on 10,000 permutations generated in Genetix. Genic differentiation was measured in GENEPOP 4.0 (Rousset 2008). Genetic distance, calculated as chord distance (Cavalli-Sforza and Edwards 1967), was calculated in Genetix (Belkhir et al. 1996), and neighbor-joining phenograms were generated from chord distance data in MEGA4.0 (Tamura et al. 2007).

Results

In total, 49 microsatellite loci were screened for polymorphism among the three blocks of Russian honey bees (representing 18 breeding lines), of which 34 were polymorphic. Based on lack of stutter and consistent amplification, nine were used for further analyses (Table 1). None of the nine were found to have null alleles ($P > 0.05$). All loci were in Hardy–Weinberg equilibrium. In total, 65 alleles at the nine loci were identified from among the 18 lines with varying allele frequencies (Fig. 2). Among the three blocks, the number of alleles was consistent (block A, 46; block B, 44; and block C, 47), however differentiation of block C was evident based on differences in the number of alleles (genic variation) (block A and

B, $P = 0.1380$; block A and C, $P = 0.0098$; and block B and C, $P = 0.0118$). The alleles for each block overlapped (i.e., were common to all blocks) but were not identical. Unique alleles were evident within each block (A = 6, B = 6, and C = 7). Allelic richness differed between blocks but was highly varied among loci (Table 1). Gene diversity also varied among loci but was similar between blocks for each locus (Table 1).

Population genetic structure measures showed no evidence ($P > 0.05$) for inbreeding but did show significant levels of diversity among blocks (Fig. 1). Block C was differentiated from both block A and B ($F_{ST} = 0.0238$, $P = 0.0494$; $F_{ST} = 0.0386$, $P = 0.0226$, respectively); however, block A did not differ from block B ($F_{ST} = 0.0074$, $P = 0.2829$). A neighbor-joining phylogram generated from chord distance (Cavalli-Sforza and Edwards 1967) data demonstrates that the 18 lines are evenly distributed relative to genetic distance, with minimal clustering (Fig. 3).

Discussion

The Russian honey bee breeding program was designed to maintain genetic diversity among the 18 selected lines and avoid problems associated with inbreeding. This plan has been successful in that diversity levels are high in all three of the breeding blocks. Overall levels of F_{IS}, gene diversity, and allelic richness were comparable with levels found in a microsatellite survey of commercial Italian bee populations in the United States and Italy (Bourgeois et al. 2008) and in commercial populations surveyed in Western Australia (Chapman et al. 2008).

Blocks A and B were not differentiated from each other; however, block C showed some genetic distinction from both block A and B, supporting the philosophy behind a block-design breeding program. In this type of breeding program, three blocks are propagated to maintain diversity among lines. Deliberate crossing of lines among blocks then improves heterozygosity (Willham and Pollack 1985). Genic differentiation, based on differences in the number of alleles, also showed significant differen-
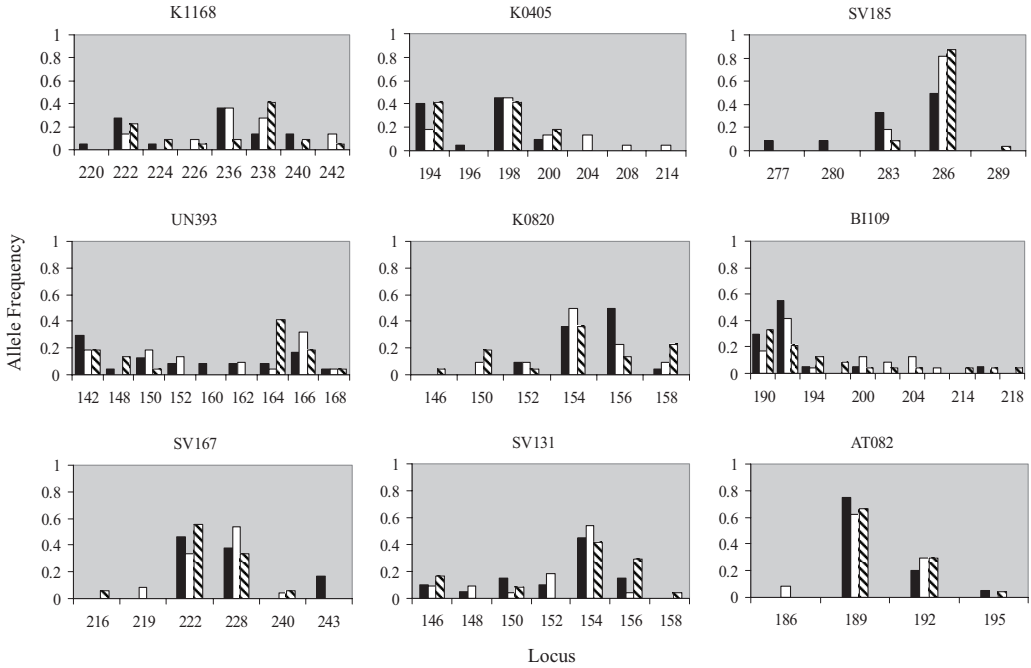
Fig. 2. Graphs of microsatellite allele frequencies of nine loci used to assess genetic diversity of 18 Russian honey bees breeding lines (in three breeding blocks of six lines each). ■, block A; □, block B; and ▨, block C.

tiation of block C lines. This may be because of genetic characteristics of the queens used to propagate the block C lines.

Conversely, chord distance data demonstrated that although the blocks may be genetically distinct with respect to both genic and genotypic differentiation,
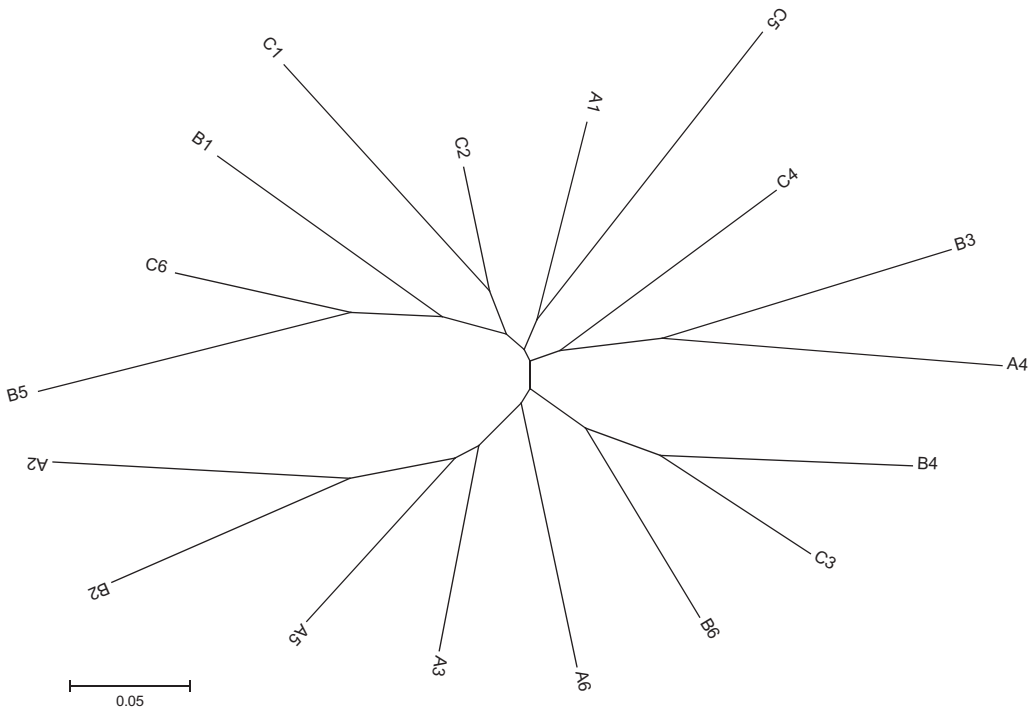

Fig. 3. Neighbor-joining phenogram of 18 Russian honey bee lines. The phenogram was generated from chord distance data (Cavalli-Sforza and Edwards 1967), based on nine microsatellite loci. Bar represents legend for genetic distance.

they were relatively homogeneous with respect to genetic distance, i.e., no clustering was evident. Chord distance takes into account all group comparisons at once, whereas genic and genotypic differentiation estimates treat each pairwise comparison separately. The lack of clustering is a result of continual cross-breeding among blocks and has met the goal of the breeding program.

From the inception of the Russian Honey Bee Breeding Program in 1996 through the final release in 2008, selective breeding has resulted in the development of 18 lines of genetically diverse bees with varroa and tracheal mite resistance and good honey production (De Guzman et al. 2001, Rinderer et al. 2001, Harris and Rinderer 2004, De Guzman et al. 2007). The implementation of the three block design has been successful in maintaining heterozygosity and high levels of diversity while keeping inbreeding levels at a minimum. This design is a good model system for honey bee breeding programs. Control of the Russian Honey Bee Breeding Program is now being transferred to the RHBA. Genetic diversity measures will be monitored to ensure that heterozygosity and allelic richness are maintained at their current levels.

Acknowledgments

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