Journal of Economic Entomology, 110(4), 2017, 1419–1423
doi: 10.1093/jee/tox175
Advance Access Publication Date: 4 July 2017
Research article

Tracking the Genetic Stability of a Honey Bee (Hymenoptera: Apidae) Breeding Program With Genetic Markers

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Subject Editor: Dr. David Tarpy

Received 27 March 2017; Editorial decision 30 May 2017

Abstract

A genetic stock identification (GSI) assay was developed in 2008 to distinguish Russian honey bees from other honey bee stocks that are commercially produced in the United States. Probability of assignment (POA) values have been collected and maintained since the stock release in 2008 to the Russian Honey Bee Breeders Association. These data were used to assess stability of the breeding program and the diversity levels of the contemporary breeding stock through comparison of POA values and genetic diversity parameters from the initial release to current values. POA values fluctuated throughout 2010–2016, but have recovered to statistically similar levels in 2016 ($POA_{(2010)} = 0.82$, $POA_{(2016)} = 0.74$; $P = 0.33$). Genetic diversity parameters (i.e., allelic richness and gene diversity) in 2016 also remained at similar levels when compared to those in 2010. Estimates of genetic structure revealed stability ($F_{ST(2009/2016)} = 0.0058$) with a small increase in the estimate of the inbreeding coefficient ($F_{IS(2010)} = 0.078$, $F_{IS(2016)} = 0.149$). The relationship among breeding lines, based on genetic distance measurement, was similar in 2008 and 2016 populations, but with increased homogeneity among lines (i.e., decreased genetic distance). This was expected based on the closed breeding system used for Russian honey bees. The successful application of the GSI assay in a commercial breeding program demonstrates the utility and stability of such technology to contribute to and monitor the genetic integrity of a breeding stock of an insect species.

Key words: *Apis mellifera*, Russian honey bee, genetic stock identification, breeding

Certification of genetically improved agricultural resources aids in maintaining stock and line integrity. Genetic stock identification (GSI) is used most commonly in commercial fisheries and agricultural plant systems (Potvin and Bernatchez 2001, Moriya et al. 2004, Skaala et al. 2004, Beacham et al. 2005, Beacham et al. 2005, Obata et al. 2006, Moriya et al. 2007, Waldbieser and Wolters 2007). The basis of GSI is a set of molecular markers that is used for stock-specific identification. Unknown samples are compared to a baseline set of samples consisting of the target stock and other possible stocks in a mixed stock analysis algorithm. GSI was adopted by the Russian Honeybee Breeders Association (RHBA) in 2008 for the Russian honey bee stock released to them by the USDA-ARS Honey Bee Breeding, Genetics & Physiology Laboratory.

Russian honey bee queens were first imported to the United States in 1997 from the Primorsky region of Russia for genetic improvement of varroa mite resistance in managed honey bee stocks (Rinderer et al. 1997, Rinderer et al. 2005). Yearly importations continued through 2002. A total of 18 selected breeding lines were developed through selective breeding for tracheal mite an varroa mite resistance and good honey production. These lines were then released to the beekeeping industry, comprising the "Russian honey bee" stock (Bourgeois and Rinderer 2009, Bourgeois et al. 2010). The lines are grouped into three blocks and crossed in a closed breeding system. This is done via natural mating in geographically isolated mating apiaries and is facilitated by drone flooding (i.e., providing more than ample drone sources in the mating apiary so as to "flood out" non-Russian drones that may enter the area from nearby colonies). Realistically mating apiaries cannot exist in complete isolation from feral colonies and neighboring apiaries, leaving open the possibility of "non-Russian" alleles introgressing into the breeding population. Hence, GSI provides a valuable check on the breeding system. A suite of 11 molecular markers (microsatellites and SNPs), used in conjunction with one another were identified for purposes of GSI of the released Russian honey bee stock (Bourgeois et al 2010). This was accomplished through probability testing where unknown honey bees were compared to a set of baseline samples consisting of two groups, Russian and "non-Russian" (i.e., a representation of the primary commercial lines of honey bees

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available in the United States). Each unknown sample was assigned a probability of assignment (POA) to either the Russian or "non-Russian" group. The results of the GSI test (specifically the POA values per colony) were compared to a minimum threshold POA value established by the RHBA and then used in conjunction with measurements of varroa mite population growth and honey production to select "certified" breeder queens for propagation of each of the lines (Bourgeois et al. 2010). In cooperation with the RHBA, the USDA-ARS Honey bee Breeding, Genetics & Physiology Laboratory continues to conduct the GSI component of the certification process.

One major concern associated with a closed breeding system with relatively few lines, such as the Russian breeding program, is maintenance of genetic diversity. At the time of the initial release, diversity parameters were assessed (Bourgeois and Rinderer 2009). Here, we compare diversity parameters from the first year post final stock release to current values. We used the microsatellite loci from the GSI assay for this comparison to monitor changes in heterozygosity and other parameters and to assess the temporal stability of the breeding program.

Materials and Methods

Sample Collection

Samples submitted for GSI testing by RHBA members, according to RHBA guidelines, were used for this study. Submissions consisted of eight individual worker bees from each breeder colony. The nature of submitted samples has changed over the 7 yr of testing. From 2009–2011 samples were recently emerged or emerging bees and pupae. From 2012 until present all samples submitted have been pupae or emerging bees to ensure colony origin. Each testing year runs from August 1 through July 31 of the next year and will be designated here as latter of the two year span (i.e., 2009 through 2010 will be designated "2010").

DNA Extraction and Genotyping

DNA was extracted from the thorax of each bee as per Bourgeois and Rinderer (2009) summarized here with the following modifications. Samples were first homogenized in lysis buffer (100 mM Tris pH 8.0, 10 mM EDTA pH 8.0, 1% SDS) and 100 mg 5-mm stainless steel beads for 3 min at a rate of 30 beats per second in a TissueLyzer II (Qiagen, Inc., Frederick, MD) and then treated with Proteinase K (20 mg/ml) at 70°C for 10 min. Protein precipitation was then completed, followed by ethanol precipitation and lyophilization. Pure genomic DNA was rehydrated in Millipore filtered and deionized dH_2O and stored at −20°C.

Microsatellites

Microsatellites and universal primers used were defined and amplified as described in Bourgeois et al. (2010). The microsatellite markers used in the current GSI assay are a subset of eight of the original set (Table 2). The reduction decreased runtime and cost while maintaining accuracy and precision. From 2009–2015, all microsatellite results were genotyped on a Beckman CEQ/GeXP Genetic Analyzer (Beckman Coulter, Inc., Fullerton, CA) platform, using WellRed dyes (Integrated DNA Technologies, Coralville, IA) using a pool-plexing strategy of PCR products for fragment analysis. Pool-plexing is a method by which PCR products are mixed together and then analyzed simultaneously. The forward primer for each locus was modified at the 5' end by the addition of the complementary sequence to the universal primer. In 2016, the genotyping platform changed to a 3500 Genetic Analyzer (Applied Biosystems) and universal primers were labeled with FAM, VIC, and JOE dyes. Fragment sizes were cross-checked for 200 individual samples to ensure equivalence of allele sizes with the Beckman platform.

Data Analysis

All GSI tests were performed in ONCOR (Kalinowski 2004), genetic stock identification and mixed stock analysis software. The reduced marker set used in 2016 was used for analysis of all samples from all year classes. Statistical analyses (one-way ANOVA, pairwise t-tests, Bonferroni correction) were performed in SAS v10.2.

Genetic distance (Cavalli-Sforza and Edwards 1967) based on microsatellite data was calculated in Genetix software (Belkhir et al. 1996). All other measures of diversity and structure (heterozygosity, allelic richness, F_{ST}, and F_{IS}) were calculated in FSTAT (Goudet 1995). The relationship of samples in the baseline, grouped by type for non-Russian bees and by line for Russian bees, was calculated using MEGA7 (Tamura et al. 2007).

Results

A total of 623 colonies (4,984 individual bees) of Russian honey bees were sampled and analyzed between 2010–2016. Of these, 446 (71.6%) would be considered as "passing," meaning they met or exceeded thresholds set by the Association each year. Differences in the mean number of "passing" colonies by year were evident ($F = 678.29$, $df = 7$, $P < 0.0001$), attributed primarily to a marked decrease ($t = 7.48$, $df = 656$, $P < 0.0001$ after Bonferroni correction) in POA values in the second year post stock release (0.82 ± 0.02 and 0.56 ± 0.03 in 2010 and 2016, respectively; Table 1; Fig. 1).

Allelic richness and heterozygosity were similar when marker suites were compared between 2010 and 2016 ($P = 0.1146$ and 0.6464, respectively; Table 2). Population genetic parameter estimates for 2010 and 2016 were $F_{ST(2009/2016)} = 0.0058$ and $F_{IS(2010)} = 0.078$, $F_{IS(2016)} = 0.149$. Neighbor-joining phenograms were generated from chord distance data for both the original release in 2008 and 2016 (Fig. 2). Branching patterns were similar, but branch lengths decreased in 2016.

Discussion

Genetic stock identification values of Russian honey bees were used to track stock purity. The microsatellite genotypes for individual bees were used for assessments of genetic diversity, population structure, and breeding line relationships over an 8-yr span. This provided an excellent opportunity to monitor the integrity of the breeding population after adoption by an industry-based breeding association.

The POA threshold value that the RHBA used for determining whether or not a colony passed the GSI test was refined several times during the testing period; however, the molecular analyses remained the same throughout. The second year post release (2011), POA values declined significantly. This decrease in POA values was not unexpected because of a reduction in the degree of isolation of nonresearch mating yards as opposed to those in the USDA apiaries. The positive trend in POA values from 2011–2016 were likely due, in part, to adjustment and refinement of beekeeper management practices. Specifically, these include increasing the degree of mating apiary isolation and proper exchange of drones among blocks in accordance with Association guidelines. By 2015, POA values increased beyond 2011–2014 levels. POA values continued to increase in 2016 to levels comparable to the onset of the program in

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Table 1. Probability of assignment (POA) scores and pairwise significance values for sampling years 2010–2016 of Russian honey bee breeder colonies

Sampling year	*n*	Mean	SD	*P*-values for pairwise comparisons between sampling years (after Bonferroni correction)					
				2011	2012	2013	2014	2015	2016
2010	126	0.82	0.02	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0002**	0.3321
2011	62	0.56	0.03		1.0000	1.0000	1.0000	**0.0292**	**<0.0001**
2012	45	0.60	0.03			1.0000	1.0000	1.0000	**0.0284**
2013	68	0.59	0.03				1.0000	0.7890	**0.0071**
2014	120	0.61	0.02					1.0000	**0.0055**
2015	110	0.67	0.02						1.0000
2016	92	0.74	0.03						

P-values in bold represent significant differences after Bonferroni correction for multiple comparisons (corrected $P < 2.33 \times 10^{-3}$).

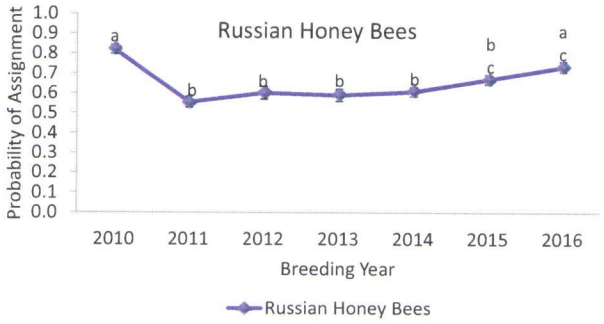

Fig. 1. Changes in probability of assignment values between 2009–2016. Lowercase letters that differ denote significant differences ($P < 0.05$, after Bonferroni correction). Data are presented as sampling year mean ± SEM.

Table 2. Genetic diversity parameters for 2010 and 2016 Russian honey bee samples submitted for genetic stock identification

Locus	Accession no.	Repeat unit	Allelic richness		Gene diversity	
			2010	2016	2010	2016
K0405	AADG02002083	(CT)9	12.923	12.913	0.634	0.606
SV185	BI507391	(AAC)12	8.984	8.998	0.548	0.621
UN393	AADG05008245	(TC)12	10.850	5.999	0.381	0.365
BI109	BI507109	(TC)13	11.796	9.355	0.528	0.469
K1168	AADG05004246	(GA)11	8.857	9.136	0.536	0.506
SV167	BI506173	(AAT)9	9.971	6.868	0.672	0.619
SV131	BI506633	(TC)9	10.000	7.889	0.646	0.602
AT082	AJ509542	(GGT)10	7.866	7.179	0.596	0.580

Comparisons over all markers by year for each parameter were not significant ($P > 0.05$).

year 2010. The data presented here represent only breeding colonies. RHBA production colonies were surveyed soon after the stock release and an updated production colony survey is planned for 2017/2018 (Bourgeois et al. 2011).

One potential pitfall of a closed breeding system, such as the program tested here, is a decrease in genetic diversity over time. This could lead to a variety of deleterious effects related to inbreeding including increased disease susceptibility and reduced brood viability due to an increase in the prevalence of homozygosity of the *complimentary sex determinating gene* (*csd*) (Beye 2004). Diversity parameters for past and present populations of Russian honeybees were estimated using the same suite of markers used for stock identification. The initial assessment of diversity (Bourgeois and Rinderer 2009) upon stock release used a different set of markers. Here we chose to use the GSI microsatellite markers for analysis of both past and present samples to assess genetic diversity and temporal stability of the GSI assay.

Genetic diversity among honey bee colonies is an important breeding population characteristic to monitor (Tarpy 2003, Mattila and Seeley 2007, Bourgeois et al. 2008, Harpur et al. 2012, Desai and Currie 2015, Niño and Cameron Jasper 2015). In a closed breeding system such as this, loss of genetic diversity could be catastrophic. Fortunately, polyandrous mating habits of honey bee queens promote colony diversity, which can have concomitant effects of improved colony survivability when faced with disease outbreaks or other colony-level stressors (Laidlaw and Page 1984, Sherman et al. 1998, Tarpy 2003, Tarpy and Seeley 2006, Seeley and Tarpy 2007, Wilson-Rich et al. 2012, Tarpy et al. 2013). Hyperpolyandry, which has been observed in Russian honey bees, improves these effects (Bourgeois et al. 2012, Wilson-Rich et al. 2012, Tarpy et al. 2013, Delaplane et al. 2015). The three-block design of the Russian breeding program used by the RHBA was designed to maintain diversity among the selected lines by regularly crossing lines among blocks (Rinderer et al. 2000, Bourgeois and Rinderer 2009, Bourgeois et al. 2010). This promotes heterozygosity.

Diversity measurements indicated that levels that were present in the initial release have not been compromised and the allelic structure in the original release has minimally changed over time. Allelic richness is an important parameter to monitor, as it is very sensitive to the effects of population bottlenecks and reveals the long term, or evolutionary, potential of the population (Nei et al. 1975, Leberg 1992, Spencer et al. 2000). While overall allelic richness values were similar, the notable decrease of 4 loci may be a response to the dramatic decrease in "passing" colonies the second year post release. This reduced the number of breeder colonies used in the subsequent season. Gene diversity is a shorter time-scale parameter, in that it represents the current heterozygosity present in each population and is predictive of immediate population response to potential bottlenecks. Gene diversity levels were similar for 2010 and 2016, indicating that the allelic framework (i.e., allelic richness and structure) of the stock has remained consistent.

Measures of population structure, with the stock representing the population, can provide additional indications of stock sustainability. F_{IS} increased between 2010 and 2016. This parameter estimates within population variation and is often referred to as the "inbreeding coefficient." An increase is indicative of a reduction of observed heterozygosity. The increase in F_{IS} observed between the 2010 and 2016 is relatively small reflecting acceptable levels of diversity within the stock. Some increase in F_{IS} was expected due to

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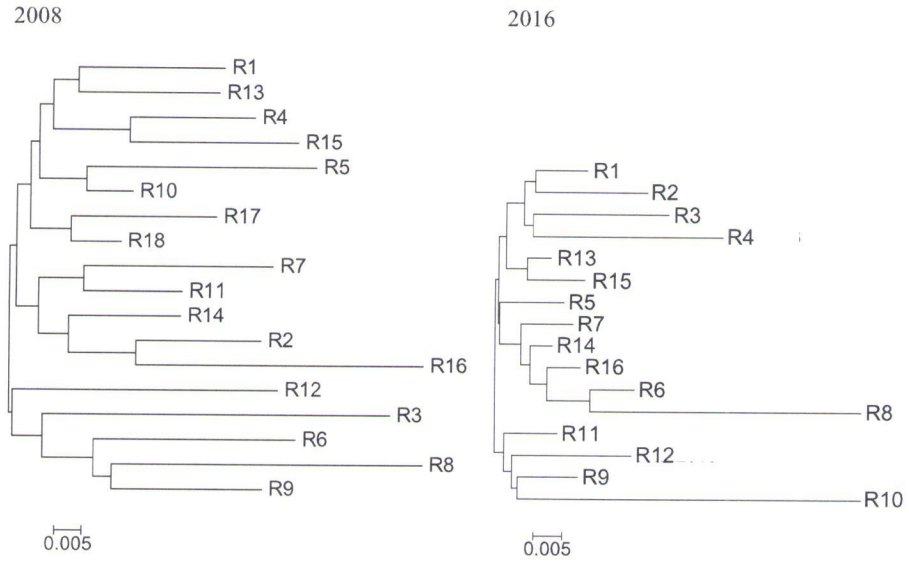

Fig. 2. Neighbor-joining phenogram of Russian honey bee lines and Non-Russian commercial bees sampled in 2008 and 2016. The phenograms were generated from chord distances (Cavalli-Sforza and Edwards 1967) based on genotypes of nine microsatellite loci. Both phenograms are of the same scale.

the nature of the closed breeding system and the decrease in passing colonies in the second year post release. F_{ST} (an estimate of population structure) was low when the 2010 and 2016 populations were compared.

Another approach to assessing population structure is to examine the relationship among breeding lines using genetic distance, as measured with chord distance in this instance (Cavalli-Sforza and Edwards 1967). Branch length represents genetic distance between the lines. The most notable attribute in this comparison between the original stock and 2016 bees is the decrease in genetic distance among all Russian lines in 2016. This was expected because lines are continually crossed as defined by the block breeding system mention previously. Line crossing also contributes to the shift in the relationship between lines.

The application of GSI tools to assist in maintenance of stock integrity and diversity in a closed breeding system has been successful. Seven years post release to the beekeeping industry, we find that diversity levels and POA values have been maintained. Introgression of alleles from unknown bee types during the interim period after stock release may have contributed to the diversity numbers measured; however, POA values recovered while not sacrificing the evolutionary potential of the stock, as measured with allelic richness. The 2016 stock is not genetically differentiated from the 2010 stock, which is also supportive of continuance of the current breeding system. Application of GSI tools to Russian bee breeding continues to serve as a model for the applicability of this approach for certification of other honey bee stocks and other beneficial insect species. Genetic certification can add economic value to breeding stock by increasing consumer confidence and by ensuring that queen and package producers are accurately representing their products.

Acknowledgments

We thank S. O'Brien and T. Adams for assistance with sample processing. We also thank the beekeepers of the RHBA for providing samples from commercial colonies. Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the U.S. Department of Agriculture and does not imply approval to the exclusion of other products that may be suitable.

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