Genetic Stock Identification of Russian Honey Bees

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ABSTRACT A genetic stock certification assay was developed to distinguish Russian honey bees from other European (*Apis mellifera* L.) stocks that are commercially produced in the United States. In total, 11 microsatellite and five single-nucleotide polymorphism loci were used. Loci were selected for relatively high levels of homogeneity within each group and for differences in allele frequencies between groups. A baseline sample consisted of the 18 lines of Russian honey bees released to the Russian Bee Breeders Association and bees from 34 queen breeders representing commercially produced European honey bee stocks. Suitability tests of the baseline sample pool showed high levels of accuracy. The probability of correct assignment was 94.2% for non-Russian bees and 93.3% for Russian bees. A neighbor-joining phenogram representing genetic distance data showed clear distinction of Russian and non-Russian honey bee stocks. Furthermore, a test of appropriate sample size showed a sample of eight bees per colony maximizes accuracy and consistency of the results. An additional 34 samples were tested as blind samples (origin unknown to those collecting data) to determine accuracy of individual assignment tests. Only one of these samples was incorrectly assigned. The 18 current breeding lines were represented among the 2009 blind sampling, demonstrating temporal stability of the genetic stock identification assay. The certification assay will be used through services provided by a service laboratory, by the Russian Bee Breeders Association to genetically certify their stock. The genetic certification will be used in conjunction with continued selection for favorable traits, such as honey production and varroa and tracheal mite resistance.

KEY WORDS honey bee, genetic stock identification, single-nucleotide polymorphism, microsatellite

Releases of genetically improved stocks and varieties in agriculture often require some means of certification to maintain and confirm stock integrity. With readily available molecular genetic methodologies, stock certification can be carried out using molecular markers that offer high reproducibility and accuracy. The use of genetic certification in conjunction with continued phenotypic selection for economically important traits will facilitate the maintenance of robust stock integrity.

Russian honey bee queens were first brought into the United States in 1997 to improve resistance to the mite *Varroa destructor* Anderson & Trueman, in managed honey bee stocks (Rinderer et al. 1997, 2005). Yearly importations continued through 2002. A closed breeding population of 18 lines was established and underwent selection for improved resistance to varroa and tracheal mites, *Acarapis woodi* (Rennie), and good honey production. The genetic improvement of these lines increased the economic value of the stock (De Guzman et al. 2001; Rinderer et al. 2001a,b, 2003, 2004; Tubbs et al. 2003). All matings occurred on an island in complete genetic isolation from outside (i.e., commercial or feral) sources. The breeding plan consisted of dividing the 18 lines into three groups and cross-breeding those groups to maintain genetic diversity (Rinderer et al. 2000). Diversity remains high among the current breeding stock with no indication of inbreeding (Bourgeois and Rinderer 2009). The final release of these lines was completed in 2008 to members of the recently established Russian Bee Breeders Association (RBBA).

A current breeding plan that incorporates the 3-block design was adopted by the RBBA. The 18 individual members each produce sibling groups of two of the 18 lines and selectively breed for further improvements. They use the appropriate drone sources for their lines that they receive from other members. Hence, although the breeding population remains closed, the breeding is done in many locations. Members all have some degree of isolation in mating yards. For the most part, "drone flooding" produces a very high proportion (≈95%) of desired mat-

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ings (Hellmich and Waller 1990, Hellmich 1991). Selection for varroa and tracheal mite resistance as well as superior honey production will be continued by the RBBA. Because Russian hybrid colonies have intermediate resistance to *V. destructor* (Harris and Rinderer 2004), colonies in selection apiaries that have queens with some degree of mismating (i.e., drone flooding was unsuccessful) should perform less well on colony evaluations for *V. destructor* population growth and show introgression of non-Russian alleles during genetic stock certification and be culled from the program. To maintain the genetic integrity of these 18 RHB lines, some method of stock identification and certification is necessary in addition to continued phenotypic selection. The most definitive method of stock certification is through the development of molecular tools for specific identification.

Genetic stock identification (GSI) using microsatellite markers is a well-established technique among salmonid fisheries in the northwestern United States and is used regularly to identify local origins of spawning populations (Potvin and Bernatchez 2001; Beacham et al. 2005a,b; Moriya et al. 2004; Skaala et al. 2004; Moriya et al. 2007). GSI using microsatellites also has been used with very high accuracy for channel catfish (Waldbieser and Wolters 2007) and swimming crabs (Obata et al. 2006). The development of a useful GSI profile rests on thorough sampling and molecular characterization of baseline populations. A baseline population constitutes the possible genotypes in the potential pool of samples for each population in question. In honey bees, *Apis mellifera* L., this would include a thorough sampling of commercially available stocks of honey bees in the United States, including those of Russian and other European origins. Identifying genetic differences between the two groups is imperative for GSI to be successful. However, some degree of uniformity within each group of the baseline also is desirable. Once the baseline population has been identified and genotyped, mixed stock analysis can provide the basis for assignment tests of individuals to their population or stock of origin. Mixed stock analysis is a comparison of allele frequencies from the unknown samples to those of the baseline population.

The purpose of this study was to develop a suitable panel of molecular markers to correctly identify Russian honey bees and distinguish them from other stocks of commercially available honey bees in the United States. Regular colony evaluations will be used in conjunction with this genetic stock certification assay to maintain the integrity of the 18 Russian lines.

Materials and Methods

Sample Collection. A baseline sampling group was established in March 2007 that provided a wide range of genotypes to examine for loci that could potentially distinguish between U.S. stocks of Russian and non-Russian origin. For the baseline samples, worker bees were sampled from colonies representing the stocks of bees that are currently available for commercial beekeeping in the United States. Non-Russian bees were supplied from 34 queen producers, representing the primary stocks sold in the United States, including Italian, Carniolan, Caucasian, SMR, MN Hygienic, and other proprietary stocks bred by specific queen breeders. All non-Russian bees were obtained from commercial queen breeders, as these are the stocks that are most likely to hybridize with Russian stock for commercial production. The Russian stock consisted of the 18 lines of Russian honey bees. These lines comprise the genetic stock that was the 2008 release to the RBBA. All Russian samples ($n = 138$) were collected from apiaries managed by the USDA–ARS Honey Bee Breeding, Genetics, and Physiology Laboratory in Baton Rouge, LA. Emerging worker bees were collected from each of the 18 Russian honey bee lines, placed on ice, and subsequently stored at −70°C until used for DNA extraction. Worker bees ($n = 116$) representing commercially produced stocks of non-Russian honey bees were submitted by commercial producers and stored either in 70% ethanol or frozen at −70°C until DNA extraction.

An additional set of 34 samples (one bee per colony) of a collection of both Russian and non-Russian colonies was collected in March 2009 and processed to further evaluate the robustness of discriminatory markers identified from the baseline samples. A subset of the Russian colonies tested represented the 18 lines of Russian bees used for the baseline. The bees were collected from the same genetic lines, but from different colonies. The origin of the individual samples in this sample set was not known by the authors until after analysis.

DNA Extraction. DNA was extracted from the thorax of individual bees (eight bees per sampling unit (i.e., line or stock). Samples were first homogenized in 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 eight 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. NH_4OAc (7.5 M) was then added for protein precipitation, followed by ethanol precipitation and lyophilization. Pure genomic DNA was rehydrated in Millipore filtered and deionized distilled H_2O (Millipore, Billerica, MA) and stored at −20°C.

Genotyping. *Microsatellites.* In total, 96 microsatellite markers (Solignac et al. 2007) were screened for polymorphism among the DNA samples. Eleven showed differences in allele frequency patterns among Russian and non-Russian stocks and were used for subsequent analyses. Primer sequences, GenBank accession numbers, and amplification conditions are listed in Table 1. In addition to the forward and reverse primers, an additional primer (Univ.) was used (Schuelke 2000). The Univ. primer sequence is M13 (GAGTTTTCCCAGTCACGAC). This primer was modified with one of three fluorescent dyes (WellRed dyes, Integrated DNA Technologies, Coralville, IA) to facilitate pool-plexing of polymerase chain reaction (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 lo-

Table 1. Microsatellite and SNP locus and primer information[a]

Locus	Forward primer	Reverse primer	Repeat unit	Fragment size	GenBank accession
Microsatellites					
6339	CGCACACGACATGCATATCC	ATCTGCTGCAGAGGGTCGAG	AAT	146	BH816339
6904	ATGACCCGATACTCGCATCTG	TGCCCATGCTTGTATCTCCC	CT	143	BH816904
A064	TCACGACCTACACCGGCTTT	GGCGAATATTCTCGAGACTCGA	TC	99	AJ509267
AC032	TGCCACCAGACGTAGGCA	TGCGTTAATTCCAGGGTGC	GT	134	AJ509641
K0457B	CCCACTATGACGTGTATCCCAT	AACCGAGGCACGTTCGAT	TC	116	AADG05001811
K0725	TGACATTCCTCTCGCTCGTC	AGCATCGTACCATTCGTATCTACC	AT	115	AADG02003867
SV131	GAGGATATTACCGCGTGAGAATC	ACTTCGTAACGCGTGGGG	TC	128	BI506633
SV167	GATCTTCGCGATTCTTCGCA	CTGGTTCACACGGGCAGGTA	AAT	221	BI506173
SV220	TTTCTCGCGTAGAATGTAGAATAGG	AAGGATTTGCCTGCTACATGAC	AAT	185	BI508658
UN099	ATCGAATCTCGCTCATCAGCT	CGTGTGTACATTGGAAGTGTGC	GA	195	AADG02008466
UN333B	GTGTAACCTGCTCACTTCTTGGA	CACGCATGCACGTTCTCA	AT	172	AADG05008174
SNPs			Allele	Melting temp (°C)	dbSNP no.
AMB-00960962	FM: CGCCGGCGCCGGCCGCACAAATATCCACCTCCACC*T*A**C**	TGTCAGCCGTTCTTTCTTCCTTC	C/T	78	rs45012879
	F-WT: ACAAATATCCACCTCCACCG**A**T			75	
AMB-00978904	FM: CGCCGGCGCCGTGGTTCGTTCACCAG*G*G**A**	TCATAGAATCGAACACGATGGAGA	A/G	77	rs45051874
	F-WT: CGTGGTTCGTTCACCAG*T*G**G**			74	
AMB-00966258	FM: CGCCGGCCGCGGACGACATCAGCGAGGAGA*A*G**T**	TCAAGTCTTCTTCGACGACATTG	T/A	80	rs45017567
	F-WT: ACGACATCAGCGAGGAGACGA			78	
AMB-01070334	FM: CGCCGCGGCGGGTCGGGACTCGATC*T*T**G**	AGGGTTGCCGTGCCATTA	G/C	81	rs45121296
	F-WT: GGTCGGGACTCGATC*G*T**C**			79	
AMB-00468928	FM: CGGCCGCGCCGACATAGAAGCTAGCCAAAGGC*G*G**T**	CGAGATAGCGCCAGAGATGC	C/T	80	rs44543186
	F-WT: ACATAGAAGCTAGCCAAAGGC*T*G**C**			78	

[a] The first primer listed for each SNP locus was modified to include the alternate allele at the terminal base (in bold), a mismatch pair three bases from the terminal base (in italics), and additional G and C bases (underlined) at the 5′ end to increase melt temperature. Microsatellite locus names and primer sequences are as determined by Solignac et al. 2007). SNP nomenclature follows the honeybee SNP database (in NCBI dbSNP).

cus was modified at the 5′ end by the addition of the complementary sequence to the Univ. primer.

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× PCR buffer II (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.01% (wt:vol) gelatin; Applied Biosystems, Foster City, CA), 1.5 mM $MgCl_2$, 0.35 U of 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.

Single-Nucleotide Polymorphisms (SNPs). In total, 96 SNPs (single nucleotide polymorphisms (Whitfield et al. 2006) were screened for polymorphism that showed differential allele frequencies for Russian and non-Russian honey bees. Of these, five showed differences in allele frequency patterns and were used for subsequent analyses. For each SNP locus three primers were designed to enable allelic discrimination using melt-curve analysis (Boniotto et al. 2005). Primers were designed using Beacon Designer 7.0 (Premier Biosoft, Inc., Palo Alto, CA). In this method of primer design, two forward primers and one reverse primer are designed. The two forward primers are designed such that the terminal base consists of one of the two bases that comprise the SNP locus. One of the forward primers has two additional modifications; a mismatch at the third vase from the 3′ end and an additional six to 10 G and C bases that function to increase the melting temperature, hence the ability to discriminate the two SNP alleles with melt-curve analysis. Melting temperature calculations were completed in Oligo Calc web-based software (Kibbe 2007). Primer sequences are shown in Table 1. PCR reactions and melt curve analyses were run on a StepOne Real-Time PCR System (Applied Biosystems) using Fast PCR methodology. The optimized amplification profile was 20 s at 95°C, followed by 40 cycles of 3 s at 95°C, 30 s at 60°C. This was followed by melt curve analysis. The temperature profile for melt-curve analysis was 15 s at 95°C followed by 1 min at 60°C and then an increase in temperature of 0.3°C every 15 s until a final temperature of 95°C. Each 12.5-μl reaction included 1.5 pmol of each primer (F-WT, F-M, and R); 1× Fast SYBR Master Mix (Applied Biosystems), and template DNA (50–100 ng).

Data Analysis. All baseline analyses, simulations, and individual assignment tests were performed in ONCOR (http://www.montana.edu/kalinowski), genetic stock identification, and mixed stock analysis software. The baseline samples were tested for accuracy using a 100% simulation test in which a single population is simulated and tested for accuracy of assignment using the method of (Anderson et al. 2008). The method simulates mixture genotypes and then estimates their occurrence in the baseline population, using 1,000 simulations and a sample size of 100. An additional test was used to determine how well individuals could be assigned to their population of origin. In this test, individual genotypes were removed from the baseline population and then assigned to a population of origin.

Table 2. Probability of genic differentiation and numbers of alleles for 11 microsatellite loci used to distinguish Russian and Non-Russian honey bees

Locus	*P*	No. alleles	
		Russian	Non-Russian
SV167	<0.0001	4	7
K0457B	<0.0001	11	9
SV220	0.0385	6	10
6339	<0.0001	6	4
K0725	<0.0001	7	7
UN333B	0.0002	4	5
UN099	0.0002	6	5
SV131	<0.0001	8	7
6904	<0.0001	8	9
A064	<0.0001	5	7
AC032	0.0156	9	7

The number of worker bees per sample that was required to achieve a high probability of group membership was determined by conducting simulations, as described above, with the addition of using varying sample sizes.

Genetic distance (Cavalli-Sforza and Edwards 1967) based on microsatellite data were calculated in Genetix software (Belkhir et al. 1996). Genic differentiation was calculated in Genepop (Rousset 2008). The relationship of samples in the baseline, grouped by type for non-Russian bees and by line for Russian bees, was calculated using MEGA4.1 (Tamura et al. 2007).

Results

Eleven microsatellite loci and five SNP loci were used to successfully distinguish RHB from commercially produced European bees in the United States (Table 1). Russian and non-Russian bees were differentiated for all loci ($P < 0.05$). Numbers of microsatellite alleles varied by locus and for each group (Table 2). Allele frequencies of SNP loci also varied by locus among groups (Fig. 1).

In the simulation assignment test, the proportion of correctly assigned individual bees was 94.2% for the non-Russian group and was 93.3% for the Russian group. The 100% simulation test yielded results of 0.9977 ± 0.0054 for the non-Russian group and 0.9972 ± 0.0061 for the Russian group for likelihood of correct assignment. These numbers represent the frequency of individual samples assigned to either the Russian or non-Russian group. The correct value was a frequency of 1.0 in each test. In all simulated samplings, the simulated genotype was correctly assigned at a minimum frequency of 0.9559.

Table 3. Probability of assignment of 34 blind-sampled honey bees

Sample[a]	Group assignment	Probability of assignment	Correct assignment
1	**Russian**	1.00	Yes
2	Non-Russian	1.00	Yes
3	Non-Russian	0.99	Yes
4	Non-Russian	0.96	Yes
5	**Russian**	1.00	Yes
6	Non-Russian	0.93	Yes
7	**Russian**	1.00	Yes
8	Non-Russian	1.00	Yes
9	**Russian**	1.00	Yes
10	Non-Russian	0.58	Yes
11	**Russian**	1.00	Yes
12	**Russian**	1.00	Yes
13	**Russian**	1.00	Yes
14	**Russian**	1.00	Yes
15	**Russian**	1.00	Yes
16	**Russian**	1.00	Yes
17	Non-Russian	1.00	Yes
18	Russian	0.93	Yes
19	Non-Russian	1.00	Yes
20	Russian	0.99	No
21	**Russian**	1.00	Yes
22	**Russian**	1.00	Yes
23	**Russian**	1.00	Yes
24	Non-Russian	1.00	Yes
25	**Russian**	1.00	Yes
26	Non-Russian	0.64	Yes
27	**Russian**	1.00	Yes
28	**Russian**	0.99	Yes
29	Non-Russian	1.00	Yes
30	Non-Russian	1.00	Yes
31	Russian	0.97	Yes
32	**Russian**	1.00	Yes
33	**Russian**	1.00	Yes

[a] Samples in bold were collected from the 18 Russian breeding lines in 2009.

In a test of the effect of sample size on accuracy of assignment, for samples sizes of 1–96, accuracy rates ranged from 0.9758 to 0.9973 for non-Russian bees and 0.9813–0.9978 for Russian bees. Within this range, increased number of worker bees per sample increased the accuracy of the assignment (Fig. 2).

Of the 34 "blind" samples tested, one was incorrectly assigned (Table 3). This incorrect assignment was at a high probability (0.98). This extreme outlying result suggests that a sampling or labeling mistake may have occurred. The probability of assignment ranged from 0.92 to 1.0 for 32 of the 34 samples (Table 3). The remaining two samples had relatively low probabilities of assignment of 0.58 and 0.64 but were correctly assigned to the non-Russian group. Such intermediate values suggest that hybridization between Russian and Italian stocks may have occurred.

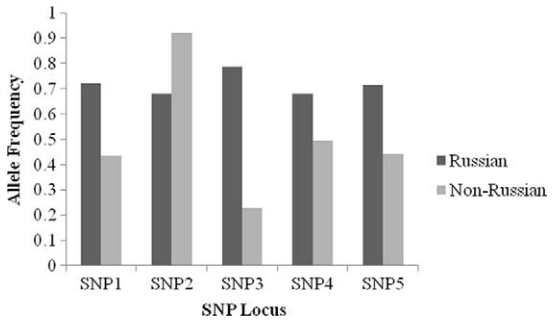

Fig. 1. Allele frequencies of five SNP loci used for stock certification of Russian honey bees. Only one allele per locus is shown. The allele selected is that with the highest frequency among Russian samples.

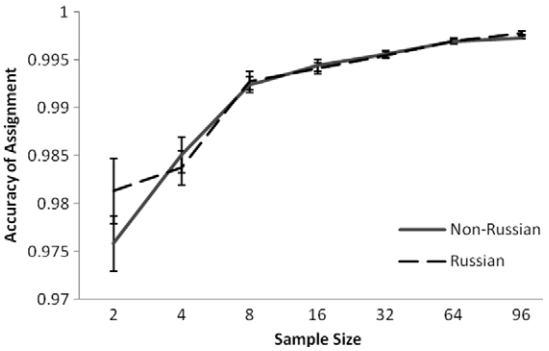

Fig. 2. Probability of assignment of Russian and non-Russian honey bees, with varied numbers of worker bees per sample based on 1,000 simulations for each stock by sample size combination. Data for each sample size are generated of from simulations of mixture genotypes and then estimates of their occurrence in the baseline population.

Chord distances were calculated for all combinations among the baseline samples. The neighbor-joining phenogram (Fig. 3) demonstrates the distinction between all Russian and non-Russian groups used in the baseline sample. The linearized tree (Fig. 4) shows more branching among the Russian lines than among the non-Russian groups.

Discussion

The GSI assay was capable of distinguishing Russian and non-Russian commercially produced honey bee stocks. Allele frequencies of 11 microsatellite and five SNP loci were used in combination to assign individual samples to one of the two groups, with high probability of correct assignment. This identification procedure had a power of identification similar to GSI procedures based on microsatellite data, used by salmonid fishery biologists who regularly use GSI to distinguish stocks from different watersheds and determine assignment of individual fish to particular stocks (Winans et al. 2004, Templin et al. 2005, Moriya et al. 2009).

The baseline sample consisted of a thorough sampling of commercially produced non-Russian stocks in the United States and the 18 lines of Russian bees recently released to the RBBA. Genetic distance measures showed a higher degree of branching among the Russian lines than among the non-Russian stocks. This may be an artifact of marker selection. The markers selected for this study differ from those used in previous studies assessing genetic diversity for both Russian and Italian honey bees (Bourgeois et al. 2008, Bourgeois and Rinderer 2009). The markers in the

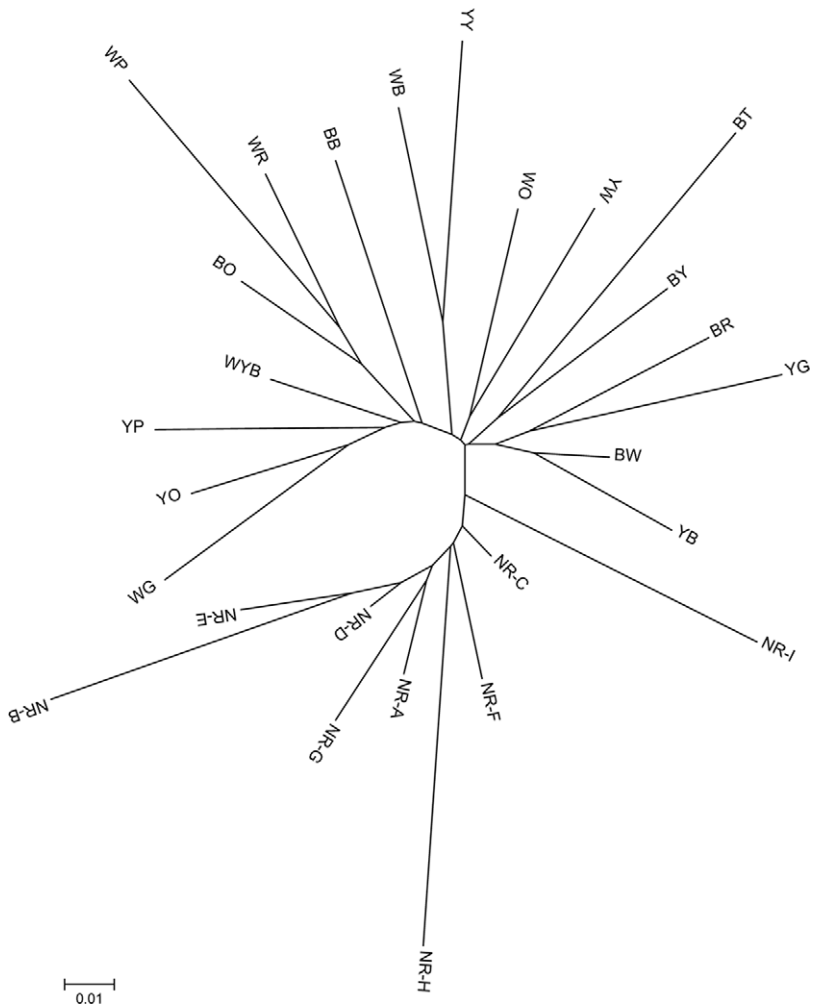

Fig. 3. Neighbor-joining phenogram of chord distances based on 11 microsatellite genotypes of Russian and non-Russian honey bees. NR, non-Russian sources.

current study were selected for homogeneity within each grouping (i.e., Russian or non-Russian). To see differences in relative diversity was unexpected. An alternative explanation is that some level of homogeneity has been produced by many years of crossbreeding among the non-Russian stocks which is manifested in relatively low levels of diversity with this suite of loci. However, this is contradictory to results from other marker suites we have used to assess diversity among Italian honey bees.

When testing the effects of sample size on accuracy of assignment, the probability of assignment of individual bees to the correct group was high, even when small sample sizes were used in the test. However, our samples were either "pure" Russian or "pure" non-Russian. Some colonies may have queens that are Russian–non-Russian hybrids, as was seen in the blind samples. Lower probabilities of group membership indicate possible hybrid queens. Also, colonies that have "pure" queens and mixed patrilines may show overall low probabilities when a colony mean is calculated, even if individuals representing separate patrilines are strongly Russian or non-Russian.

The test of "blind" samples was successful, in that only one sample was incorrectly assigned. It is unknown if Russian breeding stock has been recently introduced into the breeding program for this particular stock. Two other samples had marginal levels of Russian alleles. These stocks are known to co-exist in apiaries containing Russian bees and the opportunity for hybridization is very high. Hence, based on the possibility for hybridization between Russian and non-Russian stocks and the asymptotic shape of the probability curve, we recommend sampling a minimum of four worker bees per colony, preferably eight, and using a lower threshold of assignment probability of 0.70 when the group mean per colony is taken.

A 2009 sampling of the 18 established lines of the Russian breeding stock were included in the blind sampling. These samples were taken from the same

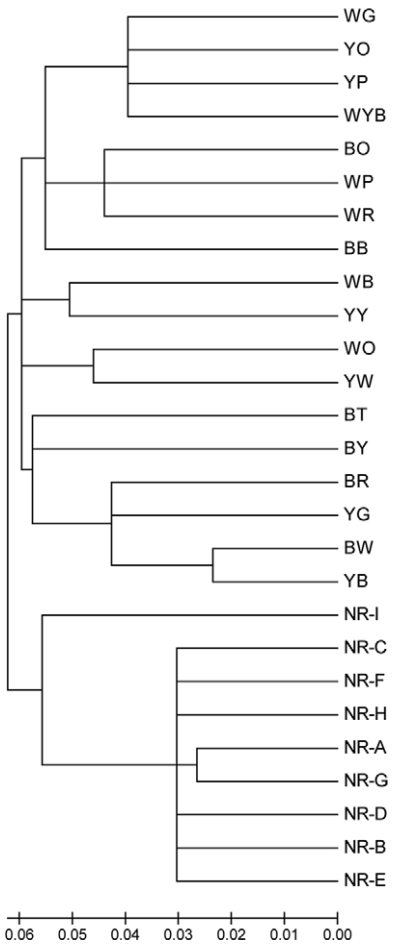

Fig. 4. Linearized phenogram of chord distances based on 11 microsatellite genotypes of Russian and non-Russian honey bees. NR, non-Russian sources.

genetic lines as the baseline samples, but from different colonies 2 yr after the original sampling. These samples demonstrate the temporal stability and robustness of the GSI assay.

This GSI tool will be used by the RBBA, via a service laboratory, for certification of Russian lines. Regular colony evaluations of economically important traits will be used in conjunction with genetic stock certification to maintain the integrity of the Russian stock over a long time interval. Maintenance of stock purity is difficult using solely a selection-based approach, because of the inability to phenotypically identify hybridized bees, just as using only GSI for stock maintenance would not be successful without continued selection pressure on favorable traits. The two approaches work well together to perpetuate a pure and selected stock.

Using GSI tools such as that described here would be appropriate and beneficial for producers of other genetically distinct stocks and will permit a major shift in how bee breeding is conducted in the United States. This particular assay has the sensitivity and robustness to facilitate alternative uses such as measurement of introgression of Russian alleles into other genetically distinct stocks or vice versa. Application of the GSI methodology to Russian bees serves as a model for the great applicability of this approach for certification of other honey bee stocks and other beneficial insect species. Genetic certification adds economic value to breeding stock by increasing consumer confidence in the product by ensuring that queen and package producers are accurately representing their production lines.

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