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RESEARCH ARTICLE

Estimation of C-derived introgression into *A. m. mellifera* colonies in the Russian Urals using microsatellite genotyping

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Abstract

Background Marker-assisted selection is well established in animal breeding method of selecting individuals with desirable traits in a breeding scheme based on DNA molecular marker patterns.

Objective Genetic diversity and C-derived admixture into local purebred gene pool of *A. m. mellifera* colonies was assessed using polymorphism of nine microsatellite loci in order to provide further marker-assisted selection of desired honey bee colonies.

Methods The genetic diversity and the level of C-derived introgression into *A. m. mellifera* colonies in the Shulgan-Tash Nature Reserve (Russia) was assessed based on nine microsatellite loci (ap243, 4a110, A24, A8, A43, A113, A88, Ap049, A28), which were analized using the fragment analysis of the PCR products in Applied Biosystems 3130 DNA Analyzer. Phylogenetic relationship of colonies was evaluated using Neighbor-Joining methods with Cavalli-Sforza and Edwards genetic distance using the PHYLIP 3.68. The model-based Bayesian clustering algorithm implemented in STRUCTURE 2.3.3 was employed to infer membership and introgression proportions (Q-value).

Results In the Shulgan-Tash Nature Reserve colonies of *A. m. mellifera* subdivided into four groups by level of C-derived introgression. Only five colonies of *A. m. mellifera* had C-derived introgression which varied from 0.5 to 2%. The genetic diversity in colonies of *A. m. mellifera* varied from 0.12 to 0.40. The Neighbor-Joining tree demonstrates the genetic relationship of *A. m. mellifera* colonies, which subdivided into three groups with different levels of C-derived introgression. Group 1 combined five honey bee colonies Bort_1, Bort_2, Bort_3, Baisalyan_1, and Kush_7 with a fraction of introgression close to 0.000 and genetic diversity from 0.20 to 0.25.

Conclusion The results showed the excellence of nine microsatellite loci genotyping in estimation of genetic diversity, distinguishing the two European evolutionary lineages M and C and estimating C-derived introgression. These genetic parameters can be applied further to perform the marker-assisted selection of purebred dark European honey bees.

Keywords *Apis mellifera mellifera* · *Apis mellifera caucasia* · Dark European honey bee · Genetic diversity · C-derived introgression

Introduction

The honey bee, *Apis mellifera*, is an essential pollinator that plays a positive role in both agriculture and ecology (Southwick and Southwick 1992; Klein et al. 2007; Hall et al. 2020). About 30 allopatric subspecies have been identified throughout Africa and Europe, 24 of which in Europe (Ruttner 1988; Hepburn et al. 1998; Engel 1999; Sheppard and Meixner 2003; Meixner et al. 2011; Ilyasov et al. 2015, 2016). Among all subspecies only the dark European honey bee *Apis mellifera mellifera* well adapted to the cold continental climate of northern Europe (Ilyasov et al. 2017; Wallberg et al. 2019). A critical adaptation of

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A. m. mellifera to the temperate climate was appeared due to the presence of efficient thermoregulation (Adam 1983; Seeley and Visscher 1985; Seeley et al. 2015), formation of a winter cluster in the colony and gathering the food for short summer (Adam 1983; Ruttner 1988; Parejo et al. 2018).

There are growing worries that intensified queen breeding and trading may induce gene flow between native and commercial colonies leading to an irremediable loss of diversity adapted to local conditions (Schneider et al. 2004; De la Rúa et al. 2009, 2013; Büchler et al. 2014; Nelson et al. 2017). The M-lineage of *A. m. mellifera*, which is a substantial portion of its native range in western Europe is heavily threatened by C-derived introgression (Jensen et al. 2005; Soland-Reckeweg et al. 2009; De la Rúa et al. 2009; Nedić et al. 2014; Pinto et al. 2014). Indeed, the influx of honeybee subspecies of the C-lineage (*A. m. ligustica*, *A. m. carnica*, *A. m. carpathica*, *A. m. caucasia*, *A. m. cecropia*) from the south to northern Europe over the past 100 years damaged the gene pool of *A. m. mellifera* (Bouga et al. 2005; Jensen et al. 2005; Soland-Reckeweg et al. 2009; Uzunov et al. 2009, 2014; Stevanovic et al. 2010; Ilyasov et al. 2015; Henriques et al. 2018). To date, M-lineage of the dark European honey bee *A. m. mellifera* would face to an endangered subspecies because of hybridization with the C-lineage subspecies (Ilyasov et al. 2007, 2016).

In an attempt to stop introgression, several conservation projects have been implemented in European countries (De la Rúa et al. 2009; Muñoz et al. 2015). Molecular genetic tools are able to identify pure-bred *A. m. mellifera* colonies reliably and rapidly and thus to check the introgression of C-derived genes in populations (Henriques et al. 2018). Microsatellite loci (Neumann et al. 1999; Scharpenberg et al. 2006; Soland-Reckeweg et al. 2009; Oleksa et al. 2011; Péntek-Zakar et al. 2015) are useful markers for identifying the level of introgression of C-derived genes into gene pools of the native honey bee and monitoring conservation projects of *A. m. mellifera* in many different areas such as the Danish island of Læsø (Jensen et al. 2005), the French region of Landes (Strange et al. 2008), the eastern part of Switzerland and the French Alps (Soland-Reckeweg et al. 2009; Parejo et al. 2018), the north-eastern part of Poland (Oleksa et al. 2011), England, France, Belgium, Denmark, the Netherlands, Switzerland, Scotland, and Norway (Muñoz et al. 2017), the Russian Ural and Volga region (Ilyasov et al. 2016), the Canary Islands for *A. m. iberiensis* (Muñoz et al. 2012), and the Filicudi and Vulcano islands for *A. m. siciliana* (Muñoz et al. 2014).

Genetic diversity is an index that defines the total number of genetic characteristics in the genetic makeup of a species (Jensen et al. 2005; Soland-Reckeweg et al. 2009; Oleksa et al. 2011; Muñoz et al. 2012, 2017). The genetic diversity closely related ecological plasticity and adaptation (De la Rúa et al. 2009; Dietemann et al. 2009; Meixner et al. 2010; Ilyasov et al. 2015; Nelson et al. 2017).

The aim of this paper is to characterize the population structure of the dark European honey bee *A. m. mellifera* from the Shulgan-Tash Nature Reserve of the Burzyanskiy region of the Republic of Bashkortostan (Ural, Russia), including the assessment of genetic diversity and C-derived admixture into local purebred gene pool based on polymorphism of nine microsatellite loci. The opportunity for artificial selection of honey bee colonies characterized by purebredness of *A. m. mellifera* with a high level of genetic diversity and adaptation to cold temperate climate was shown.

Materials and methods

Sixteen adult honey bee workers were collected from every 12 colonies of *A. m. mellifera* in the Shulgan-Tash Nature Reserve of the Burzyanskiy region of the Republic of Bashkortostan (Ural, Russia) and from every three colonies of *A. m. caucasia* in the Bee breeding station Krasnopolyanskaya of the Sochi region of the Krasnodarskiy Krai (Caucasus, Russia) and stored in 96% ethanol at minus 10 °C (Table 1, Fig. 1). The taxonomic affiliation of honey bee colonies was checked by morphometry using Alpatov's method (Alpatov 1948) and the size of mitochondrial intergenic loci COX1-COX2 (Garnery et al. 1993; Ilyasov et al. 2016).

Genomic DNA was extracted from the thoracic muscle tissue with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. DNA samples were stored at − 20 °C until further use. The quality of extracted DNA was analyzed by NanoDrop 1000 (Thermo, USA). The PCR was performed in a thermocycler BIO-RAD T100 (USA) with primers for nine microsatellites loci (ap243, 4a110, A24, A8, A43, A113, A88, Ap049, A28) (Estoup et al. 1995; Haberl and Tautz 1999; Solignac et al. 2003). The fragment analysis of the PCR products was performed in Applied Biosystems 3130 DNA Analyzer (Applied Biosystems, USA).

A neighbor-joining tree based on the microsatellite data and the chord distance of Cavalli-Sforza and Edwards (1967) was constructed using the PHYLIP 3.68 (Felsenstein, 2005) with bootstrap values computed over 2000 replications. The model-based Bayesian clustering algorithm implemented in STRUCTURE 2.3.3 (Pritchard et al. 2000) was employed to infer membership or introgression proportions (Q-value). The program was set up for 550 000 Markov chain Monte Carlo iterations after an initial burn-in of 250 000. Over 20 independent runs for each K (from 1 to 5) were performed to confirm consistency across runs. The output was exported into STRUCTURE HARVESTER 0.6.93 (Earl and Vonholdt 2012), and the estimation of the most probable number of

Table 1 The honey bee samples of *A. m. mellifera* and *A. m. caucasia* collected in Russian Ural and Caucasia

No.	Apiary name (location)	Subspecies	Colony	Number of bees	Short name
1	Tree trunk hollow Bort (Shulgan-Tash, Burzyanskiy region, Russia)	*A. m. mellifera*	1	16	Bort_1
2			2	16	Bort_2
3			3	16	Bort_3
4	Apiary Kush-Elga-Bash (Shulgan-Tash, Burzyanskiy region, Russia)	*A. m. mellifera*	7	16	Kush_7
5			25	16	Kush_25
6			29	16	Kush_29
7	Apiary Kapova Peshera (Shulgan-Tash, Burzyanskiy region, Russia)	*A. m. mellifera*	15	16	Kapova_15
8			24	16	Kapova_24
9			31	16	Kapova_31
10	Apiary Baisalyan (Shulgan-Tash, Burzyanskiy region, Russia)	*A. m. mellifera*	1	16	Baisalyan_1
11			13	16	Baisalyan_13
12			14	16	Baisalyan_14
13	Apiary of Bee breeding station Krasnopolyanskaya (Sochi, Krasnodarskiy krai, Russia)	*A. m. caucasia*	1	16	Krasnopol_1
14			2	16	Krasnopol_2
15			3	16	Krasnopol_3
	Total			240	

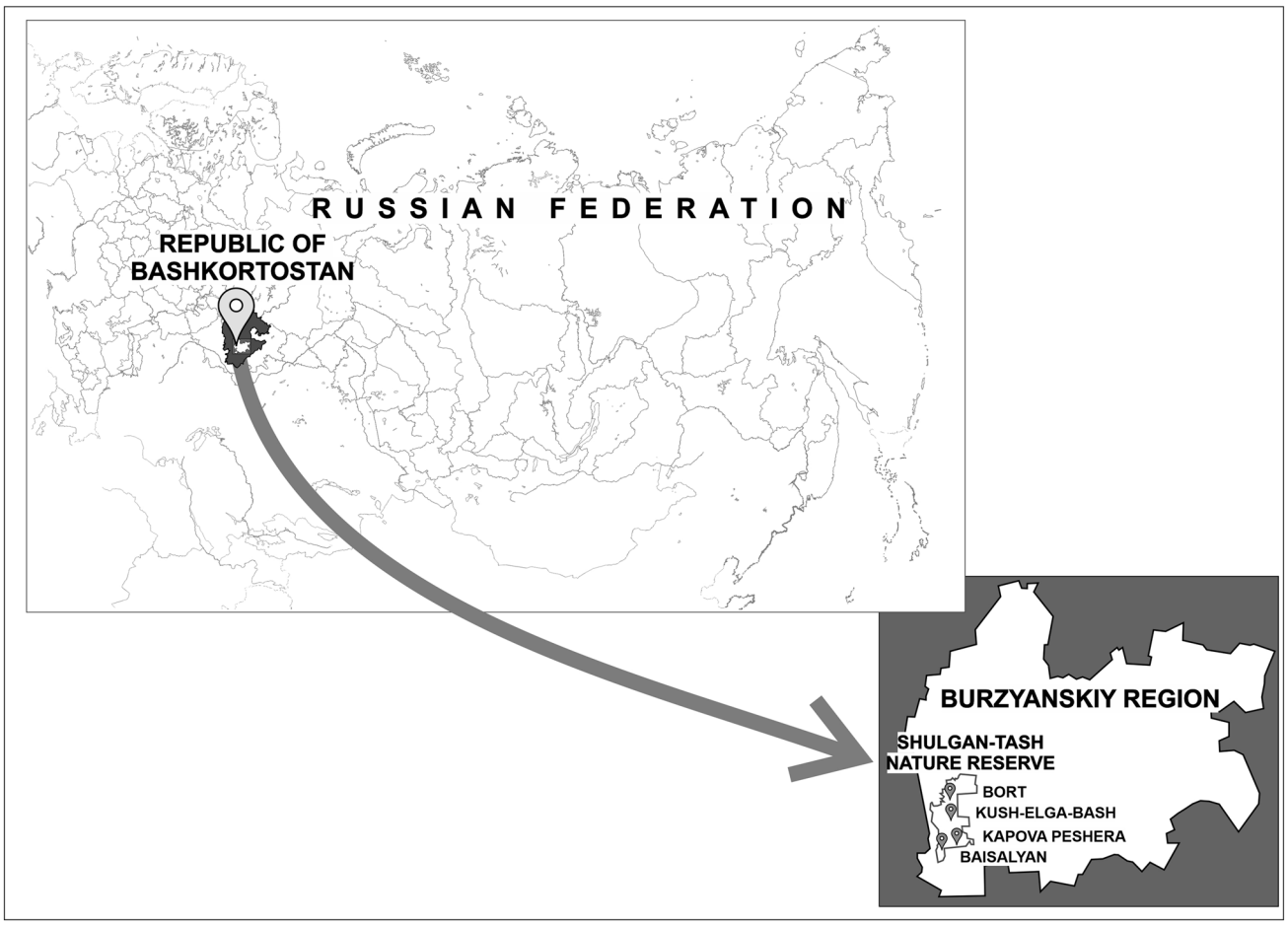

Fig. 1 The spatial geographical localization of collected honey bee colonies in Shulgan-Tash Nature Reserve of the Burzyanskiy region of the Republic of Bashkortostan, Russia

ancestral clusters (K) was calculated as described by Evanno et al. (2005).

The statistical analysis was performed using FSTAT 2.9.3.2 (Goudet 1995), GENEPOP 4.2.2 (Raymond and Rousset 1995), POPULATIONS 1.2.28 (Olivier Langella, CNRS UPR9034 1999), STRUCTURE 2.3.4 (Pritchard et al. 2000), STRUCTURE HARVESTER 0.6.93 (Earl and Vonholdt 2012), PHYLIP 3.68 (Felsenstein 1993), STATISTICA 8.0 (StatSoft, OK, USA), and EXCEL 2010 (Microsoft, CA, USA).

Results

The dark European honey bee, *A. m. mellifera,* is the most economically valuable pollinator in Russia and North European countries (Klein et al. 2007; Gallai et al. 2009; Ilyasov et al. 2016). Major losses of managed honey bee colonies prompted the need to take advantage of locally adapted subspecies and ecotypes to buffer populations against various treats (Neumann and Carreck 2010). The protection of honey bee biodiversity is, therefore, an obligatory (De la Rúa et al. 2009), since current genetic diversity harbors the adaptation potential of a species to the local environment (Frankham et al. 2002; Allendorf et al. 2012). In Russia, the main place for protection and conservation the pure breed gene pool of the native dark European honey bees *A. m. mellifera* is the Shulgan-Tash Nature Reserve located in the forestry mountain region of Urals, which is also isolated from other environments by distance. In order to characterize distinct honey bee colonies, the genetic diversity and the level of C-derived introgression into *A. m. mellifera* in the Shulgan-Tash Nature Reserve was assessed based on nine microsatellite loci.

The polymorphism of nine microsatellites loci in 192 worker bees of *A. m. mellifera* and 48 worker bees of *A. m. caucasia* was analyzed. The sizes of all alleles for each of nine microsatellite loci were evaluated: ap243—3 alleles (254, 257, 260 b. p.), 4a110—3 alleles (160, 163, 168 b. p.), a24—3 alleles (98, 106, 108 b. p.), a8—5 alleles (154, 156, 158, 164, 173 b. p.), a43—4 alleles (128, 134, 140, 142 b. p.), a113—6 alleles (216, 218, 220, 222, 228, 234 b. p.), a88—5 alleles (143, 146, 148, 152, 155 b. p.), ap049—4 alleles (123, 129, 130, 142 b. p.), a28—3 alleles (134, 140, 144 b. p.). The average number of alleles for nine microsatellite loci was 4.

The level of C-derived introgression into *A. m. mellifera* colonies (Q-value of introgression proportions) in the Shulgan-Tash Nature Reserve were assessed using nine microsatellite loci (Table 2, Fig. 2). Most of the colonies of *A. m. mellifera* in the Shulgan-Tash Nature Reserve of the Burzyanskiy region of the Republic of Bashkortostan, Russia did not contain C-derived introgression signatures, but some colonies had a slight fraction of introgression. In apiary Kush-Elga-Bash colony Kush_25 had a fraction of introgression 0.005, colony Kush_29 had a fraction of introgression 0.010. In apiary Kapova Peshera colony, Kapova_15 had a fraction of introgression 0.010, while colony Kapova_31 had a fraction of introgression 0.020. In apiary Baisalyan, colony Baisalyan_14 had a fraction of introgression 0.005. Honey bee colonies in Bort were wild colonies living deeply in the forest in pine tree trunks without human assistance while wintering and involved in natural selection. None of colonies living in tree trunk hollows in Bort did not have any C-derived introgression signatures. The average fraction of C-derived introgression throughout all *A. m. mellifera* colonies in the Shulgan-Tash Nature Reserve is 0.0002. All

Table 2 The genetic diversity and C-derived introgression into *A. m. mellifera* colonies calculated based on polymorphism of nine microsatellite loci

Colonies	Number of bees	M-lineage ± SD/C-lineage ± SD, fraction (M-lineage 90% probability intervals)	The genetic diversity ± SD, fraction
Bort_1	16	1.000 ± 0.003/0.000 ± 0.003 (0.992–1.000)	0.23 ± 0.020
Bort_2	16	1.000 ± 0.004/0.000 ± 0.004 (0.991–1.000)	0.25 ± 0.030
Bort_3	16	1.000 ± 0.003/0.000 ± 0.003 (0.990–1.000)	0.21 ± 0.025
Kush_7	16	1.000 ± 0.002/0.000 ± 0.002 (0.993–1.000)	0.40 ± 0.050
Kush_25	16	0.995 ± 0.002/0.005 ± 0.002 (0.992–1.000)	0.25 ± 0.020
Kush_29	16	0.990 ± 0.004/0.010 ± 0.004 (0.988–1.000)	0.21 ± 0.030
Kapova_15	16	0.990 ± 0.003/0.010 ± 0.003 (0.989–1.000)	0.25 ± 0.033
Kapova_24	16	1.000 ± 0.002/0.000 ± 0.002 (0.992–1.000)	0.21 ± 0.025
Kapova_31	16	0.980 ± 0.002/0.020 ± 0.002 (0.979–1.000)	0.35 ± 0.043
Baisalyan_1	16	1.000 ± 0.003/0.000 ± 0.003 (0.991–1.000)	0.20 ± 0.030
Baisalyan_13	16	1.000 ± 0.003/0.000 ± 0.003 (0.990–1.000)	0.12 ± 0.050
Baisalyan_14	16	0.995 ± 0.002/0.005 ± 0.002 (0.991–1.000)	0.30 ± 0.040
Krasnopol_1	16	0.000 ± 0.003/1.000 ± 0.003 (0.000–0.008)	0.11 ± 0.045
Krasnopol_2	16	0.000 ± 0.003/1.000 ± 0.003 (0.000–0.010)	0.15 ± 0.020
Krasnopol_3	16	0.000 ± 0.004/1.000 ± 0.004 (0.000–0.007)	0.23 ± 0.035

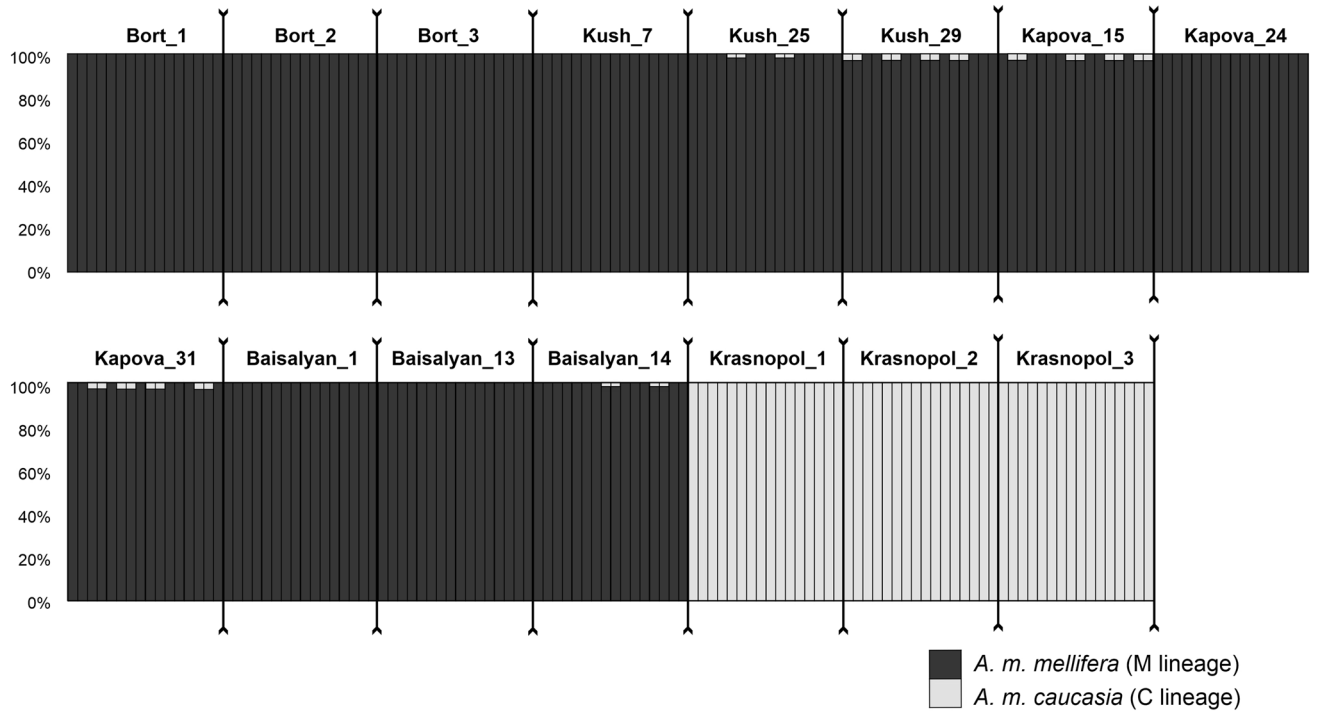

Fig. 2 The plot of C-derived introgression into *A. m. mellifera* colonies constructed in STRUCTURE 2.3.4 (Pritchard et al. 2000) using 550,000 Markov chain Monte Carlo iterations after an initial burn-in of 250,000 based on polymorphism of nine microsatellite loci

colonies of *A. m. caucasia* from the Bee breeding station Krasnopolyanskaya of the Sochi region of the Krasnodarskiy Krai, Russia was purebred without hybridization signatures of M-derived introgression into C-lineage.

The genetic diversity for each honey bee colonies was assessed based on polymorphism of nine microsatellite loci (Table 2). The genetic diversity in colonies of *A. m. mellifera* varied from 0.12 to 0.40, and in colonies of *A. m. caucasia* varied from 0.11 to 0.23, respectively. The average genetic diversity for *A. m. mellifera* colonies was 0.25, while *A. m. caucasia* colonies was 0.16. Seven honey bee colonies of *A. m. mellifera* Krasnopol_1, Baisalyan_13, Krasnopol_2, Baisalyan_1, Bort_3, Kush_29, and Kapova_24 were characterized by the lowest level of genetic diversity from 0.11 to 0.21. Five honey bee colonies of *A. m. mellifera* of Bort_1, Krasnopol_3, Bort_2, Kush_25, and Kapova_15 were characterized by a middle level of genetic diversity from 0.23 to 0.25. Three honey bee colonies of *A. m. mellifera* Baisalyan_14, Kapova_31, and Kush_7 were characterized by the highest level of genetic diversity from 0.30 to 0.40.

In order to construct Neighbor-Joining tree the Cavalli-Sforza and Edwards (1967) pairwise chord distances were calculated using polymorphism of microsatellite loci (Fig. 3). The Neighbor-Joining tree demonstrated the genetic relationship of selected colonies. Three purebred colonies of *A. m. caucasia* from the Bee breeding station Krasnopolyanskaya of the Sochi region of the Krasnodarskiy Krai, Russia were used for comparative analysis as outgroup. All colonies of *A. m. caucasia* were grouped into one common group 4 located separately from all other *A. m. mellifera* colonies. Colonies of *A. m. mellifera* were subdivided into three groups. Group 3 combined two honey bee colonies Kapova_15 and Kapova_31 with a fraction of introgression 0.010 and 0.020, respectively. Group 2 combined four honey bee colonies Kush_25, Kush_29, Kapova_24, and Baisalyan_14 with a fraction of introgression 0.005, 0.010, 0.000, and 0.005, respectively. Group 1 combined six honey bee colonies Bort_1, Bort_2, Bort_3, Baisalyan_1, Baisalyan_13, and Kush_7 with a fraction of introgression close to 0.000.

Discussion

Mass commercial movements of honey bee worldwide have the risk of breaking the genetic integrity of locally adapted ecotypes (De la Rúa et al. 2009; Meixner et al. 2010; Pinto et al. 2014). The honeybee subspecies have evolved very different adaptations to their environmental conditions. Honey bee conservation lies in limiting the beekeeping of native honey bee subspecies in a pure-breeding and conservation area (De la Rúa et al. 2009; Muñoz and De la Rúa 2012; Muñoz et al. 2014; Uzunov et al. 2014; Bertrand et al. 2015).

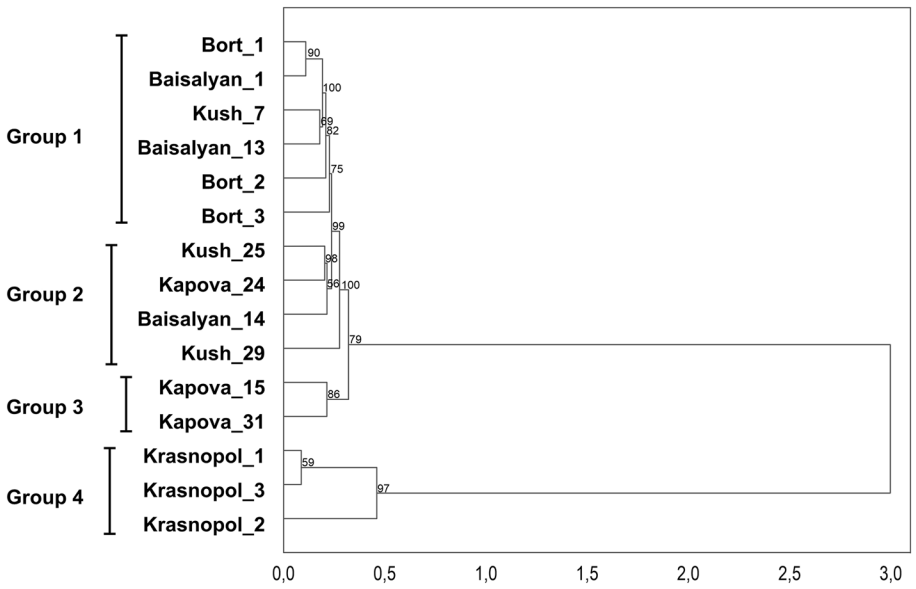

Fig. 3 Neighbor-Joining tree of genetic relationships of honey bee colonies based on the Cavalli-Sforza and Edwards (1967) chord distance based on nine microsatellite loci with 2000 bootstrap replications

Natural hybridization can be defined as the interbreeding of individuals from 2 distinct populations or groups of populations. Individuals in those populations must be distinguishable on the basis of one or more heritable characters (Harrison 1993). Natural hybridization is most easily recognized when previously allopatric populations come together in secondary contact. Renewed sympatry often results in a hybrid zone, with parental types, F1 hybrids, and multiple generation hybrids and backcrosses present in varying proportions. Introgression or introgressive hybridization is the incorporation via hybridization and backcrossing of alleles from one species or subspecies into the gene pool of a second, divergent species or subspecies (Anderson 1968). Introgression is a relative due to the alleles at one locus introgress with respect to alleles at other loci. That is, for the above definition to be applicable, some portion of the gene pool of each of the hybridizing taxa must remain constant and uncontaminated such that we can actually recognize that two distinct gene pools exist. The genes that define the two gene pools and make them distinct are those that comprise the species boundary. Introgression differs from simple natural hybridization. Introgression results in a complex mixture of parental genes, while simple natural hybridization results in a more uniform mixture, which in the first generation will be an even mix of two parental species (Harrison and Larson 2014) (Fig. 4).

Most of *A. m. mellifera* populations have been threatened by C-derived introgression: 30% in Danish island of Læsø (Jensen et al. 2005), 20% in the French region of Landes (Strange et al. 2008), 10% in the eastern part of Switzerland and the French Alps (Soland-Reckeweg et al. 2009; Parejo et al. 2018), 30% in the north-eastern part of Poland (Oleksa et al. 2011), 12% in England, France, Belgium, Denmark, Netherlands, Switzerland, Scotland and Norway (Muñoz et al. 2017), 30% in the Russian Ural and Volga region (Ilyasov et al. 2016). Our study showed the C-derived introgression into *A. m. mellifera* colonies from 0.5 to 2% in the Shulgan-Tash Nature Reserve protected by the Russian state. The low level of C-derived introgression into *A. m. mellifera* can be a result of both natural and artificial isolation.

Genetic diversity within honey bee subspecies populations deserves conservation because it is the most eminent legacy to leave to future generations (Frankham et al. 2002). Native honey bee subspecies hold high genetic diversity and significant combination of traits formed by natural selection (De la Rúa et al. 2009; Pinto et al. 2014), which in the long term are important for species and subspecies survival (Meixner et al. 2010). Once genetic diversity of honey bee subspecies is lost, it cannot be recovered, and thus it deserves conservation (Parejo et al. 2018). The genetic diversity allows long-term sustainability of honey bee subspecies populations (De la Rúa et al. 2009; Meixner et al. 2010). Genetic diversity harbors the evolutionary adaptive potential of a honey bee subspecies to adapt by natural selection to global environmental changes (Frankham et al. 2002; Allendorf et al. 2012). Thus, not only is the conservation of genetic diversity important, but genetic resources are also crucial to conserve as a genetic reservoir pool on which to build resilient agricultural productions systems for future needs and when facing global environmental change (Parejo et al. 2018).

Honey bees have a natural mechanism to increase the genetic diversity in colonies. The genetic diversity in honey bee population of *A. m. mellifera* can be increased by multiple mating of queens with 15–20 unrelated drones (Baudry et al. 1998; Oxley et al. 2010; Harpur et al. 2012).

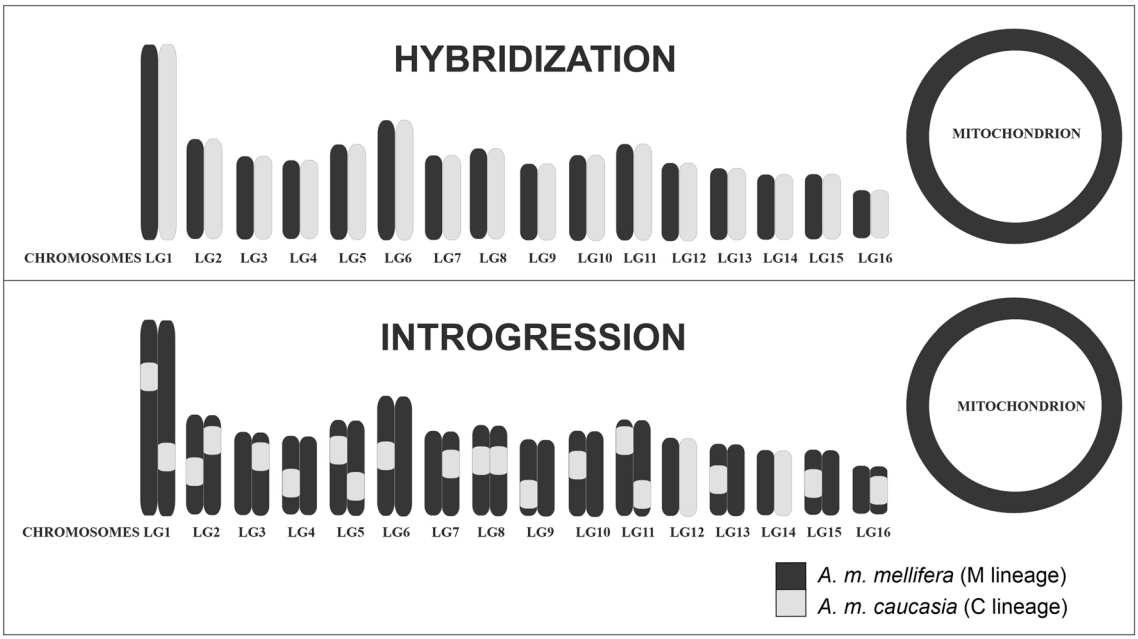

Fig. 4 The scheme of C-derived hybridization and the introgression into *A. m. mellifera* genome

The purebredness in honey bee population of *A. m. mellifera* can be protected by an assortative mating mechanism where purebred dark European honey bee queens prefer mating with only purebred dark European honey bee drones (Oleksa et al. 2013). This multiple mating and assortative mating behaviors allow honey bee colonies of *A. m. mellifera* to adapt to a rapidly changing environment because the offsprings from different purebred drones with various genotypes can live simultaneously in the same colony.

Several studies have demonstrated an advantage of high genetic diversity on reduced intracolonial parasite transmission (Shykoff and Schmid-Hempel 1991) and parasite load on colony performance in bumblebee colonies kept under natural conditions (Liersch and Schmid-Hempel 1998; Baer and Schmid-Hempel 1999). It is shown that high genetic diversity reduces the sickness rate in the honey bee colonies (Woyciechowski and Warakomska 1994; Page et al. 1995). There is a demonstration that genetic diversity reducing the parasitic load in bumblebees *Bombus terrestris* (Estoup et al. 1995). It was reported that the decrease of genetic diversity was able to reduce the immunity, adaptation, and productivity of honey bee colonies (Oldroyd et al. 1992; Page et al. 1995; Fuchs and Moritz 1999; Palmer et al. 2000). In addition, honey bee colonies with higher-level genetic diversity are characterized by the highest performance in comparison with honey bee colonies with lower-level genetic diversity (Palmer et al. 2000). Traits associated with colony fitness such as brood area (Oldroyd et al. 1992; Fuchs and Moritz 1999) and honey production (Fuchs and Schade 1994; Moritz and Neumann 2010) have been shown to be significantly greater in honey bee colonies with a high level of genetic diversity, and tasks associated with colony establishment seem to be more buffered in colonies with high genetic diversity than in colonies with low genetic diversity (Page et al. 1995).

It is known that the genetic diversity in *A. m. mellifera* populations estimated using microsatellite loci does not exceed 0.50: 0.39 in Norway, 0.44 in Sweden, 0.36 in France, 0.36 in Eastern Switzerland (Soland-Reckeweg et al. 2009), 0.49 in north-eastern Poland (Oleksa et al. 2011), 0.25 in the Burzyanskiy district of the Republic of Bashkortostan of Russia (Ilyasov et al. 2016), 0.55 in England, France, Belgium, Denmark, Netherlands, Switzerland, Scotland and Norway (Muñoz et al. 2017). Our study showed the genetic diversity in *A. m. mellifera* colonies varied from 0.12 to 0.40 in the Shulgan-Tash Nature Reserve protected by the Russian state. As the higher level of genetic diversity characterize the higher capacity for adjustments of honey bee colonies to globally changing environments, immunity, adaptation, and productivity of honey bee colonies, we supposed that the purebred dark European honey bee *A. m. mellifera* colonies with a higher level of genetic diversity should be used further as objects for molecular marker-assisted artificial selection in beekeeping. Three honey bee colonies of *A. m. mellifera* with the highest level of genetic diversity (from 0.30 to 0.40): Baisalyan_14, Kapova_31, and Kush_7 have signatures of C-derived introgression 0.5%, 2%., and 0%, respectively.

The Neighbor-Joining tree used for clustering honey bee colonies by their genetic properties. The colonies of

A. m. mellifera clearly divided from colonies of *A. m. caucasia*. Moreover, *A. m. mellifera* colonies are subdivided into three groups depending on their level of introgression. The group 1 combined six purebred honey bee *A. m. mellifera* colonies Bort_1, Bort_2, Bort_3, Baisalyan_1, Baisalyan_13, and Kush_7 with middle level of genetic diversity 0.23, 0.25, 0.21, 0.20, 0.12, 0.40, respectively. All colonies of the dark honey bee *A. m. mellifera* from group 1 excluding colony Baisalyan_13 with the lowest genetic diversity could be recommended for further purebred keeping the dark European honey bees. We assume that honey bees living in the natural conditions in the wild pine tree forests in the tree trunk hollow are able to increase own genetic independently and preserve their own purebredness by evolutionarily fixed mechanisms as multiple mating and assortative mating, which allows adapting to rapidly changing environment.

In summary, the conservation of honeybee diversity is decisive for future needs in apiculture and the valuable pollination services to crops and wild plants (De la Rúa et al. 2009). Colony losses worldwide and the replacement of local honeybees by commercial selected stock endanger the genetic integrity of native subspecies *A. m. mellifera* (Muñoz et al. 2015). Fortunately, the dark European honey bee conservational program has been initiated in the Shulgan-Tash Nature Reserve of the Burzyanskiy region of the Republic of Bashkortostan (Ural, Russia). Our results indicate the excellence of microsatellite loci genotyping in distinguishing the two European evolutionary lineages M and C and estimating C-derived introgression, especially when they are selected by their genetic diversity. The estimation of C-derived introgression into dark European honey bee using nine microsatellite loci genotyping allows developing methods of marker-assisted breeding of purebred colonies of *A. m. mellifera*. The simplicity of analysis, transferability between laboratories, low genotyping error, and low per locus genotyping cost, make microsatellite markers more compliant to the test of tracking C-derived introgression into *A. m. mellifera* across Europe.

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Compliance with ethical standards

Conflict of interest Rustem Ilyasov, Myeong-Lyeol Lee, Ural Yunusbaev, Alexey Nikolenko, and Hyung-Wook Kwon declare that they do not have a conflict of interest.

Informed consent The authors' consent to publish and a copyright transfer.

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