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ORIGINAL ARTICLE

Vocal phenotype of male rutting roars and genetic markers delineate East European red deer (*Cervus elaphus*) from Central and West European populations

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Abstract

This study investigates a population of red deer *Cervus elaphus*, founded by 10 individuals introduced in the nineteenth century from Germany to the Voronezh region of the European part of Southern Russia and then developed without further introductions. We characterize for the first time the vocal phenotype of the Voronezh red deer male rutting calls in comparison with similar data on the Pannonian (native Central European) and Iberian (native West European) red deer obtained by the authors during preceding studies. In addition, we provide for the first time the genetic data on Pannonian red deer. In Voronezh stags, the number of roars per bout (2.85 ± 1.79) was lower than in Pannonian (3.18 ± 2.17) but higher than in Iberian (2.11 ± 1.71) stags. In Voronezh stags, the duration of main (the longest within bouts) roars was longer (2.46 ± 1.14 s) than in Pannonian (1.13 ± 0.50 s) or Iberian (1.90 ± 0.50 s) stags. The maximum fundamental frequency of main roars was similar between Voronezh (175 ± 60 Hz) and Pannonian (168 ± 61 Hz) but higher in Iberian stags (223 ± 35 Hz). Mitochondrial cytochrome *b* gene analysis of red deer from the three study populations partially supports the bioacoustical data, of closer similarity between Voronezh and Pannonian populations. In contrast, microsatellite DNA analysis delineates Voronezh red deer from either Pannonian or Iberian red deer. We discuss that population bottlenecking might affect the acoustics of the rutting roars, in addition to genotype.

Keywords Acoustic variables · Call bouts · Cytochrome *b* · Rutting vocalization · Microsatellites · Voronezh deer

Introduction

Red deer *Cervus elaphus* stags produce their rutting calls for attracting potential mates and deterring competitive males (Clutton-Brock and Albon 1979). Studies of acoustic variation of stag rutting calls (Frey et al. 2012; Passilongo et al. 2013; Della Libera et al. 2015; Volodin et al. 2015a,b, 2019; Golosova et al. 2017) are in agreement with the subdivision of red deer to phylogenetic lineages (Mahmut et al. 2002; Ludt et al. 2004; Skog et al. 2009; Zachos and Hartl 2011; Zachos et al. 2016). The acoustics of stag rutting calls proved to be helpful population markers in red deer (Frey et al. 2012; Passilongo et al. 2013; Volodin et al. 2019) in addition to the genetic markers, such as mtDNA and microsatellites (Feulner et al. 2004; Niedziałkowska et al. 2012; Krojerova-Prokešova et al. 2015; Carranza et al. 2016; Zachos et al. 2016).

Many European populations of red deer were investigated for the acoustics of stag rutting roars (*C.e. scoticus*:

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McComb 1988; Long et al. 1998; Reby and McComb 2003; *C.e. corsicanus*: Kidjo et al. 2008; *C.e. italicus*: Della Libera et al. 2015; *C.e. hispanicus* Frey et al. 2012; Passilongo et al. 2013; Volodin et al. 2015a; *C.e. hippelaphus*: Hurtado et al. 2012; Bocci et al. 2013; Volodin et al. 2019). However, for the easternmost red deer populations of European parts of Russia (Caucasian and Voronezh), data on the acoustics of stag rutting roars are scarce or lacking. For the native Caucasian red deer *C.e. maral* population of the Caucasian region of Russia (Ludt et al. 2004; Trepet and Eskina 2017), only a few spectrograms of stag rutting roars were published (Nikol'skii et al. 1979). For the introduced Voronezh red deer *C.e. hippelaphus* population of Southern Russia (Kuznetsova et al. 2012, 2013; Likhatsky et al. 2012), only the dynamics of male roaring were investigated (Rusin et al. 2021), whereas the acoustics of the rutting roars have yet to be studied.

Until the beginning of the eighteenth century, red deer became extinct in large areas of European parts of Russia except in the Kaliningrad region, the Caucasus, and the Crimea, where the small populations of red deer survived (Heptner et al. 1988). The so-called Voronezh red deer population was established at the end of the nineteenth century from 10 German individuals of unknown origin. They were released at the hunting facility of Prince Oldenburg near the town Voronezh, Russia, to restore the red deer population which became extinct in the European Plain part of Russia due to overhunting to the middle of the nineteenth century (Likhatsky et al. 2012). In 1960–1990, the growing population of Voronezh red deer in this facility (since 1927, "Voronezh State Nature Reserve") was distributed over several regions of the European part of Southern Russia: Voronezh, Belgorod, Lipetsk, Rostov, Krasnodar (Danilkin 1999; Likhatsky et al. 2012).

Aside from Voronezh red deer, two other red deer populations are present in Southern Russia. A population of native Caucasian red deer, also present in the southern European part of Russia, is limited within the borders of the Caucasian Reserve (which is located in the mountains). These animals do not come down to the plains and do not interact with the introduced Voronezh red deer (Danilkin 1999; Trepet and Eskina 2017). A population of red deer retained on the Crimea Peninsula has no direct contacts with Voronezh red deer population; in contrast, some individuals from Voronezh red deer population were released in the Crimea territory, then mixed with remnants of the local red deer population and the introduced Caucasian red deer (Danilkin 1999; Kuznetsova et al. 2013).

Rutting calls of red deer and wapiti (*C. (elaphus) canadensis*) are emitted in bouts from one to several rutting roars (Reby and McComb 2003; Kidjo et al. 2008; Frey et al. 2012; Passilongo et al. 2013; Golosova et al. 2017). The longest calls within bouts represent "main roars" (Frey et al. 2012; Volodin et al. 2019). Consequently, the interpopulation comparison of stag rutting roars can be conducted at the levels of bouts and main roars (Volodin et al. 2019). The variables of main roars used for characterizing populations are the duration, fundamental frequency (f0), and formants (Fant 1960; Titze 1994; Fitch and Reby 2001; Reby and McComb 2003; Taylor and Reby 2010; Frey et al. 2012; Frey and Riede 2013; Volodin et al. 2019). Bout composition can also be used to characterize red deer populations (Frey et al. 2012; Volodin et al. 2019). Rutting stags produce roars of two types: common roars, with a visible f0 and harmonics, and harsh roars, with a f0 masked by deterministic chaos and subharmonics (Reby and McComb 2003; Frey et al. 2012; Passilongo et al. 2013; Volodin et al. 2019).

Red deer stags translocated from their native grounds to other territories retain the population-specific traits of their rutting calls (Volodin et al. 2015a; Golosova et al. 2017). For instance, the acoustics of rutting calls are similar between the aboriginal Scottish red deer stags (Long et al. 1998) and those translocated to New Zealand (McComb 1988). Rutting roars are similar between the aboriginal Pannonian stags (Volodin et al. 2019) and the Austrian-Hungarian stags that originated in Pannonia and then translocated to Argentina (Hurtado et al. 2012). Consistently, similar rutting calls are produced by aboriginal and translocated Siberian wapiti (Volodin et al. 2013b; Golosova et al. 2017). We therefore expected that Voronezh red deer of Central European (German) origin would retain the vocal phenotype of the roars closer to Central European red deer (e.g., Pannonian red deer from southern Hungary, Volodin et al. 2019) and more different from those of West European red deer (e.g., Iberian red deer from southern Spain, Frey et al. 2012; Passilongo et al. 2013). The Pannonian (native Central European, Banwell 1998) and Iberian (native West European, Carranza et al. 2016) red deer are relevant for the bioacoustical comparison with Voronezh red deer, because detailed data on bout structure and the acoustics of main roars only are available for these two populations (Frey et al. 2012; Volodin et al. 2019). An additional advantage is that Pannonian and Iberian red deer represent two large native European red deer populations to which no introgressions of alien red deer were made by humans (Frantz et al. 2017).

After the Late Pleistocene glacial maximum occurred 25–12 thousand years ago (Clark et al. 2009), the species *Cervus elaphus* recolonized Europe (Ludt et al. 2004; Skog et al. 2009; Zachos and Hartl 2011; Niedziałkowska et al. 2021). Recolonization started from the three main refugia, corresponding to red deer mitochondrial DNA (mtDNA) lineages A, B, and C (Ludt et al. 2004; Skog et al. 2009; Niedziałkowska et al. 2011, 2021). Red deer belonging to the A lineage recolonized Western Europe from the Iberian glacial refugium (Ludt et al. 2004; Skog et al. 2009). Red deer B lineage originated in the Italian Peninsula and

formed a relict population, nowadays inhabiting mainly Sardinia and Corsica (Doan et al. 2017). Red deer of C lineage recolonized Eastern and Southern Europe from the Balkan glacial refugium (Ludt et al. 2004; Skog et al. 2009; Niedziałkowska et al. 2011, 2012; Krojerova-Prokešova et al. 2015). Although Voronezh red deer originate from Germany (lineage A), available genetic data suggest that they are close to the C lineage (Kuznetsova et al. 2012, 2013). The Voronezh red deer (mainly from Voronezh and Krasnodar regions) form a separate haplogroup (W5 or E) with other red deer from Europe (Doan et al. 2018). The Iberian red deer belong to A lineage (Carranza et al. 2016). The Pannonian red deer have yet to be studied genetically.

In this study, we characterize the acoustic variables of the bouts and main roars of rutting male Voronezh red deer in comparison with similar data on Pannonian and Iberian red deer, obtained by the authors in preceding studies (Frey et al. 2012; Volodin et al. 2019). In addition, for population genotyping, we analyze mtDNA cytochrome *b* and microsatellite markers independent from the acoustical data samples of animals representative for the Voronezh, Pannonian and Iberian red deer populations.

Material and methods

Acoustic methods

Study sites and data collection

For the Voronezh red deer population, audio recordings of unmarked wild-living mature males were conducted in a subpopulation of about 1500 individuals (Rusin et al. 2021) in the "Belgorod" study site of South Russia (50° 37′ N, 36° 52′ E) during the rut from 30 August to 26 October 2016. This subpopulation was established between 1971 and 1990 by 127 individuals translocated from Voronezh State Nature Reserve (Likhatsky et al. 2012). The "Belgorod" study site was an unfenced 20,000-hectare area of a forest-crop field mosaic habitat, with about 30% forest cover, about 15 km to the east of Belgorod.

Automated acoustic recordings (22.05 kHz, 16 bit, stereo) of Voronezh red deer stag rutting calls were collected with two Song Meter SM2 + devices (Wildlife-Acoustics Inc., Maynard, MA, USA). The devices were mounted on singly standing trees 2–4 m above the ground, at two sites of active rut separated by a distance of 1650 m. The devices were equipped with two omnidirectional microphones fixed horizontally at 180° to each other. The devices were set at maximum possible sensitivity, potentially enabling the collection of all rutting roars produced by stags within a radius of about 0.5 km around the device. Automated recordings provided high-quality recordings of the rutting roars, as the stags mostly vocalized at a close distance (within 100 m) to the recording device. A high rotation of rutting males at the recording sites (Rusin et al. 2021) could be expected to decrease potential pseudoreplication by repeatedly recording the same individual.

Acoustic recording was scheduled from 18:00 to 07:00, which included 5 min of recording followed by a 25-min pause. Each 5-min recording was stored as a wav-file. To avoid recording the same roars with both devices simultaneously, we de-synchronized the schedule of their work within each half-hour: the interval between the start of recording of the two devices comprised 15 min; therefore, each device recorded the calls during the pause of another device. Thus, every 24 h, each system collected twenty-six 5-min audio files. In total, we collected 251.3 h of recordings in 3016 audio files, each file of 5-min duration.

To compare the acoustic data from Voronezh red deer with Pannonian and Iberian red deer, we used data obtained by the authors in previous studies (Frey et al. 2012; Volodin et al. 2019). For the native wild-living Pannonian red deer, we used data of acoustic measurements from the 1740 bouts of rutting calls, containing a total of 5535 roars obtained in the study by Volodin et al. (2019). For the native wild-living or free-ranging Iberian red deer, we used data from the acoustic measurements of 1146 bouts, containing a total of 2928 roars obtained in the study by Frey et al. (2012).

Acoustic analyses

For acoustic analysis of Voronezh red deer rutting calls, we used Avisoft SASLab Pro software (Avisoft Bioacoustics, Berlin, Germany). Only high-quality calls with clearly visible spectral structure and not superimposed by wind or other noises were included in analyses. In total, we analyzed 467 high-quality bouts comprising 1335 rutting roars, selected evenly throughout the rutting period, with no more than two bouts taken per 5-min wav-file. Before the analysis, the wav-files were downsampled to 11.025 Hz for better frequency resolution.

Following Frey et al. (2012) and Volodin et al. (2019), a call sequence was registered as a bout only when we were sure that all calls of the sequence came from the same animal and did not contain concurrently produced calls of other stags. The concurrently produced calls of other stags are commonly well visible as overlapping bands in the spectrogram. Bouts with two or more roars were termed multi-roar bouts. Bouts containing only one roar were termed single-roar bouts.

For each bout of rutting calls, we selected the longest roar within the bout to analyze these calls separately as "main roars" of the bouts (Frey et al. 2012; Volodin et al. 2019). For multi-roar bouts, we determined the position of the main roar within a bout and then classified the

roars accordingly as first main roars, last main roars, and intermediate main roars. For single-roar bouts, all roars were treated as the main roars of these bouts. Main roars were classified as either common roars, with a clearly visible f0 and its harmonics, or harsh roars, without a clearly visible f0 (Reby and McComb 2003; Kidjo et al. 2008; Frey et al. 2012; Volodin et al. 2019). In addition, we selected the highest-frequency roar in each bout irrespective of whether it was a main roar or a different roar. We scored each main roar for the presence of nonlinear phenomena: deterministic chaos or subharmonics (Wilden et al. 1998; Fitch et al. 2002). Sections of these nonlinear phenomena may comprise up to 50% of the duration of common roars and from 50 to 100% of the duration of harsh roars (Reby and McComb 2003; Frey et al. 2012; Volodin et al. 2019).

For each main roar ($n = 467$), we measured the duration on the screen with the standard marker cursor in the spectrogram window (Hamming window, FFT 1024 points, frame 50% and overlap 96.87%) using Avisoft SASLab Pro software (Volodin et al. 2019). For 459 of 467 main roars where the maximum fundamental frequency (f0max) could be tracked, we measured f0 with the harmonic cursor from the power spectrum created in the 100 ms section of the f0-maximum area of the roar (Volodin et al. 2019). All measurements were exported automatically to Microsoft Excel (Microsoft Corp., Redmond, WA, USA).

Statistics

Statistical analyses were carried out with STATISTICA, v. 8.0 (StatSoft, Tulsa, OK, USA). Means were given as mean $\pm SD$, all tests were two-tailed, and differences were considered significant whenever $p < 0.05$. As most distributions did not meet the assumption of normality, we log-transformed the data before using an ANOVA. After transformation, most (26 of 33) distributions did not depart from normality (Kolmogorov–Smirnov test, $p > 0.05$). As the ANOVA test is relatively robust concerning departures from normality (Dillon and Goldstein 1984), this was not an obstacle to applying the parametric tests.

We used a Student t test to compare the acoustic variables between main common and main harsh roars. We applied a one-way ANOVA with Tukey HSD (honestly significant difference) test separately for main common and main harsh roars to compare the acoustics of the roars in different positions within a bout. In addition, we used a one-way ANOVA with Tukey HSD test and χ^2 test with Yates correction to compare the acoustics of stag main roars between the Voronezh, Pannonian, and Iberian populations.

Genetic methods

Study sites and data collection

For Voronezh red deer, 44 samples (feces, ear cartilages, and muscles, all 96% alcohol-preserved) were collected in 2016–2017 in three localities/subpopulations of the European part of South Russia: "Belgorod" ($n = 15$), "Lipetsk" ($n = 20$) and "Reserve" ($n = 9$) (Table S1). The "Belgorod" was the locality (50° 37′ N, 36° 52′ E; "Russky les") where a wild-living subpopulation of red deer numbering about 1500 individuals in 2016–2017 was originally founded between 1971 and 1990 by 127 released Voronezh red deer (Likhatsky et al. 2012). This is also where the rutting calls of wild-living Voronezh red deer stags were collected for this study. The "Lipetsk" was the locality (52° 58′ N, 38° 34′ E; "Oleniy Nature Park") where a captive subpopulation of Voronezh red deer numbering about 100 individuals in 2016–2017 was originally founded in 2013 by 10 individuals from the Voronezh State Nature Reserve. The "Reserve" was the locality (52° 02′ N, 39° 41′ E; "Voronezh State Nature Reserve") with a subpopulation of wild-living Voronezh red deer numbering only a few dozen animals in 2016–2017. This subpopulation of wild-living Voronezh red deer was originally founded from the 10 individuals translocated from Germany in the nineteenth century (Likhatsky et al. 2012).

From the native wild-living Pannonian red deer of southern Hungary, 10 samples (blood dried on paper) were collected from the animals legally killed by hunters in the Inner-Somogy landscape, near the city of Nagyatád (46° 04′ N, 17° 29′ E) in September 2015 (Table S1). In January 2018, 24 samples (blood buffered in EDTA) were collected from the native captive Iberian red deer originating from the Las Dehesas public game reserve in Alpera near the town Albacete (38° 59 N, 1° 51′ W) and from Cabañeros National Park near the town Toledo, (39° 51′ N, 4° 01′ W) (Table S1).

DNA extraction and sequencing

From blood and tissue samples, DNA was extracted using the DIAtom™ DNAPrep Kit (Isogen Laboratories Ltd., Russia). From feces, DNA was extracted using QiaAmp® Fast DNA Stool mini Kit (Qiagen GmbH, Germany). Extraction was conducted according to the manufacturer's protocols. Each polymerase chain reaction (PCR) was conducted using 5xMasterMix Kit (Dialat, Russia) PCR buffer with the addition of the SmartTAQ polymerase (Dialat; concentration 2.5 units/μl). For PCR-amplification of cyt *b* gene, we used Cytb-ung-F (forward) (5′-GAAAAACCATCGTTGT(C/T)ATTCA-3′) and Cytb-ung-R (reverse) (5′-TTTTCTGGTTTACAAGACCAGT(G/A)T-3′) primers to get products of about 1031 bp (Zvychaynaya et al. 2013). The amplification conditions were as follows: initial denaturing at 95 °C (3 min);

35 cycles of denaturing at 94 °C (20 s); annealing at 55 °C (20 s); and extension at 72 °C (130 s) with a final extension at 72 °C (5 min). For samples with degraded DNA (feces and dry blood), we used Glu (L14724; forward) (5′-TGATAT GAAAAACCATCGTTG-3′) and CB2 (H15174; reverse) (5′-CCCTCAGAATGATATTTGTCCTCA-3′) primers to get products of about 355 bp (Palumbi et al. 2002). The amplification conditions were as follows: initial denaturing at 94 °C (3 min); 45 cycles of denaturing at 94 °C (15 s), annealing at 50 °C (15 s), and extension at 72 °C (45 s) with a final extension period at 72 °C (6 min).

PCR products were purified using precipitation with 70% ethanol and Na acetate (3 M) or with ExoSAP-IT® *Express* PCR Product Cleanup (Termofisher scientific, USA). Purified PCR products were sequenced with ABI PRISM® BigDye™ Terminator v. 3.1 Kit (Termofisher scientific, USA) in both forward and reverse directions using the amplification primers. Products of sequence-PCR were purified using precipitation with 70% ethanol and Na acetate (3 M) and loaded onto an automated ABI PRISM 3500 Gene Analyzer (Termofisher scientific, USA).

Cytochrome b analyses

The alignment and editing of the mitochondrial cyt *b* sequences were performed by unaided eye using the BioEdit algorithm (Hall 1999) followed by manual analysis. In total, 74 cyt *b* sequences were obtained: 44 sequences from the Voronezh red deer (ten of 355 bp and 34 of 1031 bp), 7 sequences from the Pannonian red deer (six 355 bp and one 1031 bp), and 23 sequences from the Iberian red deer (all 1031 bp) (Table S1). Alignments were done using the BLAST algorithm (Altschul et al. 1990). Fifteen haplotypes have been deposited to GenBank (NCBI) (Banklt2316702) under respective accession numbers MT119264–MT119278, and alignments are available on request. From 74 cyt *b* sequences (58 long sequences of 1031 bp and 16 short sequences of 335 bp), we created two different alignments. One alignment contained 58 cyt *b* long (1031 bp) sequences. Another alignment contained 74 cyt *b* short (355 bp) sequences (16 initially short cyt *b* sequences and 58 cyt *b* sequences shortened from long (1031 bp) ones, Table S1).

Phylogenetic relationship was inferred by using the maximum likelihood (ML) method and the Kimura two-parameter model (Kimura 1980) in MEGA X (Kumar et al. 2018). Node support values in the phylogenetic tree were estimated according to bootstrap support (1000 replicates). As outgroups, we included one sequence of *C.e. sibiricus* (GenBank AY044862.1), one sequence of *C.e. xanthopygus* (GenBank AY070224.1), one sequence of *C. canadensis nelsoni* (GenBank AY347753.1), and one sequence of *C.e. bactrianus* (GenBank AY142327.1) in the alignments. Median-joining haplotype networks were constructed using the Network v. 5.0 software (Fluxus Technology Ltd, UK, www.fluxus-engineering.com). In order to reveal the position of Voronezh red deer relative to the other European populations, 29 complete cyt *b* sequences of European red deer were obtained from GenBank and shortened to 1031 bp (Table S2). The haplotype (h) and nucleotide (π) diversity frequencies and the genetic distances among the populations (Φst) were computed using Arlequin v. 3.5.2.2 (Excoffier and Lischer 2010).

Microsatellite fragment analysis

Microsatellite analysis was based on 57 samples: 24 samples from Voronezh red deer (8 samples from locality Belgorod and 16 samples from locality Lipetsk), 9 samples from Pannonian red deer, and 24 samples from Iberian red deer (Table S1). All 57 samples were genotyped at 8 microsatellite loci (Table S3) following Kuehn et al. (2003) and Bishop et al. (1994). For all samples, we analyzed each locus separately. Each PCR was conducted using 5xMasterMix Kit (Dialat, Russia) PCR buffer with the addition of the SmartTAQ polymerase (Dialat; concentration 2.5 units/µl). PCR amplification consisted of an initial denaturing at 94 °C (3 min) followed by n cycles of denaturing at 93 °C (30 s), annealing at 56 °C (x sec), and extension at 72 °C (30 s) with a final extension at 72 °C (30 min) (for MM12 and CSSM14 $n=30$, $x=60$; for BMS757 and BM1818 $n=38$, $x=60$; for BM4107, CSPS115, CSSM19, and CSSM22 $n=35$; $x=15$). PCR products were separated using an automated ABI PRISM 3500 Gene Analyzer (Termofisher scientific, USA), and the data were analyzed using GeneMapper version 4.1 (Termofisher scientific, USA). For Pannonian red deer samples (dry blood), we repeated the analyses at least two times (three times if the results were disputable) to ensure the authenticity of the results.

All loci were tested for deviations from Hardy–Weinberg equilibrium (HWE) in GenAlEx 6.5 (Peakall and Smouse 2006, 2012). The frequencies of null alleles were estimated by CERVUS 3.0 (Kalinowski et al. 2007). We estimated genetic diversity measures (mean number of alleles per locus (Na), number of effective alleles (Ne), observed (Ho) and expected (He) heterozygosity, fixation index (F), and Shannon's information index (I) with GenAlEx 6.5). GenAlEx was also used to identify private alleles and their frequencies and estimate Nei genetic distances between pairs of populations. The matrix of genotypic distances based on individual genotypes was obtained in GenAlEx 6.5, and the multidimensional scaling method (MDS) was obtained with STATISTICA, v. 8.0 (StatSoft, Tulsa, OK, USA). Mean allelic richness per locus (A_R) for each predefined European population was calculated with FSTAT v. 2.9.3.2 (Goudet 1995); the minimum sample size was 8 individuals.

We also used STRUCTURE v2.3.4 (Pritchard et al. 2000) to estimate the number of subpopulations (K). Five independent runs of $K = 1 - 10$ were carried out with 500,000 Markov chain Monte Carlo (MCMC) iterations after a burn-in period of 150,000 iterations, using the model with correlated allele frequencies and assuming admixture. The most probable number of subpopulations was decided based on the Evanno method (Earl and von Holdt 2012).

Results

Acoustic results

Rutting calls of Voronezh stags

In Voronezh red deer stags, rutting calls represented bouts of 1–13 roars (Fig. 1). Of the 467 bouts, one-roar bouts comprised 20.99% and the two-roar bouts comprised 29.98% of the bouts. Among 229 bouts, consisting of three or more roars, main roars of the bouts were in the first position in 17.91% of the bouts, the last position in 37.56% of the bouts, and the intermediate position in 44.54% of the bouts. Among the 467 main roars of the bouts, 389 were common roars (83.30%) and 78 were harsh roars (16.70%). Harsh main roars were shorter than common main roars ($t = 6.37$, $df = 465$, $p < 0.001$) but did not differ from common roars in f0max ($t = 1.39$, $df = 457$, $p = 0.17$) (Table 1).

One-way ANOVA revealed the effect of main roar position within bout (first vs other position) on f0max for common ($F_{3,385} = 11.09$, $p < 0.001$) but not for harsh roars ($F_{3,66} = 0.48$, $p = 0.70$). Main common roars in the first position (including main roars of one-roar-bouts) were higher in f0max compared to the main common roars in a different position ($0.001 < p < 0.02$ for all cases, Tukey HSD test). Main common roars of one-roar bouts and main roars in the first position within multi-roar bouts did not differ by f0max ($p = 0.60$, Tukey HSD test). The durations of both common and harsh main roars were not influenced by their position within bouts ($F_{3,385} = 2.68$, $p = 0.05$, and $F_{3,66} = 1.26$, $p = 0.29$, respectively). The number of roars per bout affected f0max of both main common roars ($F_{9,379} = 4.73$, $p < 0.001$) and main harsh roars ($F_{8,61} = 11.01$, $p < 0.001$) but did not affect the duration of either main common roars

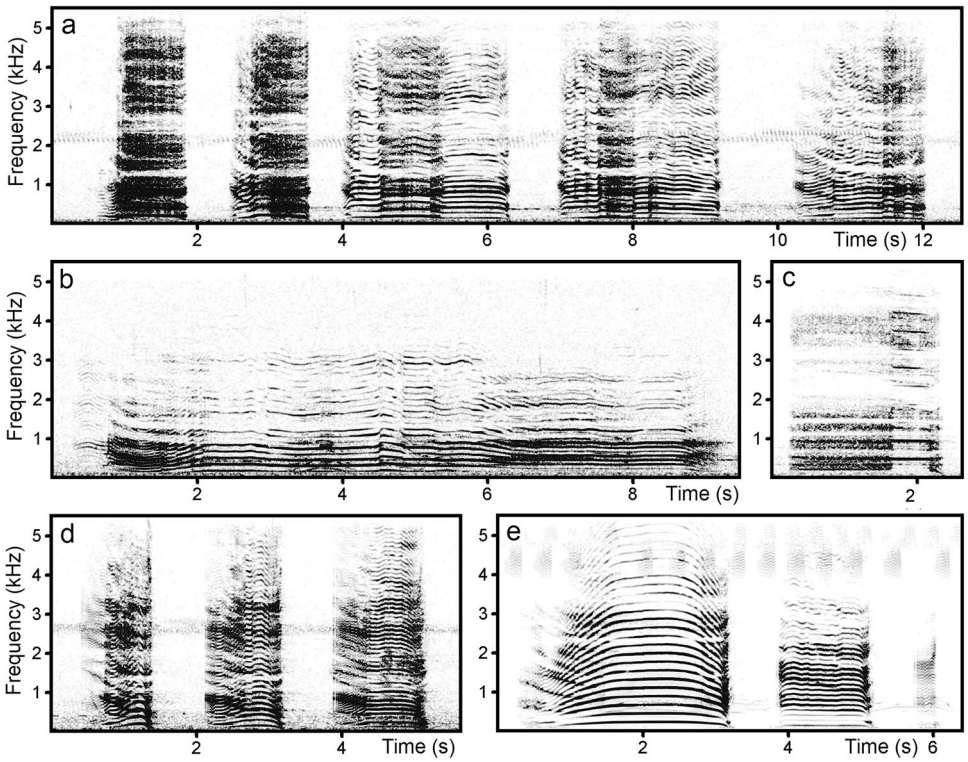

Fig. 1 Spectrogram of rutting roars of red deer stags. **a** Voronezh red deer five-roar bout of rutting calls; the first and second calls are harsh roars and the third, the fourth and the fifth calls are common roars; the third call is the main roar of the bout. **b** Voronezh red deer single-roar bout, the longest rutting call recorded from Voronezh red deer. The nasal onset is well visible until approximately 0.7 s; furthermore, the call is oral. **c** Voronezh red deer main roar, the second half of the roar displays a section with source-filter interaction (coupling). **d** Pannonian red deer three-roar bout of rutting calls, all three calls are common roars; the third call is the main roar of the bout. **e** Iberian red deer three-roar bout of rutting calls, all the three calls are common roars; the first call is the main roar of the bout. The spectrogram was created at 11,025 Hz sampling frequency, Hamming window, FFT 1024, frame 50%, overlap 93.75%

Table 1 Values (mean ± *SD*) of acoustic variables of main (the longest) roars of bouts of rutting calls in Voronezh, Pannonian, and Iberian red deer stags and one-way ANOVA results for their comparison; percentages of main common roars and main harsh roars of all main roars (results of χ^2 test for their comparison are given in text). The same superscripts indicate that acoustic variables do not differ significantly ($p > 0.05$, ANOVA; Tukey HSD test, or χ^2 test). Designations: Duration = main roar duration; f0max = main roar maximum fundamental frequency; Main common roars = roars with a clearly visible f0 and its harmonics; Main harsh roars = roars without a clearly visible f0; Highest-frequency main roars = main roars which are the highest in fundamental frequency within bouts, both common and harsh; Main roar position = roar position within bouts containing more than 2 roars

Acoustic variable	Voronezh stags (this study)	Pannonian stags (Volodin et al. 2019)	Iberian stags (Frey et al. 2012)	ANOVA
All main roars				
Roars per bout (*n*)	2.85 ± 1.79^{a}	3.18 ± 2.17^{b}	2.11 ± 1.71^{c}	$F_{2,2914} = 30.37$, $p < 0.001$
Duration (s)	2.46 ± 1.14^{a}	1.13 ± 0.50^{b}	1.90 ± 0.50^{c}	$F_{2,2914} = 1220.50$, $p < 0.001$
f0max (Hz)	175 ± 60^{a}	168 ± 61^{a}	224 ± 34^{b}	$F_{2,2914} = 343.38$, $p < 0.001$
Main common roars	83.3% [a]	66.3% [b]	89.1% [c]	
Roars per bout (*n*)	2.85 ± 1.81^{a}	3.51 ± 2.23^{b}	2.51 ± 1.76^{c}	$F_{2,2197} = 63.07$, $p < 0.001$
Duration (s)	2.61 ± 1.16^{a}	1.27 ± 0.55^{b}	1.88 ± 0.50^{c}	$F_{2,2197} = 746.16$, $p < 0.001$
f0max (Hz)	173 ± 57^{a}	179 ± 61^{a}	223 ± 35^{b}	$F_{2,2197} = 231.39$, $p < 0.001$
Main harsh roars	16.7% [a]	33.7% [b]	10.9% [c]	
Roars per bout (*n*)	2.79 ± 1.71^{a}	2.52 ± 1.88^{a}	2.60 ± 1.75^{a}	$F_{2,714} = 2.08$, $p = 0.13$
Duration (s)	1.75 ± 0.67^{a}	0.87 ± 0.25^{b}	2.12 ± 0.49^{c}	$F_{2,714} = 567.06$, $p < 0.001$
f0max (Hz)	184 ± 75^{a}	147 ± 54^{b}	236 ± 29^{c}	$F_{2,714} = 101.12$, $p < 0.001$
Highest-frequency main roars	56% [a]	57% [a]	94% [b]	
Duration (s)	2.58 ± 1.27^{a}	1.12 ± 0.53^{b}	no data	$F_{1,1252} = 812.06$, $p < 0.001$
f0max (Hz)	192 ± 67^{a}	183 ± 69^{b}	no data	$F_{1,1252} = 6.30$, $p = 0.012$
Main roar position				
First	26.6% [a]	31.6% [a]	49.9% [b]	
Intermediate	27.6% [a]	34.0% [b]	20.5% [c]	
Last	45.8% [a]	34.4% [b]	29.7% [c]	

($F_{9,379} = 1.06$, $p = 0.39$) or main harsh roars ($F_{8,61} = 1.11$, $p = 0.37$). In the bouts containing 2–13 roars (369 bouts, 1237 roars), main roars were also the highest frequency within bouts only in 165 (44.7%) of bouts.

Voronezh red deer stags were capable of producing very long roars. Among the 467 main roars, 45 (9.6%) roars were longer than 4 s. Of these 45 roars, 32 roars ranged in duration from 4 to 5 s, 10 roars ranged in duration from 5 to 6 s, and 3 roars were longer than 6 s. The longest rutting roar recorded from Voronezh red deer lasted 8.89 s (Fig. 1b).

In Voronezh red deer, 6 of 1335 roars contained sections with source-filter coupling: an acoustic phenomenon resulting from vibrations of the vocal folds at the formant frequency (Fig. 1c), previously documented for rutting roars of both Iberian and Pannonian stags (Volodin et al. 2013a, 2019). The duration of the roars with sections of source-filter coupling varied from 1.21 to 3.09 s (mean = 1.80 ± 0.72 s). The f0max (coinciding with f0max of the coupling part) varied from 408.8 to 529.0 kHz (mean = 473.8 ± 41.0 kHz). In Voronezh stags, the bouts including the roars with source-filter coupling consisted of 1 to 9 roars. Among 6 roars with source-filter coupling, 5 were main roars of their bouts. Two of the 6 roars with source-filter coupling occupied the first position in the bout, and the remaining 4 roars occupied the last position within a bout.

Comparison of rutting calls of Voronezh, Pannonian, and Iberian stags

One-way ANOVA revealed the significant effects of the population (Voronezh, Pannonian and Iberian) on stag main roar duration, maximum fundamental frequency (f0max), and the number of calls per bout (Table 1). The duration differed between Voronezh, Pannonian, and Iberian stags ($p < 0.001$ for all comparisons). The longest main roars were produced by Voronezh stags (2.46 ± 1.14 s), and the shortest main roars were produced by Pannonian stags (1.13 ± 0.50 s) (Table 1). The main roars highest in f0max were produced by Iberian stags (224 ± 34 Hz), whereas f0max did not differ between the main roars of Voronezh (175 ± 60 Hz) and Pannonian stags (168 ± 61 Hz) (Table 1).

For both common and harsh main roars, ANOVA revealed the effect of population on duration and f0max, whereas the effect of population on the number of roars per bout was only revealed for common roars (Table 1). For main common roars, duration differed between all three populations. The

f0max differed between Iberian and Voronezh and between Pannonian and Iberian stags, but not between Pannonian and Voronezh stags. For main harsh roars, the f0max and duration differed among all the three populations (Table 1). The longest main common roars (2.61 ± 1.16 s) were found in Voronezh stags, whereas the longest main harsh roars were found in Iberian stags (2.12 ± 0.49 s). For bouts where the main roar was also the highest frequency, the roars of Voronezh stags were significantly longer than the roars of Pannonian stags and higher in f0max (Table 1). For Iberian stags, comparative data were not available.

Common roars were more frequent than harsh roars in all populations (Table 1), but occurred significantly more often in Iberian than either in Voronezh ($\chi^2 = 9.61$, $p = 0.002$) or Pannonian stags ($\chi^2 = 191.73$, $p < 0.001$), and more often in Voronezh than in Pannonian stags ($\chi^2 = 46.24$, $p < 0.001$). In Iberian stags, 94% of main roars were also the highest-frequency in their bouts, significantly more than in Pannonian (57%; $\chi^2 = 69.23$, $p < 0.001$) or Voronezh (56% $\chi^2 = 63.80$, $p < 0.001$) stags, which did not differ from each other ($\chi^2 = 0.09$, $p = 0.76$) (Table 1).

Main roars in the first position within bouts were more frequent in Iberian than in Pannonian ($\chi^2 = 66.76$, $p < 0.001$) or Voronezh ($\chi^2 = 54.19$, $p < 0.001$) stags and did not differ between Pannonian and Voronezh stags ($\chi^2 = 3.24$, $p = 0.07$). Main roars in the last position within bouts were more frequent in Voronezh than in Pannonian ($\chi^2 = 15.53$, $p < 0.001$) or Iberian stags ($\chi^2 = 27.67$, $p < 0.001$) and more frequent in Pannonian than in Iberian stags ($\chi^2 = 4.73$, $p = 0.03$). Main roars in the middle position within bouts were more frequent in Pannonian than in Voronezh ($\chi^2 = 4.96$, $p = 0.03$) or Iberian stags ($\chi^2 = 41.58$, $p < 0.001$) and in Voronezh than in Iberian stags ($\chi^2 = 6.81$, $p = 0.009$) (Table 1).

Genetic results

Cytochrome b

In the alignment of 58 cyt *b* 1031 bp sequences (34 of Voronezh, 1 of Pannonian and 23 of Iberian red deer), we found 11 cyt *b* haplotypes (5 in Voronezh, 1 in Pannonian, and 5 in Iberian red deer) (Fig. 2a). The alignment contained 30 (2.9%) substitutions; 24 loci (2.3%) were parsimonious informative. The transitions/transversions ratio (R) was 7.04. No insertions or deletions were present in this alignment. In the alignment of 74 cyt *b* 335 bp sequences (44 of Voronezh, 7 of Pannonian, and 23 of Iberian red deer), we found 12 cyt *b* haplotypes (4 in Voronezh, 4 in Pannonian, and 4 in Iberian red deer) (Fig. S1). The alignment contained 12 (3.4%) substitutions; 10 loci (2.8%) were parsimonious informative. The transitions/transversions ratio (R) was 4.01.

Analysis of 58 cyt *b* 1031 bp sequences revealed a clear distinction between haplotypes from different populations. The median-joining haplotype network showed 3 haplogroups (Fig. 2a). One of them contained all Iberian red deer haplotypes, the second contained Voronezh red deer (1LIP, 26VOR, 15BELG) haplotypes, while the third contained haplotypes of Pannonian (2PAN) and Voronezh (12LIP) red deer. Haplotype 6LIP of Voronezh red deer took an intermediate position between the latter. The Iberian red deer haplotypes group was separated by 12 mutational steps from the closest haplotype 6LIP (Fig. 2a).

Phylogenetic analysis based on the maximum likelihood (ML) method showed a similar topology with significant bootstrap support for the major clades (Fig. 3a). The ML tree showed clear division into three clades; one of them contained all Iberian red deer haplotypes, another contained all Voronezh red deer haplotypes, and the third one contained Voronezh 12LIP and Pannonian 2PAN red deer haplotypes. Haplotype 6LIP took a separate position. The Eastern red deer (*C.e. sibiricus* and *C.e. xanthopygus*) and wapiti (*C. canadensis nelsoni*) used as outgroups, formed a separate clade with 100% bootstrap support. The Bactrian red deer *C.e. bactrianus* also formed a separate branch which was basal to all European red deer with high (99%) bootstrap support.

In order to include more Pannonian red deer haplotypes, we conducted the analysis based on 74 cyt *b* 335 bp sequences (Fig. S1). Despite the short length of the cyt *b* fragment, the network based on short sequences showed clear division into three haplogroups, the same as in Fig. 2a. Pannonian red deer haplotypes formed a separate group, including haplotype 12LIP from the Voronezh population. Haplotype 6LIP held its intermediate position (Fig. S1). The ML tree based on the short sequences also showed the division of European haplotypes into three groups. The haplotype 12LIP entered the group formed by the Pannonian red deer haplotypes and haplotype 6LIP was placed on a separate branch, closer to the group of Pannonian haplotypes (Fig. 3b).

To reveal the position of Voronezh red deer relative to other European populations, we created a median-joining haplotype network based on 30 cyt *b* haplotypes from this study and GenBank (Table S2, Fig. 2b). All haplotypes of Voronezh red deer (both from this study and GenBank) formed a separate haplogroup E (according to Doan et al. 2018) except for haplotype 12LIP, which belonged to the same group with haplotypes from Hungary, Slovakia, and partly the Czech Republic (haplogroup C, Table S2, Fig. 2b). Haplotype 6LIP retained its intermediate position between these two groups. All haplotypes from Spain, France, Germany, Poland, and partly the Czech Republic formed haplogroup A (Table S2; Fig. 2b). The distance (number of mutations) between Voronezh red deer haplogroup (E) and Western haplogroup (A) (containing haplotypes from Western and Central Europe) was much larger than the distance

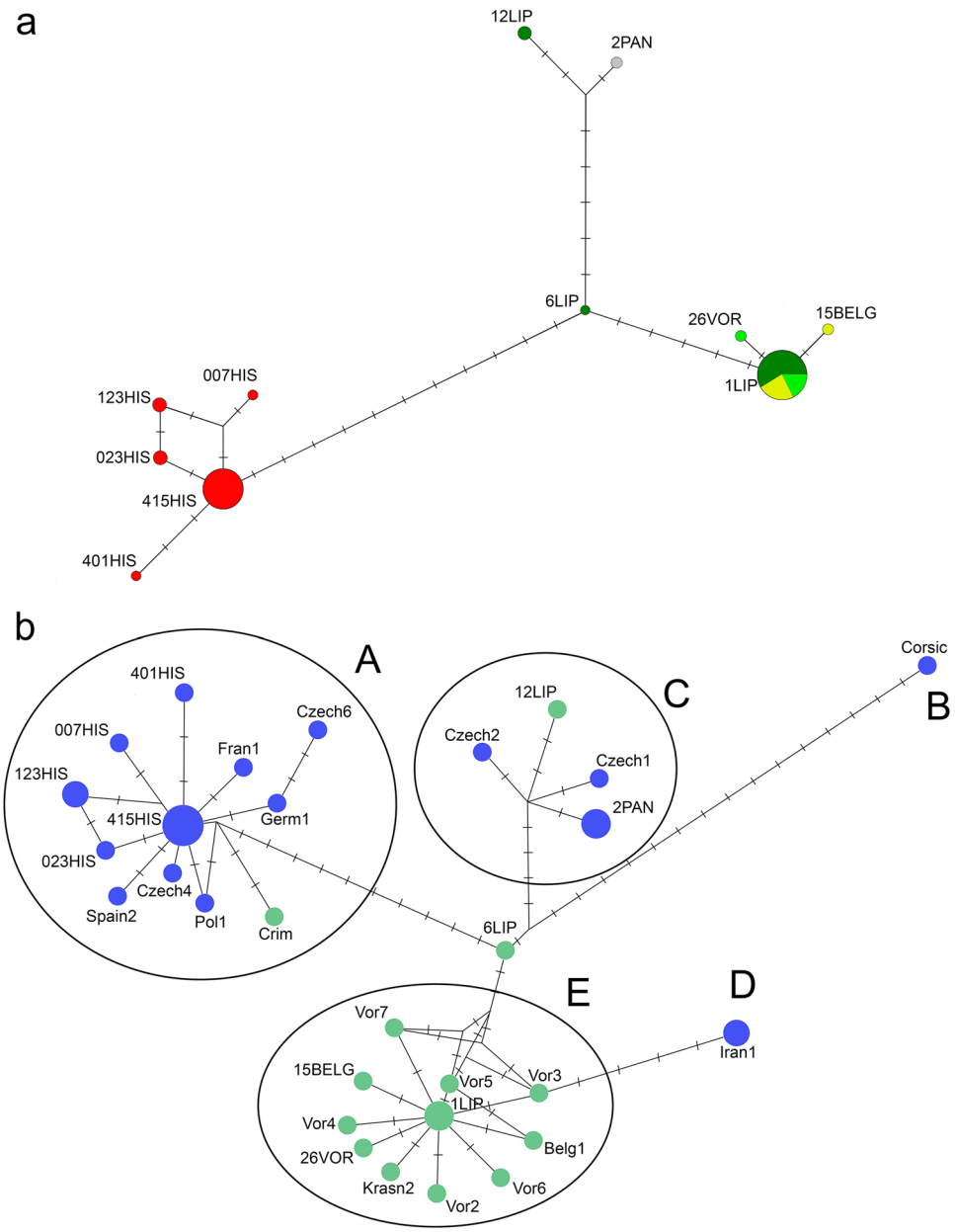

Fig. 2 Median-joining network of red deer cyt *b* haplotypes. The hatches on the lines connecting haplotypes represent nucleotide substitutions. Circle sizes are proportional to the haplotype frequency. Colors represent geographical origin of haplotypes. **a** Network is based on 58 cyt *b* 1031 bp sequences from this study. Dark green color indicates Lipetsk (Voronezh red deer); light green color indicates Reserve (Voronezh red deer); yellow color indicates Belgorod (Voronezh red deer); gray color indicates Pannonian red deer, and red color indicates Iberian red deer. **b** Network is based on 30 cyt *b* 1031 bp haplotypes from this study and from GenBank. Green color indicates haplotypes from Russia (both this study and GenBank); blue color indicates haplotypes from other countries (both this study and GenBank)

between Voronezh red deer haplogroup and Eastern haplogroup (C) (containing haplotypes mainly from Eastern and Central Europe) (Fig. 2b). Noticeably, we did not find any single common haplotype for Voronezh and other European red deer.

Genetic differentiation (Φst) among the three populations was considerable (Table 2). The greatest distance was found between Voronezh and Iberian red deer (0.745), while the distance between Voronezh and Pannonian red deer was slightly lower (0.710). The distance between Iberian and Pannonian red deer was the least (0.505). Despite the large Φst differentiation between Voronezh and Pannonian red deer, the results of cyt *b* analysis suggest that Voronezh red deer is indeed closer to lineage C, as the number of mutations was less between these two groups, and also the Voronezh red deer haplotype 12LIP was part of C haplogroup (Figs. 2a, 3, S1).

Comparison of genetic diversity between Voronezh, Pannonian, and Iberian red deer populations revealed the lowest haplotype (h) and nucleotide (π) diversities in Voronezh red deer. The highest haplotype and nucleotide diversities were found in Pannonian red deer despite having the smallest number of available specimens (Table 3).

Microsatellites

Fragment analysis of 8 microsatellite loci, based on the total of 57 specimens of Voronezh, Pannonian and Iberian red

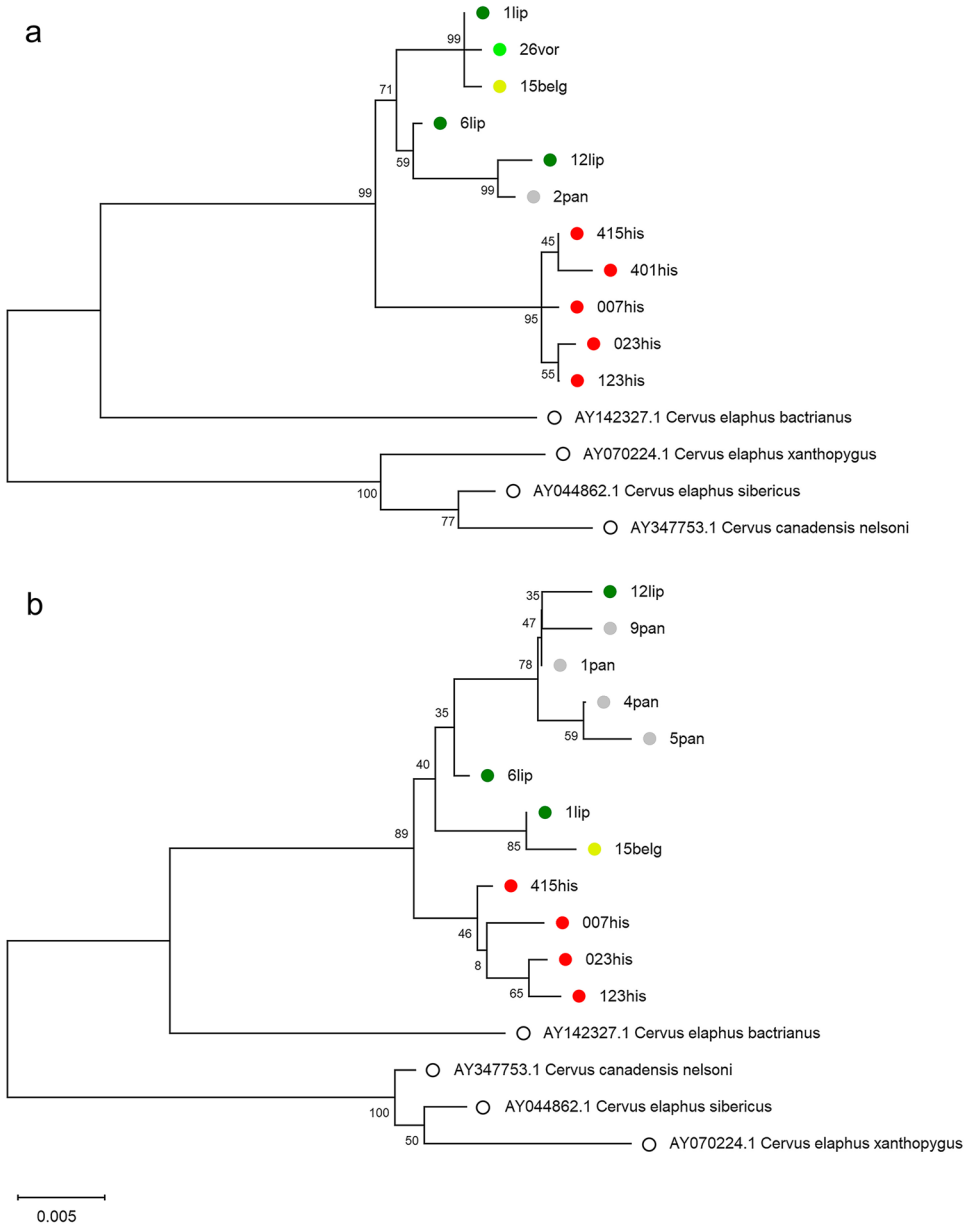

Fig. 3 Phylogenetic relationships of the cyt *b* haplotypes based on (**a**) long (1031 bp; $n=58$), and (**b**) short (355 bp; $n=74$) sequences. Numbers on branches indicate bootstrap support for maximum likelihood (1000 replicates) algorithms. Genetic distances are calculated by the Kimura's two-parameter model. Colors represent geographical origin of haplotypes. Dark green color indicates Lipetsk (Voronezh red deer); light green color indicates Reserve (Voronezh red deer); yellow color indicates Belgorod (Voronezh red deer); gray color indicates Pannonian red deer, and red color indicates Iberian red deer

deer, revealed that the number of alleles per locus varied from 5 to 16 (Fig. S2, Table S4 and S5,). The most conservative loci were MM12 and CSSM14 (represented by 5 alleles). The most variable loci were BM757 (15 alleles) and BM4107 (16 alleles). For CSSM14 and CSPS115 loci, relatively high frequencies of null alleles were estimated (26 and 34%, respectively). For BM757 and CSSM19, the frequencies of null alleles were lower (13 and 11%, respectively). The exclusion of CSSM14 and CSPS115 loci with high frequencies of null alleles did not affect the general patterns of population differentiation, so we used all loci for further statistical analyses.

Table 2 Genetic differentiation (Φst) calculated among the pairs of populations based on cyt *b* haplotype frequencies in red deer populations. For all pairs $p<0.001$

Population	Voronezh red deer	Iberian red deer
Iberian red deer	0.745	
Pannonian red deer	0.710	0.505

Deviations from Hardy–Weinberg equilibrium with deficiency of heterozygotes were detected in four loci in all sample sets: two loci in the sample set from locality Lipetsk (Voronezh red deer) (CSSM14 and CSPS115), three loci in the sample set of Iberian red deer (CSSM14, CSPS115, and BM757), and two loci in the sample set of Pannonian red deer (CSSM14 and CSSM19). Deviations from HWE

Table 3 Genetic diversity measures (mean ± *SD*), based on 74 short (355 bp) cyt *b* sequences. Designations: $\hbar$ haplotype diversity; π nucleotide diversity; *PD* the mean number of pairwise differences

Population	*N* specimens	*N* haplotypes	N polymorphic loci (% of the sequence length)	Haplotype diversity ($\hbar$)	Nucleotide diversity (π)	PD
Voronezh red deer	44	4	6 (1.7%)	0.174 ± 0.076	0.001 ± 0.002	0.572 ± 0.473
Iberian red deer	23	4	3 (0.9%)	0.387 ± 0.122	0.002 ± 0.002	0.625 ± 0.509
Pannonian red deer	7	4	3 (0.9%)	0.714 ± 0.181	0.003 ± 0.003	1.048 ± 0.785

Table 4 Genetic diversity measures (mean ± *SE*), based on microsatellites of the red deer populations/localities. Designations: *Na* number of alleles per locus, *Ne* number of effective alleles, *Ho* observed heterozygosity, *He* expected heterozygosity, *F* fixation index [F = (He – Ho)/He], I – Shannon's information index, A_R mean allelic richness per locus

Population/locality	*N* specimens genotyped	Na	Ne	H_o	H_e	F	I	*N* private alleles	A_R
Voronezh red deer (Lipetsk))	16	3.875 ± 0.350	2.202 ± 0.172	0.555 ± 0.072	0.522 ± 0.044	−0.052 ± 0.081	0.947 ± 0.075	0.125 ± 0.125	3.332
Voronezh red deer (Belgorod)	8	3.750 ± 0.453	2.503 ± 0.261	0.531 ± 0.066	0.561 ± 0.057	0.029 ± 0.097	1.016 ± 0.121	0.250 ± 0.250	3.750
Iberian red deer	24	6.375 ± 0.778	4.222 ± 0.717	0.581 ± 0.075	0.714 ± 0.045	0.200 ± 0.068	1.487 ± 0.151	2.250 ± 0.526	5.018
Pannonian red deer	9	5.125 ± 0.934	3.515 ± 0.682	0.507 ± 0.091	0.656 ± 0.048	0.254 ± 0.122	1.311 ± 0.172	1.625 ± 0.653	4.979

could be caused by relatively high frequencies of null alleles in these loci. They could also be explained for Iberian and Voronezh red deer from Lipetsk by non-random mating on red deer farms.

In the entire sample set of 57 specimens, 74 alleles were found. Of those found, 13 were private for Pannonian red deer, 18 for Iberian red deer, 2 for locality Belgorod (Voronezh red deer), and 1 for locality Lipetsk (Voronezh red deer). Forty other alleles were shared between populations. Both the expected heterozygosity (He) and the diversity index (I) were the highest for Iberian red deer and the lowest for locality Lipetsk (Voronezh red deer) (Table 4). The allelic richness (A_R) and the average number of alleles per locus (Na) were both higher for Iberian and Pannonian red deer compared to Voronezh red deer (Table 4). The Fixation index (F) was relatively high for the populations of Iberian and Pannonian red deer (Table 4). However, the small sample size of these populations did not allow us to make reliable conclusions about the actual level of diversity and inbreeding.

According to the multidimensional scaling (MDS) of individual animal genotypes, the three studied populations were clearly separated, which confirmed genetic differences among them (Fig. 4). Nei genetic distances between pairs of populations/localities had values ranging from

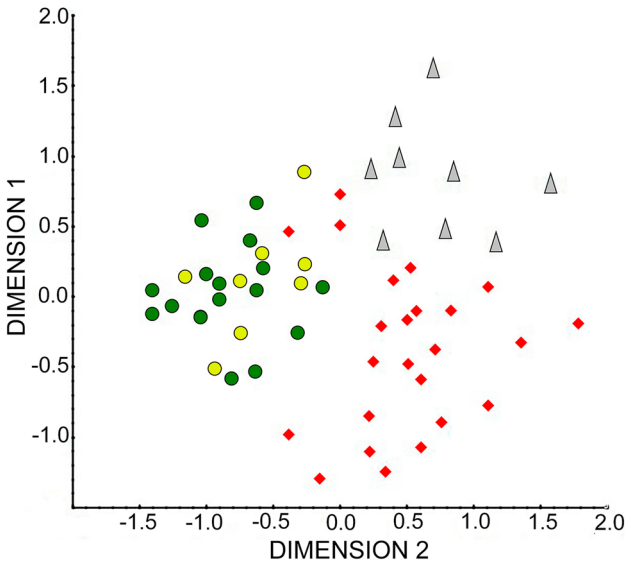

Fig. 4 MDS plot based on the genetic distance matrix of red deer individual genotypes. Green circles indicate Lipetsk (Voronezh red deer); yellow circles indicate Belgorod (Voronezh red deer), gray triangles indicate Pannonian red deer, and red diamonds indicate Iberian red deer

0.141 to 0.943 (Table 5). The largest distances were found between Voronezh and Pannonian red deer, while the distance between Iberian and Pannonian red deer populations was shorter. The distance between Voronezh red deer from two localities was relatively small (0.141), and it could be clearly demonstrated by the MDS plot that there were no pronounced genetic differences between Belgorod and Lipetsk localities (Table 5, Fig. 4).

The results of the STRUCTURE analysis with the Evanno algorithm showed that K = 2 was the most probable number of clusters in our study (Fig. 5, Table S6). One of the clusters included all genotypes of Voronezh red deer, while the other contained all Iberian and Pannonian genotypes. For

Table 5 Nei genetic distances between pairs of populations/localities. For all pairs $p < 0.001$

Population/locality	Voronezh red deer (Lipetsk)	Voronezh red deer (Belgorod)	Iberian red deer
Voronezh red deer (Belgorod)	0.141		
Iberian red deer	0.708	0.555	
Pannonian red deer	0.943	0.617	0.430

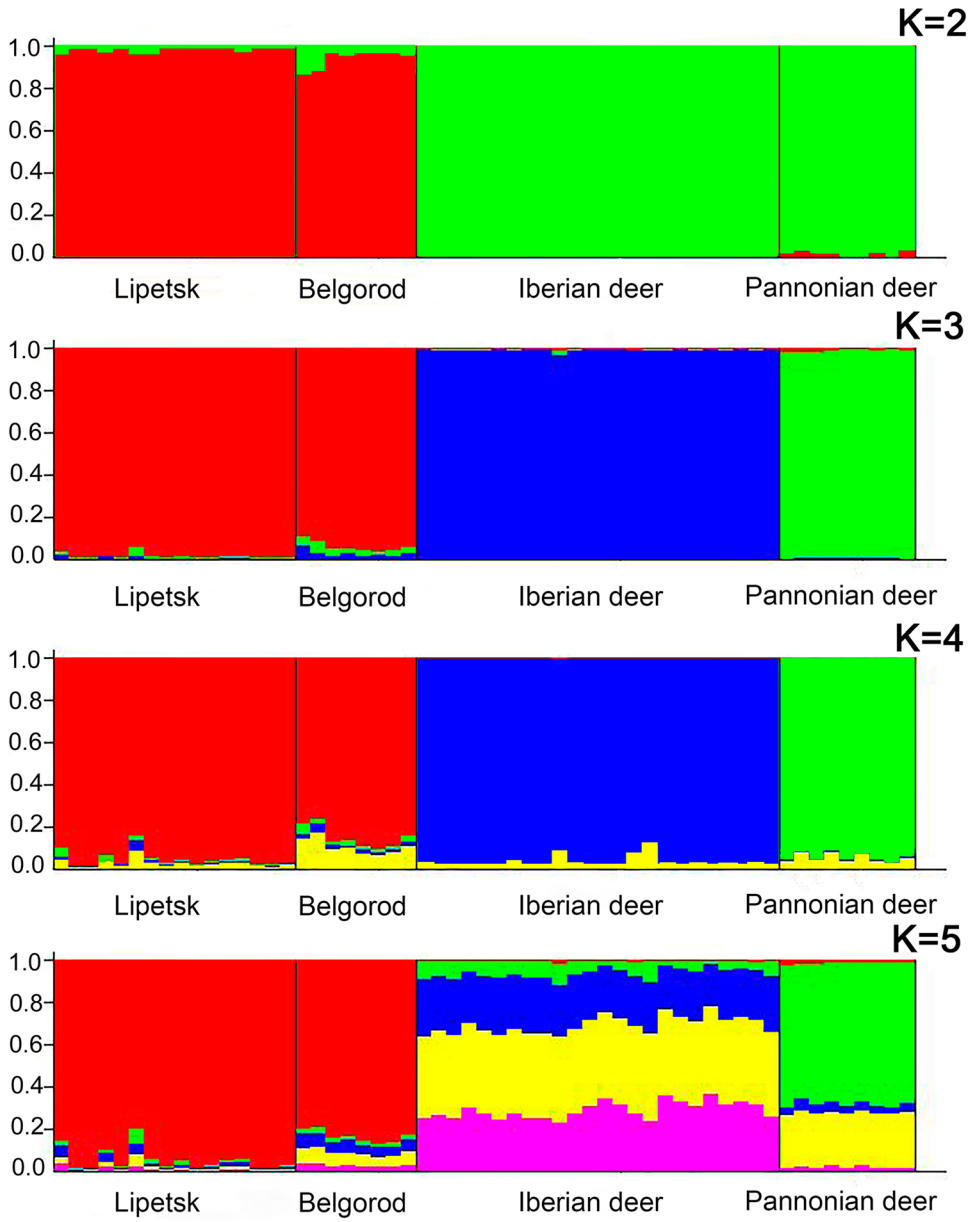

Fig. 5 Analysis of population genetic structure of the 8-loci dataset using the STRUCTURE algorithm. The admixture model with independent allele frequencies was applied

the further clusterization on K = 3 and K = 4, all Voronezh red deer clustered together with high (close to 1) probability, while Iberian and Pannonian red deer formed two separate clusters (Fig. 5).

Discussion

Rutting calls

This is the first study describing the acoustic variables of the rutting bouts and main roars of male Voronezh red deer, which historically originate from red deer translocated to the European part of South Russia from Germany (Likhatsky et al. 2012). Our data are in good agreement with the results of other studies focused on the acoustics of rutting calls in different European populations of red deer. Bouts of rutting calls of Voronezh red deer could contain up to 13 roars per bout, whereas, in the bouts of other European subspecies of red deer, the number of roars per bout could reach up to 22 calls (Kidjo et al. 2008; Frey et al. 2012; Passilongo et al. 2013; Volodin et al. 2019). The average maximum fundamental frequency of main roars in Voronezh red deer (175 Hz) was within the range of frequencies characteristic for other European populations of red deer, from 52 Hz in the Corsican (Kidjo et al. 2008) to 274 Hz in the Alpine Italian population (Bocci et al. 2013) (Fig. 6). The duration of main roars in Voronezh red deer (2.46 s) was also within the range of durations found in other European populations, from 1.07 s in Central European (Hurtado et al. 2012) to 2.49 s in Iberian red deer (Volodin et al. 2015a).

At the same time, Voronezh red deer were distinctive from other European populations by the large amount of very long roars, with the longest registered roar of 8.89 s (Fig. 1b). For comparison, in Iberian red deer, the maximum documented main roar duration was 5.90 s (Volodin et al. 2015a). Thus, we can conclude that male Voronezh red deer produce the longest roars among European populations of red deer.

In Voronezh red deer, we documented a rare acoustic phenomenon, the source-filter coupling, which was previously found in one single roar of 2928 investigated roars of Iberian stags (Volodin et al. 2013a) and in 19 of 5535 investigated roars of Pannonian stags (Volodin et al. 2019). In Voronezh red deer, the main roars which contained the sections with source-filter coupling were distinctive from other main roars with a very high maximum fundamental frequency (up to 529 Hz) (Fig. 1c). Source-filter coupling is recognizable by the call parts displaying the coincidence of the fundamental frequency with the call formant frequency and the strong increase of call amplitude, indicating the effect of resonance (Titze 2008; Volodin et al. 2013a). We, therefore, may conclude that this rare acoustic

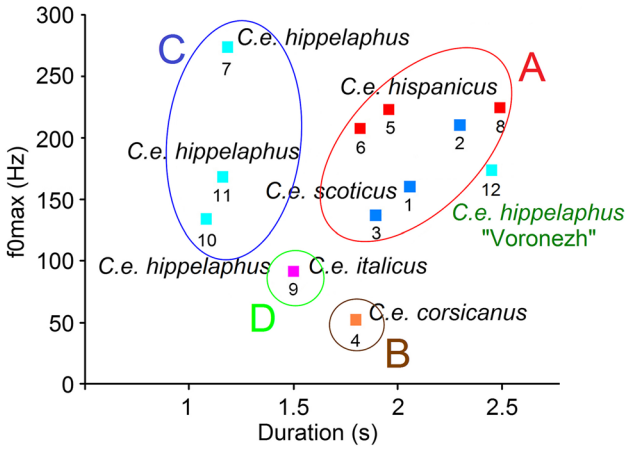

Fig. 6 Two acoustic variables of stag rutting roars (duration and maximum fundamental frequency) across the European subspecies of red deer. A, B, C, D Mitochondrial cyt *b* lineages according to Skog et al. (2009) and Doan et al. (2017). Different square colors correspond to different subspecies: dark blue *C.e. scoticus*; red *C.e. hispanicus*; orange *C.e. corsicanus*; light blue *C.e. hippelaphus*; purple *C.e. italicus*. Data were taken from the following: (1) McComb (1988); (2) Long et al. (1998); (3) Reby and McComb (2003); (4) Kidjo et al. (2008); (5) Frey et al. (2012); (6) Passilongo et al. (2013); (7) Bocci et al. (2013); (8) Volodin et al. (2015a); (9) Della Libera et al. (2015); (10) Hurtado et al. (2012); (11) Volodin et al. (2019); (12) This study. Modified after Volodin et al. (2019)

phenomenon occurs in European red deer regularly, but it can only be detected in a small number of calls in any red deer population. Distinctive from Iberian and Pannonian red deer, in Voronezh red deer, most main roars with source-filter coupling occupied the last position within bout.

Main roars of Voronezh red deer differed from those of either Iberian (European cyt *b* mtDNA lineage A, Skog et al. 2009) or Pannonian population (European cyt *b* mtDNA lineage C, this study) by duration, the number of roars per bout, the ratio of harsh to common roars, and the position of the main roar within bout (Table 1). However, in a plot of duration vs maximum fundamental frequency of stag rutting roars across European populations of red deer (Fig. 6), the roars of Voronezh red deer are closer to the roars of the mtDNA lineage A red deer than to the roars of mtDNA lineage C red deer. Although the main roars of Voronezh and Pannonian red deer did not differ by the maximum fundamental frequency, the maximum fundamental frequency of the roars varied substantially both within and between mtDNA lineages A and C. At the same time, the duration of the main roars of Voronezh red deer is the longest among European red deer and is similar to the duration of the roars of mtDNA lineage A stags (Fig. 6). Thus, in some acoustic variables, the roars of Voronezh red deer are closer to mtDNA lineage A; in other acoustic variables, they are closer to mtDNA lineage C, and in other acoustic variables,

they are intermediate between lineages A and C (Table 1, Fig. 6).

Although the founder effect could potentially shape these vocal differences, this is not an outcome directly from our data. So far, no data on the impacts of the founder effect on acoustics is available for any mammalian species. Some data supporting the effect of gene drift on vocalization were obtained for rodents (Campbell et al. 2010; Matrosova et al. 2016). At the same time, a potential bottleneck effect on vocalization was reported for songbirds: e.g., a reduced vocal diversity has been observed after colonization of new habitats by a limited number of founders (Baker and Jenkins 1987; Hill and Pawley 2019) . However, data for birds are contradictory, as, e.g. in a parrot species, the bottleneck effect on vocalization was lacking (Baker 2014).

Phylogenetic position of Voronezh red deer among European populations

The main aim of the current genetic analysis is to support the acoustic results and to show a clear distinction between three studied populations, which belong to different mitochondrial lineages: A (Iberian red deer), C (Pannonian red deer), and E (Voronezh red deer) (Skog et al. 2009; Doan et al. 2018). It is important to indicate that we used individuals from the same populations (although independent sample sets of animals) for acoustic and genetic analyses.

Previous data assigned Voronezh red deer to C haplogroup (Kuznetsova et al. 2013). However, in our study, most Voronezh red deer haplotypes formed a separate haplogroup (E or W5) (Table S2; Fig. 2b). This haplogroup was first revealed during the research by Doan with coauthors (2018) and contained mainly haplotypes of Voronezh red deer from different parts of the distribution area (Voronezh, Vladimir, and Krasnodar Regions). Our results confirm this detached position of Voronezh red deer relative to other European red deer populations. Other haplotypes from Europe that formed two haplogroups (A and C) are in accordance with their geographical distribution (Table S2; Fig. 2b). The Crimean haplotype (Crim) was included in haplogroup A, containing haplotypes from Western and Central Europe. Both A and C lineages are presented in the Crimean Peninsula, and multiple human-aided translocations of red deer to this region (including Voronezh red deer, Siberian wapiti, and red deer from the Caucasus) occurred during the last two centuries (Kuznetsova et al. 2013; Doan et al. 2018).

According to the population history of the Voronezh red deer and its German origin, it would be expected to find haplotypes close to A haplogroup. However, we did not find any evidence for lineage A in the population of Voronezhred deer. The founder effect could explain this finding, i.e., fixing the haplotypes of the few ancestors of the Voronezhpopulation and conservation of these haplotypes during a long period of time (about 150 years). The haplotypes of Voronezhpopulation founders could have been lost in Europeover this relatively long period.

In the European part of Russia, the presence of lineage A could only be found in regions where massive translocations and introductions have occurred during the last two centuries, e.g., in Bryansk and Tver regions (Central Russia), and where Voronezh red deer were mixed with red deer from Białowieża Forest (Poland, Belarus) (Danilkin 1999; Sitnikova and Mishta 2006; Kuznetsova et al. 2013). Here, we want to emphasize that most modern red deerpopulations in the European part of Russiaare the result of human-managed translocations and reintroductions. Therefore, we cannot draw conclusions based on the analysis of modern samples regarding the natural distribution of red deerin these territories after glaciation (Heptner et al. 1988; Danilkin 1999). Analysis of microsatellites showed large genetic distances between the studied populations, with the smallest distance between Iberian and Pannonian red deer (Table 5). According to the population history, no massive translocations or reintroductions were applied to the Voronezhred deerpopulation, so gene flow was low or lacking (Likhatsky et al. 2012). Distribution of individuals on the MDS plot, based on their genetic distances, and STRUCTURE analysis support this (Figs. 4 and 5, Fig. 5). All individuals of Voronezhred deerfrom both localities clustered together and demonstrated a high level of genetic homogeneity (Fig. 5).

Mitochondrial and microsatellite diversity of Voronezh red deer

Our study advances the knowledge concerning genetic diversity of red deerin the European part of South Russia. The status of this population is peculiar because of the lack of gene flow between Voronezhred deerand the other red deerpopulations. All our sample sets from different localities (Belgorod, Lipetsk, and Reserve) demonstrated the presence of common haplotypes (Fig. 2a, Fig. S1) and low mtDNA diversity, with nucleotide diversity $\pi = 0.001$ and haplotype diversity $h = 0.174$ (Table 3). Generally, European red deerdemonstrate low nucleotide diversity ($\pi = 0.02$) but high haplotype diversity ($h = 0.95$) (Skog et al. 2009). At the same time, low nucleotide and haplotype diversities were expectable for Voronezhred deerbecause of their population history and is common for populations originating from a small number of individuals and passing through several bottlenecks (Feulner et al. 2004; Hmwe et al. 2006a,b; Niedziałkowska et al. 2012).

The results of the 8-loci microsatellite analysis also confirmed the low genetic diversity in Voronezh red deer. Six of these 8 loci were previously used in the largest-scale microsatellite analysis of European red deer (Zachos et al. 2016; Frantz et al. 2017), so the obtained data are well comparable

with those on other red deer populations. It was expected that all indices of genetic diversity would be the lowest for Voronezh red deer compared to Pannonian and Iberian red deer (Table 4). Only 3 alleles were unique to the Voronezh red deer, despite the large sample size of animals. This result corresponds to data on populations of red deer that have passed through the bottleneck (Zachos et al. 2016). The degree of allelic richness A_R for Voronezh red deer (3.332 for the locality Lipetsk, 3.750 for the locality Belgorod) was higher than the A_R of highly isolated and severely bottlenecked red deer from Sardinia (2.69) and Mesola (2.76) but lower compared to the A_R of other European red deer populations (Niedziałkowska et al. 2012; Zachos et al. 2016). However, the degree of expected heterozygosity for Voronezh red deer (He = 0.522 for Lipetsk and He = 0.561 for Belgorod) remained within the average values of He for European red deer populations, which range from 0.33 to 0.83 (Kuehn et al. 2003; Dellicour et al. 2011; Niedziałkowska et al. 2012; Krojerova-Prokešova et al. 2015; Zachos et al. 2016).

Our study confirmed a detached position of Voronezh red deer relative to other European red deer populations both in acoustics and genetics. This population has developed from several individuals in conditions of genetic isolation due to the absence of autochthonous populations in this part of the country (Heptner et al. 1988; Likhatsky et al. 2012). It spread to almost the entire European part of Russia over the past century by human-managed translocations and natural migrations (Danilkin 1999; Likhatsky et al. 2012; Kuznetsova et al. 2013). The contribution of the Voronezh deer to the restoration of local populations is enormous, as red deer from the Voronezh Reserve were introduced not only in Russia but also in Belarus, Ukraine, Moldova, and the Baltic states (Likhatsky et al. 2012; Kuznetsova et al. 2013). The uniqueness of this population is that most of its history is documented, from the origin to the present time. However, the collapse of the USSR led to a decline in state control over the import of animals and their translocations. This resulted mixed origin populations in hunting grounds and private farms (Kuznetsova et al. 2013). The only way to avoid further dilution of population structure in Voronezh red deer is to control translocations, which is always a problem in game species. Our research provides a background for the conservation of Voronezh red deer using bioacoustics and molecular techniques, as the lack of knowledge about the modern population structure of Russian red deer makes wildlife management difficult.

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Author contribution OG, IV, and EV conceived and designed this study and analyzed the data. MK managed the genetic part of research. EL, AN, and TT performed the field study. OG, EV, and IV wrote the manuscript, with contributions from all of the authors.

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Data availability All data needed to evaluate the conclusions in the paper are present in the paper and the supplementary materials and in GenBank under respective accession numbers; alignments are available on request.

Declarations

Ethics approval This study has been conducted in cooperation with the staff of the facilities in accordance with the rules of the facilities and in accordance with ethical and animal welfare standards and the laws of the Russian Federation, where material for the current study was collected. Animal disturbance was kept at a minimum, as the recording was conducted automatically in the absence of people. Samples from Pannonian stags were obtained from animals legally killed by hunters under observation of facility managers. The data collection protocol no. 2011–36 was approved by the Committee of Bioethics of Lomonosov Moscow State University.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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