Can skull morphology-morphometry discern Russian wolf-dog hybrids from wolves (*Canis lupus*) and dogs (*Canis familiaris*)?

Pavel N. Korablev*, Nikolay P. Korablev, Andrei V. Zinoviev & Miroslav P. Korablev

ABSTRACT. Although wolves, dogs and their hybrids can be discerned by genetic analysis, the study of morphology-morphometry to discern the three groups remains important as genetic analysis is not always possible or too expensive. In this study we aim to differentiate the three subgroups by analyzing two morphometric and ten morphological characteristics in 329 canid skulls. After morphometric-morphologic allocation, we applied genetic analysis on 108 skulls based on 11 autosomal microsatellites to verify the morphometric-morphologic results. In 30 specimens genetic tests were unsuccessful. In addition, 23 samples from wolves (14 skins and 9 muscle samples) as well as 32 samples from modern dogs (8 hair and 24 blood samples) were used as reference data in the genetic analysis. Based on morphology-morphometry we diagnosed 322 wolves, four dogs and three hybrids. Genetic testing was done on 78 specimens: one presumed dog, three presumed hybrids and 74 of the wolves, as diagnosed morphologically before. All, but one, morphologically diagnosed wolves, were confirmed as being wolves genetically. That one was identified a hybrid genetically. From the four morphological dogs one was confirmed genetically, the other three had failed genetic tests. Of the three morphological hybrids one was genetically a dog. The results of this study indicate the absence of reliable morphological criteria for identifying skulls of hybrids, which is a consequence of the high morphological variability in dogs and wolves. However, the use of morphometric and morphological characteristics, helps to narrow the numbers of skulls that require genetic analysis for more precise identification.

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KEY WORDS: *Canis lupus*, *Canis familiaris*, hybrids, skull, morphology, genetic analysis.

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Можно ли по морфологии и морфометрии черепа отличить российских волко-собачьих гибридов от волков (*Canis lupus*) и собак (*Canis familiaris*)?

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РЕЗЮМЕ. Несмотря на то что волки, собаки и их гибриды могут быть определены с использованием генетического анализа, изучение морфологических и морфометрических признаков для различения этих трех групп остается важным, поскольку генетическая идентификация не всегда возможна или слишком затратна. В настоящем исследовании на основании изучения 329 черепов мы попытались дифференцировать три указанных группы, используя анализ двух морфометрических и десяти морфологических характеристик. С целью верификации результатов краниологического анализа 108 черепов были проанализированы на основе 11 аутосомных микросателлитных локусов, однако образцы от 30 черепов показали неудовлетворительное качество генотипирования и были исключены из исследования. Помимо этого, в генетический анализ были включены образцы от 23 волков (14 — засушенная кожа и 9 — мышцы в этаноле) и 32 собак (8 образцов шерсти и 24 образца крови), которые использовали в качестве референсных данных. На основании краниологического анализа были диагностированы 322 черепа волков, четыре — собак и три — гибридов. Генетический анализ был успешно проведен для 78 черепов (один предположительно череп собаки, три черепа

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гибридов, 74 — волков), ранее диагностированных морфологически. Практически все морфологически диагностированные черепа волков были генетически подтверждены как волки, за исключением одного черепа, который генетически был определен как гибрид. Из четырех морфологически определенных черепов собак один был подтвержден генетически, генетический анализ трех остальных не дал результата. Из трех морфологически определенных черепов гибридов диагностика двух подтвердилась генетически, один по результатам генетического анализа оказался собакой. Результаты настоящего исследования свидетельствуют об отсутствии надежных морфологических критериев определения черепов гибридных особей, что является следствием высокой морфологической изменчивости волков и собак. Однако использование морфометрических и морфологических признаков помогает сократить количество черепов, требующих генетического анализа для более точной идентификации.

КЛЮЧЕВЫЕ СЛОВА: *Canis lupus*, *Canis familiaris*, гибриды, череп, морфология, генетический анализ.

Introduction

Hybridization of the wolf (*Canis lupus* Linnaeus, 1758) and dog (*Canis familiaris* Linnaeus, 1758) is a widespread phenomenon (Bibikov, 1985; Vila *et al*., 1999; Vila & Wayne, 1999; Mech & Boitani, 2003; Pilot *et al*., 2018). Recently, this problem has acquired a particularly acute conservation significance in regions where wolves and dogs co-occur (Bibikov, 1985; Vila & Wayne, 1999; Boitani, 2000; Donfrancesco *et al.*, 2019).

On the territory of the former Soviet Union wolf-dog hybrids have been observed since long (Barabash-Nikiforov, 1957). Wolves with an atypical (black, white, piebald or intensely red) coat color, or animals different in behavior compared to their wild relatives (e.g., behaving more boldly in relation to people, making lairs in dilapidated houses or in the outskirts of villages, as well as attacking livestock during daylight hours and in the presence of people) are probably hybrids (Ryabov, 1973, 1978, 1985). The low population numbers, between 1950–1970, of highly hunted wolves in the USSR, led to a significant number of hybrids that shared the ecological wolf niche (Ryabov & Bibikov, 1982). However, as the number of wolves increased since the 1970s, hybridization events have become much less common (Ryabov, 1985).

The true extent of the effect of hybridization on the gene pool of wolf populations remains unknown. For example, previous publications have evaluated the proportion of hybrids as 5% in Italy (Verardi *et al*., 2006) and 4% in Spain (Godinho *et al*., 2011). Recent studies argue much higher hybridization rates, reaching 26% in the whole wolf distribution range in Italy (Caniglia *et al.*, 2020) and even reaching 30–50% in Central Italy (Salvatori *et al*., 2019). Moreover, Pilot *et al*. (2018) used 61K genome-wide SNPs to show that 62% of Eurasian wolves have small blocks of dog ancestry, evidencing hybridization as a common phenomenon, consistently occurring in human-dominated areas. Luckily the recent rapid development of molecular genetic (DNA) techniques, can contribute significantly to better mapping of wolf-dog hybridization (Vilà *et al*., 2003; Verardi *et al*., 2006; Godinho *et al*., 2011; Hindrikson *et al*., 2012; Khosravi *et al*., 2013; Kopaliani *et al*., 2014; Pacheco *et al*., 2017; Pilot *et al*., 2018, 2021; Salvatori *et al*., 2019; Caniglia *et al*., 2020; Korablev *et al*., 2021a).

Genetic methods provide the most precise criteria to distinguish between purebred and hybrid individuals (Donfrancesco *et al*., 2019; Caniglia *et al*., 2020). However, morphological analysis, specifically of skulls, may still be of value to identify hybrids, as in cases where samples do not contain enough DNA nor high-quality DNA. Also, it is not always possible to study all available specimens in collections genetically, due to their large sample size and related costs. The morphological approach, thus, has a potential to identify atypical skulls with questionable species affiliation and thus reduce the number of required genetic analyzes. To our knowledge, and up to now, only one study has used morphometric and morphological methods to define wolf-dog hybrids (Milenković *et al*., 2006).

When searching for wolf-dog hybrids the first step is to define anatomical markers that can separate pure wolves from dogs. The major ones reported in the literature are: shape of the coronoid process of the mandible (straight morphology of caudal border of the vertical ramus in wolves or "turned back" morphology in dogs; Olsen & Olsen, 1977), differences in orbital angles (low angle in wolves (39.5°–46.5°) or high angle in dogs (49°–55°); Studer, 1901; Bockelmann, 1920; Iljin, 1941; Aaris-Sørensen, 1977), shape of the ventral margin of the mandible (straight mandible in wolves versus a convex one in dogs; Lawrence & Reed, 1983; Germonpré *et al*., 2015), contact points of the skull on a horizontal plane (skull rests on canines and bulla tympanica in wolves and on P4 and bulla tympanica in dogs; Zeuner, 1963; Benecke, 1987), caudal shifting of the border of the hard palate (the caudal hard palate border is rostral to the line touching the caudal sides of M2 in wolves or caudal to the M2 line in dogs; Iljin, 1941; Benecke, 1987). Recently, the diagnostic value of most proposed characteristics has been analyzed, which allowed to discard most of them as non-informative or of low diagnostic value (Janssens *et al*., 2019).

Also eight following characteristics were proposed by Stubbe (1981): outline of the presphenoid bone

(arrow-shaped with extensions in wolves and evenly wedge-shaped narrowed in dogs), shape of jugular foramen (narrow bean-shaped in wolves or varying from ovoid to teardrop in dogs), shape of the opening located on the occipitotemporal suture dorsal to the base of the jugular process (large, visible and irregular in wolves versus small, oval or round, often scaly, in dogs), position of the cavity of the intermaxillary canal (closer to the alveolar margin of the incisors in wolves or to the line of the nasal opening in dogs), outline of the process of the maxillary bone located caudal to M2 (similar to an isosceles triangle in wolves or longer and with a tendency to coalesce with the palate in dogs), shape of the middle part of the scales of the occipital bone (flat in wolves or having pronounced vertically elongated bulge in dogs), shape of the vomer (extended in central part in wolves or gradually tapering in dogs), and width of the mandible in the area of incisors (relatively narrow with closely spaced incisors in wolves versus relatively wide with rarely spaced incisors in dogs). Based on a comparative analysis of 100 wolf and 150 dog skulls, the author stated that this morphological features are informative to discern dogs from wolves. However, they were not part of comparative studies of canid skulls by other authors, and thus their real diagnostic value has not yet been evaluated.

Our study here, uses both morphology-morphometry and genetics, and aims to analyze the diagnostic value of two morphometric and 10 morphological markers, as well as 11 microsatellite genetic markers, for the identification of two important cranial wolf collections.

Material and methods

The study consisted of two main stages: a) craniological analysis (measurement of skulls and description of non-metric characteristics) and b) genetic analysis based on autosomal microsatellite loci.

Material used

In total, 329 skulls, collected between 1950–2016, from two Russian collections were used for craniological analysis. The collections of Central Forest Biosphere Reserve (CFR — Tver region, Russia) (n = 303, accession numbers 13.1.1–13.190.1, 13.1.2–13.36.2, 13.1.3–13.77.3) and Tver State University (TSU — Tver region, Russia) (n = 23, accession numbers M 1/1–M 1/7, M 1/9, M 1/10, M 1/12, M 1/13, MI 1/1, MI 1/3, MI 1/5, MI 1/7–MI 1/9, MI 1/11, MI 1/17, MI 1/18, MI 15, MI 16, MI 19) contain skulls from Tver, Smolensk and Vologda regions in Central Russia (Fig. 1). Additional three CFR specimens (1C.l., 2C.l. and 3C.l.) different in size and form from typical wolf skulls were also examined. Skull 1C.l. belonged to an animal killed together with wolves in 2007; skull 2C.l. belonged to an animal similar to a wolf in behavior and coat color, killed in 2009; skull 3C.l. belonged to an animal killed in 1994. The other skulls from the CFR and TSU collections which were defined as atypical in craniological and/or genetic analyses (see below) were collected under different circumstances. Skull 13.102.1 belonged to an animal killed in 1994; skull 13.63.1 belonged to an animal killed together with wolves in 1994; skull MI 1/5 belonged to an animal killed in 1996; collection history for skulls MI 15 and MI 19 is unknown.

We conducted an age study of the material, taking into account the significant age-related variability in the size of wolf skulls (Landon *et al*., 1998; Mech *et al*., 2011; Korablev *et al*., 2021b). The age groups of skulls were determined using tooth wear (Gipson *et al*., 2000). If this method could not be applied (e.g., severe injures of teeth), we estimated the age based on cranial sutures closure as well as by the development of the cranial crests *crista sagittalis* and *crista occipitalis* (Klevezal, 2007). We considered two main groups: juveniles (≤1-year old) and adults (≥1-year old). Skulls of juvenile wolves were excluded from morphometric statistics to reduce the effect of age variability on the results of morphometric analysis.

Genetic testing was initially attempted for 108 skulls, including 8 atypical skulls (1C.l., 2C.l., 3C.l., 13.102.1, 13.63.1, MI 1/5, MI 15 and MI 19). However, 30 specimens (including atypical skulls MI 1/5, MI 15 and MI 19) failed amplification at most loci and were excluded from DNA analysis. Thus, for DNA analysis the following samples were successfully analyzed: 1) 78 skulls (74 from presumed wolves, three from presumed hybrids, one from presumed dog), 2) 14 skins from presumed wolves, 3) 9 muscle tissue from presumed wolves, 4) 24 blood samples from modern dogs and 5) 8 hair samples from modern dogs.

Samples from presumed wolves and modern dogs were used as reference data in DNA hybridization analyses. We used samples from owned mongrel dogs of a medium or relatively large size, originating from the study area, which could theoretically participate in wolf breeding.

Morphometric analysis

Condylobasal length (CbL) and zygomatic width (ZW) were measured and the index square of the skull was calculated (IS = CbL × ZW) (Fig. 2).

Morphological analysis

We used 10 non-metric characteristics for describing the structural features of the skull: outline of the presphenoid bone (Stubbe, 1981), shape of jugular foramen (Stubbe, 1981), shape of the opening located on the occipitotemporal suture dorsal to the base of the jugular process (Stubbe, 1981), position of the cavity of the intermaxillary canal (Stubbe, 1981), outline of the process of the maxillary bone located caudal to M2 (Stubbe, 1981), the middle part of the scales of the occipital bone (external occipital eminence (Stubbe, 1981), shape of the maxillary process of zygomatic bone (our data), contact points of the skull on a horizontal plane (Zeuner, 1963; Benecke, 1987; Yudin, 1992), caudal shifting of the border of the hard palate

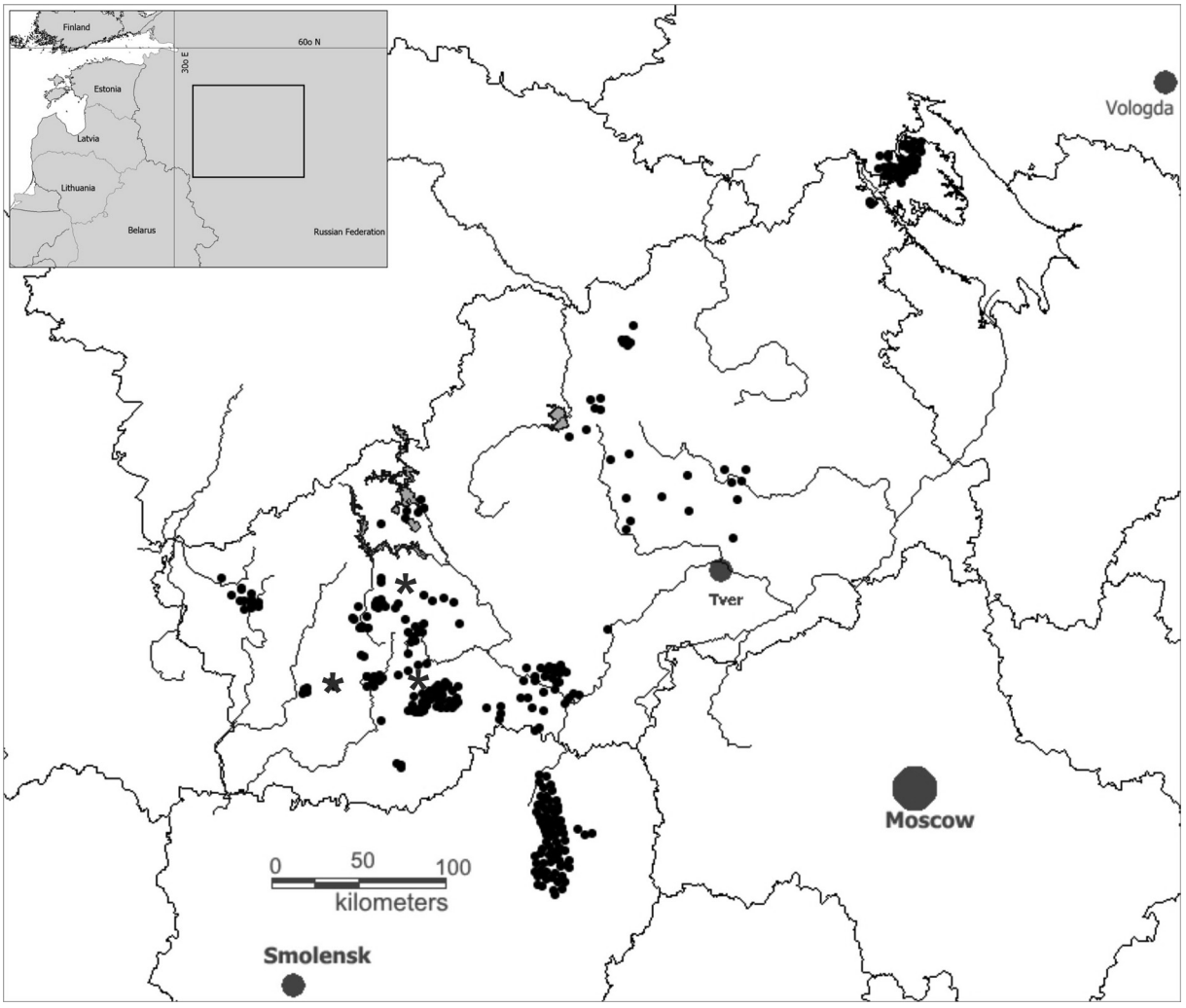

Fig. 1. Study area. Black circles indicate the places where the studied skulls were collected. Grey asterisks indicate the location of identified hybrids.

(Iljin, 1941; Benecke, 1987), and shape of the ventral margin of the mandible (Lawrence & Reed, 1983; Yudin, 2013). Variations of the characteristics typical for wolves and dogs are described in Table 1.

We coded a certain morphological trait typical for the wolf as "l" (*Canis **l**upus*), for the dog, as "f" (*Canis **f**amiliaris*). If a characteristic could not be clearly attributed to one or the other, it was defined as intermediate "l-f".

Genetic analysis

DNA was extracted using Diatom DNA Prep 100 kit (ISOGENE Laboratory, Russia) according to the manufacturer's protocol, with an extension to the lysis step of 24 hours. 70 µl of whole blood or about 100 µl of crushed muscle tissues, dried skins, bone shavings, pulp scrapings, or hair follicles cut from a bundle of hair were used for DNA extraction.

We analyzed 11 autosomal canine microsatellites: cph2, cph5, cph8, cph12 (Fredholm & Wintero, 1995), C09.250 (Ostrander *et al*., 1993), fh2004, fh2079, fh2088, fh2096, fh2132, fh2137 (Francisco *et al*., 1996). A negative DNA isolation and PCR control were used to detect possible DNA contamination. All PCR reactions were prepared in a laminar flow box. PCR was performed separately for each locus in 10 µL volumes with a final concentration of: 0.05 mM of each dNTP, 2.5 mM of MgCl2, 0.5 pM of the forward and reverse primers, 1 unit of Hot Start Taq DNA polymerase (SibEnzyme, Russia), 10× PCR buffer and 1.0 µL of DNA template. The forward primer for each locus was labelled with fluorescent dye: fam (cph2, cph8, fh2004, fh2132), rox (cph5, c09.250, fh2137), tamra (cph12, fh2088, fh2096), r6g (fh2079). The allele length was determined on an ABI 3500 genetic analyzer (Applied Biosystems, USA) with the addition of the SD-450 size standard (SYNTOL, Russia). Results were analyzed in GeneMapper v. 4.0 (Applied Biosystems, USA). All samples were amplified in at least three independent PCRs, after which the obtained genotypes were visually

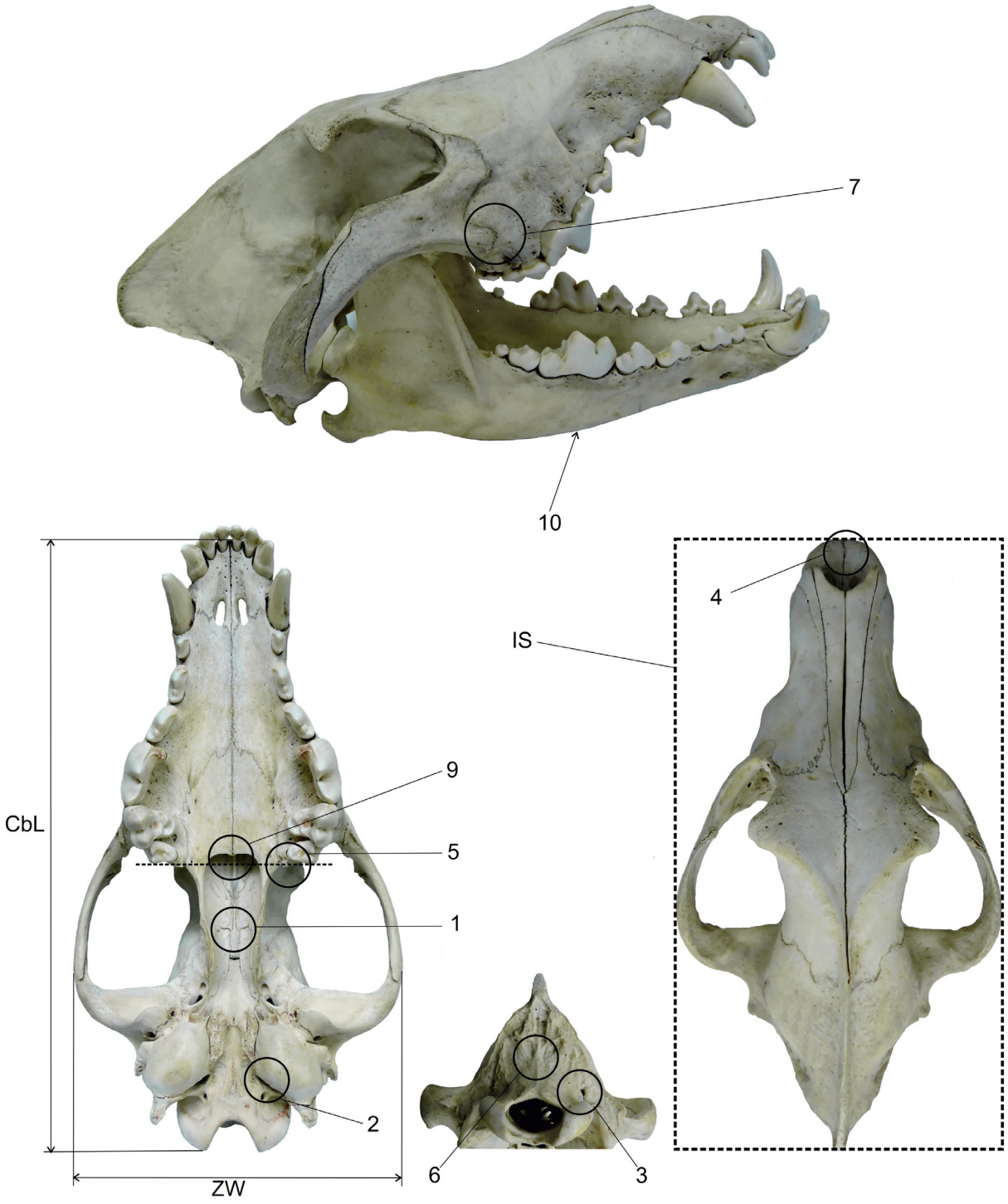

Fig. 2. Morphometric and morphological skull characteristics studied. See text and Table 1 for details.

checked. If the allele composition in a sample at a locus remained unclear (mainly in low-quality samples — skulls, dried skins or hair), such samples were re-amplified at that loci, in one or two additional independent PCRs. Three identical homozygote profiles or two identical heterozygote profiles were required for acceptance of single-locus genotypes.

To identify possible hybrids, genotypes both from the wild wolf population and dogs were first clustered in Structure 2.3.4 (Pritchard *et al*., 2000) using an ad-

Table 1. The morphological characteristics of the skull used to identify canid species.

	Characteristic	Wolf (1)	Dog (f)
1	Outline of the presphenoid bone (Stubbe, 1981)	Arrow-shaped (with extensions)	Evenly wedge-shaped narrowed
2	Shape of jugular foramen (Stubbe, 1981)	Narrow bean-shaped	From ovoid to teardrop
3	The shape of the opening located on the occipitotemporal suture dorsal to the base of the jugular process (Stubbe, 1981)	Large, visible and irregular	Small, oval or round, often scaly
4	Position of the cavity of the intermaxillary canal (Stubbe, 1981)	Closer to the alveolar margin of the incisors	Closer to the line of the nasal opening
5	Outline of the process of the maxillary bone located caudal to M2 (Stubbe, 1981)	Close to the shape of an isosceles triangle	Longer and with a tendency to coalesce with the palate
6	The middle part of the scales of the occipital bone (external occipital eminence) (Stubbe, 1981)	Flat (in young animals, bulge in isolated cases)	Pronounced vertically elongated bulge
7	The shape of the maxillary process of the zygomatic bone (our data)	Forms a protrusion towards M1 about 1 cm long or more	Forms a slight protrusion (no more than 0.5 cm) or does not form at all
8	Contact points of the skull on a horizontal plane (Zeuner, 1963; Benecke, 1987; Yudin, 1992)	The skull rests on canines and bulla tympanica	Skull rests on P4 and bulla tympanica
9	Caudal shifting of the border of the hard palate (Benecke, 1987; Iljin, 1941)	The caudal hard palate border is rostral to the line touching the caudal sides of M2	The caudal hard palate border is caudal to the line touching the caudal sides of M2
10	Shape of the ventral margin of the mandible (Lawrence & Reed, 1983; Yudin, 2013; Germonpré *et al*., 2015)	The ventral margin is relatively straight, has a smooth curve at the level of P4	The ventral margin is rounded, has a sharp bend at the level of P4

mixture model with correlated allele frequencies, and no prior population information. Analysis was performed assuming two populations (K = 2) with 1000000 MCMC iterations after a burn-in period of 100000 iterations. Wolves which showed individual membership proportions $q_{wolf} \geq 0.98$ and 90% Bayesian credible intervals (BCI) ≥ 0.90, with no missing data (n = 43) and dogs with $q_{dog} \geq 0.98$, BCI ≥ 0.90, and no missing data (n = 22), were used as references to simulate 50 genotypes for each of the 6 predefined ancestry classes (pure wolves, pure dogs, F1 and F2 hybrids as well as first generation backcrosses) in Hybridlab 1.0 (Nielsen *et al.*, 2006). The simulated genotypes were then analyzed in Structure under the above-mentioned parameters, in order to define threshold levels of the individual membership proportions that we further used to distinguish between purebred individuals and hybrids.

Results

Morphometric analysis

1) CbL and ZW

The minima and maxima for adult males (n = 108) from the two collections are: CbL 205.1–256.6 (mean 236.5 ± 1.1), ZW 124.8–162.8 (mean 142.4 ± 0.7). The limits for adult females (n = 48) are: CbL 209.4–242.2 (mean 227.3), ZW 122.9–149.1 (mean 135.3 ± 0.9). The values for adults without gender separation (n = 156) are: CbL 205.1–256.6 (mean 233.7 ± 0.9) (Fig. 3), ZW 122.9–162.8 (mean 140.2 ± 0.6) (Fig. 4).

Measurements of the CFR skulls 1C.l., 2C.l., 3C.l., are: CbL 226.8 mm and ZW 123.0 mm for 1C.l. (male); CbL 196.2 mm and ZW 121.1 mm for 2C.l. (sex uncertain); CbL 209.8 mm and ZW 113.2 mm for skull 3C.l. (sex uncertain).

Regarding the C.l. skulls, CbL from skull 1C.l. fits in the CbL range for males, while ZW is below the minimum value of males and fits into the lower limit of the range for females. Measurements for skull 2C.l. (CbL, ZW) are lower than the minimum value of males and females. CbL from skull 3C.l. fits in the lower limit of the CbL range for both sexes; ZW is lower than the minimum value of males and females.

Skull 13.63.1 (female) from the CFR collection, defined a hybrid genetically (see below), has relatively small size showing morphometric dog signature (CbL 189.1 mm, ZW 119.0 mm).

Skull 13.102.1 (male) from the CFR collection, which is genetically defined a hybrid (see below), has morphometric wolf signature (CbL 244.6 mm, ZW 144.4 mm).

Skulls differing in proportions from typical wolves are also found in the TSU collection (skulls MI19, MI15, MI1/5).

Skull MI 19 has CbL 218.4 mm, ZW 116.4 mm.

Skull MI 15 has CbL 205.3 mm, ZW 101.8 mm.

Skull MI 1/5 has CbL 205.1 mm, ZW 118.4 mm.

The data show that the sizes of these three skulls contrast with the mean measurements of the studied collections (Fig. 3–4).

2) IS

Descriptive IS statistics for adult male wolves (n = 108) averages 33698 ± 252 (min–max: 27707–41130); adult females (n = 48) — 30763 ± 310 (min–max: 26909–35427). Average IS for adults without gender

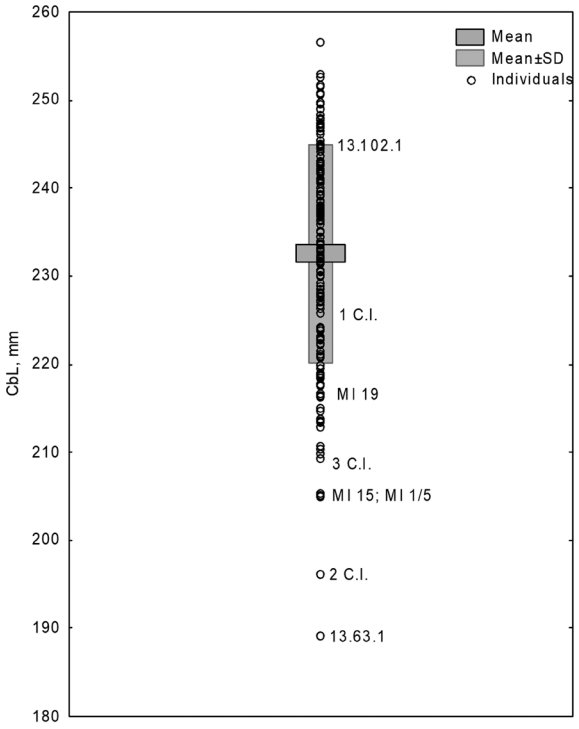

Fig. 3. CbL for wolves and atypical animals from the studied skull collections.

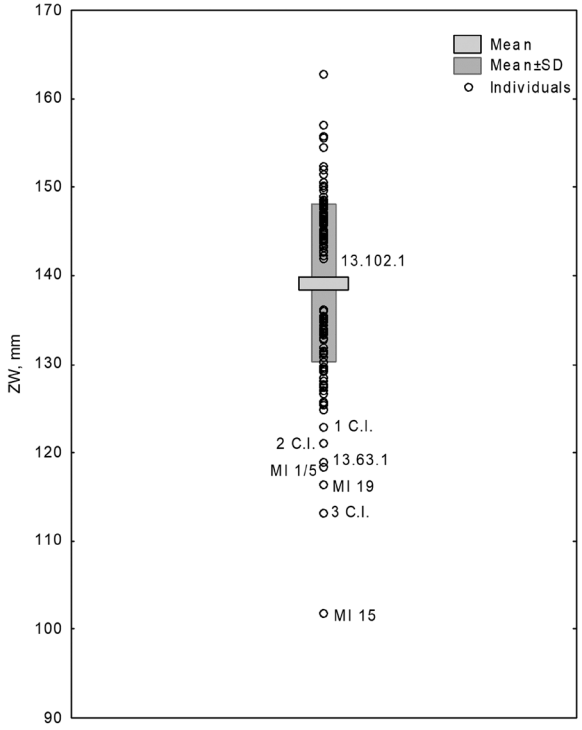

Fig. 4. ZW for wolves and atypical animals from the studied skull collections.

separation (n = 156) is 32795 ± 227 (min–max: 26909–41130) (Fig. 5). The sexual dimorphism of IS is significant (one-way ANOVA F (1;160) = 49.2; $p < 0.0001$).

IS for atypical skulls (n = 8) averages 25463 ± 1536 (min–max: 20902–35164). The difference in IS between skulls of wolves and atypical skulls averages 22%.

Morphological analysis

All examined non-metric characteristics are polymorphic, subjective and prone to variable interpretation by different investigators. Frequencies of variations for each characteristic are reported in Table 2.

Among all material studied, 5.8% did not show typical dog characteristics. One dog characteristic was observed in 50.2% of skulls, 33.4% had two such characteristics, and 6.4% had three. Proportion of skulls with 4 to 10 dog characteristics is 0.6% each.

Variation "f" was recorded in majority of characteristics in most atypical skulls (Table 3), which adds arguments to their dog nature.

Skull 1C.l., which has the only typical wolf variation, six dog variations and two intermediate ones, can be assigned to hybrids morphologically.

Skull 2C.l. shows almost all characteristics in typical dog variation and can be identified as a dog morphologically.

Skull 3C.l. shows two typical wolf variations, five dog variations and three intermediate forms and can be assigned to hybrids morphologically.

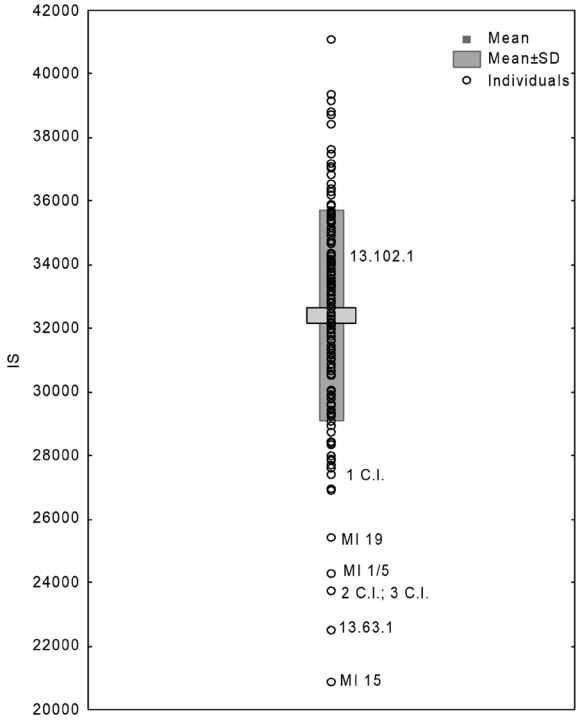

Fig. 5. IS for wolves and atypical animals from the studied skull collections.

Table 2. The frequency of variations of morphological characteristics in the studied skulls.

Character No.	Variation	Frequency
1	l	0.89
	f	0.03
	l-f	0.08
2	l	0.98
	f	0.004
	l-f	0.01
3	l	0.59
	f	0.32
	l-f	0.09
4	l	0.75
	f	0.03
	l-f	0.22
5	l	0.94
	f	0.01
	l-f	0.05
6	l	0.48
	f	0.38
	l-f	0.14
7	l	0.95
	f	0.01
	l-f	0.04
8	l	0.25
	f	0.75
9	l	0.78
	f	0.02
	l-f	0.20
10	l	0.93
	f	0.02
	l-f	0.05

Skull 13.102.1 identified as a hybrid genetically (see below), has two typical wolf variations, three dog variations and five intermediate ones and can be attributed to hybrids morphologically.

Skull 13.63.1 identified as a hybrid genetically (see below), has seven typical wolf variations with only two intermediate forms and thus can be assigned to wolves morphologically.

In atypical skulls from the TSU collection (MI 19, MI 15, MI 1/5), the vast majority of variations are typically canine, which allows these specimens to be morphologically identified as dogs.

Genetic analysis

Admixture analysis of wolves and dogs in Structure shows a sharp genetic differentiation between the two subgroups with an average q_i = 0.986 for wolves and q_i = 0.984 for dogs (Fig. 7).

The average membership probability is q_{wolf} = 0.947 (0.866–0.967, 90% BCI 0.630–1.000) for simulated wolves and q_{dog} = 0.942 (0.867–0.970, 90% BCI 0.564–1.000) for simulated dogs. Therefore, we established a threshold of $q_i \geq 0.860$ to distinguish purebred individuals from hybrids, for both wolves and dogs.

Under this threshold level, we are able to confidently detect among the atypical skulls from the CFR collection three hybrids (1C.l., 13.63.1 and 13.102.1) as well as two presumably feral dogs (2C.l. and 3C.l.) (Fig. 6).

Discussion

The history of studies related to the comparative morphology of the wolf and dog spans over 150 years. Results of these studies are the most relevant for the archaeozoologists to identify fossil finds when studying the origin of early dogs (Germonpré *et al*., 2012). Careful critical analysis of commonly used diagnostic criteria has shown that they are often based on small samples, without reliable statistical support (Janssens *et al*., 2019). Traits that were considered typical for the dog are often found in the wolf and *vice versa*. Our study generally agrees with this conclusion.

At the same time our data definitely indicate that the skulls of dogs and wolves differ in the set of characteristics, and hybridization also leaves its signature on the morphology of the skull. First of all, it can be seen in the proportions of the skull. Skulls of adult dogs or hybrids visually differ even from young wolves by smaller size (Figs. 7–8), which is confirmed by the IS values. The IS graph (Fig. 5) shows that the skulls assigned to dogs based on the combination of morphological traits are located at the bottom with a large interval from the main group of skulls. Specimens 1C.l. and 13.63.1 identified as hybrids genetically, occupy an intermediate position between presumably wolves and dogs or placed together with dogs on the graph (Fig. 5). Such a decrease in skull size was also reported by Milenković *et al.* (2006) in two hybrid females from Serbia. But the decrease in skull size in hybrids is not absolute as proved by the skull size of a male hybrid from Serbia, that did not differ from the typical wolf skull size of this region (Milenković *et al*., 2006). And also skull 13.102.1 in our study, identified as a hybrid genetically, has an IS value above the mean (Fig. 5).

Most of the morphological characteristics used in this study have intermediate forms, which complicates objective definition of variations. A good example of this is the definition of variation for characteristic 10 (Fig. 9).

Using three characteristics (8, 9 and 10) among diagnostic criteria critically analyzed by Janssens *et al*. (2019), we found certain similarity in the frequencies of variations in characteristic 9 both in our study and in the cited work. Janssens *et al*. (2019) indicate that characteristic 9 in wolves exhibited wolf-like variation in 69% and dog-like variation in 8% of skulls. Our data show similar proportion of the variants (78% and 2%, accordingly). It is remarkable that some characteristics appeared in the "f" variation more often than "l" (characteristic 8) or were close to the frequency of 50% (characteristics 3 and 6) (Table 2).

Table 3. Variations of morphological characteristics in atypical skulls.

Skull No.	Characteristic									
	1	2	3	4	5	6	7	8	9	10
1C.l.	l-f	-	l	f	f	l-f	f	f	f	f
2C.l.	f	l	f	f	f	f	-	f	f	f
3C.l.	l-f	l	l-f	f	l-f	l	f	f	f	f
13.102.1	f	l	l-f	l+f	l-f	l-f	f	f	l-f	l
13.63.1	l-f	l	l	l-f	l	l	l	f	l	l
MI 19	f	-	f	f	f	-	-	f	f	f
MI 15	f	f	f	f	f	f	f	f	l	f
MI 1/5	f	l	f	l-f	f	f	f	-	f	-

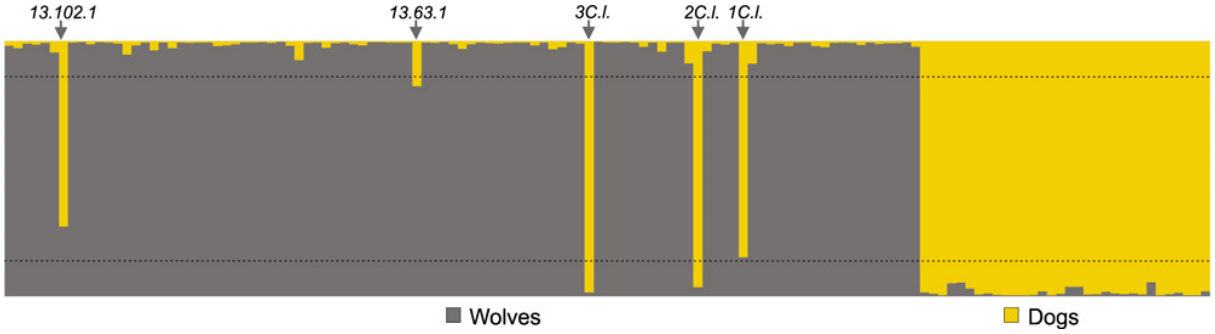

Fig. 6. Results of the Structure admixture analysis. Samples of the canids confidently assigned to hybrids (1C.l., 13.63.1 and 13.102.1) or feral dogs (2C.l. and 3C.l.) are marked. The dashed lines represent the threshold probability of 0.860 to distinguish purebred individuals from hybrids.

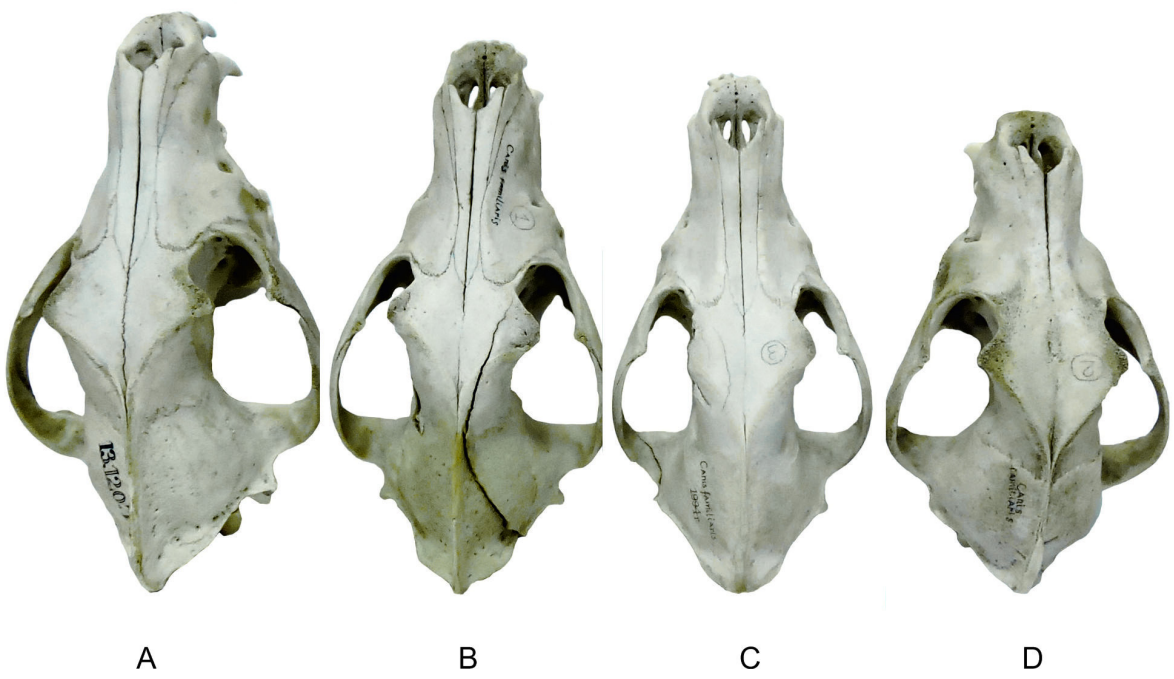

Fig. 7. Skulls from the CFR collection: A – wolf 13.120.1; B–D – atypical skulls 1C.l., 3C.l. and 2C.l., accordingly.

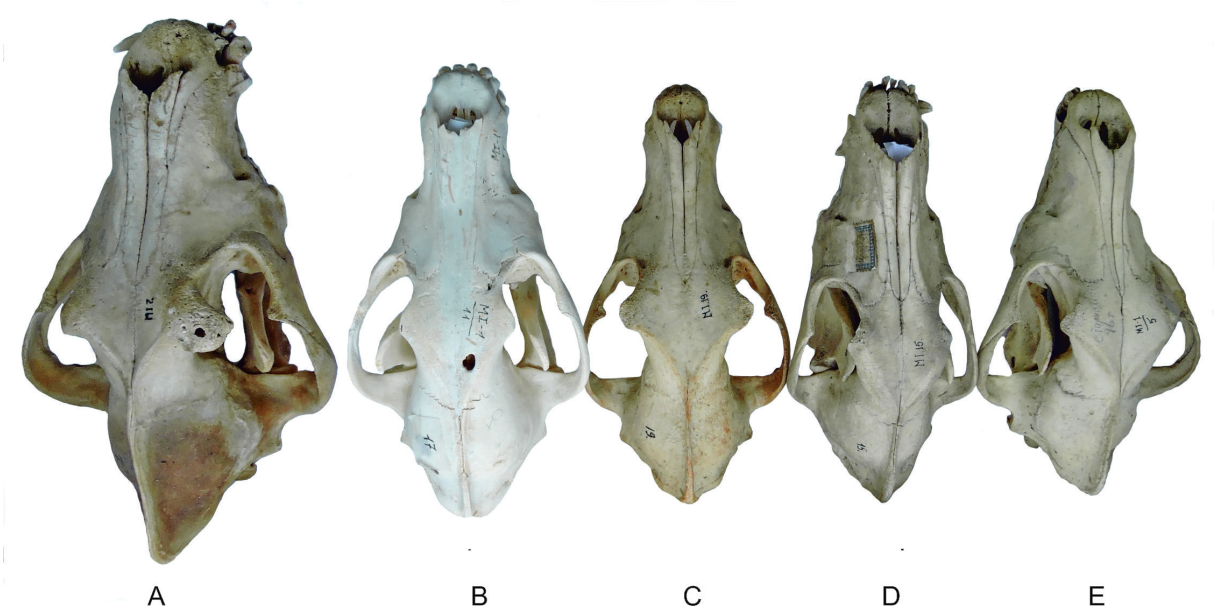

Fig. 8. Skulls from the TSU collection: A – old wolf M 1/7; B – young wolf MI 1/11; C–E – atypical skulls MI 19, MI 15 and MI 1/5, accordingly.

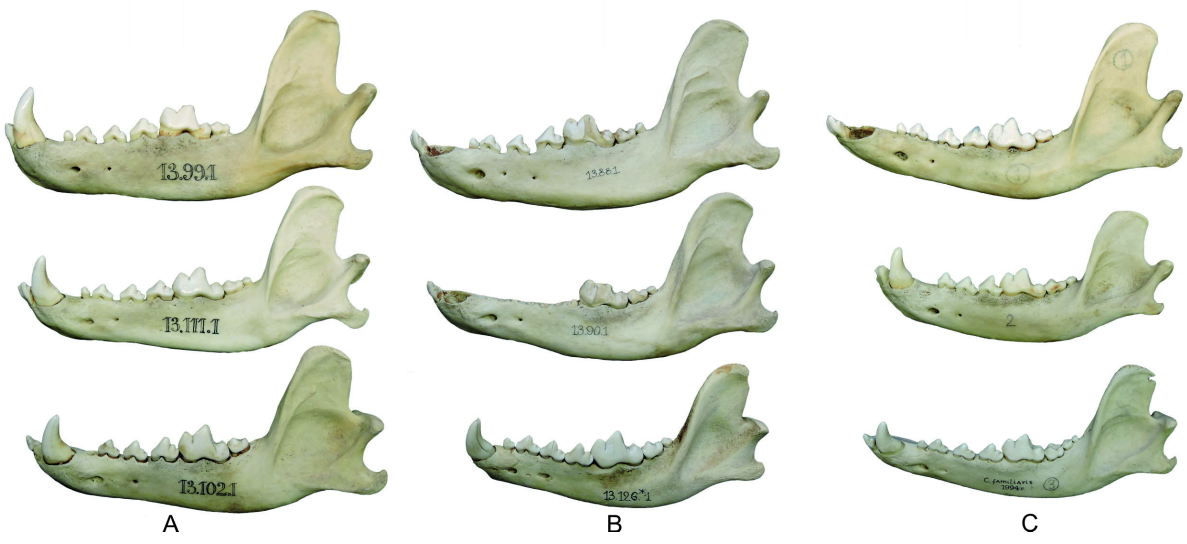

Fig. 9. Canine lower jaws: A – ventral margin in the "l" variation; B – ventral margin in the "l-f" variation; C – ventral margin in the "f" variation.

Not only archaeozoologists face difficulties in identifying the species by the skull morphology. For example, a canid, shot in the Swiss Alps in 1954, was morphologically identified by the London British Museum as a dog. Yet several decades later based on DNA analysis it was beyond doubt defined as a genuine wolf (Dufresnes *et al*., 2019).

The results of the present study further confirm data from Janssens *et al*. (2019) on the absence of a reliable morphological criterion for telling apart wolf and dog skulls. The use of the proposed criteria in identifying hybrids is probably even less effective due to the possible intermediate nature of the inheritance of traits (Milenković *et al*., 2006).

Studies on the introgression of dog genes into the wolf populations in Western Europe suggest that both the discriminating power of ancestry-informative microsatellite markers and their number are important in the analysis of hybridization (Godinho *et al*., 2011; Randi *et al*., 2014; Salvatori *et al*., 2019; Caniglia *et al*., 2020). These studies generally show that increasing the number of markers increases the reliability of hybrid

identification, while limiting the number of markers leads to an underestimation of introgression.

The panel of 11 microsatellites used in this study, has clearly only limited power to confidently identify introgression (Korablev *et al*., 2021a). This uncertainty increases the probability of incorrect assignment of hybrids to the group of purebred individuals. Certainly an increase in the number of ancestry-informative markers will identify more certain and thus additional hybridization events. However, given the low probability of the presence of dogs in the study area (Korablev *et al*., 2021a), we must expect a relatively low level of introgression, and thus the obtained data of our study here are probably accurate regarding the actual hybridization rate.

Conclusion

The results of our study show that the CbL and ZW of the skulls of dogs or hybrids are generally lower than the mean values for wolves. The product of these measurements (IS) reflects the generalized morphological differences between wolves, dogs, and hybrids, and can be used as a simple tool for primary species identification of *Canis* skulls.

In addition, our study indicates that there is not one reliable morphological criterion to distinguish between the skulls of wolves, dogs, or their hybrids, which confirms the conclusions of Janssens *et al*. (2019) for characteristics 8, 9, and 10. Characteristics 2 and 6 proposed as by Stubbe (1981) are difficult to determine due to the lack of clear criteria and the presence of intermediate forms.

Characteristics 1, 3, 4 and 5 for dogs appear in wolf skulls with a frequency of 1 to 32%, which, together with the intermediate form "l-f" it makes them also unreliable for testing. Thus, there is not a single nonmetric characteristic that is specific exclusively to wolf or dog.

At the same time a study of morphological and morphometric characteristics allows to identify skulls that require additional genetic diagnostics to confirm an allocation to one of the three groups.

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