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Genetic variation in Przewalski's horses, with special focus on the last wild caught mare, 231 Orlitza III

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Abstract. In our continuing efforts to document genetic diversity in Przewalski's horses and relatedness with domestic horses, we report genetic variation at 22 loci of blood group and protein polymorphisms and 29 loci of DNA (microsatellite) polymorphisms. The loci have been assigned by linkage or synteny mapping to 20 autosomes and the X chromosome of the domestic horse (plus four loci unassigned to a chromosome). With cumulative data from tests of 568 Przewalski's horses using blood, hair or tooth samples, no species-defining markers were identified, however a few markers were present in the wild species but not in domestic horses. Inheritance patterns and linkage relationships reported in domestic horses appeared to be conserved in Przewalski's horses. A derived type for the last wild caught mare 231 Orlitza III provided evidence for markers apparently not found in (or not currently available by descent from) the other species founders that were captured at the end of the nineteenth century. This information has been critical to the development of parentage analyses in the studbook population of Przewalski's horses at Askania Nova, at one time the largest herd of captive animals and the source of stock for reintroduction efforts. Some horses in the study showed genetic incompatibilities with their sire or dam, contradicting published studbook information. In many cases alternative parentage could be assigned from living animals. To assist in identification of correct parentage, DNA marker types for deceased horses were established from archived materials (teeth) or derived from offspring. Genetic markers were present in pedigreed animals whose origin could not be accounted for from founders. Genetic distance analysis of erythrocyte protein, electrophoretic and microsatellite markers in Przewlaski's horses and ten breeds of domestic horse place the Przewalski's horse as an outgroup to domestic horses, introgression events from domestic horses not withstanding.

Genetic studies of Przewalski's horse *(Equus ferus przewalskii)* have principally been motivated by two purposes: 1) documenting the differences between Przewalski's horse (PH) and the closely related and interfertile species, the domestic horse *(E. caballus)* (DH) and 2) making breeding management decisions for PHs, extinct in the wild and increasingly faced with inbreeding concerns in captive populations. Previous extensive genetic studies with blood groups and protein polymorphisms showed a considerable amount of variation within the species (e.g., Scott, 1979; Ryder et al., 1979, 1981, 1984; Putt and Whitehouse, 1983; Bowling and Ryder, 1987; Bowling and Dileanis, 1990; Patterson et al., 1990; Bowling, 1992; Bowling et al., 1992). A limited microsatellite survey provided additional evidence of polymorphism (Breen et al., 1994). Most genetic markers were shared with domestic horses, but markers apparently unique to PH were present. Recently, PH mitochondrial DNA (mtDNA) haplotypes have been documented through D-loop sequencing (Oakenfull and Ryder, 1998). Among representatives of the four extant female lines, only two haplotypes

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were present, one in three lines and the other in the remaining line, neither reported in DHs.

Recorded pedigrees (e.g., Volf and Kus, 1991; Kus, 1997) help provide information for breeding management decisions, but inbreeding can be minimized only if the pedigrees are correct. Animal identities can be switched as a consequence of the similarity of appearance between animals and inadequate use of permanent, reliable animal marking methods. The availability of genetic marker profiles, originally recorded for purposes of phylogeny studies, reestablished appropriate identities of switched animals (e.g., Bowling and Ryder, 1988).

In this report, we focus on deriving the genetic type for 231 Orlitza III, the last wild caught mare (captured in Mongolia in 1947) whose offspring were bred at Askania Nova (AN) in Ukraine. For breeding management of a species whose pedigrees otherwise include only 11 animals captured from the wild in 1901–1903, Orlitza's genome potentially provides significant contributions of genes and combinations critical to species survival. We also present an extensive genetic marker survey incorporating erythrocyte antigens, protein polymorphisms and microsatellite data from 152 PHs bred or used at AN. The data include 51 loci, covering at least 20 autosomes plus the X of the DH (and four loci for which the autosomal assignment is unknown). An additional 400 PHs from zoos throughout Europe and North America, although not as comprehensively profiled as those of the AN group, are combined with information from AN animals for a comprehensive marker assessment of the phylogenetic distance relationship between PH and breeds of DH.

Materials and methods

Samples

Blood, teeth and hair were used as sources of antigens, proteins and DNA for blood group, protein and microsatellite variation assays. Tissue cultures established from skin biopsies and blood were used as a source of DNA for mtDNA studies. Blood samples were collected from 145 living horses bred or used at AN. Teeth from another seven animals provided from museum collections at AN and Kiev were used as a DNA source to assist in ascertaining and deriving AN founder types. Genetic profiles, including data on erythrocyte antigens, protein variants detected by electrophoresis and/or microsatellite markers were also obtained from 416 animals from zoos throughout North America and Europe. Animals in this report are identified according to their studbook identity (Volf and Kus, 1991) at the time of sampling. DH data used for comparison was from the database information of the Veterinary Genetics Laboratory, which included tests of over one million horses from at least 50 breeds, although not all samples or breeds were tested for every system described here.

Loci analyzed

Using standard protocols for serology, protein electrophoresis and fragment length analysis of fluorescent-tagged primer PCR-amplification of DNA, seven loci of blood groups, 15 loci of protein polymorphisms and 29 loci of microsatellites were analyzed for genetic variation (references provided in Juneja et al., 1984, 1989; Bowling and Clark, 1985; Bowling and Dileanis, 1990; Bowling et al., 1990, 1992, 1997) mtDNA sequence was determined according to the protocol of Oakenfull and Ryder (1998). The locus abbreviations and allelic nomenclature provided in the tables represent a consensus standard nomenclature used for DH by member laboratories of the International Society for Anim Genet (ISAG). Conclusions of parentage exclusion followed robust implementation of laboratory procedures and included extensive retesting to verify results. Due to the large number of highly polymorphic loci tested for each animal from the AN program (usually 51 loci, except for animals tested from tooth samples for which only microsatellite profiles were obtained), a qualification of parentage was tantamount to proof of parentage (Bowling, et al., 1997). Chromosome assignments for genes and microsatellites are from linkage maps assembled for DH by Lindgren et al. (1998) and Guérin et al. (2003) and from synteny mapping by Shiue et al. (1999). While PH has two more chromosomes than DH (2n = 66 vs 64), the difference involves a Robertsonian translocation, in which chromosome 5 of DH (ECA5) (a metacentric) is present in PH as two acrocentric chromosomes (Ryder et al., 1985; Yang, et al., 2003; Myka, et al., 2003).

Deriving types

Although a blood sample was not available from 259 Pegas, his blood and microsatellite DNA type could be derived using available offspring and their dams. On first analysis, Pegas' putative offspring provided evidence for a profile that would have more than two alleles at several loci, but subsequently a subset of offspring was identified that provided a consistent genotype with no more than two alleles per locus. The derived DNA profile matched that later obtained using an archived tooth from Pegas. No archived material was available from Orlitza III, but her type could be derived using genetic profiles obtained directly from her son 285 Bars (blood), directly and by derivation from her son Pegas, from 146 Robert Orlik (tooth) and from her grandson 313 Vizor (tooth).

Genetic distance

The genetic distance values for pairwise considerations of ten DH breeds and PH, based on 38 loci were calculated using DISPAN, Ota (1993). These included seven blood group loci (A, C, D, K. P. Q and U; 15 protein polymorphisms (at the AP, CA, CAT, HBA, PGD, PGM, GPI, ALB, C3, ES, GC, .PLG, TF, PI and XK loci); and 16 microsatellite loci (ASB17, VHL20, HTG10, HTG7, HTG4, AHT5, AHT4, HMS6, HMS3, HMS7, HMS1, LEX3, LEX33, ASB2, UCDEQ425 and HTG). Thirteen additional microsatellites were tested for the PH diversity study, but only limited DH data were available for these loci when the studies were undertaken, so they are not included in the genetic distance calculations. Ten breeds representative of draft horses, light (riding) horses, racehorses and ponies were used for calculating allele frequencies: Percheron (PN), Arabian (AR), Paso Fino (PF), Iberian (IB), Lipizzaner (LI), Morgan Horse (MH), Trakehner (TK), Thoroughbred (TB), Norwegian Fjord (NF) and Miniature (MI). The number of DH and PH analyzed for each locus are listed in the Supplemental Table (available from the corresponding author or at www.karger.com/doi/10.1159/000075754). Each of these breeds consists of a larger population than the PHs, and has a larger number of founders. Probably the LI data in this study provide the closest comparison to PH in terms of population parameters (founders, current size, number of animals tested). As a means of visualizing these distance data, a dendrogram was constructed based on a neighbor joining algorithm (NEIGHBOR) using PHYLIP (Felsenstein, 1993).

Results

Genetic markers of PH and variant comparisons with DH

Blood groups: In tests that detect 52 alleles across ten breeds of DH, 19 alleles were found, none unique to PH (Table 1). PH is particularly restricted at EAD with three alleles compared with 25 in DH. The only fixed locus was EAK – no PHs were positive for the single antigen (Ka) identified for this locus.

Protein polymorphisms: In tests that detect 100 alleles across ten breeds of DH, 43 variants were found, seven (at five loci) unique to PH (ES-P, GPI-T, XK-P, TF-d, PI-Prz,-Pzl, -Pzk) (Table 2). These variants have been described previously (Scott, 1979; Bowling and Ryder, 1987; Patterson et al., 1990). The only fixed loci (PGD, GC) were those with low allelic diversity in DH.

Microsatellites: In tests that detect at least 297 alleles across ten breeds of DH, 137 alleles were found, eight (at seven loci) unique to PH (HMS15-223, ASB17-Y, HMS3-T, HMS2-S, -U,

EB2E8-T, UCDEQ502-T and LEX22-111) (Table 3). Due to the limited information available across breeds for some of the microsatellite loci, the number of alleles given for DHs might be low. The identification of alleles not found to be represented in DH at HMS15, HMS2, EB2E8, UCDEQ502 and LEX22 might prove incorrect, but the data are not thought to provide a significant misrepresentation.

Table 1. Blood groups in Przewalski's horses: origins by founder among Askania Nova horses

ECA[a]	Locus	No. of alleles[b]		AN source						
		DH	PH	RO plus O (derived)[c]			Hosana (derived)[d]		Am[e]	Vjuga[f]
2	EAK	2	1	-/						
8	EAQ	6	4	b	c	/-			ac	
(14)	EAD	25	3	adn	cg	d				
20	EAA	12	5	bce	/-		bc	adf		adg
24	EAU	2	2	a	/-					
U	EAC	2	2	a	/-					
U	EAP	3	2	/-			acd			
	Total	52	19							

[a] *Equus caballus* chromosome assignment, provisional in brackets.
[b] DH = domestic horse; PH = Przewalski's horse.
[c] RO = Robert Orlik, O = Orlitza.
[d] Only partial type derived.
[e] This blood group found in American horses (Sigor, Sibol, Lisa, Boleta) at AN, not in other sources.
[f] This blood group found in Vjuga and descendants, not in other sources at AN.

Comparison of variants: Most of the genetic variants in PHs are shared with DHs (184/199 alleles, 92%). No species defining loci were identified. Nonetheless, profiles of individual animals are usually distinctive from DHs, especially by blood typing, due to the high frequency of unique PH blood protein variants at TF, ES and PI (data not shown). Calculations of average heterozygosity based on allelic frequencies (Table 4) suggest that heterozygosity is within the estimated range for all the DH breeds, except the breed with the highest heterozygosity (MIs). These heterozygosity comparisons are also provided in Bowling and Ruvinsky (2000).

Deriving the genetic profile for Orlitza

The derived type for Orlitza's DNA (microsatellite) markers is presented in Table 3. Lacking direct blood typing information for Robert Orlik, the sire of her offspring, it was difficult to derive Orlitza's markers in blood group and protein systems. However, from consideration of genotypes of PHs worldwide, it is clear that Orlitza provided two new alleles for PH, TF-D and CA-E, both found in DH. Considering the DNA markers, she contributed two other new variants to PHs, HMS3-T, EB2E8-T, neither one shared with DH. For the microsatellite loci, she was heterozygous at 14 of 25 autosomal loci (56%), a minimum estimate since it is based only on transmission data to three offspring.

Linkage relationships among loci

Several of the tested loci are known to be syntenic in DH, specifically loci on ECA1, 4, 10, 15, 16, 24 and X (see chromosome assignments given in Tables 1–3). Among those known to be linked in DH, the same relationships appeared to be true for

Table 2. Protein polymorphisms in Przewalski's horses: origins by founder among Askania Nova horses

ECA[a]	Locus	No. of alleles[b]		AN: derived types[c]			AN		Elsewhere, not in AN founders			Introgression at AN?)	
		DH	PH (u)	(RO +	O)	(H)	American[d]					RO dam[e]	Vjuga
02	PGD	3	1	F									
03	GC	2	1	F									
03	ALB	3	2	B			A						
03	ES	12	4 (1)	P[f]		H			I	#[g]			
05	PGM	3	2	F					S			S	
07	C3	5	3	2			3	4					1
(07)	CAT	3	3	F	S		X						
(07)	APOA4	4	2	F	S								
09	CA	6	2	E	I								
10	GPI	5	2 (1)	I			T						
10	XK	4	3 (1)	F	K		P						
13	HB	5	2	I	II								
16	TF	15	7 (1)	d	D	F3	F2		E	I	J		
24	PI	25	6 (3)	Prz	Pzl		L	S1	Pzk	P		L2	
U	SP3	5	4	F1	F2	I							S
	Total	100	44 (7)										

[a] *Equus caballus* chromosome assignment, provisional in brackets.
[b] DH = domestic horse, PH = Przewalski's horse, u = unique to PH, not in DH.
[c] RO = Robert Orlik, O = Orlitza, H = Hosana.
[d] Additional variation at AN contributed by Sigor, Sibol, Lisa, Boleta.
[e] Dam of Viola, Vira, Vetla, Vorot.
[f] Crosshatching denotes PH allele.
[g] Null allele.

PH (Bowling, unpublished results). Linkage disequilibrium among linked loci on ECA4, 10, 15, 24 and X allowed the added power of haplotyping to point to parentage solutions.

Pedigree analysis

Initially, to derive the genetic profile of Orlitza III we used only blood samples obtained from animals at AN in 1991 and 1992 and from AN-bred horses elsewhere (285 Bars, 601 Vira, 605 Vorot, 606 Vulkan, 831 Varna, 826 Vata, 832 Plunzer, 1118 Garant and 1048 Moros). Analysis of blood group and protein polymorphisms and a limited set of DNA markers identified pedigree incompatibility problems that needed to be sorted out before the profiles could be derived. For example, the collection of horses assigned to be sired by Pegas could not all be offspring of a single stallion (data not shown). Likewise, offspring attributed to Volga, daughter of Orlitza III, could not all be the offspring of a single mare (data not shown). Additionally, among offspring that represented inbred combinations of Robert Orlik and Orlitza III with no other founder input, more alleles were present at some loci than could be provided by two horses (e.g., six alleles at ASB17, that is, two more than could be provided by two horses; four alleles at LEX3 (X-linked), one more than could be provided by a single mare and stallion) (Table 5). While the data clearly provided evidence to exclude parentage relationships, correct parentage could not be assigned without additional information. For this task we increased the number of loci tested and tested samples (teeth) from AN genetic founder animals. We had biological samples (blood or teeth) from all AN founders except Orlitza III (samples from Robert Orlik, 283 Hosana, 396 Vada, 533 Sigor, 1128 Sibol, 812 Boleta and 846 Lisa), although in the last analysis we were unable to obtain results from Vada's tooth sample. We also obtained a profile from a tooth from 295 Sixtus, an infertile stallion imported to AN from Germany with no studbook-assigned foals. We had no material from another stallion at AN with no assigned foals (79 Tornado/Vasik), but with a similar pedigree to Hosana and Vada.

Table 3. Microsatellite polymorphisms in Przewalski's horses: origins by founder among Askanis Nova horses

ECA[a]	Locus	No. of alleles[b]		Allelic variation in Przewalski's horses at AN and elsewhere									At AN, not elsewhere (Introgression?)		
				At AN							Outside AN				
		DH	PH (u)	Robert Orlik		(Orlitza)[c]		Hosana		American horses[d]	Additional variants		Unkn RO **dam**[e]	Vjuga	Vika, Sosna, Moros, Potok
01	HMS7	10	5	L	Q	(O)		L	O		N	S			
01	HMS15	15	8 (1)	229	231	(217)	(#)[f]	195	225	219	223[g]				
01	UCDEQ487	9	6	P	#	(M)	(O)	P	Q	L			N		
02	ASB17	22	8 (1)	H	S	(D)	(T)	U	Y		F	W	N		
04	HTG7	5	4	M	N	(K)	(P)	N					O		
04	LEX33	12	5	P	S	(R)	(S)	P	S	Q					
04	HMS6	8	3	L	N	(L)		N			M		O		
08	AHT5	11	4	K	N	(K)	(O)	K	N	M			Q		
08	UCDEQ46	5	2	M		(L)		nt[h]							
08	LEX23	11	4	250		(236)	(246)	250		240	226				
09	HTG4	8	5	N	Q	(K)		M	P					O	
09	HMS3	11	6 (1)	P	R	(M)	(T)	I	P	L					
10	ASB9	9	5	O		(M)	(O)	N	P		L		I		
10	HMS2	9	6 (2)	J	K	(M)	(N)	S	U						
10	UCDEQ412	12	3	K	P	(P)		K	P	R					O
15	ASB15	10	4	E	N	(E)		I			P			M	P
15	ASB2	14	5	B	M	(K)	(M)	B	M	N	R		I		
15	HTG6	11	3	N		(N)		N		R	O		J	I	
15	HMS1	8	3	K	M	(M)	(N)	K					J		
16	UCDEQ505	10	4	M	N	(Q)		N	Q		G				
21	HTG10	12	7	K	O	(J)		N		I	Q	R	M		
24	AHT4	11	5	H	N	(N)	(J)	K	N	L					
26	EB2E8	8	3 (1)	K	N	(T)		K							
28	UCDEQ425	11	6	K	L	(J)		K	O		F	I			I
30	VHL20	10	4	M	R	(O)	(R)	P							I
X	UCDEQ502	9	6 (1)	T		(O)		F			N	P	S	K	
X	LEX27	6	3	200		(198)		202							194
X	LEX22	6	3 (1)	115		(105)		111	115				113		
X	LEX3	14	6	I		(L)	(O)	H	M		F				
	Total	297	136 (8)												

[a] *Equus caballus* chromosome assignment.
[b] DH = domestic horse, PH = Przewalski's horse, u = unique to PH, not in DH.
[c] Derived using types of Robert Orlik, Pegas, Bars and Vizor.
[d] Additional variation at AN contributed by Sigor, Sibol, Lisa, Boleta.
[e] Dam of Viola, Vira, Vetla, Vorot.
[f] Null allele.
[g] Crosshatching denotes PH allele.
[h] Not tested.

Table 4. Estimated average heterozygosity at 38 polymorphic loci for ten breeds of horses and Przewalski's horse, arranged from lowest to highest values. Sixteen erythrocyte antigen loci, seven protein polymorphisms and 15 microsatellite loci were analyzed (see Materials and methods). Ten individuals of each breed were used for estimating genetic diversity

Breed or taxon[a]	Estimated average heterozygosity *(± SD)*
TB	0.461 *(± 0.047)*
LI	0.473 *(± 0.042)*
PH	0.474 *(± 0.044)*
AR	0.478 *(± 0.045)*
IB	0.491 *(± 0.046)*
TK	0.511 *(± 0.043)*
NF	0.531 *(± 0.039)*
PN	0.535 *(± 0.041)*
MH	0.537 *(± 0.042)*
PF	0.551 *(± 0.045)*
MI	0.579 *(± 0.038)*

[a] AR: Arabian; IB: Iberian; LI: Lipizzan; MI: Miniature Horse; MH: Morgan Horse; NF: Norwegian Fjord; PF: Paso Fino; PN: Percheron; PH: Przewalski's horse; TB: Thoroughbred; TK: Trakehner.

Table 6. Examples of genetic analysis of parentage, considering the studbook pedigree and the likely true pedigree based on up to a total of 51 loci of blood type and DNA markers

	Year of birth	Studbook pedigree		Genetic analysis[a]			Genetic pedigree[b]	
		Sire	Dam	Mating	Sire	Dam	Sire	Dam
490 Vetka	71	*Vizor*	Volga	*EXC*	*EXC*	nt	Pegas	Viola
524 Viola	72	*Vizor*	Volga	*EXC*	*EXC*	nt	Gordyj	? §
548 Vena	73	*Pegas*	Volga	*EXC*	*EXC*	nt	Gordyj	Vetla
601 Vira	74	Pegas	Volga	*EXC*	Q	nt	Pegas	? §
606 Vulkan	74	Pegas	*Vetka*	*EXC*	Q	*EXC*	Pegas	Hosana
765 Vetla	78	*Pegas*	*Vira*	*EXC*	*EXC*	*EXC*	Vizor	? §
766 Veska	78	Pegas	*Vena*	*EXC*	Q	*EXC*	Pegas	Vetla
826 Vata	79	*Pegas*	*Vena*	*EXC*	*EXC*	*EXC*	Gordyj	? *
831 Varna	79	Pegas	*Vetka*	*EXC*	Q	*EXC*	Pegas	Viola
832 Plunzer	79	Pegas	*Vira*	*EXC*	Q	*EXC*	Pegas	?
843 Vaflja	79	Pegas	Volga	*EXC*	Q	nt	Pegas	?
893 Volsebnik	80	Pegas	*Vena*	*EXC*	Q	*EXC*	Pegas	Vira
970 Paris	81	Pegas	Volga	*EXC*	Q	nt	Pegas	? *

[a] nt = not tested; EXC = excluded as parent; Q = qualifies as parent.
[b] ? § = mare by Robert Orlik out of unknown dam; ?* = mare by Vizor out of Hosana (i.e., Golubka).

Table 5. Selected loci of microsatellites for horses that by studbook trace only to Robert Orlik plus Orlitza III showing the presence of more alleles than can be accounted for by a single breeding pair

Horses	Alleles present at microsatellite loci			
	ASB17	*HTG7*	*HTG4*	*LEX3*
490 Vetka	NT	N	KN	LM
524 Viola	NY	MN	NP	IM
548 Vena	SY	NO	KN	MO
601 Vira	HS	MN	N	L
606 Vulkan	TU	KN	MN	M
765 Vetla	HS	MO	N	IO
766 Veska	HS	KM	KN	IL
826 Vata	HT	NP	MP	M
831 Varna	TY	KM	KP	LM
832 Plunzer	H	KM	KN	O
843 Vaflja	H	NP	KQ	LO
893 Volsebnik	ST	MN	KN	L
970 Paris	T	KP	MN	M
Alleles	H,N,S,T,U,Y	K,M,N,O,P	K,M,N,P,Q	I,L,M,O

From pedigree analysis of 139 animals, genetically compatible parentage matches were identified for 110. Another 29 could not be confirmed since we did not have two qualifying parents. In a few cases, a studbook-listed parent was dead or not tested. In others the studbook-listed parentage was tested and excluded, but no qualifying parent could be found. Primarily the foals were sired by Pegas (28), as well by his sons 391 Gordyj (3), 821 Parad (26) and 970 Paris (27). Due to the extensive genetic profiles obtained for all the horses, despite the similarity in pedigrees, only a single qualifying sire was identified for each offspring (all others could be excluded). No offspring were attributed to Sixtus by studbook record or through genetic testing. The pedigree corrections have been provided to the management staff at AN and to the studbook keeper.

Primarily the insoluble problems appeared to be in the pedigrees of animals foaled prior to the mid-1980's. For example, among the 20 tested animals foaled from 1971 to 1980, none could be verified to have a correctly assigned pedigree. For the most part, by pedigree these animals represented crosses of descent from Robert Orlik and Orlitza III, but from genetic tests, the founder inputs were more complex and included Hosana as well as unidentified horses. Among several of the older horses (e.g., Viola, Vira, Vetla) for which a sire could be identified (see Table 6), a genetic profile could be established for a dam that could be a daughter of Robert Orlik, but included alleles not found either in the profile of Robert Orlik, of Hosana or the derived type of Orlitza III. For example, see ASB17-N and HTG7-O among profiles in Table 5, not present in AN founder types provided in Table 3. Accounts of horses at AN suggest that PH/DH hybrids sired by Robert Orlik were present (Treus, 1962; Garrutt et al., 1966). A possible explanation for the presence of alleles at AN not present in AN PH founders was that one of these hybrid mares had been misidentified as a PH, probably as Volga. A critical test of this hypothesis was mtDNA analysis for horses tracing to Volga in matrilineal line. A mtDNA type had been determined for Bars, Oakenfull and Ryder (1998) which should be shared by his sister Volga and her matrilineal descent. In support of the hybrid hypothesis, control region mtDNA haplotypes of 490 Vetka, 601 Vira and 831 Varna were identical but did not match that of Bars (Fig. 1).

Another problem foundation pedigree was that of Vjuga (by pedigree: Vizor × Vada). She was excluded to Vizor and no alternative sire could be identified. Unfortunately the Vada tooth did not yield PCR-amplifiable DNA so we had no genetic profile for Vada. Vjuga had several distinctive variants, not

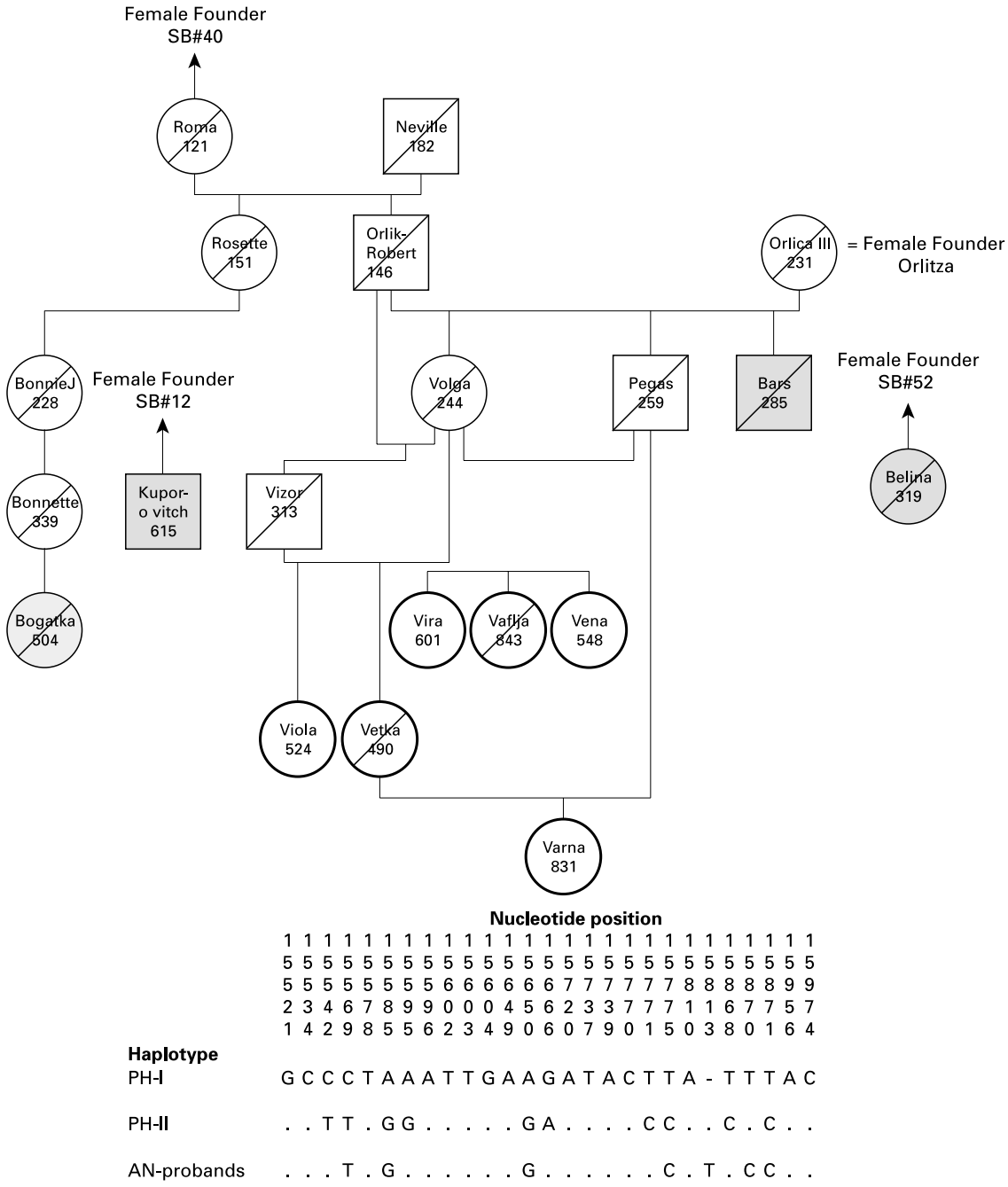

Fig. 1. Partial pedigree of Askania Nova Przewalsk's horses. The mitochondrial haplotype of Orlitza III (231) was determined by evaluating her offspring, Bars (285). This haplotype corresponds to a known PH haplotype (PH-II). Reputed descendants of Volga (244) displayed a single haplotype not observed in Przewalski's horses (denoted AN-probands in the haplotype diagram below the pedigree). Nucleotide positions in the haplotype diagram correspond to the numbering of the *E. callabus* complete mitochondrial sequence of Xu and Arnason (1994).

present in other pedigrees at AN, and also not present in PHs outside of AN (for example, C3-1, HTG6-I, UCDEQ502-K; Tables 2 and 3). The pedigree elements of Vada and Hosana are common in PH pedigrees worldwide and we could anticipate that factors present in Vada but not Hosana would be found outside of AN. Thus, we also propose introgression from a second source into the Vjuga line, possibly through the sire, since no qualifying stallion was identified.

Finally, as also presented in Tables 2 and 3, there was a third set of factors in the horses 898 Potok, 966 Vika, 1048 Moros and 1215 Sosna that could not be accounted for from AN founders.

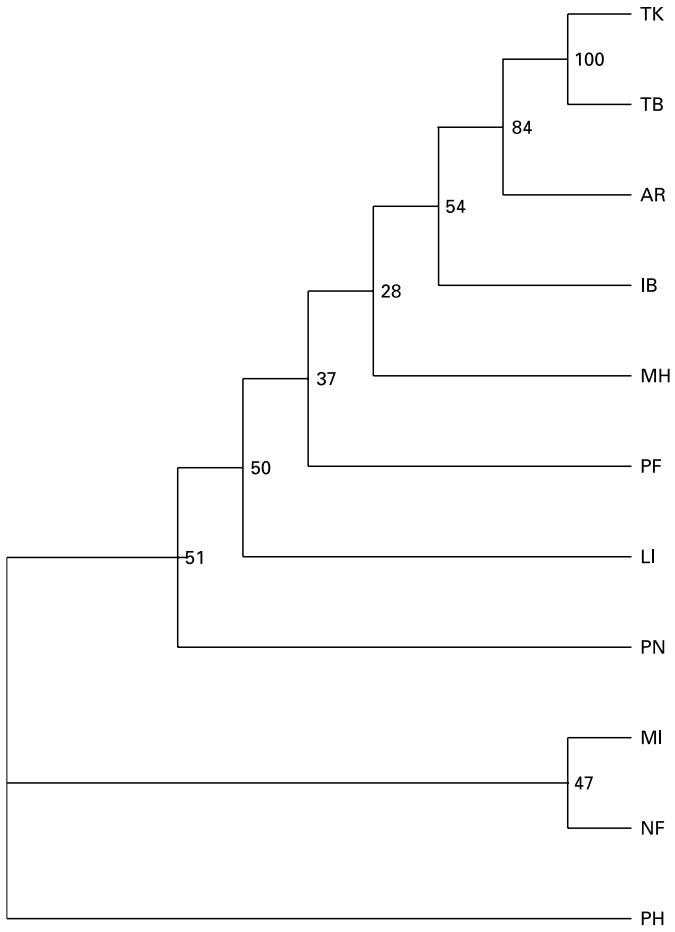

Fig. 2. Neighbor-Joining dendrogram of ten breeds of domestic horses and Przewalski's horse. Genetic distances were calculated and dendrograms produced as described in Materials and methods. Numbers adjacent to nodes represent boostrap percent values from 1,000 replications of resampled loci. Breed designations are abbreviated as in Table 4.

Phylogeny

As in other genetic distance studies, in our present effort PH provides the most dissimilar of the paired comparisons between DH breeds or between PH and DH breeds (Table 7). As a means of visualizing these distance data, a dendrogram was constructed based on a neighbor-joining algorithm (Fig. 2), likewise showing the outgroup position of the PHs compared with DHs. These data and the figure are also presented in Bowling and Ruvinsky (2000).

Discussion

Genetic diversity among PHs

Genetic diversity is of special significance for endangered species such as PH. The potential for inbreeding problems is a constant concern due to the small founder numbers and the restricted breeding bases conveniently available for zoos. Inbreeding leads to homozygosity or the possibility of pairing recessive deleterious or lethal alleles. Since the species is extinct in the wild, genetic variation cannot be augmented from that resource. Zoological societies have agreed to manage the genetic variation in the captive populations to minimize inbreeding and maintain approximately the present levels of variability (Ryder et al., 1984; Princée et al., 1990; Zimmermann, 1997). While the total number of alleles found in PHs for the loci analyzed in this study is slightly under half that of DHs (199 *vs* 449), nonetheless the allelic frequency distributions are such that theoretically the animals can exhibit as much heterozygosity as within breeds of DH (see Table 4). While individual animals, especially the products of inbred pedigrees, may be relatively homozygous, management schemes whose goals are to minimize inbreeding should be able to generate animals with comparatively high levels of heterozygosity.

Table 7. Standard genetic distances (± SD) between ten breeds and Przewalski's horse based on 38 polymorphic loci as described in Materials and methods

	MH	NF	PF	TB	TK	AR	LI	PN	IB	MI
NF	0.109 ± *0.02*									
PF	0.057 ± *0.01*	0.124 ± *0.02*								
TB	0.114 ± *0.03*	0.214 ± *0.05*	0.129 ± *0.03*							
TK	0.069 ± *0.01*	0.152 ± *0.04*	0.094 ± *0.02*	0.041 ± *0.01*						
AR	0.078 ± *0.02*	0.175 ± *0.04*	0.099 ± *0.02*	0.105 ± *0.02*	0.065 ± *0.02*					
LI	0.113 ± *0.02*	0.194 ± *0.05*	0.126 ± *0.02*	0.202 ± *0.05*	0.155 ± *0.03*	0.139 ± *0.03*				
PN	0.079 ± *0.02*	0.115 ± *0.03*	0.092 ± *0.02*	0.194 ± *0.04*	0.150 ± *0.03*	0.156 ± *0.03*	0.132 ± *0.03*			
IB	0.107 ± *0.03*	0.168 ± *0.03*	0.092 ± *0.02*	0.170 ± *0.04*	0.137 ± *0.03*	0.109 ± *0.03*	0.199 ± *0.04*	0.139 ± *0.03*		
MI	0.091 ± *0.02*	0.092 ± *0.02*	0.083 ± *0.02*	0.182 ± *0.04*	0.141 ± *0.03*	0.151 ± *0.03*	0.136 ± *0.03*	0.089 ± *0.02*	0.137 ± *0.03*	
PH	0.345 ± *0.08*	0.354± *0.08*	0.323± *0.08*	0.382± *0.09*	0.382± *0.09*	0.394± *0.09*	0.394± *0.09*	0.344± *0.08*	0.389± *0.09*	0.308± *0.07*

Genetic profile for 231 Orlitza III

In the earliest samples we tested in the mid-1980's from AN representatives tracing to Orlitza III, we noted the conspicuous presence of TF-D and CA-E, which we had not previously detected in PHs (Bowling and Ryder 1987). Recently, studies showed that the mtDNA type from Bars (son of Orlitza III) matched the haplotype of two of the earlier wild caught horses, so there was also evidence for genetic similarity of Orlitza III with other PHs. Although Orlitza III died in 1973, we were able to derive a comprehensive genetic profile at 29 microsatellite loci based on the markers that she transmitted to her offspring Bars, Pegas and Volga (to Vizor). From her derived type considering 51 loci (see Tables 1–3), it is apparent that although she brought in new variants for the species at four loci, Orlitza III shared most of her alleles with those of the earlier PHs. The genetic legacy of Orlitza III survives in current pedigrees through three offspring Bars, Pegas and Volga, each of which contained a distinctive sampling of her genome.

Pedigree problems and reassignments

Unanticipated pedigree problems were encountered in the process of deriving the genetic profile for Orlitza III. In our final analysis we were able to sort out pedigrees for most but not all of the animals. We found genetic variants other than those provided by the AN foundation stock. Our data suggested that the Orlitza III daughter Volga may have been represented only by her son Vizor. mtDNA typing substantiated the hypothesis of introgression into PHs through horses alleged to be offspring of Volga. Additional possibilities of introgression were also identified.

As increasingly revealed by molecular studies in other taxa, maintaining without introgression a wild species that is interfertile with other species or subspecies is a difficult proposition. Other examples of introgression in endangered species include Asiatic lions (O'Brien, et al., 1987; Driscoll, et al., 2002), Bornean and Sumatran orangutans (Ryder and Chemnick, 1993), and American bison (Ward, et al., 2001). Species preservation programs are built on a complex platform of priorities. The specter of introgression is but one of the issues facing conservation projects. In the case of PHs, the animals without either the previously Mongolian domestic documented introgression, (Volf and Kus, 1991) or the introgression reported here, represent a restricted breeding group with documented fertility problems (Bader et al., 1990; Hegel et al., 1990).

Phylogenetic relationships

The extra chromosome pair in PH compared with DH makes it conceptually difficult to put them on a direct line of relationship. Perhaps not surprisingly, the extended genome coverage of this study does not contradict previous dendrograms based on nuclear genes – PH remains as an outgroup to the domestic horse breeds (Bowling and Ryder, 1987; Dubrovskaya et al., 1992; Tikhonov et al., 1998). This conclusion persists, despite the accepted presence of introgression from DH represented in published pedigrees by the Mongolian domestic mare and the additional introgression proposed here. Furthermore, comparison of PH and DH mtDNA control region sequences with those of Pleistocene horses revealed a closer similarity of PH and DH mitochondrial DNA than either has to Pleistocene horses (Vila, et al., 2001).

Conclusion

Orlitza III is the only PH species founder from which a derived genetic profile has been obtained. The genetic profile for Orlitza compared with that of the descendants of the earlier 11 wild caught animals provides evidence that she contributed new genetic variants to PHs, including two alleles apparently unique to PH. She had overlap with domestic horses at substantially all genetic loci, as have previously tested animals. In the efforts to derive her type using her descendants bred at AN, incorrect pedigrees were found. With extended genetic profiling it was possible to ascertain the correct parentage for most of the horses. However, overall more alleles were present than could be accounted for from the AN founders, findings that are most straightforwardly explained by introgression from DH. The pool of PHs without introgression is small and there are serious concerns about whether that subset can maintain the species. Despite introgression, our data indicate that the Przewalski's horse stands as an outgroup to breeds of domestic horses in the dendrograms based on nuclear genes (Fig. 2). The preservation of the Przewalski's horse gene pool necessitates incorporation of all the animals with all founder ancestries, including those involving introgression.

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