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Genetic diversity of donkey populations from the putative centers of domestication

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Summary

Donkey domestication drastically changed ancient transport systems in Africa and Asia, enabling overland circulation of people and goods and influencing the organization of early cities and pastoral societies. Genetic studies based on mtDNA have pointed to the African wild ass as the most probable ancestor of the domestic donkey, but questions regarding its center of origin remain unanswered. Endeavoring to pinpoint the geographical origin of domestic donkey, we assessed levels and patterns of genetic diversity at 15 microsatellite loci from eight populations, representing its three hypothesized centers of origin: northeast Africa, the Near East and the Arabian Peninsula. Additionally, we compared the donkey genotypes with those from their wild relative, the African wild ass (*Equus africanus somaliensis*) to visualize patterns of differentiation among wild and domestic individuals. Obtained results revealed limited variation in levels of unbiased expected heterozygosity across populations in studied geographic regions (ranging from 0.637 in northeast Africa to 0.679 in the Near East). Both allelic richness (Ar) and private allelic richness presented considerably higher values in northeast Africa and in the Arabian Peninsula. By looking at variation at the country level, for each region, we were able to identify Sudan and Yemen as the countries possessing higher allelic richness and, cumulatively, Yemen also presented higher values for private allelic richness. Our results support previously proposed northeast Africa as a putative center of origin, but the high levels of unique diversity in Yemen opens the possibility of considering this region as yet another center of origin for this species.

Keywords allelic richness, centers of origin, domestic donkey, microsatellite variation

Introduction

Domestication of plants and animals has drastically transformed the course of human history. It provided early farmers with predictable food sources and influenced the density and mobility of human settlements, enabling the rise of civilization. Among the earliest centers of domestication, the Fertile Crescent stands out as the most important source of livestock species, with goat, sheep, cattle and pig undergoing domestication events in a short period of time, between 11 000 and 10 000 years ago (Zeder 2008). This first wave of domestication provided the food resources for a rapid demographic expansion of human populations and triggered overland transport of people and goods. Increasing needs for efficient means of transportation led to a second wave of domestication, which is believed to have started in the Eurasian grasslands about 6000 years ago and from which have resulted the domestic horse, donkey and camel. The rise of the first organized conglomerates of people across Eurasia and northern Africa and consequently the need of large amounts of supplies might have fomented the use of these species for transportation. Another reason is related to the climate changes suffered after the last maximum glaciation (around 18 000 years ago), from which resulted

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an increase of aridity in northern Africa and southwest Asia. This fact might have propelled some herder communities to domesticate animals to help in moving around to seek water sources and green pastures to replace those that had become patchier and more distant (Beja-Pereira *et al.* 2004; Rossel *et al.* 2008).

Donkey domestication has been a controversial theme, with zooarcheological, ethnographic and, more recently, genetic data providing new insights into a complex scenario. Two alternative hypotheses arose for an African domestication of the donkey. The 'Egyptian hypothesis' states that, due to the presence of donkey bones in Predynastic Egyptian sites (6000–5000 BP), donkeys were most likely domesticated from resident Nubian wild ass (*E. africanus africanus*) by Egyptian villagers in the Nile Valley (Epstein 1971; Clutton-Brock 1992). A more recent hypothesis argues that donkey domestication occurred as a response by early pastoralists in northeastern Africa to the increasing aridity in the Sahara (7000–6500 BP). This 'pastoralist hypothesis' is well supported by ethnographic, climatic and linguistic data and has become increasingly accepted (Marshall 2007). Besides these two African hypotheses for the domestication of the donkey, the identification of putative *Equus africanus* remains at sites in the Levant and the Arabian Peninsula raised the possibility of a west Asian domestication of the donkey (Zeder 1986; Uerpmann 1987; Meadow & Uerpmann 1991; Clutton-Brock 1992).

Genetic studies on donkey domestication up to now have relied on the analyses of mitochondrial DNA (mtDNA) variation from putative ancestors and contemporary domestic donkeys. The first molecular study, by Beja-Pereira *et al.* (2004), identified the African wild ass as the probable ancestor of the domestic donkey, clearly ruling out the Asiatic wild ass as a putative progenitor. The existence of two clearly defined mtDNA clades among domestic donkeys suggested the occurrence of two independent domestication events, involving two distinct wild populations. Additionally, it was possible to identify one of the African wild ass subspecies – the Nubian wild ass – as the putative ancestor of Clade I donkeys. Ancestry of Clade II donkeys remains unknown; however, it has been proposed that a relative of the Somali wild ass, probably already extinct, would be the most probable candidate (Beja-Pereira *et al.* 2004; Kimura *et al.* 2011). Besides northeastern Africa, both the ancient range of the Atlas wild ass in the Maghreb and the coast of Yemen remain potential geographical areas for the origin of the wild ancestor of Clade II donkeys (Kimura *et al.* 2011).

The African wild ass is critically endangered and facing a high risk of extinction (Moehlman *et al.* 2008); however, populations of the Somali wild ass (*Equus africanus somaliensis*) still subsist in Ethiopia and Eritrea. The Nubian wild ass (*Equus africanus africanus*), formerly distributed in Sudan and northern Eritrea, is currently very rare or even extinct.

Archeological data concerning the presence of donkeys in ancient societies is difficult to obtain because, unlike cattle, donkeys were neither ceremonially buried nor a common subject in important art work (Marshall 2007). Nonetheless, there are numerous sites containing donkey remains both in Africa (from the North African coast to the Horn) and in Asia (Arabian Peninsula and Near East). In fact, the largest known sample of *Equus africanus* or early *Equus asinus* from an archeological site is located in the Ash Shumah site in Yemen (Cattani & Bokonyi 2002), confirming the presence of African wild ass or an early domesticated form by 7770 ± 95 BP in the Arabian Peninsula.

Centers of origin are expected to retain more ancestral variation (Troy *et al.* 2001). As populations expand from centers of origin, genetic diversity is lost as a consequence of the limited numbers of individuals involved in this expansionist movements ('founder effect'). This pattern of decline in genetic diversity with increasing distance from proposed centers of origin has been found in cattle studies using autosomal markers (Loftus *et al.* 1999; Cymbron *et al.* 2005).

Hotspots of diversity, such as centers of origin, can often present a similar signature in terms of diversity as areas considered as melting pots (ancient trading areas or routes). Distinguishing hot spots from melting pots can be done by analyzing allelic patterns and frequencies to discriminate shared diversity and unique diversity, typical in centers of origin. This study stands as the first to assess levels of genetic variation among domestic donkeys from putative centers of origin and their wild counterpart (African wild ass) using nuclear markers.

Materials and methods

In this study, we assessed levels of genetic diversity at 15 autosomal microsatellite loci in donkey populations from eight countries within the three hypothesized centers of origin of the domestic donkey; northeastern Africa (Ethiopia, Sudan, Egypt), the Arabian Peninsula (Oman, Yemen) and the Near East (Syria, Turkey, Jordan). Populations were sampled to assess levels and patterns of genetic diversity. Additionally, we compared obtained domestic donkey genotypes with those obtained from captive Somali wild ass ($n = 20$) (Rosenbom *et al.* 2012), at a subset of 10 microsatellite loci, to visualize patterns of differentiation among wild and domestic individuals.

DNA extraction and microsatellite genotyping

Blood and tissue samples belonging to donkeys from putative centers of origin were collected from a total of 129 individuals (Table 1). DNA was extracted with the DNeasy Blood & Tissue kit (Qiagen GmbH), according to standard protocols. Samples were then diluted in elution

Table 1 Summary statistics of sampled domestic donkeys. Represented parameters are mean values per geographic region, across loci. Number of samples is indicated (*n*) as well as observed heterozygosity (H_O), expected heterozygosity (H_E), unbiased expected heterozygosity (uH_E), allelic richness (Ar) and private allelic richness (PAr). Allelic richness (Ar) and private allelic richness (PAr) were calculated using the rarefaction algorithm for the minimum sample size in the Near East region ($n = 20$).

Region[1]	*n*	H_O	H_E	uH_E	Ar ($n = 20$)	PAr ($n = 20$)
Northeast Africa	60	0.579 ± 0.055	0.631 ± 0.055	0.637 ± 0.056	5.81	0.53
Near East	20	0.564 ± 0.052	0.658 ± 0.047	0.679 ± 0.049	5.67	0.27
Arabian Peninsula	49	0.613 ± 0.048	0.656 ± 0.041	0.665 ± 0.041	5.93	0.65

[1]Northeast Africa: Egypt, Sudan, Ethiopia; Near East: Turkey, Syria, Jordan; Arabian Peninsula: Oman, Yemen.

buffer, according to the amount of DNA visible on the gel and stored at –20 °C.

Genomic DNA was amplified by polymerase chain reaction (PCR), for 15 autosomal microsatellite loci (Table S1). Forward primers were modified by end labeling with fluorescent dyes (6-FAM™, VIC®, NED™, PET®) at the 5′ end. Each 15-μl reaction consisted of water, DNA, primers and fluorescent labels (0.06 μM of forward primer, 0.6 μM of reverse primer and dye), dNTPs (30 mM each), 10× buffer [200 mM of Tris–HCl (pH 8.4), 500 mM of KCl], BSA (0.4 μg/μl), MgCl2 (variable between 1.5 and 3 mM, according to the locus) and Platinum® Taq DNA Polymerase [(0.3 U); Invitrogen™]. Samples were amplified in a Dual 96-Well GeneAmp® PCR System 9700 thermocycler (Applied Biosystems) in the following conditions: initial denaturation at 95 °C for 10 min, followed by 35 cycles of 30 s at 95 °C, 30 s at temperatures between 52 °C and 60 °C (variable with the locus) and 30 s at 72 °C; a final elongation step was held for 10 min at 72 °C. PCR products were checked in 2% agarose gel stained with GelRed™ and, according to the quality of the amplification, diluted in water, mixed with formamide and LIZ® 500-bp internal size standard (Applied Biosystems) or detected by capillary electrophoresis using a 3100 Genetic Analyzer® (Applied Biosystems) sequencer. GENEMAPPER® v4.0 software (Applied Biosystems™) was used to score individual genotypes.

Statistical analyses

Genetic diversity

Deviations from Hardy–Weinberg equilibrium proportions were tested, using the GENEPOP v4.2 program (Raymond & Rousset 1995) for each population–locus combination. Departure from Hardy–Weinberg expectations was assessed by exact tests with unbiased *P*-values estimated using a Markov chain method (set to 1000 batches of 10 000 iterations each and with 10 000 steps of dememorization). A global test across loci and populations was performed using Fisher's method. The null hypothesis of no genotypic linkage disequilibrium was tested between all pairs of loci in each population, and additionally, a global test (Fisher's method) for each pair of loci was performed across samples. Statistical significance was adjusted for multiple comparisons using sequential Bonferroni correction (Rice 1989).

For geographic comparisons, donkey samples were grouped in three regions corresponding to three putative centers of origin: the Near East, the Arabian Peninsula and northeast Africa. Levels of genetic diversity were assessed by calculating unbiased expected (uH_E), expected (H_E) and observed (H_O) heterozygosities per each proposed geographic region using GENALEX 6.5 software (Peakall & Smouse 2006, 2012).

Allelic richness (Ar) and private allelic richness (PAr) were estimated, per each geographic region, using the rarefaction algorithm implemented in HP-RARE 1.0 (Kalinowski 2005) to account for potential biases arising from unequal sample sizes. Estimates of these two measures were standardized to the smallest sample size (Near East, $n = 20$).

Statistical significance of obtained differences for calculated diversity measures (uH_E, Ar and PAr) was assessed by conducting Mann–Whitney tests as implemented in MINITAB® statistical software.

Levels of genetic diversity were further investigated by focusing on individual countries in regions corresponding to putative centers of origin (Near East, Arabian Peninsula and northeast Africa) by calculating the above-mentioned diversity parameters, using GENALEX 6.5 software (Peakall & Smouse 2006, 2012). We also used HP-RARE 1.0 (Kalinowski 2005) to estimate Ar as well as PAr by country, correcting these values for the smallest sample size (Egypt, $n = 15$).

Allelic patterns

Allelic frequencies and distributions, by locus, were analyzed across geographic regions using GENALEX 6.5 software (Peakall & Smouse 2006, 2012). A Mann–Whitney test was conducted using MINITAB® statistical software to test if differences in allelic frequencies among regions were statistically significant. To distinguish unique from shared diversity, we looked at putative private alleles by geographic region, with frequencies between 2% and 5%, to exclude rare alleles caused by random sampling. Additionally, we looked for divergent alleles in the frequency distribution plot, by locus, to identify their geographical origin.

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Population differentiation

Analysis of molecular variance (Excoffier *et al.* 1992) was performed using GENALEX 6.5 software (Peakall & Smouse 2006, 2012). Weir & Cockerham (1984) hierarchical *F*-statistics were used to estimate the proportion of genetic variability found among domestic donkey populations (F_{ST}), among individuals belonging to the same population (F_{IS}) and within individuals (F_{IT}). To determine whether the observed level of differentiation was significantly greater than that expected by chance, we compared the obtained value against the outcomes of 1000 permutations.

In order to visualize similarities and dissimilarities among sampled donkey populations belonging to countries of interest (Egypt, Ethiopia, Sudan, Yemen, Oman, Syria and Turkey) and between these and their wild counterpart (African wild ass), factorial correspondence analyses (FCA) were performed using GENETIX v. 4.05 (Belkhir *et al.* 1996–2004).

Results

Genetic diversity

Two locus–population combinations significantly ($P < 0.001$) deviated from Hardy–Weinberg expectations (locus *VHL20* in Sudan and locus *CA425* in Yemen); however, no locus significantly deviated from Hardy–Weinberg expectations proportions across populations. Results showed no significant gametic (linkage) disequilibrium between all possible loci pairs, across populations, after Bonferroni corrections for multiple tests.

A total of 120 alleles were detected in the 15 surveyed loci, giving a mean number of eight alleles per locus. Obtained heterozygosity values (both unbiased expected and observed) showed some variation across regions. Unbiased expected (uH_E) heterozygosity ranged from 0.637 ± 0.056 in northeast Africa to 0.679 ± 0.049 in the Near East (Table 1), however, values for observed heterozygosity (H_O) were higher in the Arabian Peninsula region (0.613 ± 0.048). Patterns of diversity given by Ar and PAr were somewhat different. Values of both Ar and PAr were higher in the Arabian Peninsula (Ar = 5.93; PAr = 0.65) and northeast Africa (Ar = 5.81; PAr = 0.53) regions, which might be indicative of a source of unique, unshared genetic diversity, consistent with what is expected in centers of origin. Results for Mann–Whitney tests showed no support for significant differences ($P < 0.05$) in diversity parameters (uH_E, Ar and PAr) among geographic regions.

Genetic diversity was further investigated by focusing on countries in the regions of interest. Obtained results showed Sudan as possessing the highest levels for calculated genetic diversity parameters (H_O, H_E, uH_E and Ar) (Table 2). Yemen possessed the highest values for PAr (PAr = 0.55) and the second highest values for Ar (Ar = 5.78). Sudan and Yemen stand out, among countries in putative centers of origin, as those possessing the highest values for analyzed diversity parameters, indicating these countries in particular as potential sources of genetic diversity.

Allelic patterns

Analyses of allelic patterns across geographic regions revealed the existence of 24 private alleles across regions, with 96% of those alleles found in the northeast Africa and Arabian Peninsula regions. However, looking at allelic frequencies, approximately 64% presented frequencies between 2% and 5% in the Arabian Peninsula, whereas only one private allele in the northeast Africa region showed a frequency above 2%. Additionally, we observed the relative position of these private alleles in the frequency plots, by locus. Obtained results showed that private alleles identified in the Arabian Peninsula at frequencies between 2% and 5% were also the most divergent, presenting a marginal distribution in the frequency plots (Fig. 1). However, when testing for significant differences between allele frequency distributions across regions, using Mann–Whitney tests, no significant differences were found ($P < 0.05$).

Population differentiation

Analyses of molecular variance among domestic donkey populations revealed low differentiation, with only 10% of found variation justifying differences among populations (F_{ST}) and 22% of obtained variation being justified by

Table 2 Summary statistics of domestic donkeys from countries in putative centers of origin. Represented parameters are mean values, across loci. Number of samples is indicated (*n*) as well as allelic richness (Ar), private allelic richness (PAr), observed heterozygosity (H_O), expected heterozygosity (H_E) and unbiased expected heterozygosity (uH_E).

Country	*n*	H_O	H_E	uH_E	Ar (*n* = 15)	PAr (*n* = 15)
Egypt	15	0.538 ± 0.06	0.537 ± 0.058	0.557 ± 0.059	4.78	0.12
Turkey + Syria	17	0.555 ± 0.06	0.580 ± 0.057	0.600 ± 0.058	4.64	0.12
Oman	20	0.590 ± 0.064	0.622 ± 0.048	0.648 ± 0.05	5.26	0.28
Sudan	20	0.647 ± 0.054	0.640 ± 0.051	0.667 ± 0.053	6.07	0.29
Yemen	29	0.565 ± 0.066	0.598 ± 0.062	0.610 ± 0.063	5.78	0.55
Ethiopia	25	0.464 ± 0.075	0.486 ± 0.077	0.496 ± 0.079	5.26	0.27

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differences among individuals belonging to the same population (F_{IS}). Variation within individuals (F_{IT}) was responsible for approximately 68% of obtained genetic diversity.

Pairwise F_{ST} values among domestic populations from countries in putative centers of origin and the African wild ass varied between 13% and 17% (for the Sudanese and Egyptian populations respectively). Pairwise F_{ST} between the Yemeni donkey population and the African wild ass population was the second lowest (13.6%), after Sudan (Table S2). Also, the FCA revealed clear differentiation among domestic donkeys and their wild counterpart (Fig. 2). Obtained pairwise F_{ST} values among Yemeni donkeys and the remaining domestic populations were also the highest among all possible domestic population pairs (Table S2).

Discussion

Donkey domestication has proved to be a complex process, with different putative ancestors being proposed along the course of history, as well as different geographic regions being pinpointed as potential areas for donkey domestication, on the basis of historical and archeological data. Genetic studies clearly pointed to the African wild ass as the ancestor of the domestic donkey; in particular, the Nubian wild ass (*Equus africanus africanus*) was identified as the putative ancestor of Clade I donkeys (Beja-Pereira *et al.* 2004; Kimura *et al.* 2011).

Despite being currently critically endangered or even extinct in the wild, the Nubian wild ass historically was distributed in Sudan and northern Eritrea (Moehlman *et al.* 2008). Based on the geographical distribution of this subspecies and its role in domestic donkey ancestry, our hypothesis was that domestic populations occurring in close proximity to geographic areas where the Nubian wild ass was distributed would present higher levels of genetic diversity. Obtained results clearly pinpointed Sudan as presenting the highest values of uH_E, as well as Ar, supporting our initial hypothesis. Levels of diversity in other analyzed countries in northeast Africa, namely in Egypt and Ethiopia, were considerably lower for all diversity parameters (Table 2). In light of the newly obtained genetic

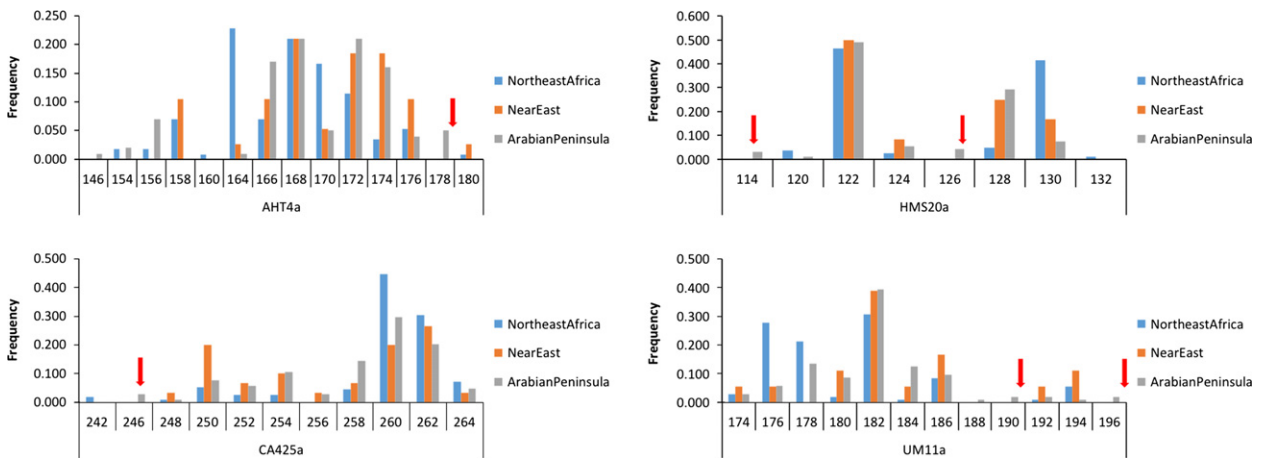

Figure 1 Allelic frequencies distribution across regions for the four loci showing a large number of private alleles identified at frequencies between 2% and 5% (*AHT4, HMS20, CA425* and *UM11*). Red arrows denote private alleles.

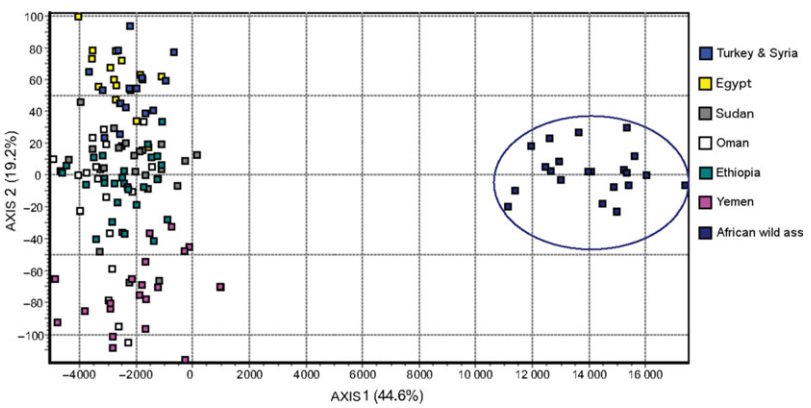

Figure 2 Factorial correspondence analyses of domestic donkey populations belonging to putative centers of origin and an African wild ass (*Equus africanus somaliensis*) population. African wild ass individuals are identified in the circle.

diversity data, the alternative pastoralist hypothesis for donkey domestication in northeast Africa is favoured in detriment of an Egyptian domestication.

Focusing on individual countries, Yemen's population stands out as the one presenting higher values of PAr, pinpointing this country as a potential source of unique, unshared diversity among studied populations. The presence of African wild ass or early domesticated donkeys at a site in Yemen is well documented and considered reliable among archeologists (Marshall 2007). Dating of excavated bones at about 7770 ± 95 BP predates the proposed date for donkey domestication at approximately 5000 years ago (Beja-Pereira *et al.* 2004; Kimura *et al.* 2011), making plausible the existence of a wild population in the Arabian Peninsula during ancient times. This scenario favors a possible domestication event in the Arabian Peninsula from an already extinct wild ass population, as suggested by archeological data (Cattani & Bokonyi 2002).

The absence of geographical structure among studied domestic donkey populations, using a set of nuclear markers, is consistent with previously reported the absence of structure in mtDNA donkey haplotypes (Beja-Pereira *et al.* 2004; Pérez-Pardal *et al.* 2014). This pattern can be explained by some specificity of the donkey and its domestication process, namely the mobile character of the species and the absence of intensive management (artificial selection) in most regions of the word, unlike the closely related horse. Despite the short time span since domestication, domestic donkeys and the African wild ass can be clearly differentiated.

Conclusion

As the first study using nuclear markers and samples belonging to domestic donkey populations from putative centers of origin and their wild counterpart (African wild ass), the obtained results point to populations from two geographic regions as possessing the highest genetic diversity values: northeast Africa and the Arabian Peninsula. Also on a finer scale, Sudan and Yemen are the two countries where the highest values of diversity were displayed. Although these results confirm previously reported studies on mtDNA that pointed toward northeast Africa as the potential center of origin of the donkey, the high-diversity levels found in the Arabian Peninsula, namely in Yemen, are not negligible and suggest a possible involvement of this region in donkey domestication. Such similar high-diversity levels also point toward a much more complex domestication process by which wild animals from several geographically distinct populations might have been recruited. This, and the fact that the domestic donkey's purpose was the transportation of goods and people, led to different gene pools getting swiftly mixed and distributed all over the ancient world.

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Conflict of interests

The authors declare no conflict of interests to declare.

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Primer sequences, dye labels and bibliographic reference for the 15 amplified microsatellite loci.

Table S2 Pairwise F_{ST} distances among donkey populations from countries of interest and the African wild ass (Somali wild ass).