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Summary A feral donkey population (*Equus asinus*), living in the Asinara National Park (an island north-west of Sardinia, Italy), includes a unique white albino donkey subpopulation or colour morph that is a major attraction of this park. Disrupting mutations in the *tyrosinase* (*TYR*) gene are known to cause recessive albinisms in humans (oculocutaneous albinism Type 1; OCA1) and other species. In this study, we analysed the donkey *TYR* gene as a strong candidate to identify the causative mutation of the albinism of these donkeys. The *TYR* gene was sequenced from 13 donkeys (seven Asinara white albino and six coloured animals). Seven single nucleotide polymorphisms were identified. A missense mutation (c.604C>G; p.His202Asp) in a highly conserved amino acid position (even across kingdoms), which disrupts the first copper-binding site (CuA) of functional protein, was identified in the homozygous condition (G/G or D/D) in all Asinara white albino donkeys and in the albino son of a trio (the grey parents had genotype C/G or H/D), supporting the recessive mode of inheritance of this mutation. Genotyping 82 donkeys confirmed that Asinara albino donkeys had genotype G/G whereas all other coloured donkeys had genotype C/C or C/G. Across-population association between the c.604C>G genotypes and the albino coat colour was highly significant ($P = 6.17E-18$). The identification of the causative mutation of the albinism in the Asinara white donkeys might open new perspectives to study the dynamics of this putative deleterious allele in a feral population and to manage this interesting animal genetic resource.

Keywords Asinara island, coat colour, deleterious mutation, equid, oculocutaneous albinism Type 1, pigmentation, population genetics

Asinara (of which one of its middle age etymology seems to recall the meaning of 'land of the donkeys') is a small Mediterranean island (about 52 km²) located closely north-west of Sardinia (Fig. 1a). This island was inhabited until 1885, at which time it was closed as it became an Italian quarantine site and subsequently a highly secured prisoner colony. The island was re-opened to the public in 1999 after the constitution of the Asinara National Park in 1998 (<http://www.parcoasinara.org>; Gazzetta Ufficiale della Repubblica Italiana 1997). Among the species living on the island, the Asinara white donkey (Fig. 1b) or 'Asino dell'Asinara' (*Equus asinus*) is the most representative and

peculiar component of the Park's fauna and is the symbol of Asinara. The origin of the Asinara white donkeys is uncertain and based only on legends, from which it is possible to date the occurrence of the first white donkeys back to before the closure of the island in the 19th century (Ministero di Agricoltura, Industria e Commercio 1905; Vinceti 2007). The Asinara white donkey subpopulation or colour morph (accounting for ~100–120 animals) lives together with coloured (usually grey) donkeys that can be attributed to the Asino Sardo population (Pinna *et al.* 1993). All these donkeys can mate, producing a hybrid population (whose number of heads is not known). The whole donkey population of Asinara island (white and coloured) can be considered a feral population, as no human direct intervention has been managing these animals for more than a century (Kugler & Broxham 2014). The white coat coloured animals are also considered by the Food and Agriculture Organization (Sherf 2000) and by the register of equine and asinine Italian local breeds

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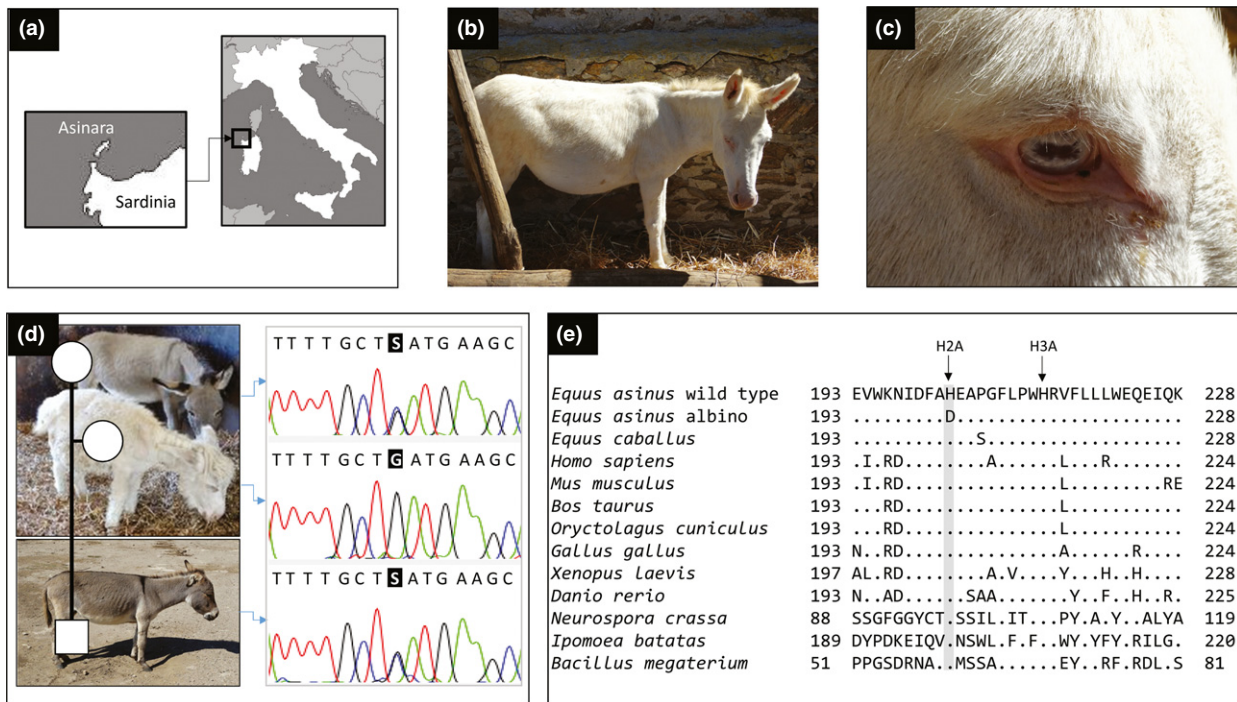


Figure 1 Geographical position of the Asinara island, phenotypic details of white Asinara donkeys and the causative mutation determining their albinism. (a) Geographical location of the Asinara island. (b) Asinara white albino donkey. (c) A close-up of the depigmented eye of an Asinara white albino donkey. (d) Recessive Mendelian inheritance of the albino phenotype demonstrated in a trio. Two grey parents (with heterozygous genotype H/D at the p.His202Asp site or C/G in the nucleotide sequence at the c.604C>G nucleotide position, indicated with S, according to the IUPAC nomenclature) gave birth to an albino donkey (D/D genotype or G/G at the nucleotide position). Microsatellite analysis (data not shown) confirmed the relationship among the three donkeys. (e) Alignment of the donkey tyrosinase protein region containing the p.His202Asp substitution with the corresponding region in different species. The grey region indicated with an arrow corresponds to the position of the p.His202Asp substitution in donkeys (H2A position in the CuA site). The other arrow indicates the histidine of the H3A position in the CuA site (the H1A position is not included in this alignment). Protein accession numbers for the sequences used in the alignment are as follows: *Equus caballus*, F6Y1A2; *Homo sapiens*, P14679; *Mus musculus*, P11344; *Bos taurus*, Q8MIU0; *Oryctolagus cuniculus*, G15YA0; *Gallus gallus*, P55024; *Xenopus laevis*, F7CL37; *Danio rerio*, F1QDZ4; *Ipomoea batatas*, Q9MB14; *Neurospora crassa*, P00440; *Bacillus megaterium*, B2ZB02. Numbers in the alignments indicate the starting and ending amino acid residues of the corresponding protein.

(Ministero delle Politiche Agricole, Alimentari e Forestali 2010) as a donkey breed in critical status.

Despite the uniqueness of the Asinara white donkeys, only a few authors have investigated this subpopulation. As far as we know, these animals have been analysed at the DNA level using microsatellites to evaluate genetic variability together with other donkey breeds in only two studies (Cosseddu *et al.* 2001; Colli *et al.* 2013). Pinna *et al.* (1993) described the Asinara white donkeys at the morphological level and reported that these animals resemble those of the Asino Sardo breed in terms of size and body shape, confirming their genetic closeness determined by microsatellite markers (Colli *et al.* 2013). The differentiating trait is only the complete white coat colour, lacking pigmentation in the skin, hair, eyelashes and eyebrows, and eyes that are light blue, as also described for several forms of human oculocutaneous albinism Type 1A and 1B (OCA1A and OCA1B) defects (e.g. Grønskov *et al.* 2007; Fig. 1c). These donkeys have low visual acuity, and during sunny hours they hide inside the unused

buildings of the prisoner colony. These traits and their evasive behaviour away from the sun indicate that Asinara white donkeys are affected by albinism (Pinna *et al.* 1993). The albinism in these animals is one of the few cases of this type of pigmentation defect that is maintained in a wild or feral vertebrate population (Protas *et al.* 2006; Xu *et al.* 2013), as fitness is expected to be lower, especially in a sunny Mediterranean environment.

In many different species, the albino locus allelic series (formally identified as the *C* locus; Searle 1968) is determined by mutations in the *tyrosinase* (*TYR*) gene that lead to completely white coat colour and lack of pigmentation in the case of disrupting mutations (Aigner *et al.* 2000; Oetting 2000; Beermann *et al.* 2004; Schmutz *et al.* 2004; Blaszczyk *et al.* 2005; Imes *et al.* 2006; Blaszczyk *et al.* 2007; Anistoroaei *et al.* 2008), determining the recessive *c* allele(s) (Searle 1968). Tyrosinase (EC 1.14.18.1) is the key enzyme involved in the melanogenesis process in which both melanins (eumelanins and pheomelanins) are produced. This enzyme has an active site

composed of a pair of antiferromagnetically coupled copper ions, CuA and CuB, which are coordinated by six histidine residues, three per each copper-binding site (Claus & Decker 2006; Kanteev *et al.* 2015). Removal of only one of the copper-binding histidine residues results in loss of the corresponding copper ion, thereby abolishing enzyme activity (e.g. Jackman *et al.* 1991).

In this study, we used a candidate gene approach to identify the causative mutation of the albinism in Asinara white donkeys. For this aim, six primer pairs (Table S1) were designed on the assembled donkey *TYR* gene (Bertolini *et al.* 2015) and used to amplify and sequence by Sanger and Ion Torrent sequencing technologies (as described in Fontanesi *et al.* 2015) all coding exons, portions of the intronic regions (downstream and upstream of the exons), 5'- and 3'- untranslated regions of the donkey *TYR* gene in 13 animals of different coat colours (seven Asinara white donkeys, expected to have the *c/c* genotype at the albino locus, and six coloured donkeys: two grey Asinara donkeys, phenotypically considered as Asino Sardo donkeys; one Asino Sardo donkey; one Martina Franca; one Sicilian Grey; and one Ragusano; EMBL accession numbers LN880531 and LN880532). Seven single nucleotide polymorphisms (SNPs) were identified (Table S2). Four SNPs were in exonic regions (three in exon 1 and one in exon 2), and the remaining polymorphisms were in intronic regions (two in intron 2 and one in intron 4; Table S2). Of the four missense mutations, two (c.274G>A or p.Val83Ile in exon 1 and c.987G>A or p.Glu316Lys in exon 2) were identified only in the heterozygous condition in one coloured donkey (Ragusano). The SIFT score (Kumar *et al.* 2009) indicated that these two amino acid substitutions are tolerated (Table S2). For the c.18G>C or p.Leu6Phe mutation, the genotype for three coloured donkeys of different breeds (Martina Franca, Grigio Siciliano and Ragusano) was G/G (L/L), whereas it was heterozygous G/C (L/F) in two grey donkeys sampled in the Asinara island (resembling Asino Sardo donkeys) and homozygous C/C (F/F) in the third grey Asino Sardo donkey sampled in Sardinia. Genotype C/C or F/F was fixed in all Asinara white albino donkeys as well (Table S2). SIFT analysis indicated that this missense mutation is not deleterious ($P = 0.48$). The second missense mutation (c.604C>G or p.His202Asp; Fig. 1d), identified only in donkeys from Asinara island that were homozygous D/D in all sequenced white donkeys, had a highly significant SIFT score ($P < 0.001$) supporting the deleterious effect of this substitution (Table S2).

The amino acid at position 202 of the wild-type *TYR* protein is one of the three highly conserved histidine positions of the first copper-binding site (CuA) of the *TYR* catalytic domain (Fig. 1e). This histidine is the second copper-binding histidine residue within the CuA site (indicated as H2A) that is always present at this position in all

tyrosinase protein sequences available, even across kingdoms (Fig. 1e; García-Borrón & Solano 2002; Claus & Decker 2006). The 3D structure of the wild-type and mutated donkey *TYR* proteins obtained following the homology modelling strategy (template protein: PDB entry 4P6R of *Bacillus megaterium*; Goldfeder *et al.* 2014) with MODELLER software (version 9.14; Eswar *et al.* 2006) confirmed the disruptive effect of the p.His202Asp substitution (Fig. S1).

According to the sequencing data, as grey donkeys sampled on the Asinara island were heterozygous at the c.604C>G (p.His202Asp) missense mutation, it was possible to presume a recessive mode of inheritance of the effect of the mutated allele, as expected for mutations causing albinism (Searle 1968). Mendelian recessive inheritance of this mutation was strengthened by sequencing and genotyping (Table S1) a trio family sampled on the Asinara island composed of a grey father (genotype C/G or H/D), a grey mother (C/G or H/D) and a white albino foal (G/G or D/D; Fig. 1d).

To further confirm the role of the p.His202Asp substitution, the c.604C>G mutation was genotyped (Table S1) in a total of 65 donkeys (including the animals already sequenced to confirm the sequencing determined genotype) from eight coloured breeds or populations in addition to 17 feral Asinara white albino donkeys (Table 1). All Asinara white albino donkeys were homozygous for the mutated allele. Only four grey Asino Sardo donkeys were heterozygous (three sampled in Asinara National Park, of which the sequencing of two have already been described and the third being from a farm in the province of Sassari, in the north of Sardinia). Considering all genotyped donkeys of

Table 1 Distribution of c.604C>G (p.His202Asp) genotypes obtained from PCR-RFLP and sequencing analyses among the investigated breeds.

Donkey breeds/populations	No. of donkeys	c.604C>G genotypes ¹		
		C/C	C/G	G/G
Amiata (coloured)	2	2	–	–
Asinara (white albino)	17	–	–	17
Asino Sardo (coloured) ²	7	3	4	–
Coloured hybrids (coloured)	13	13	–	–
Martina Franca (coloured)	13	13	–	–
Pantesco (coloured)	1	1	–	–
Ragusano (coloured)	19	19	–	–
Romagnolo (coloured)	2	2	–	–
Sicilian Grey (coloured)	8	8	–	–
Total	82	61	4	17

¹Including also grey donkeys sampled in Asinara island (see text for details).

²The number of donkeys with the corresponding genotype is reported. Genotypes are indicated for the c.604C>G single nucleotide polymorphism: allele C corresponds to the deduced amino acid histidine and allele G corresponds to the deduced amino acid aspartic acid for the missense mutation indicated as protein position (p.His202Asp).

different breeds and populations and the occurrence of homozygous G/G (D/D) animals only in albino donkeys, across-population association between the genotype at the c.604C>G mutation and the albino phenotype was highly significant ($P = 6.17E-18$; two-tailed chi-squared test).

The phylogenetic tree produced including the five donkey TYR haplotypes (obtained from the sequenced donkeys using PHASE program v. 2.1; Stephens *et al.* 2001) and the horse sequence (Wade *et al.* 2009), generated with the UPGMA method available in MEGA6 software (Tamura *et al.* 2013), supported the hypothesis that the albino mutation occurred in a 'grey' haplotype also present in Sardinia donkeys (Fig. S2). This hypothesis might exclude the legendary origin of the white donkeys of Asinara that had them deriving from white Egyptian donkeys imported by Marchese di Mores, Duke of Asinara Island, in the 19th century, or from a French shipwreck in the same period (Vinceti 2007).

The isolation of the Asinara donkey population and the consequent putative high inbreeding level might have been the causes of the increased frequency of the TYR-mutated allele in the Asinara island donkey population. The presence of many small uninhabited tumbledown buildings left over from previous uses of the island that are used as shelters by the white donkeys during the sunniest period of the year and the low activity of these animals during daylight might reduce the negative effects of this mutation. However, we cannot be sure whether these hypotheses are sufficient to explain the conservation of a mutation determining a potential deleterious effect in a free-living population (Page-McCaw *et al.* 2004). We did not investigate whether the albino TYR haplotype is in linkage disequilibrium with other variant(s) conferring advantages in a wild, marginal and harsh environment.

The identification of the causative mutation of the albinism in the Asinara white donkeys adds a new natural animal model for human OCA1 defects and might open new perspectives to study the dynamics of this putative deleterious allele in a feral population and to manage this interesting animal genetic resource that is the symbol of the Asinara National Park.

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Conflict of interest

The authors declare they have no conflict of interests.

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Supporting information

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

Figure S1 3D modelled structure of the (a) wild type (allele H – His at position 202) and (b) mutated (allele D – Asp at position 202) TYR proteins in the CuA and CuB copper-binding sites.

Figure S2 Phylogenetic tree of the donkey TYR gene haplotypes.

Table S1 PCR primers used in this study, sequencing and genotyping.

Table S2 TYR gene polymorphisms and genotypes of the sequenced donkeys.