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ORIGINAL INVESTIGATION

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# Striking differentiation of sub-populations within a genetically homogeneous isolate (Ogliastra) in Sardinia as revealed by mtDNA analysis

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**Abstract** Since the reduced genetic diversity found in isolates should simplify the study of complex traits, analyses of patterns of homogeneity within populations are of particular interest. We analysed the mtDNA haplogroups and hypervariable segment I (HVS-I) sequences of 475 individuals from a geographically restricted and isolated area (Ogliastra) within Sardinia, comprehending 175 random samples from 20 out of 23 villages. The remaining 300 subjects were chosen from the other three villages, Talana, Urzulei and Perdasdefogu, by sampling all maternal lineages. A comparison with other European populations reveals that Ogliastra ranks among the most genetically homogenous population and that it has been small and isolated throughout its history. The lack of variation and the high genetic homogeneity indicate that an important founder event and a demographic expansion took place during the Neolithic (~7,700 years before present) in Ogliastra's mtDNA gene pool. We present highly resolved phylogenetic networks for Ogliastra and for the three sub-isolates. MtDNA differentiation in the sub-populations versus Ogliastra is revealed by a strong demarcation in their genetic pools due to distinctive founder effects and genetic drift. We found that genetic homogeneity strictly depends on a scale factor in population size and on sampling methodology. The outstanding homogeneity and the reduced female gene pool observed in Ogliastra, in the European context, hide an extremely marked differentiation in sub-isolates originated from the same archaic population. Although Ogliastra can be considered a genetically homogeneous isolate, small villages' divergent genetic histories underline the importance of more systematic analysis of DNA variation between and within populations.

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## Introduction

The relative patterns of genetic diversity in isolated European populations (i.e., Sardinian, Saami, Finnish and Icelandic) are of particular interest, since isolates could provide a significant advantage in studying complex traits. Specifically, the accurate knowledge of demographic history, which includes the number of founders, population size, consanguinity, immigration, population expansion rate and genetic drift, improve the understanding of the genetic structure (Freimer et al. 1997; Kruglyak 1999; Risch 2000). All these bio-demographic parameters need to be taken into account to establish the usefulness of a population isolate for mapping complex traits (Peltonen 1999, 2000; Angius et al. 2001). Several studies indicated that Sardinia, once considered as genetically isolated, is no more valuable than major continental populations for linkage disequilibrium (LD) mapping of variants associated with common disorders (Eaves et al. 2000; Taillon-Miller et al. 2000). Recent findings suggest that LD is more extensive in sub-population isolates than in admixed urban samples (Wright et al. 1999; Zavattari et al. 2000; Angius et al. 2001).

Some culturally or geographically isolated regions of Europe (i.e., Sardinia, Finland, and the Basque Country), in spite of their relative genetic homogeneity, show a variable degree of sub-population variability, and homogeneity increases with the relative isolation of the considered population (Bertranpetit et al. 1995; Meinila et al. 2001). Mitochondrial DNA (mtDNA) analysis can be important in genetic isolate studies, for what it can reveal about population origins, migrations and demographic history. Several mtDNA studies were directed at the characterization of specific populations that show unusual genetic and/or linguistic characteristics (Rodriguez-Delfin et al. 2001; Finnila and Majamaa 2001; Tolk et al. 2000; Larruga et al 2001; Meinila et al. 2001). Inter-populations comparison

and phylogeny can be useful to reveal complex processes that shaped the current genetic structure of population isolates.

Previous works provide evidence that within Sardinian sub-populations differences were created by genetic drift, since geographical barriers kept certain areas extremely isolated (Piazza et al. 1988; Workman et al. 1975). Studies of mtDNA diversity within the island were used to assess genetic differentiation and to reveal signals of demographic expansions, migrations or isolation (Morelli et al. 2000).

Ogliastra is a very well characterized geo-political and cultural area in eastern Sardinia. It is a sparsely populated area, with about 60,000 inhabitants (34 inhabitants/km$^2$) clustered into 23 villages or small towns. This preserved area represents an isolated sub-region characterized by genetic and linguistic differentiations. Ogliastra's steep hills and rocky mountains help create geographical isolation for some of the villages encouraging the preservation of cultural resources and even linguistic variability. Dialectal variety within Ogliastra shows features that are intermediate between two main linguistic groups: Sardo Logudorese in the north and Sardo Campidanese in the south (Blasco Ferrer 1988). However, even if geographic and linguistic boundaries appear to be often discontinuous in the region, its population seems to be genetically differentiated from the rest of the island (Cappello et al. 1996).

Comparison of mtDNA data from Ogliastra with other European populations could allow inferences on population history and the phylogenetic analysis could be a compelling source of evolutionary information. To evaluate the relative configuration of mtDNA genetic diversity among populations that differ with regard to their demographic histories, we first compared Ogliastra with other European populations. The aim of this study was not limited to gaining insight into the overall level of Ogliastra genetic diversity based on mtDNA data, but to detail the differences found in three sub-isolates within the same restricted geographic area. Our data suggest that drift or other demographic factors might cause mitochondrial DNA variation in the sub-populations within Ogliastra.

## Materials and methods

### Populations and sampling

Two different sampling designs were used: one for the Ogliastra area and a more detailed one for Talana, Urzulei and Perdasdefogu villages.

The overall Ogliastra sample was obtained by recruiting random subjects (with proven Ogliastra origins) from 20 villages within the Ogliastra area, Fig. 1. We recruited 175 healthy maternally unrelated individuals. In attempt to take into account different population sizes in the Ogliastra villages, whenever possible bigger samples were recruited from the more populated villages in the area. Within Ogliastra, Tortolì (~9,700 residents), Lanusei (~6,200 residents) and Jerzu (~3,500 residents) are three major local towns that represent the main socio-economic centres in the area.

The three small villages of Talana, Urzulei and Perdasdefogu were studied in more detail and considered separately from the larger Ogliastra population, Fig. 1. Talana and Urzulei, located in the northwestern mountains, have 1,161 and 1,419 residents, respectively. Even if today a 12-km road connects them, in the past they maintained few contacts and very few inter-marriages with each other. They even speak different dialects. The third village studied in detail, Perdasdefogu, is located on a highland plateau in the south of Ogliastra. It has today about 2,400 residents, but during the last 50 years it has undergone a great immigration wave because of the opening of a military base nearby.

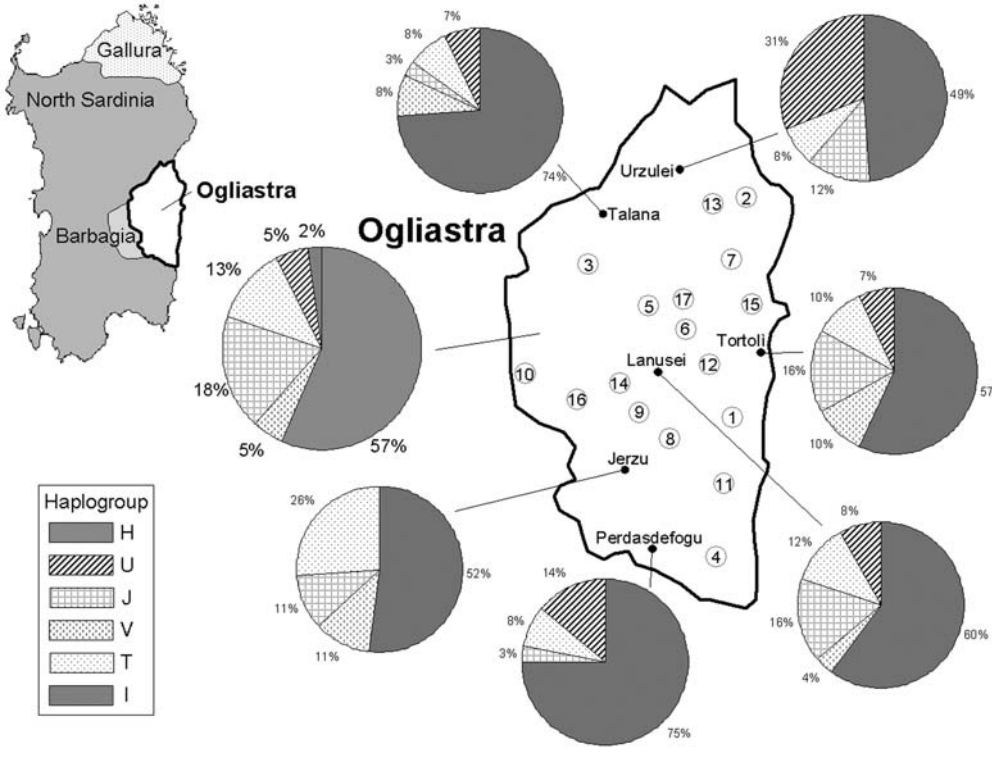


**Fig. 1** Geographical localization of Ogliastra and origin of all studied samples (sample size is indicated *in parentheses*). *1* Barisardo (7), *2* Baunei (3), *3* Villagrande (17), *4* Tertenia (3), *5* Arzana (13), *6* Ilbono (12), *7* Lotzorai (3), *8* Gairo (6), *9* Ulassai (6), *10* Seui (3), *11* Cardedu (2), *12* Loceri (4), *13* Triei (5), *14* Osini (1), *15* Girasole (2), *16* Ussassai (2), *17* Elini (3). Haplogroups frequencies for Ogliastra (175), Tortolì (31), Lanusei (25), Jerzu (27), Talana (100), Urzulei (100) and Perdasdefogu (100) are reported

For Talana, Urzulei and Perdasdefogu a different sampling design was utilized using all genealogical information collected in those villages. Systematic ecclesiastical records of births, deaths, marriages, and people's origin have been registered since 1589 in the parochial "Quinque libri" and since 1861, also municipalities have kept accurate archives (Angius et al. 2001). All data were transferred into an appropriate database, allowing the reconstruction of all the female and male genealogical lineages for Talana, Urzulei and Perdasdefogu through a proprietary tool (Mancosu et al. 2003). Maternal lines were reconstructed, starting from the present-day population and following the female lines back to their arrival in the village, if immigrants, or up to the beginning of the archives. Maternal lines with less than three generations were not considered, in order to exclude extremely shallow lineages (introduced after 1950) that did not expand in the population.

To avoid a possible over-representation of uneven expanded maternal lines, using genealogical information, we calculated the proportion of individuals in the present population which belong to each maternal line as follows. Consider $n$ distinct genealogical maternal lines: $ML_1$, $ML_2$,..., $ML_i$,..., $ML_n$ which are actually present in a village, and let $k_i$ be the number of individuals (in the present-day population) which belong to each $i$-th line. For each line $ML_i$, the proportion of individuals is calculated as $k_i / N$, where $N$ is the present-day population size. This proportion was used to select the appropriate number of individuals to draw from each distinct genealogical maternal line in order to obtain a balanced sample, which is thus representative of the entire village. Accordingly, we typed 100 individuals for each village. A few maternal genealogical lines that account for less than 0.02% were sampled and analysed as well, but they were not included in the results.

Informed consent was obtained from each individual in this study and all samples were taken in accordance with the Declaration of Helsinki.

### Genotyping and DNA sequencing

MtDNA sequence of each sample was amplified in eight fragments. PCR products were digested with specific restriction endonucleases to detect 14 polymorphic restriction sites defining nine haplogroups (Torroni et al. 1996). Digested PCR products were separated on 2% agarose gel in TBE buffer and restriction patterns were visualised by ethidium bromide staining and UV light. HVS-I (16024–16383) of the control region was amplified according to Thomas et al. (2002) and compared with the reference sequence described by Anderson et al. (1981). Sequencing was performed using ABI BigDye Terminator Cycle Sequencing kits (Applied Biosystems, USA). The fluorescently labelled extension products were loaded on ABI 3100 DNA analyser (Applied Biosystems, USA).

### Statistical and inter-population analyses

Gene diversity values (GDV) were calculated as: $GDV=n/(n-1)(1-\Sigma^{k}_{i=1} p_i^2)$, where $n$ is the total number of sequence, $k$ the number of distinct lineages, and $p_i$ is the sample frequency of the distinct lineages. This index represents the probability that two randomly chosen sequences from a sample would be non-identical by state.

Three population parameters were estimated: $\theta_\pi$, $\theta_s$ and $\theta_k$. The three $\theta$ indices, based on different assumptions, estimate $2N_{fc}\mu$, where $N_{fc}$ represents the female effective population size and $\mu$ the mutation rate. The $\theta_\pi$ value is the mean pairwise difference between sequences, calculated as $\theta_\pi=\Sigma^{k}_{i=1} \Sigma_{j<i} p_i p_j d_{ij}$, where $d_{ij}$ is the number of mutational differences between lineages $i$ and $j$ in a sample, $k$ is the number of distinct lineages, and $p_i$ and $p_j$ are the respective frequencies of lineages $i$ and $j$. The $\theta_s$ value was estimated as $\theta_s=S/\Sigma^{n-1}_{i=1} 1/i$, where $S$ is the number of polymorphic sites in a sample of sequences and $n$ is the number of sequences. The $\theta_k$ value was estimated by use of the formula $E(k)=\theta_k \Sigma^{n-1}_{i=0} (1/\theta_k+i)$, where $k$ is the number of distinct lineages observed in a sample size of $n$. Differences in $\theta$ values between populations should reflect differences in female effective-population size when the mtDNA control-region mutation rate is considered to be the same in all populations. However, $\theta_\pi$ is strongly affected by ancient demographic fluctuations (Rogers and Harpending 1992), while $\theta_s$ and $\theta_k$ are more sensitive to the effects of lineage sorting during recent demographic history (Helgason et al. 2000).

We also test Tajima's D statistic, using the nucleotide sequence variations of HVS-I in mtDNA (Tajima 1989a, 1989b). All $\theta$ indices, Tajima's D statistic and GDV values were calculated with the software package ARLEQUIN 2.0 (Schneider et al. 1997).

### Phylogenetic analysis

Reduced-median networks (Bandelt et al. 1995) of the haplotypes, defined by their HVS-I sequence plus additional RFLP information, were constructed by use of the program NETWORK 3.0 (available at the Life Sciences and Engineering Technology Solutions website). Median-joining networks were generated with $\varepsilon$ values of 0, because this yields a network containing a limited number of plausible evolutionary pathways among haplotypes. Because of the presence of mutation-rate heterogeneity between HVS-I and RFLPs markers, a weighting scheme was used. According to Macaulay et al. (1999), we obtained the relative weighting scheme from the formula $R=(n_2-1)/(n_1+1)$, where $n_2$ and$n_1$ are mutations observed in two systems (RFLPs and HVS-I, respectively) evolving along the same genealogy. The relative weighting schemes are as follows: Ogliastra $R=\mu_{RFLP}/\mu_{HVS\text{-}I}=0.66$; Talana $R=\mu_{RFLP}/\mu_{HVS\text{-}I}=0.22$; Urzulei $R=\mu_{RFLP}/\mu_{HVS\text{-}I}=0.50$; Perdasdefogu $R=\mu_{RFLP}/\mu_{HVS\text{-}I}=0.50$. (Note that certain HVS-I mutations – e.g., 16065 T→C≡16065 *Hin*fI – also generate RFLP site gains or losses and thus contribute to both counts).

### Age estimate

To estimate the expansion time of Ogliastra subsets of mtDNAs, we considered the HVS-I sequence diversity accumulated on top of the assumed ancestral sequence type within the region covered by nucleotide pairs 16024–16383. For Ogliastra star-like haplogroups, such as H in this study, the number of transitions on the reconstructed phylogeny from the ancestral type to each sample ($\rho$) was calculated. According to Forster et al. (1996), we used a calibration of the rate of transitional mutations of $\mu=4.96\times10^{-5}$ transitions/year. To summarize the uncertainty in this point estimate, we followed the approach of Saillard et al. (2000).

## Results

### MtDNA diversity and haplogroup frequencies in Ogliastra

We found 26 different HVS-I lineages, characterized by 21 variable sites, in 175 Ogliastra sequences. Summary statistics of mtDNA variation for Ogliastra and other European populations (where sample sizes are >100, to minimize the effect of sampling error) are reported for comparison in Table 1. Gene diversity, $\theta_k$ and $\theta_s$ values for Ogliastra and Saami are significantly lower than the values for the other considered populations. The Tajima's D values for Ogliastra (–0.95) and Saami (–0.95) should indicate that these populations have kept their sizes small and constant over time.

Haplogroup frequencies observed in Ogliastra are reported in Table 2. We also report frequencies of haplogroups H, V, J, T, U and I for Sardinia considered as a whole (Simoni et al. 2000) and for other Sardinian subpopulations, such as Barbagia, Gallura (Morelli et al. 2000) and North Sardinia (Torroni et al. 1996). In comparison

**Table 1** Summary statistics of mtDNA variation in Ogliastra and in other European population samples as reported, after reanalysis of primary data from original sources, in Arnason (2003). The table is ordered by gene diversity values; $\theta_\pi$ mean number of pairwise differences, $\theta_k$ estimate of $2N_{fc}\mu$ based on number of haplotypes, $\theta_s$ estimate of $2N_{fc}\mu$ based on number of segregating sites, *D* Tajima's test statistic

| Populations | Sample size | No. of lineages | No. of variable sites | Gene diversity | $\theta_\pi$ | Rank by $\theta_\pi$ | $\theta_k$ | Rank by $\theta_k$ | $\theta_s$ | Rank by $\theta_s$ | Tajima's D |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 Icelandic | 520 | 128 | 75 | 0.97 | 4.32 | 2 | 53.94 | 7 | 10.98 | 8 | –1.73 |
| 2 British | 169 | 101 | 81 | 0.97 | 3.93 | 5 | 104.94 | 3 | 14.20 | 3 | –2.25 |
| 3 Austrian | 117 | 74 | 75 | 0.96 | 4.41 | 1 | 85.47 | 5 | 14.06 | 4 | –2.2 |
| 4 Russian | 103 | 62 | 54 | 0.96 | 4.00 | 3 | 64.91 | 6 | 10.37 | 7 | –1.96 |
| 5 Finnish | 175 | 74 | 64 | 0.96 | 3.70 | 8 | 47.84 | 8 | 11.15 | 6 | –2.04 |
| 6 German | 423 | 217 | 106 | 0.96 | 3.64 | 9 | 178.11 | 1 | 16.00 | 1 | –2.27 |
| 7 Spanish | 181 | 105 | 80 | 0.95 | 3.81 | 6 | 103.57 | 4 | 13.86 | 5 | –2.24 |
| 8 Norwegian | 216 | 122 | 89 | 0.95 | 3.72 | 7 | 115.24 | 2 | 14.96 | 2 | –2.30 |
| 9 Basque | 106 | 53 | 50 | 0.94 | 2.86 | 10 | 41.52 | 9 | 9.55 | 9 | –2.21 |
| 10 Saami | 115 | 26 | 31 | 0.82 | 3.99 | 4 | 10.17 | 10 | 5.83 | 10 | –0.95 |
| 11 Ogliastra | 175 | 26 | 21 | 0.82 | 2.38 | 11 | 7.80 | 11 | 3.66 | 11 | –0.95 |

**Table 2** Frequencies of haplogroups H, V, J, T, U and I for Ogliastra and for Sardinia, North Sardinia, Gallura and Barbagia

| Haplo-group | Populations | | | | |
|---|---|---|---|---|---|
| | Sardinia[a] (*n*=73) | North Sardinia[b] (*n*=133) | Gallura[c] (*n*=51) | Barbagia[c] (*n*=45) | Ogliastra[d] (*n*=175) |
| H | 38.4% | 45.8% | 41.2% | 64.4% | 56.6% |
| V | 2.7% | 6.0% | 0.0% | 8.9% | 5.1% |
| J | 6.7% | – | 9.8% | 2.2% | 18.3% |
| T | 11% | – | 9.8% | 8.9% | 12.6% |
| U | 8.5% | – | 27.5% | 4.4% | 5.1% |
| I | 2.7% | – | 3.7% | 0.0% | 2.3% |

[a]Simoni et al. (2000)
[b]Torroni et al. (2001)
[c]Morelli et al. (2000)
[d]Present study

with the rest of Sardinia, Ogliastra shows a remarkably high frequency in haplogroups H (56.6%) and J (18.3%).

Tortolì, Lanusei and Jerzu were compared with Ogliastra as a whole. Haplogroup frequencies for Tortolì and Lanusei are similar to those observed in Ogliastra as a whole, whereas Jerzu appears to be lacking the U haplogroup (Fig. 1). Haplogroup frequencies for Talana, Urzulei and Perdasdefogu are also shown. By using mtDNA analysis we highlight inter-village differences, together with intra Ogliastra differences. In Talana we observed a remarkably high frequency of H haplogroup (74%), whereas in Urzulei the U haplogroup reaches 31%. Perdasdefogu shows high frequencies in H (75%) and U (14%) haplogroups.

## Phylogenetic networks

All analysed variable positions within the HVS-I (16024–16383) sequences for Ogliastra, Talana, Urzulei and Perdasdefogu samples are reported in an appendix table.

A reduced-median network of the sub-haplogroups of Ogliastra samples (175 individuals typed for HVS-I and RFLP markers) is shown in Fig. 2. Notwithstanding the large number of study samples, we found only 26 distinct mtDNA lineages grouped in haplogroups H, J, V, T, I and U and thus the Ogliastra phylogenetic network appears quite comprehensible. These haplogroups appear separated from each other, and only few reticulations remain unresolved. The H haplogroup network is rather star-like and clearly differentiated from other haplogroups by site 7025. H sub-haplogroup (Fig. 2) is the most represented in our sample (40%), and only few H sub-haplogroups originate from it. For haplogroup H the mean distance from the putative ancestral haplotype (sub-haplogroup H) is $\rho_{HVS\text{-}I}$=0.384± 0.241. Therefore the age of the H haplogroup is estimated to be 7,700±4,800 years.

We reconstructed the phylogenetic mtDNA portrait of Talana, Urzulei and Perdasdefogu populations. We found 22, 13 and 13 mtDNA lineages for Talana, Urzulei and Perdasdefogu, respectively; the relative median-joining networks are shown in Fig. 3a, b, c, respectively. All villages considered in detail show a limited number of maternal genealogical lineages that coalesce in few sub-haplogroups. We found complete concordance between genealogical data reconstruction and genetic analysis in all the three villages. The Talana (Fig. 3a) H-cluster is the most represented haplogroup (74%), and it appears highly star-like with 13 mtDNA lineages deriving from reference sequence. Other haplogroups are less frequent. Some of Talana H-derived haplogroups (H2, H6, H7, H8, H9, H11 and H15) are typical of this village and are not found in Ogliastra, Urzulei or Perdasdefogu. Five out of 13 mtDNA lineages are derived by single mutations from the H haplogroup, while eight of them underwent at least two mutations. Five sub-haplogroups (H1, H3, H4, H8 and H12) are quite common (>6%) and the H3 sub-haplogroup in particular shows a remarkable high frequency of 18%. In Urzulei, haplogroups appear separated from each other, and only a few reticulations remain unresolved. Also in this case, haplogroups U, J and T are quite distant from the central H core of the network. Some haplogroups

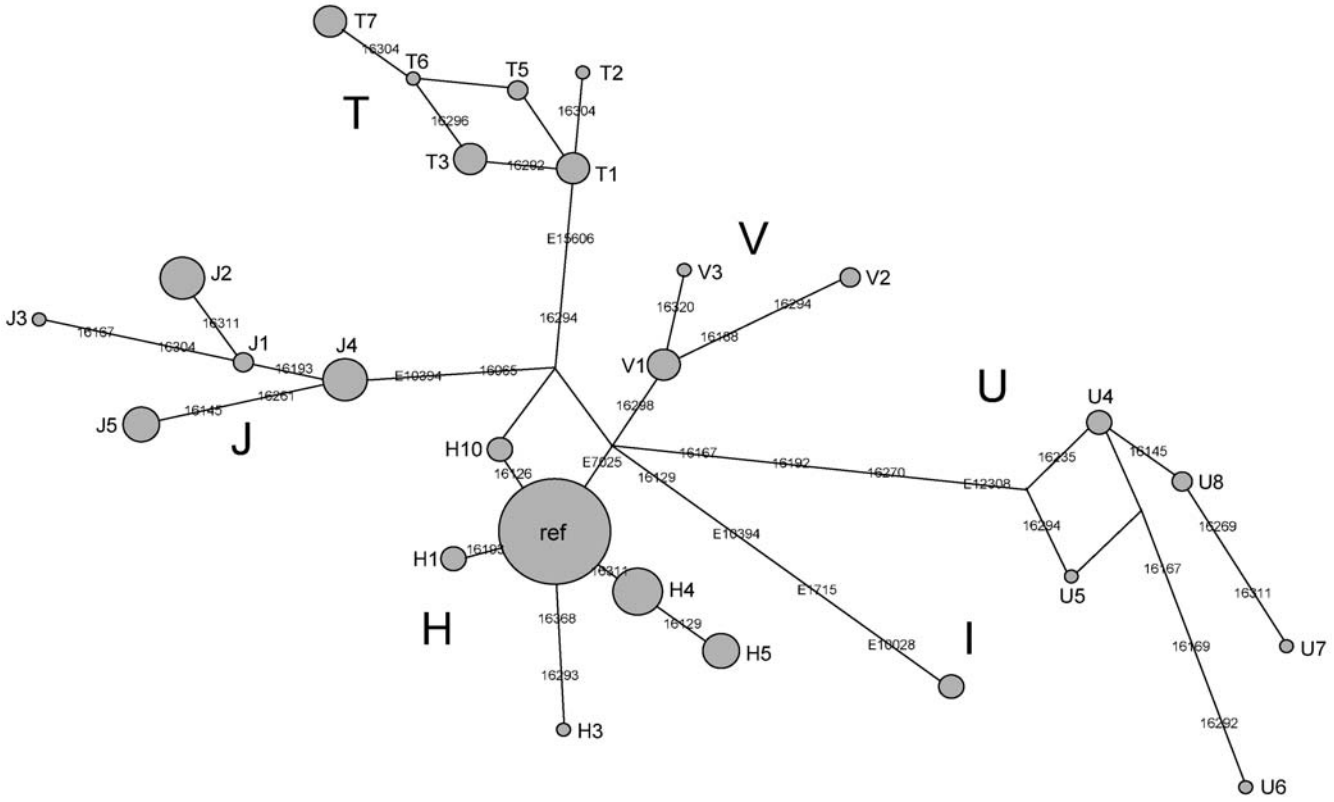


**Fig. 2** Median Joining Network of Ogliastra mtDNA lineages for HVSI (16030–16368) and RFLP markers 1715, 7025, 10028, 10394, 12308, 15606 and 16065. *Circle area* is proportional to sequence frequency. *Ref* reference sequence (Anderson et al. 1981)

reach a high frequency: 49% for the H, 31% for the U and 12% for the J, but the spectrum of conserved H-derived sub-haplogroups observed in Talana was not so wide. Some sub-haplogroups are typical of the village and are not observed elsewhere in Ogliastra (Fig. 3b). Perdasdefogu median network exhibits only an unresolved reticulation within haplogroup H (Fig. 3c), and does not appear highly star-like in haplogroup H, such as in the case of Talana or Urzulei. Haplogroups V and I are absent; reference sequence reaches 40% and some sequences belonging to haplogroups H and U are typical of this village.

## Discussion

The present study offers evidence of population differentiation within Sardinia, within Ogliastra and among sub-population isolates through mtDNA analysis. The influence of factors such as founder effects, genetic drift, as well as sample selection, are accurately investigated.

### Genetic differentiation of Ogliastra within Europe

Genetic homogeneity of the Ogliastra population and the relationships between Ogliastra and other European populations were analysed. GDV estimates show a clear difference existing in Saami and Ogliastra populations versus all other European populations under consideration for genetic homogeneity. Our data may suggest that Ogliastra have been a small and isolated population throughout its history. Ogliastra HVS-I mtDNA sequences are characterized by a smaller than average number of polymorphic sites and a relatively small number of distinct lineages, reflected in small $\theta_s$ and $\theta_k$ values, respectively. All $\theta$ values calculated for Ogliastra are substantially lower than those for other European populations. In particular, $\theta_k$ suggests that Ogliastra, as the Saami, has a small recent female effective-population size. The differences are substantial also when $\theta_s$ values are compared with recently reported data on other European populations (Arnason 2003). Moreover, when considering $\theta_\pi$, which primarily provides information about ancient female demographic history, Ogliastra has a $\theta_\pi$ of 2.38, interestingly similar to the Basque ($\theta_\pi$= 2.86). All these results imply a very small female effective-population size for Ogliastra. Even after a recent reanalysis of mtDNA variation using primary data from sources on 26 European samples, reported by Arnason (2003), our data indicate that Ogliastra belongs with the Saami in the lowest diversity population group. Considering all the genetic diversity measures, Ogliastra ranks among the most genetically homogenous European populations. The Tajima's D statistics for Ogliastra should indicate that it has kept its size small and constant over time. Small D values are typical of populations that did not undergo important demographic expansions (Tajima 1989a, 1989b). However, in comparison with the Saami (D=–0.95), a relatively young sub-population (200–400 generations) (Peltonen et al.

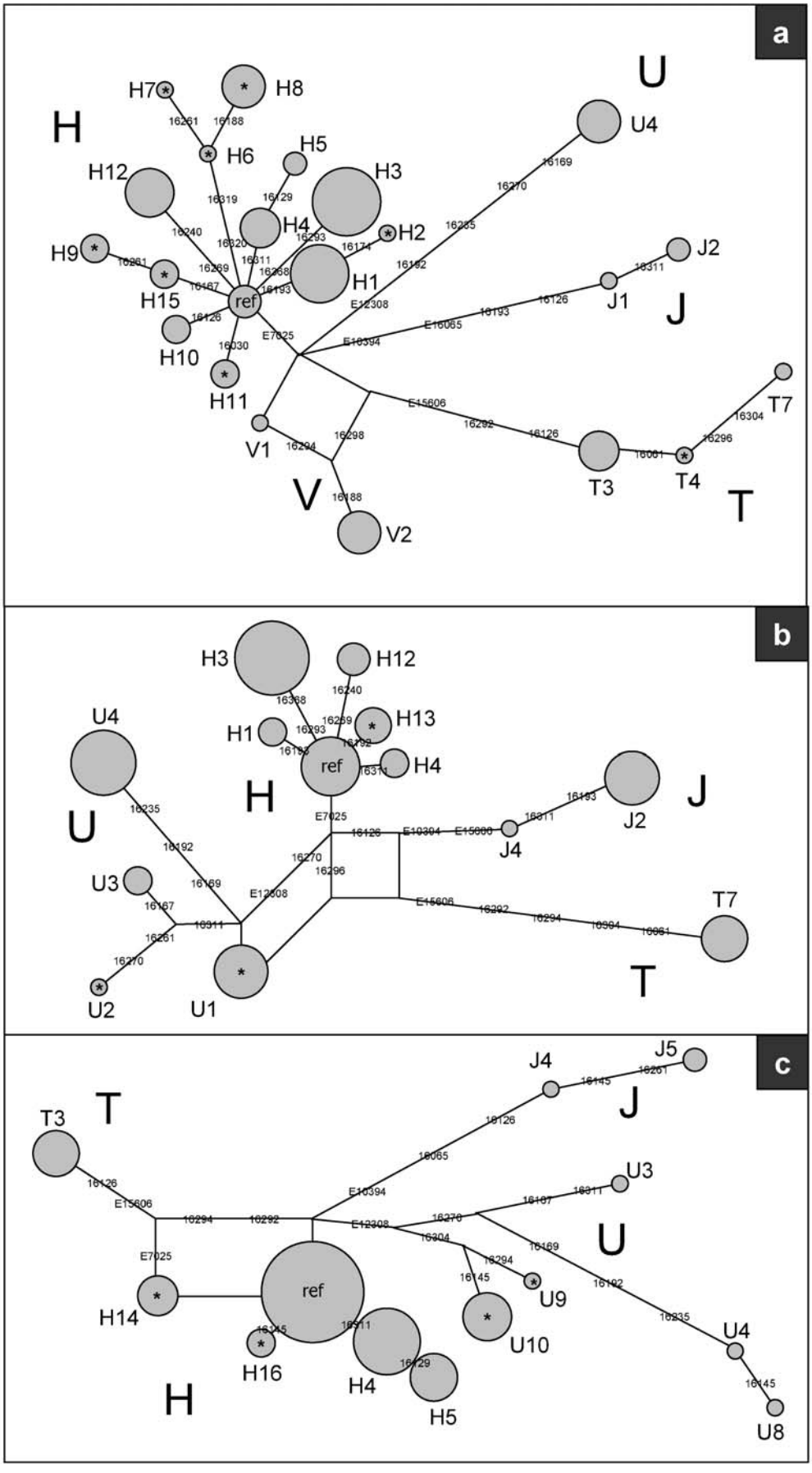


**Fig. 3** Median joining network of **a** Talana, **b** Urzulei and **c** Perdasdefogu mtDNA lineages for HVSI (16030–16368) and RFLP markers 1715, 7025, 10028, 10394, 12308, 15606 and 16065. *Stars* indicate typical sequences found in the village and not shared with Ogliastra sample. *Circle area* is proportional to mtDNA sequence frequency. *Ref* reference sequence (Anderson et al. 1981)

2000), there are indicators supporting at least a Neolithic origin for Ogliastra and an ancient peopling of the area. We estimate that the H haplogroup, distributed through all the Caucasoid populations, underwent expansion in the Ogliastra population 7,700±4,800 years ago. The age estimates for H haplogroup found in Ogliastra imply that a demographic expansion took place some time between 12,500 and 2,900 years before present (YBP). During the Neolithic age, Sardinia had trade relations with extra-insular communities in Corsica, Liguria and Provence as attested by the presence of obsidian artefacts from Sardinia in those regions (Tykot 2002). The Earliest Neolithic, although best attested at coastal sites, is also found in the island central areas (Lilliu 1988; Fadda 1993). Moreover, archaeological findings ("Corbeddu cave" dated at about 15,500 YBP) indicate the peopling of Sardinia from at least the Upper Paleolithic (Balmuth 1992; Sondaar et al. 1995; Hofmeijer 1997). The lack of variation ($\rho_{\text{HVS-I}}$= 0.384±0.241) and the high genetic homogeneity indicate that limited lineage variations occurred early in Ogliastra prehistory (more than 7,500 YBP), and this has remained relatively stable since its origin. Our data indicate that the Neolithic population that lived in Ogliastra area had contributed to the actual mitochondrial gene pool of Ogliastra population.

## Genetic differentiation of Ogliastra within Sardinia

Comparing mtDNA haplogroups frequencies among the populations of Sardinia, North Sardinia, Ogliastra, Barbagia, and Gallura, inter-population differences can be revealed. Genetic differentiation from Sardinia as a whole appears evident for Ogliastra, Barbagia and Gallura, where some haplogroups reach high frequencies (Table 2). In more detail, Ogliastra and Barbagia show a remarkable high frequency in the H haplogroup (56.6% and 64.4%, respectively), whereas the U haplogroup is over-represented in Gallura (27.5%). Geographical and linguistic isolation (Cappello et al. 1996), together with genetic drift, could explain these data. As reported by Torroni et al. (2001), the H haplogroup in North Sardinia shows a frequency that is comparable to that observed in Sardinia as a whole. Nevertheless, it is interesting to note the differences observed between Gallura and North Sardinia where the V haplogroup is absent in the first, whereas it goes up to 6% in the latter (Morelli et al. 2000). We draw particular attention to samples origin of reported data because genetic features (such as haplogroups frequencies and homogeneity) can be very different, depending on population history, foundation and geographical location.

## Genetic differentiation within Ogliastra

In order to reveal intra-population differences within Ogliastra we analysed samples from six sub-populations because of their different characteristics: Tortolì, Lanusei, Jerzu, Talana, Urzulei and Perdasdefogu. In Fig. 1 we summarize the mtDNA haplogroup results obtained. Tortolì and Lanusei haplogroup frequencies are almost identical to Ogliastra's as a whole. Tortolì and Lanusei, being the main socio-economic centres in the area, in fact underwent recent immigration from most Ogliastra villages and, as expected, they exhibit today the same mtDNA portrait as the larger Ogliastra population. Jerzu, the other main economic centre of Ogliastra, did not undergo important immigration trends and it differentiates itself for a remarkably high frequency of the T haplogroup (26%). Making allowances for the limited sample size we considered, the effect of genetic drift could be inferred for the T haplogroup in Jerzu population.

Through accurate genealogical reconstructions we were able to establish all the maternal lineages present in Talana, Urzulei and Perdasdefogu in order to analyse all the mtDNA types present today in these populations. The accuracy of genealogy was confirmed by genetic analysis of several individuals that represent the maternal lines. For Talana we estimated that 11 maternal lineages, accounting for about 77% of the present-day population, were introduced before the 18th century (Angius et al. 2001) and, of these, seven maternal lines representing 40% of today's population were present in the village since 1690. In Urzulei we identified 12 ancient maternal lineages (accounting for 58% of actual female population) introduced in the village before 1690; ten lineages (accounting for 33%) were introduced before 1800, and 9% of the present-day population is originated by lineages introduced in the period 1800–1950. Perdasdefogu has similar characteristics: 12 ancient maternal lineages (accounting for 39% of present day female population) were introduced in the village before 1690; 13 lineages (accounting for 38%) were introduced before 1800, and 23% of today's population is originated by lineages introduced in the period 1800–1950. In all these villages only a small number of founders was assessed to have contributed to the actual female gene pool. The limited number of genetic (mtDNA) lineages present in Talana, Urzulei and Perdasdefogu give a picture of very reduced female genetic pools.

Founder and genetic drift effects come into view while investigating the different patterns of mtDNA variations found in the villages. Considering the phylogenetic networks, in Talana, Urzulei and Perdasdefogu we sampled all the female genealogical lineages in the attempt to depict a comprehensive mtDNA representation, whereas for Ogliastra we carried out a random sampling at the population level. In the Ogliastra population as a whole the reference sequence is the most represented and only few H-derived sub-haplogroups can be observed (Fig. 2). On the contrary, in the Talana samples we found 13 H derived sub-haplogroups and H3 is the most frequent one (Fig. 3a). The spectrum of highly conserved variations within the H haplogroup can be explained by several factors. MtDNA variation maintenance in Talana can be justified by the village's long-standing isolation, the high endogamy and the low immigration rate during its history (Angius et al. 2001). The H3 sub-haplogroup high frequency (in contrast to the poorly represented H sub-haplogroup) and the pres-

ence of other distinctive mtDNA types is clear evidence of strong genetic drift taking place in Talana. For Urzulei, we observe genetic drift mainly for the haplogroups U (36 %) and H (49%) and the H haplogroup does not have so many conserved variations as those observed in Talana. In Perdasdefogu, a strong genetic drift mainly occurred within haplogroup H where the reference sequence has a frequency of 40%. Several frequent U sub-haplogroups were found only in Urzulei and Perdasdefogu (Fig. 3b, c). Probably, in Urzulei the founder effect is evident for the U haplogroup, or maybe this haplogroup was by chance over represented in the founding female gene pool. However, other evolutionary forces, such as selective pressure, could have acted as a possible factor, shaping the female genetic pool of the villages.

We emphasize how these small populations remained extremely isolated and genetically differentiated, although, as in the case of Talana and Urzulei, they inhabited neighbouring villages. These populations have a strong demarcation in their genetic pools, probably because of different founders. A possible explanation is that the Ogliastra population, ancestral to Talana, Urzulei and Perdasdefogu, was already genetically differentiated long before the villages were settled and the current (few) relationships were established. On the other hand, the different female founders contribution could account for the important differences observed in the current genetic makeup of the villages. Studying small population isolates, differentiation created by isolation can be revealed only if comprehensive phylogeny is reconstructed. At a larger population level, as is the case of Ogliastra as a whole, a random sampling is sufficient to depict the genetics of that population, whereas when small founder populations are compared a more detailed exploration is necessary. Genetic homogeneity strictly depends on the population scale factor and on sampling methodology. The outstanding homogeneity and the reduced female gene pool observed for the Ogliastra region, in the context of European populations, hide an extremely marked differentiation in peculiar sub-isolates (Talana, Urzulei, Perdasdefogu) originating from that archaic Neolithic population and residing in the same restricted area. Different bio-demographic histories determined by population expansion, immigration rate and endogamy depict peculiar genetic portraits.

These results clearly indicate that, even if Ogliastra can be considered one of the most genetically homogeneous populations in Europe, caution is needed in studying complex traits by LD mapping. We have already demonstrated that LD levels in Ogliastra as a whole are much lower than those observed in sub-populations living in this area (Angius et al. 2002). This can be explained by the divergent demographic histories of small villages, confirmed by this study through mtDNA analyses. All these findings underline the importance of examining in detail its demographic and genetic history before claiming a population as genetically homogeneous.

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## Electronic-database information

The URLs for data in this article are as follows:

- Genome Data Base, http://www.gdb.org/;
- Helsinki declaration, http://www.wma.net/e/policy/b3.html;
- Arlequin ver. 2.000: a software for population genetics data analysis – by Stefan Schneider, David Roessli, Laurent Excoffier of the Genetics and Biometry Laboratory, University of Geneva, Switzerland, http://lgb.unige.ch/arlequin/;
- Life Sciences and Engineering Technology Solutions, http://www.fluxus-engineering.com/ (for Network 3.0 software).

## Appendix table

The variable positions within the HVS-I (16024–16383) sequences that were analysed for the Ogliastra, Talana, Urzulei and Perdasdefogu samples are as follows (for Table see following page):

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| | Ref | 16030 C | 16061 T | 16126 T | 16129 G | 16145 G | 16167 C | 16169 C | 16174 C | 16188 C | 16192 C | 16193 C | 16235 A | 16240 A | 16261 C | 16269 A | 16270 C | 16292 C | 16293 A | 16294 C | 16296 C | 16298 T | 16304 T | 16311 T | 16319 G | 16320 C | 16368 T |
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| | 2 | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | G | ∘ | ∘ | T | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ |
| | 3 | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | G | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | C |
| | 4 | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | C | ∘ | ∘ | ∘ |
| | 5 | ∘ | ∘ | ∘ | A | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | C | ∘ | ∘ | ∘ |
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| H | 7 | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | T | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | A | T | ∘ |
| | 8 | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | T | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | A | T | ∘ |
| | 9 | ∘ | ∘ | ∘ | ∘ | ∘ | T | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | T | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ |
| | 10 | ∘ | ∘ | C | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ |
| | 11 | G | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ |
| | 12 | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | G | ∘ | G | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ |
| | 13 | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | T | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ |
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| | 16 | ∘ | ∘ | ∘ | ∘ | A | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ |
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| U | 4 | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | A | ∘ | ∘ | T | ∘ | G | ∘ | ∘ | ∘ | T | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ |
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| | 6 | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | A | ∘ | ∘ | T | ∘ | G | ∘ | ∘ | ∘ | T | T | ∘ | T | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ |
| | 7 | ∘ | ∘ | ∘ | ∘ | A | ∘ | A | ∘ | ∘ | T | ∘ | G | ∘ | ∘ | T | T | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | C | ∘ | ∘ | ∘ |
| | 8 | ∘ | ∘ | ∘ | ∘ | A | ∘ | A | ∘ | ∘ | T | ∘ | G | ∘ | ∘ | ∘ | T | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ |
| | 9 | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | T | ∘ | ∘ | C | ∘ | ∘ | ∘ | ∘ |
| | 10 | ∘ | ∘ | ∘ | ∘ | A | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ |
| | 1 | ∘ | ∘ | C | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | T | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ |
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| | 4 | ∘ | ∘ | C | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ |
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| | 5 | ∘ | A | C | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | T | ∘ | T | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ |
| | 6 | ∘ | ∘ | C | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ | T | ∘ | T | T | ∘ | ∘ | ∘ | ∘ | ∘ | ∘ |
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