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RESEARCH PAPER

Influence of evolutionary forces and demographic processes on the genetic structure of three Croatian populations: A maternal perspective

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***Background*: Many Croatian islands are examples of genetic isolates, with low level of heterozygosity and high level of inbreeding, due to practice of endogamy.**
***Aim*: The aim was to study the genetic structure of two insular and one mainland population through high-resolution phylogenetic analysis of mitochondrial DNA (mtDNA).**
***Subjects and methods*: MtDNA polymorphisms were explored in 300 unrelated individuals from Mljet, Lastovo and the coastal city of Dubrovnik, based on SNP polymorphisms.**
***Results*: All mtDNA haplogroups found in the sample were of typical European origin. However, the frequency distribution of their subclades differed significantly from other Croatian and European populations. MtDNA haplotype analysis revealed only two possible founder lineages on Mljet and six on Lastovo, accounting for almost half of the sample on both islands. The island of Mljet also has the lowest reported haplotype and nucleotide diversity among Croatian isolates and the island of Lastovo, a new sublineage of a usually quite rare U1b clade.**
***Conclusion*: The results can be explained by the effect evolutionary forces have on genetic structure, which is in line with the specific demographic histories of the islands. An additional research value of these two island isolates is the appearance of certain Mendelian disorders, highlighting their importance in epidemiological studies.**

Keywords: Island isolates, mainland, mtDNA, evolutionary forces, demography

INTRODUCTION

The Croatian islands, situated in the eastern Adriatic Sea (Figure 1), have for decades been a focus of diverse and wide-ranging research. They represent well-characterized genetic isolates with regard to their ethnohistory, biological trait measurements, disease prevalence, migration patterns and environmental and sociocultural characteristics. The results of numerous studies (Rudan P et al. 1987, 2003, 2004; Rudan I (2001), Rudan I et al. 1999, 2002, 2003, 2006) have indicated that village populations on the Croatian islands have preserved certain genetic specifics over the course of history up to the present day, and measures of kinship and genetic distances have revealed isolation of such communities from each other and from the mainland. Since such small communities can reflect large demographic processes that occurred in human pre-history and history, the scientific value of genetic isolates has also been proven in the field of population genetics and archeogenetics. Their geographical and reproductive isolation has kept their genetic and demographic history preserved over a long period of time and can therefore give us insight into the ancient migratory paths and forming of the Croatian mtDNA gene pool, as already indicated in Tolk et al. (2000) and Peričić et al. (2005). We can trace micro-evolutionary processes and see how evolutionary forces, such as genetic drift, founder effect and population bottlenecks shaped the current genetic landscape of the Croatian population. Also, isolates have been recognized as ideal tools for mapping Mendelian, and (to a certain extent) even complex disease traits (Rudan I et al. 1999; Peltonen et al. 2000; Deka et al. 2008), because of their environmental homogeneity and common practice of consanguinity.

As a part of the Mediterranean area, this region was of high importance for the colonization of the wider European area, based on both archeological and genetic evidence, and it served as an important highway of communication and maritime connections among Adriatic communities (Rootsi et al. 2004; Forenbaher 2009). The Adriatic archipelago gained its present form during the Neolithic period (ca. 6000

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*(at the time of research)

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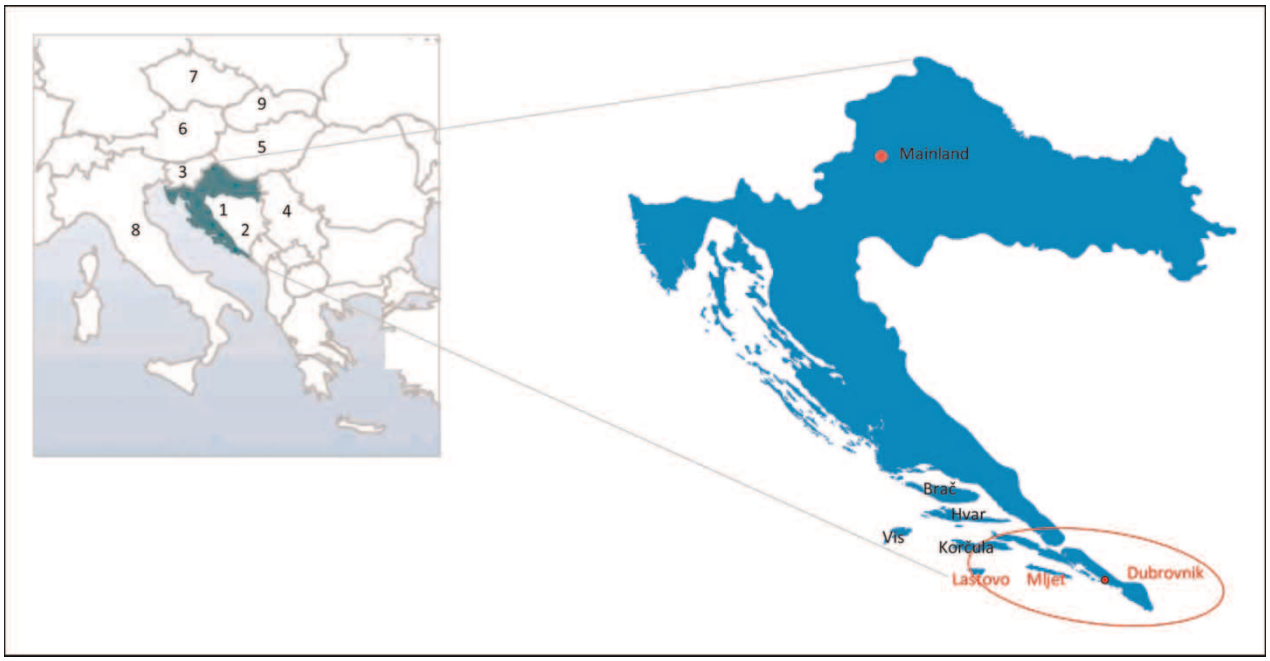

Figure 1. Map of Croatia with the location of Dubrovnik, investigated islands of its archipelago (south Dalmatian islands) and central Dalmatian islands used for comparison; surrounding populations taken for comparison marked with numbers (1–Bosnia, 2–Herzegovina, 3–Slovenia, 4–Serbia, 5–Hungary, 6–Austria, 7–Czech Republic, 8–Italy, 9–Slovakia); former Republic of Ragusa in the circle.

BP), at a time when farming and new technologies began to spread into Europe, with the Adriatic route as an important pathway by which immigrants, domesticates and other innovations were dispersed. Radiocarbon dates for Impresso sites from both sides of the Adriatic suggest that farming was introduced into Dalmatia (southern part of the eastern Adriatic coast) from southern Italy and then spread northwards along the coast (Forenbaher 1999). The existence of such ancient migratory paths, proposed by archeological evidence, can now be confirmed by uniparental markers such as mtDNA and Y chromosome, which offer us a new and different insight into historic events that took place in this area (Underhill and Kivisild 2007). Studies of isolates such as Basques (Bertranpetit et al. 1995), Ashkenazi Jews (Behar et al. 2006) or Saami (Tambets et al. 2004) have already revealed the influence of specific historic and demographic events on the genetic structure of such populations. Due to their geographic and reproductive isolation, island isolates are considered among the most suitable populations for studying the process of human microevolution and population structuring (Jeran 2010) and since the effective population size of mtDNA is 4-times smaller than that of autosomal loci, fluctuations in population size reflect themselves in mtDNA diversity.

Our aim was to assess the genetic diversity of two south Dalmatian Croatian island populations, based on high-resolution analysis of their mtDNA, and to compare it with a population structure of a coastal, mainland sample (city of Dubrovnik). The islands of Mljet and Lastovo are both examples of population isolates, since they display a high inbreeding level and, hence, a low level of genetic diversity resulting from their isolation. Their isolation is not only geographic, but also reproductive (practice of consanguinity) and historic (reduced gene flow caused by the autonomy of Lastovo, while being a part of Republic of Dubrovnik between the 14th and 19th century, and the role of Mljet as a quarantine station against the spread of plague in the same period). Also, these populations went through several bottlenecks (mostly connected with different epidemic waves), resulting in a reduction of population size and an increased influence of genetic drift, which led to the expression of specific autochthonous diseases.

SAMPLE AND METHODS

Blood samples were taken from a total of 300 adult individuals after giving their informed consent—68 from Mljet, 51 from Lastovo and 181 from Dubrovnik. According to the last census (2001), the island of Mljet numbers 1111 and Lastovo 835 inhabitants. Hence, our sample covered 6% of the total population on each island. Individuals were chosen based on an extensive questionnaire with genealogical information that allowed exclusion of potentially related individuals up to the grandparent level, in line with the sampling strategy used in previous studies of Croatian isolates (Tolk et al. 2000; Cvjetan et al. 2004; Peričić et al. 2005; Jeran et al. 2009; Jeran 2010).

Genomic DNA was extracted from whole blood samples using the 'salting out' method (Miller et al. 1988) at the Institute for Anthropological Research in Zagreb, Croatia. All further laboratory analyses were performed at the Estonian Biocentre and Department for Evolutionary Biology, Institute of Molecular and Cell Biology, University of Tartu, Tartu, Estonia.

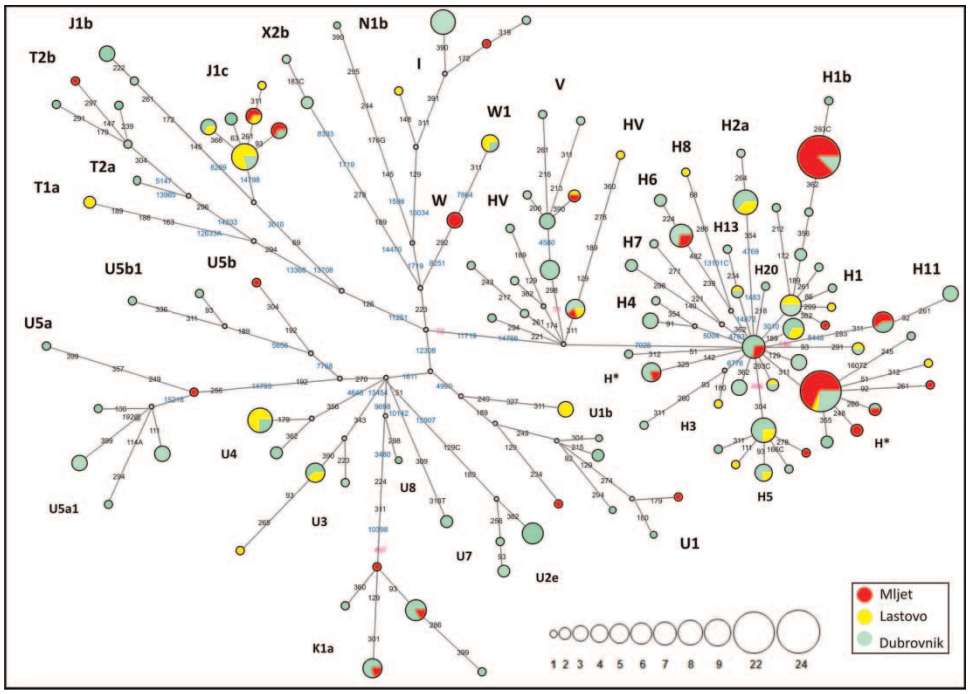

Figure 2. Reduced-Median-Joining phylogenetic network of all mtDNA haplotypes in a sample gathered by high-resolution analysis. The size of the node is proportional to the number of individuals.

The hypervariable segment I (HVS-I) of the control region of mtDNA was PCR amplified, purified and sequenced on an Applied Biosystems 3730xl DNA Analyzer using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Warrington, UK). Exact haplogroups and subhaplogroups were determined based on SNP polymorphisms specific for main Eurasian lineages, with a combined usage of the RFLP method and sequencing (Supplementary table; online version only). Sequences were aligned and analyzed according to rCRS (NC_012920), using ChromasPro software (Technelysium Pty Ltd, Tewantin QLD, Australia). The Global Human Mitochondrial DNA Phylogenetic Tree, based on both coding and control region mutations and including haplogroup nomenclature, was consulted while defining haplogroup affiliations (www.phylotree.org) (Van Oven and Kayser 2009). Phylogenetic networks of mtDNA haplotypes were constructed using program Network 4.502 and Network Publisher (www.fluxusengineering.com) applying both reduced median and median joining algorithms and corrected by hand, if needed. Different weights were assigned to substitutions (Hasegawa et al. 1993; Allard et al. 2002; Soares et al. 2009). Haplotype diversity index, nucleotide diversity and mean number of pairwise differences were calculated using Arlequin 3.5 software (Excoffier and Lischer 2010). Principal Component Analysis (PCA) was performed as a visual representation of differences/similarities between the populations based on mtDNA subhaplogroup frequencies, using the software POPSTR. Only haplogroups with noticeable impact on the scatterplot were visualized in the plot. Genetic distances (Fst) were estimated by the pairwise difference method and visualized as a multidimensional scaling (MDS) plot, using the Primer 6.0. software (Clarke and Gorley 2006). Genetic relationships between populations were further explored based on haplotype frequencies by analysis of molecular variance (AMOVA), as implemented in Arlequin 3.5 software (Excoffier and Lischer 2010). Coalescence ages were calculated on networks, by means of average distance (in terms of number of mutations) from the root haplotypes (*rho*–ρ). One transitional step between nucleotide positions 16090–16365 was taken as equal to 18 845 years (Soares et al. 2009). Standard deviations for the estimates from networks were calculated as in Saillard et al. (2000). Complete sequencing of three U1 mitochondrial genomes and their phylogeny construction was performed using the methodology described in details by Torroni et al. (2001).

RESULTS

The frequency distribution pattern of mtDNA haplogroups in Croatia is consistent with the typical European maternal gene pool (Richards et al. 1998, 2000; Macaulay et al. 1999; Cvjetan et al. 2004; Malyarchuk et al. 2008a; Soares et al. 2010). However, its many island isolates show somewhat deviated frequencies and haplogroup proportions, as already suggested in previous publications (Tolk et al. 2000; Peričić et al. 2005; Jeran et al. 2009). The first obvious island–mainland difference in our sample was the subclade diversity—16 subhaplogroups were present on the islands, in comparison with exactly twice as many in the mainland sample (Figure 2). These results are in accordance with the implication that gene flow and influx of women to

the islands were limited, as a result of geographic isolation and specific demographic history. Distribution of (sub)-haplogroups in our sampled populations is visualized in Figure 2 and presented in detail in Tables I and II.

Haplogroup (hg) H is the most prevalent clade in the whole sample of 300 individuals, with a wide frequency range of 39.2–73.5%. Hg H is a dominant European haplogroup and rather uniformly distributed throughout the continent, suggesting its major role in the peopling of Europe (Richards et al. 1998; Torroni et al. 1998; Achilli et al. 2004; Loogvali et al. 2004; Roostalu et al. 2007). Recent studies gave this haplogroup additional importance, since new evidence suggests H hg also had a significant role in the peopling of Northern Africa, especially the H1 clade (Ennafaa et al. 2009; Ottoni et al. 2010; Garcia et al. 2011). H1 haplogroup is the most frequent H subclade in Europe, Near East and North Africa, accounting for ~ 30% of the H hg gene pool in Slavic populations of Eastern Europe (Loogvali et al. 2004; Roostalu et al. 2007). From the two most common H1 subclades in Europe, subclade H1a is most abundant in this area and H1b is in general a rather scarce branch scattered around Europe, with peak frequencies in southern Iberia (Garcia et al. 2011). H frequency on Mljet is extremely high (73.5%) and, when compared with other population studies, only the Spanish Basques have such elevated H hg frequency (62.6%) (Richards et al. 1998). The extreme increase in the portion of H hg on Mljet is due to the elevated frequency of one specific subclade, H1b (30.9%), represented solely by haplotype 16189–16356–16362, otherwise rare in our insular populations and in this area in general, as mentioned before. Besides on Mljet, it was found in only one individual on the islands of Lopud, Brač and Pašman and in none of the Croatian mainland samples (unpublished data, database of the Institute for Anthropological Research, Zagreb). In the Dubrovnik sample, three individuals carry the exact motif, suggesting it came to the island of Mljet from the nearest coastal region. This is an excellent example of a founder effect, showing the strong influence of micro-evolutionary processes, such as genetic drift, on the population structure. Hg H encompasses 39.2% of the Lastovo mtDNA gene pool, which is in range with the usual H prevalence in Europe, but still lower than the rather typical percentage found in Dubrovnik (47%). Hg H has a star-like phylogeny (Figure 2) and is the most diversified haplogroup in general, with more than 40 determined subclades (Brandstätter et al. 2008; Álvarez-Iglesias et al. 2009) and many more awaiting detection. In this context, haplogroup H* has to be mentioned as a specific, heterogeneous sub-group that consists of many not yet identified and classified H subclades. In our sample, samples tested for H1, H2, H3, H4, H5, H6, H7, H11, H12, H13 and H19 that could not be assigned to any of them were designated H* hg. Our results show that H* frequency was the highest on Lastovo and in Dubrovnik and second highest on Mljet, right after the H1b clade. Interestingly, although 13 different H subclades were found in our sample, the only ones present in all three populations were H1, H5 and few potential lineages from the H*group. Since those were H subclades with the highest haplotype diversity, we calculated the coalescence age for H1 (excluding H1b) and H5 and obtained values of 13 460 (±5384) and 9422 (±5267), respectively. Such values are expected and suggest a connection of these H clades with the Late Upper Paleolithic expansions (14 500 YBP), as stated previously by Malyarchuk et al. (2008a).

Table I. Frequency distribution of mtDNA (sub)haplogroups in studied populations (%).

(Sub)hg	Mljet (n = 68)	Lastovo (n = 51)	Dubrovnik (n = 181)
H	*73.5*	*39.2*	*47.0*
H*	32.4	11.8	17.7
H1	1.5	7.8	5.0
H1b	30.9		2.2
H2		5.9	3.3
H3		2.0	0.6
H4			2.2
H5	1.5	7.8	6.1
H6	2.9		3.9
H7			1.7
H8		2.0	
H11	4.4		2.8
H13		2.0	1.1
H20			0.6
J	*5.9*	*19.6*	*6.1*
J1b			2.2
J1c	5.9	19.6	3.9
T	*1.5*	*3.9*	*2.3*
T1a		3.9	
T2a			0.6
T2b	1.5		1.7
U	*5.9*	*23.5*	*19.9*
U1a			2.3
U1b		5.9	
U1c	2.8		0.6
U2e			5.0
U3		5.9	2.2
U4		11.8	2.2
U5a	1.5		5.0
U5b	1.5		1.1
U7			1.1
U8a			0.6
K	*4.4*		*6.1*
K1a	4.4		6.1
W	*4.4*	*5.9*	*0.6*
W*	4.4		
W1		5.9	0.6
HV	1.5	*3.9*	*7.7*
V	*1.5*	*2.0*	*3.3*
I	*1.5*	*2.0*	*5.0*
X2b			*1.7*
N1b			*0.6*

Considering the second most prevalent haplogroup in Europe, haplogroup U, results differ greatly. A relatively low frequency has been recorded for the island of Mljet (5.9%). Conversely, the island of Lastovo (23.5%) and Dubrovnik (19.9%) display a significantly higher U frequency, however with differences in subclade diversification. While the Lastovo sample has only three (U1, U3 and U4), Dubrovnik's hg U sample harbours all eight subclades, including very rare subclades such as U1, U7 and U8.

Table II. Distribution of mtDNA haplotypes (HVSI sequences) of all subhaplogroups in the three studied populations.

HG	SUBHG	MLJ	LA	DBK	HVSI (+16000 to the polymorphic site)
H	H*	0	0	1	72-245-311
	H*	0	2	4	189
	H*	0	0	3	362
	H*	2	0	5	CRS
	H*	0	0	3	129
	H*	15	1	6	311[a]
	H*	1	0	4	142–325
	H*	0	0	2	311–355
	H*	0	0	1	51–312
	H*	0	1	1	93–291
	H*	2	0	0	248–311
	H*	0	1	0	51–311–312
	H*	1	0	0	92–261–311
	H*	0	1	1	293C
	H*	1	0	1	260–311
	H1	1	0	0	362
	H1	0	1	0	209
	H1	0	0	1	261
	H1	0	0	1	86
	H1	0	0	2	189
	H1	0	0	1	172–212
	H1	0	0	1	189–356
	H1	0	3	3	CRS[b]
	H1b	21	0	3	189–356–362[a]
	H1b	0	0	1	189–293C–356–362
	H2a	0	3	5	354[b]
	H2a	0	0	1	264–354
	H3	0	0	1	93–260–311
	H3	0	1	0	180
	H4	0	0	1	354
	H4	0	0	3	93
	H5	0	2	6	304
	H5	0	0	1	166C–304
	H5	1	0	0	278–304
	H5	0	1	3	93–304
	H5	0	1	0	111–304
	H5	0	0	1	304–311
	H6	2	0	5	362
	H6	0	0	2	224–362
	H7	0	0	2	140–298
	H7	0	0	1	221–271
	H8	0	1	0	68–288–362
	H11	3	0	2	293–311
	H11	0	0	3	92–261–293–311
	H13	0	1	1	CRS
	H13	0	0	1	234
	H20	0	0	1	218
HV		0	0	1	261
		0	0	1	217–243–261
		0	1	0	129–189–278–311–360
		0	0	5	298
		0	0	1	221–294
		1	1	4	311
		0	0	1	129–174–189
		0	0	1	174–362
I		0	0	1	129–172–223–311–319–391
		0	1	0	129–148–223
		0	0	8	129–223–311–390–391
		1	0	0	129–172–223–311–391
J	J1b	0	0	3	69–126–145–172–222–261
	J1b	0	0	1	69-126-145-172-261
	J1c	0	0	2	63-69-126
	J1c	2	1	0	69-126-261
	J1c	2	0	1	69-93-126
	J1c	0	7	2	69-126[b]
	J1c	0	1	0	69-126-261-311
	J1c	0	1	2	69-126-366
K	K1a	1	0	0	224-311
	K1a	1	0	4	129-224-301-311
	K1a	0	0	1	93-224-286-311-399
	K1a	1	0	5	93-224-311
	K1a	0	0	1	224-311-360
N	N1b	0	0	1	145-176G-223-244-295-390
T	T1a	0	2	0	126-163-186-189-294
	T2a	0	0	1	126-294-296
	T2b	1	0	0	126-147-294-296-297-304
	T2b	0	0	1	126-294-296-304
	T2b	0	0	1	126-179-291-294-296-304
	T2b	0	0	1	126-239-294-296-304
U	U1	0	0	2	189-215-249
	U1	0	0	1	92-189-249-294
	U1	0	0	1	189-249-304
	U1	1	0	0	129-179-189-249-274
	U1	1	0	0	129-189-234
	U1	0	0	1	129-180-189-249-274
	U1b	0	3	0	249-311-327[b]
	U2e	0	0	6	51-129C-189-362
	U2e	0	0	1	51-129C-189-256
	U2e	0	0	2	51-93-129C-189-256
	U3	0	0	1	223-343
	U3	0	1	0	93-265-343-390
	U3	0	2	3	343-390
	U4	0	0	2	356-362
	U4	0	6	2	179-356[b]
	U5a	0	0	1	192-249-256-270-357-399
	U5a	1	0	0	192-256-270
	U5a1	0	0	3	111-192-256-270
	U5a1	0	0	1	114A-192-256-270-294
	U5a1	0	0	1	136-192-256-270
	U5a1	0	0	3	256-270-399
	U5b	1	0	0	192-270-304
	U5b1	0	0	1	189-270-311-336
	U5b1	0	0	1	93-189-270
	U7	0	0	2	309-318T
	U8	0	0	1	298
V		1	1	0	298-390
		0	0	1	213-298-311
		0	0	1	216-261-298
		0	0	1	206-298
		0	0	3	298
W	W*	3	0	0	223-292
	W1	0	3	1	223-292-311[b]
	X2b	0	0	1	183C-189-223-278
X	X2b	0	0	2	189-223-278

MLJ, Mljet; LA, Lastovo; DBK, Dubrovnik. [a] supposed founder lineages on Mljet; [b] supposed founder lineages on Lastovo.

In general, hg U is the oldest European haplogroup (with a coalescence age of ~ 50 000 years ago) and its subclade U5 encompasses most of the hg U diversity in Europe (Richards et al. 1998). Also, it is the most frequent U-clade in Adriatic islanders in general (Jeran 2010). In this context, it is interesting that the U5 subhaplogroup is completely absent on Lastovo, where the general proportion of U haplogroup is the highest in all Adriatic isolates. The second largest subclade of U in Europe and Croatia and the most prevalent

hg on Lastovo is U4 (Jeran 2010; Soares et al. 2010). However, its elevated frequency (11.8%) and low diversity indicates a founder event. Also, the presence of U1 samples on Lastovo (5.9%), Mljet (2.9%) and in Dubrovnik (2.8%) is surprisingly high. This subhaplogroup is very rare in Europe and most common in North Caucasus (Macaulay et al. 1999; Richards et al. 2000), where its frequency reaches 5.5%. Although it displays an increased value in this region, it is still less than the observed percentage on Lastovo, where it is presented with a single U1b haplotype (16249–16311–16327). The haplogroup is more resolved in the mainland sample, where it harbours four different U1 HVS-I motifs; however, none of them matches the Lastovo one. Although this suggests that the Lastovo U1 variant did not arrive to the island from the nearest coastal city of Dubrovnik, the presence of such a rare haplogroup in southern Dalmatia and its high diversification rate indicates a common gene flow from long-distance migrations. Although this influx was most probably of minor impact, it enabled a surprisingly diverse and outspread U1 haplogroup in the south Croatian coastal and insular area. Besides on Mljet and Lastovo, individuals carrying U1 hg have been found on another four Dalmatian islands (Pag, Hvar, Brač and Korčula) (Jeran 2010). Coalescence age was estimated for the U1 subclade in our sample and the obtained value was 43 343 (±13 458), which is consistent with the age of U1 estimated previously by Richards et al. (2000). However, in our Croatian and Balkan database (unpublished data, Institute for Anthropological Research, Zagreb) only one U1b sample with the same HVSI-motif as on Lastovo has been found on the island of Brač. Also, in a wider European context, this exact U1b haplotype has previously only been recorded in one sample from Greece and Italy and three Russian Cossaks (Oleg Balanovsky, personal communication). The scarcity of this U1b haplotype in Europe has led us to sequence the entire mitochondrial genome of the three Lastovo samples defined as U1b and the results are visualized in a form of a phylogenetic tree in Figure 3. Since we have detected additional mutations (one in the HVSI region, two in the coding region and three in the HVSII region) that are not yet recorded in The Global Human Mitochondrial DNA Phylogenetic Tree (Van Oven and Kayser 2009) and we have confirmed their existence in all three samples, we suggest a higher resolution for the Ub1 lineage based on our results. However, since there is a possibility these mutations are only local and island-specific, further full-sequencing of this subhaplogroup from neighbouring and more distant regions is needed in an attempt to trace its spread and origin on this island.

Elevated frequency of the third major European haplogroup, hg J, has been observed only on Lastovo (19.6%), in comparison with its average occurence on Mljet, in Dubrovnik and other European and Croatian populations. The highest frequency of J hg in Europe recorded up till now has been in the eastern Mediterranean (14%) (Richards et al. 2000). This increase of J lineage on Lastovo is due to a significantly higher occurrence of J1c subclade, with a single haplotype (16069–16129) representing most of the J portion. This subclade has been found on all of the previously analysed islands, however in a significantly lower percentage (Jeran 2010) and one unique J1c haplotype (16069–16126–16261) has been found only on Mljet and Lastovo.

Concerning haplogroup T, whose average frequency in Europe varies around 8% (Torroni et al. 1998), it is

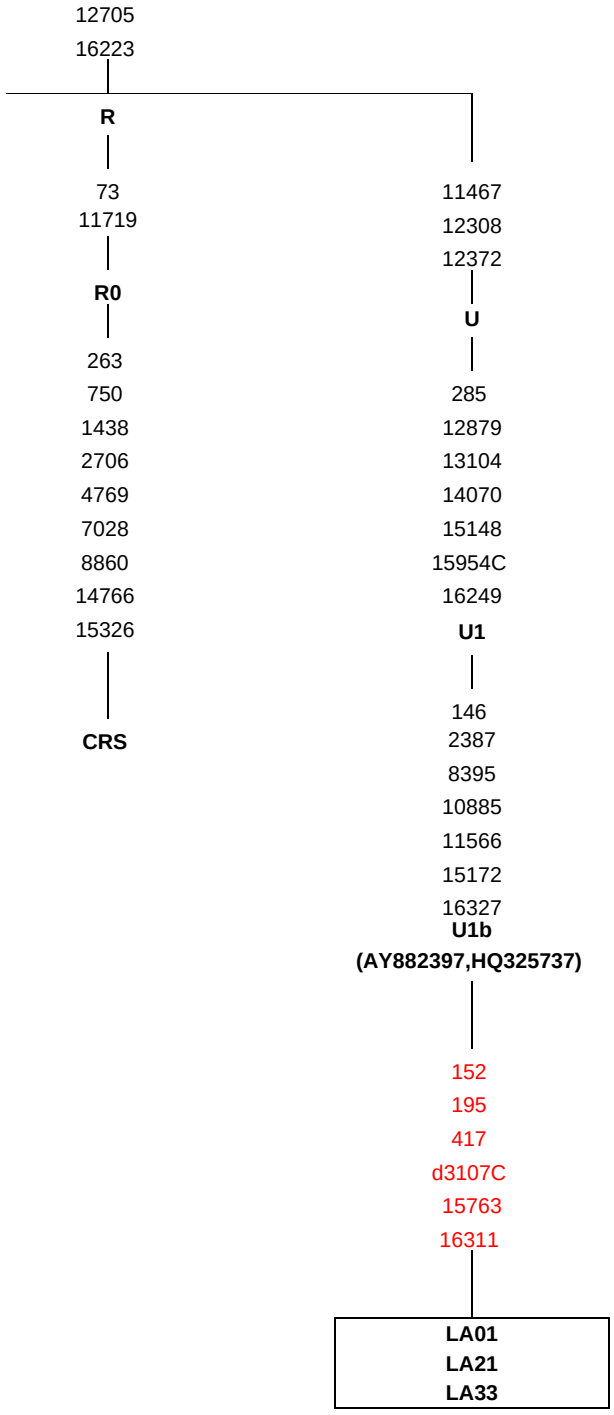

Figure 3. Maximum parsimony phylogenetic tree for three U1b samples from the Island of Lastovo. The tree is rooted in rCRS (Andrews et al. 1999). All mutations are shown on branches; they are transitions unless a base change is indicated and deletions have a 'd' prefix. No recurrent mutations were identified. Additional, previously not reported mutations are marked in red.

under-represented on both the islands and mainland, especially the T2 clade. Mljet and Lastovo show opposite results concerning subclade portion—no T1a samples were reported on Mljet and, conversely, no T2b samples on Lastovo. The Dubrovnik sample also lacked the T1a subclade, but both T2a and T2b sub-groups have been detected, although with extremely low frequencies (see Table I).

An interesting connection between Mljet and Lastovo has been found inside the V haplogroup. V hg is a younger sister clade of haplogroup H and in most European populations its frequency ranges from 1–7% (Torroni et al. 2001). Both of our islands exhibit a decrease in the frequency of this clade (1.5–2.0%), but they share one unique V haplotype (16298–16390), found on none of the other Croatian islands (Jeran 2010).

MtDNA haplogroups that derive directly from the super clade N (N1a, N1b, N1c and I, W, X) are relatively rare in Europe and do not usually exceed the level of 5% (Richards et al. 1998). In our insular samples, due to the effect of genetic drift, N1 subclades and haplogroup X were completely absent, while I and W clade displayed relatively small percentages, similar to HV and V lineages (1.5–5.9%). Unlike them, the Dubrovnik sample harboured all mentioned clades, with HV frequencies even somewhat higher than expected.

Haplogroup frequencies vary notably between these three populations, due to the action of evolutionary forces on the islands (as shown in Table I). However, even haplogroups presented with similar frequencies in our populations differ regarding haplotype composition. On Lastovo, six haplotypes were defined as possible founder lineages, while there were only two on Mljet. Possible founder lineages were considered to be ones encompassing more than 5% of the sample, namely four persons on Mljet or three on Lastovo. The effect of genetic drift is most clearly observed on Mljet, since the elevated frequency of haplogroup H1b on the island (30.9%) is represented only by one HVS-I haplotype (16189–16356–16362). This haplotype, together with an H* haplogroup HVS-I motif 16311, accounts for more than 50% of the sample and they therefore present founder haplotypes on Mljet. Although Lastovo is more diverse in haplotypes, it also harbors the highest frequency of J1c 16069–16126 with 13.7% and U4 16179–16356 with 11.8% of all eastern Adriatic island populations (Jeran 2010). Table II offers a more detailed insight into the founder lineages on each island and the distribution of HVSI haplotypes of all (sub)haplogroups detected.

As for the mainland sample from the city of Dubrovnik, all values fit within a typical European maternal gene pool. The only increase in frequency has been observed for the HV clade (7.7%). In Europe, its frequency ranges from 0.3% in North Europe, 2.6% in Southeast Europe, up to 3.4% in the eastern Mediterranean and 4.4% in populations of the central Mediterranean (Torroni et al. 1998; Richards et al. 2000). However, since it can not generally be assigned to the effect of genetic drift as in our island isolates, it could suggest a slightly higher genetic impact from the Near East, where this clade is predominantly present (Macaulay et al. 1999), probably through trading paths.

To visualize the relationships between our sampled populations and other surrounding populations, we performed PCA and MDS plots. Neighbouring islands of Brač, Hvar, Vis and Korčula (Peričić et al. 2005; Jeran 2010) were chosen for comparison and according to their geographic position named 'central Dalmatian islands', to distinguish them from Mljet and Lastovo ('southern Dalmatian islands'). In addition to Dubrovnik, mtDNA results from 10 other mainland populations were included in the visualizations—Croatian mainland, Bosnia, Herzegovina, Slovenia, Serbia (Institute for Anthropological Research, personal communication; Cvjetan et al. 2004), Hungary (Irwin et al. 2007), Austria (Brandstätter et al. 2007), Czech Republic (Malyarchuk et al. 2006), Slovakia (Malyarchuk et al. 2008b) and Italy (Balanovsky Oleg, personal communication). All populations were chosen for comparison based on their neighbouring location and historic connections with the sampled populations.

The PCA plot was performed based on (sub)haplogroup frequencies, with hgs responsible for the position of a population on the scatterplot marked in blue colour (Figure 4). The outlying position of Mljet is evident and due to the extremely increased frequency of haplogroup H1b. Similarly, owing to the high prevalence of haplogroups J1c and U1, Lastovo is distant from the mainland sample of Dubrovnik and other continental populations, but close to the islands, forming an insular cluster. In a wider European context, the closest mainland populations to the insular cluster, based on (sub)hg frequencies, are the Croatian mainland and neighbouring Balkan populations (Slovenia, Bosnia, Herzegovina and Serbia), as expected.

Genetic distances, obtained by the pairwise difference method, are presented in Table III and visualized by a multidimensional scaling (MDS) plot in Figure 5. They are significant between all tested insular population pairs and the island of Mljet clearly differs from all other insular and continental populations. Also, all three sampled populations are significantly distant from the Croatian mainland. This finding is rather surprising, but can be interpreted as a result of genetic drift for the islands. As for the sampled mainland population, the distance between Dubrovnik and Bosnia and Herzegovina (B&H) is insignificant, implying that the gene flow between these populations was stronger than between Dubrovnik and the rest of the continental Croatia. This can be explained by Dubrovnik's specific position, making it more accessible to neighbouring B&H than to other Croatians. Interestingly, our analysis of genetic distances has also shown that Lastovo is closer to some other European countries (Czech Republic, Slovakia and Hungary) than to the Croatian mainland or neighbouring countries. However, we are inclined to interpret this finding as not significant (since historical records do not offer any data confirming this connection) and assign it to the randomness of the genetic drift. As in the PCA plot, MDS representation nicely shows the central cluster of continental populations, with insular populations dispersed around

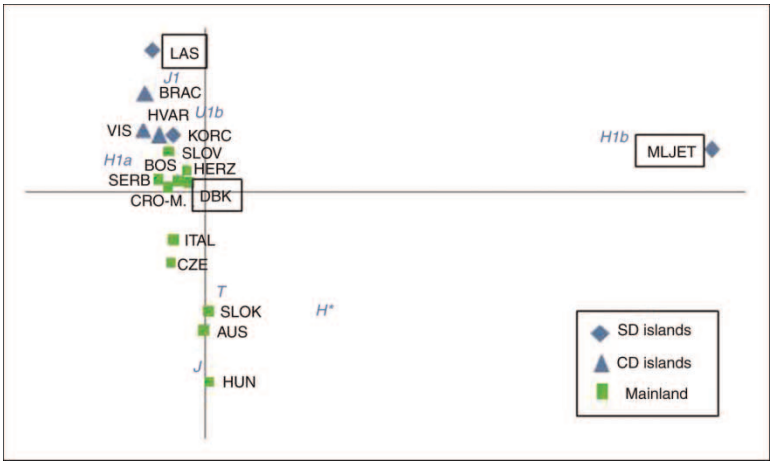

Figure 4. PCA plot based on frequencies of (sub)haplogroups in six island populations and 11 continental populations. Abbreviations: Brac, Brač, Las, Lastovo; Korc, Korčula; Dbk, Dubrovnik; Cro-M, Croatian mainland; Bos, Bosnia; Herz, Herzegovina; Cze, Czech Republic; Ital, Italy; Slov, Slovenia; Slok, Slovakia; Aus, Austria; Serb, Serbia; Hun, Hungary; SD islands, south Dalmatian islands; CD islands, central Dalmatian islands.

them in different directions and with the island of Mljet as a clear outlier.

Genetic diversity for our sampled populations was evaluated by haplotype diversity index (HDI), nucleotide diversity (ND) and mean number of pairwise differences (MNPD). They were calculated in order to assess the level of isolation and inbreeding in our sampled populations and to compare them with the calculations obtained for other populations (Table IV). The values obtained for Mljet were surprising, since we expected higher values for this island than for the open-sea island of Lastovo. On the contrary, our results indicate that the island of Mljet displays the lowest level of diversity among all studied Croatian isolates and can also be placed right after the Saami (Tambets et al. 2004) and the Ogliastra isolate in Sardinia (Fraumene et al. 2003) in a more global context, followed by the island of Vis, Korčula and Lastovo.

In order to test the genetic structure of sampled populations, analysis of molecular variance (AMOVA) was performed. Typically, most of the variation was due to differences between individuals within populations (98.49%), whereas a small percentage of variation was due to differences among populations within the same group (0.88%) and differences among the six established groups of populations (0.63%) (Table V).

A haplotype sharing analysis was also performed in order to assess the proportion of shared matrilineal ancestry between all pairs of island populations and showed interesting results. The geographic position of the sampled island populations would suggest that the mainland sample shares more haplotypes with the island of Mljet, then with Lastovo. However, as already shown through plots and the HD, inverse values are more likely to be obtained. We only took into account the presence of identical lineages in two populations, regardless of their frequencies, and the results were in concordance with the values obtained by other analysis. From 25 haplotypes detected on Mljet and 28 on Lastovo, only four were shared

Table III. Population pairwise Fsts (above diagonal) and Fst p-values (below diagonal) between the populations gained by pairwise difference distance method. Non-significant values ($p > 0.05$) are in italics.

	Dbk	Last	Mljet	Brač	Hvar	Korč	Vis	Bos	Herz	Cro-M.	Serb	Slov	Hung	Aust	Slok	Ital	Czech
Dbk		0.005	−0.001	0.002	−0.001	0.004	0.005	0.003	0.006	0.008	0.017	0.032	0.016	0.012	0.067	0.007	0.014
Last	0.000		0.005	0.001	0.000	0.011	0.004	0.007	0.007	0.009	0.012	0.044	0.010	0.015	0.086	0.018	0.015
Mljet	0.000	0.000		0.003	0.008	0.007	0.002	0.003	0.004	0.007	0.013	0.031	0.015	0.014	0.060	0.009	0.011
Brač	0.009	0.009	0.000		0.002	0.005	0.003	0.005	0.004	0.006	0.011	0.040	0.012	0.015	0.069	0.012	0.012
Hvar	0.000	0.000	0.000	0.000		0.005	0.006	0.004	0.007	0.010	0.018	0.040	0.015	0.015	0.082	0.010	0.017
Korč	0.000	0.000	0.000	0.009	0.000		0.016	0.013	0.018	0.021	0.017	0.057	0.008	0.028	0.096	0.012	0.031
Vis	0.000	0.009	0.000	0.000	0.018	0.000		0.004	0.003	0.001	0.009	0.039	0.014	0.009	0.067	0.010	0.004
Bos	*0.252*	0.018	0.000	*0.126*	0.000	0.000	0.000		0.002	0.001	0.012	0.021	0.021	0.007	0.059	0.011	0.006
Herz	*0.342*	0.036	0.000	*0.144*	0.000	0.000	0.009	*0.603*		−0.000	0.015	0.017	0.023	0.002	0.051	0.012	0.000
Cro-M.	0.018	0.009	0.000	0.018	0.000	0.000	0.018	*0.153*	*0.144*		0.012	0.022	0.025	0.002	0.057	0.014	0.000
Serb	0.045	0.027	0.000	0.027	0.000	0.000	0.027	*0.315*	*0.063*	0.027		0.050	0.013	0.021	0.091	0.032	0.020
Slov	0.000	0.045	0.000	0.009	0.027	0.000	0.018	0.000	0.000	0.000	0.000		0.070	0.017	0.043	0.050	0.020
Hung	0.000	*0.054*	0.000	0.000	0.000	0.000	0.000	0.000	0.036	0.009	0.045	*0.126*		0.032	0.105	0.026	0.030
Aust	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.018	*0.054*	0.000	*0.090*	*0.090*	*0.054*		0.064	0.019	0.007
Slok	0.000	*0.072*	0.000	0.000	0.000	0.000	0.000	0.009	*0.063*	*0.054*	*0.099*	0.045	*0.243*	*0.054*		0.094	0.045
Ital	0.000	0.027	0.000	0.000	0.036	0.000	0.009	0.009	0.009	0.000	*0.072*	0.009	*0.288*	*0.108*	0.018		0.020
Czech	0.000	*0.090*	0.000	0.000	0.000	0.000	0.000	0.009	0.027	0.027	0.018	*0.126*	*0.621*	*0.063*	*0.810*	0.027	

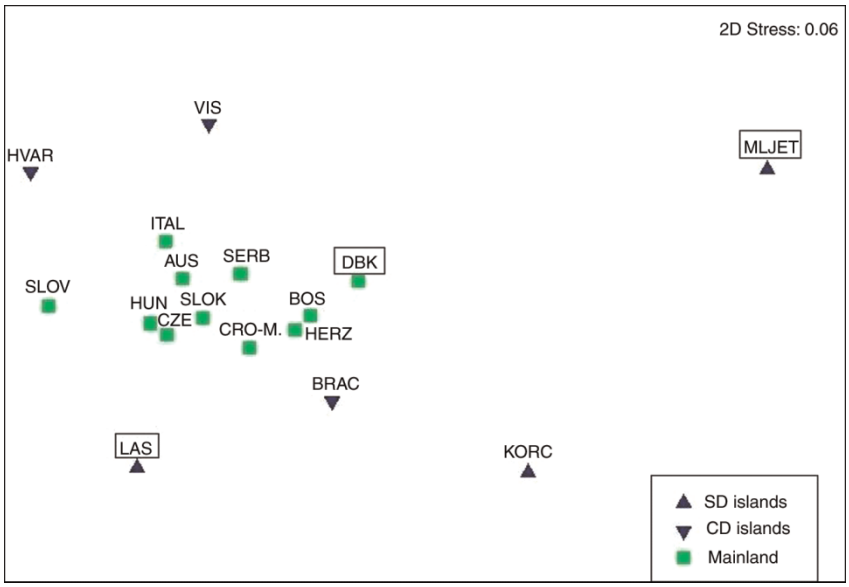

Figure 5. Multidimensional scaling plot based on Fst distances (pairwise difference method) between six island populations and 11 continental populations. Abbreviations: Brac, Brač; Las, Lastovo; Korc, Korčula; Dbk, Dubrovnik; Cro-M, Croatian mainland; Bos, Bosnia; Herz, Herzegovina; Cze, Czech Republic; Ital, Italy; Slov, Slovenia; Slok, Slovakia; Aus, Austria; Serb, Serbia; Hun, Hungary; SD islands, south Dalmatian islands; CD islands, central Dalmatian islands.

between the two islands (7.5%), suggesting different haplogroups and haplotypes were subject to genetic drift on each island or different migratory paths shaped their genetic landscape. Interestingly, the mainland city of Dubrovnik shares more haplotypes with Lastovo (12.8%), then with Mljet (9.6%), although the distance of these two islands from mainland would indicate otherwise.

Observed private haplotypes, confined to a particular population and occurring in only one or few individuals, were considered as young and of recent origin and could, therefore, not tell us much about the genetic history of these populations. A high percentage has been found on Mljet (36%), but they were mostly singletons and account altogether for only 14.5% of the population, suggesting a limited, but regular influx of women carrying these motifs.

DISCUSSION

This study aimed to clarify the effect evolutionary forces have had on the population structure of Mljet, Lastovo and Dubrovnik in pre-history and history, through the analysis of their mtDNA variability. The results from the previous

Table IV. Genetic diversity measures for 17 populations—three central Dalmatian islands, four southern Dalmatian islands and 10 mainland populations.

	N[a]	Nhgs[b]	Nhap[c]	HDI[d]	ND[e]	Nps[f]	MPD[g]	Reference
Mljet	68	17	25	0.8569 ± 0.031	0.029 ± 0.015	62	7.06 ± 3.35	present study
Lastovo	51	16	28	0.9592 ± 0.013	0.039 ± 0.020	64	9.34 ± 4.36	present study
Dubrovnik	181	32	90	0.9880 ± 0.001	0.040 ± 0.020	127	9.70 ± 4.46	present study
Brač	95	27	50	0.9742 ± 0.006	0.040 ± 0.020	89	9.61 ± 4.44	Jeran (2010)
Hvar	161	31	71	0.9804 ± 0.003	0.045 ± 0.023	102	10.87 ± 4.97	Jeran (2010)
Korčula	89	25	39	0.9568 ± 0.009	0.031 ± 0.016	80	7.54 ± 3.55	Jeran (2010)
Vis	129	27	45	0.9539 ± 0.008	0.037 ± 0.019	88	8.97 ± 4.16	Jeran (2010)
Cro-Mainland	389	44	196	0.9873 ± 0.001	0.036 ± 0.018	170	9.85 ± 4.52	Cvjetan et al. (2004); unpub. data*
Bosnia	231	39	146	0.9902 ± 0.0022	0.034 ± 0.017	142	9.38 ± 4.32	Cvjetan et al. (2004); unpub. data*
Herzegovina	128	36	74	0.9881 ± 0.002	0.041 ± 0.020	119	9.80 ± 4.51	Cvjetan et al. (2004); unpub. data*
Serbia	118	33	86	0.9930 ± 0.002	0.037 ± 0.018	124	10.13 ± 4.66	Cvjetan et al. (2004); unpub. data*
Slovenia	99	29	73	0.9878 ± 0.005	0.043 ± 0.021	110	11.70 ± 5.34	Institute for Anthropological Research, personal communication
Hungary	204	38	145	0.9811 ± 0.005	0.036 ± 0.018	144	10.011 ± 4.59	Irwin et al. (2007)
Austria	266	31	154	0.9737 ± 0.005	0.037 ± 0.019	149	10.28 ± 4.71	Brandstätter et al. (2007)
Slovakia	186	29	131	0.9934 ± 0.001	0.037 ± 0.019	143	10.32 ± 4.73	Malyarchuk et al. (2008b)
Italy	181	25	125	0.9765 ± 0.007	0.034 ± 0.017	138	9.47 ± 4.36	Balanovsky, personal communication
Czech Repub.	177	31	111	0.9878 ± 0.0032	0.039 ± 0.019	132	10.66 ± 4.87	Malyarchuk et al. (2006)

[a] Number of gene copies; [b] Number of different haplogroups; [c] Number of different haplotypes; [d] Haplotype diversity index; [e] Nucleotide diversity; [f] Number of polymorphic sites; [g] MPD, Mean number of pairwise differences (based on SNP data). * - unpublished data, database of the Institute for Anthropological Research, Zagreb.

Table V. AMOVA—pairwise difference distance method among six groups of populations (1 = Dubrovnik, 2 = Lastovo, 3 = Mljet, 4 = other Dalmatian islands, 5 = neighbouring Balkan countries and Croatian mainland, 6 = other European countries).

Source of variation	d.f.	Sum of squares	Variance components	% of variation	Fixation indices	p-value
Among groups	5	113.943	0.03138 Va	0.63	Fct = −0.00629	0.00978
Among populations within groups	11	135.060	0.04392 Vb	0.88	Fsc = 0.00886	0.0000
Within populations	2699	13 265.182	4.91485 Vc	98.49		
Total	2715	13 514.184	4.99015		Fst = 0.01509	0.0000

section showed notable differences between them, mostly attributed to genetic drift/founder effect. However, these micro-evolutionary processes came into action as a result of specific demographic events these populations experienced, such as fluctuations in population size (e.g. bottlenecks resulting from introduced diseases) and post-founding migrations. In general, the first period in history that brought significant input to the gene pool of eastern Adriatic islanders was probably between the 6th and 8th century AD, in the time of the colonization by the Slavic tribes Croats. The second migration period occurred between the 15th and the 18th century, at a time characterized by Turkish expansion into southeast Europe and the Balkan Peninsula and their conflicts with Venice, resulting in great migrations from the mainland to the Adriatic islands. Important historical data in our context is that both islands of Mljet and Lastovo were under the rule of the Republic of Ragusa (historical name for Dubrovnik) from the beginning of the 15th until the end of the 18th century. During this period the island of Mljet was used as 'lazaretto' (quarantine station) in times of epidemics, which were common in medieval times (Saftić et al. 2006). The island was perfect for this role since it was only scarcely inhabited and quite inhospitable—a large number of poisonous snakes was at that time occupying the island (Gjurašić 2010). The globally well-known term 'quarantine' was first used in 1377 in the Republic of Ragusa and was related primarily to plague. Subjects suspected to carry these contagious diseases were segregated and sent to isolation, mostly to Mljet, where the first 'lazzareto' in the world was opened in 1397 (Gensini et al. 2004; Gjurašić 2010). Apart from those carrying plague, Mljet was also home for people with leprosy and *Mal de Meleda*. *Mal de Meleda* represents Mljet's own indigenous disease, which made the island very well known in the world of epidemiology (Saftić et al. 2006). This dermatologic condition is autosomal, recessive and originated on the island between the 14th and early 19th century, at the time of the Republic of Ragusa. As mentioned, the island's reproductive isolation and high consanguinity level enabled the increased frequency of homozygous genotypes and higher prevalence of this medical condition among Mljet's inhabitants. However, the disease is not restricted only to Mljet. Cases have also been reported in Italy, Northern Africa and the Middle East and the most wide-spread hypothesis is that the mutation originated on Mljet and was then spread by sailors through trading routes of the medieval Republic of Ragusa (Saftić et al. 2006). In a clinical study conducted by Kolčić et al. (2006), Mljet was also shown to be a great outlier, since it presented a population with extremely high occurrence of metabolic syndrome (53%), which is a cluster of cardiovascular risk factors that greatly increase the risk for developing diabetes, cardiovascular and renal disease. These results, the highest reported for a Croatian population, suggest a connection with inbreeding and the isolation level of the island, which is now also confirmed at the maternal level and that can be interpreted in the light of the role the island played in the past.

The island of Lastovo is the southernmost open-sea Croatian island, situated ~ 50 km away from the mainland. Because of its geographical position, it has been an important point of the sailing routes connecting the western and eastern coast of the Adriatic since Neolithic times (Forenbaher 2009). An important historical event that affected the demography of the island is also connected to the Republic of Ragusa. In 1310, through the proclamation of the Lastovo Constitution, the island accepted the rule of the Republic and in return was freed from immigration and land purchase. That resulted in the island's complete isolation for the next 500 years and, thus, it escaped the main medieval epidemic waves (Jeran 2010). Lastovo's oral tradition states that initial attempts to establish a 'lazzareto' on the island (because of its suitable distance from the mainland) soon failed (Gjurašić 2010). The connections with the mainland were poor and it was hard to ensure enough food supplies for the ill, so the island didn't follow Mljet's destiny. Its main role remained the strategic importance of its position for the sailing routes going through the Adriatic. General scarcity of historical documents (or their complete absence in the Middle Ages) and existence of strong oral tradition are signs of the great autonomy Lastovo had in that period. The island is also specific from an epidemiological point of view, since a striking 7-times higher prevalence of ovarian cancer has been reported for its female inhabitants, in comparison to the general Croatian and European populations (Rudan I 2001). The disease affects women younger than 45 years of age and does not in any sense differ from ovarian cancer cases in the rest of the population. However, the difference in the prevalence is not negligible and further analysis has shown a strong familial clustering (Saftić et al. 2006). Similarly high prevalence has been observed for familial congenital hip dislocation, found in unusually high frequency on Lastovo (Maričević 1995; Saftić et al. 2006). In the previously mentioned study by Kolčić et al. (2006), this island was also investigated in the context of metabolic syndrome research and the result showed a 30% high prevalence of this disease. Considering the fact that the same

study reported a considerably lower prevalence of metabolic syndrome in other Mediterranean countries (Greece, Italy, Spain), observed increased results on Lastovo can be interpreted in the light of consanguinity practice and inbreeding. Significantly high inbreeding levels for the three Dalmatian islands (Mljet, Vis and Lastovo) have been determined in 2006, based on a study of 26 autosomal STR loci by Vitart et al. (2006) and other genetic specifics of Croatian islands have been described even earlier, in studies conducted by Rudan I, Rudan P and collaborators (Rudan P et al. 1987, 2003, 2004; Rudan I 2001; Rudan I et al. 1999, 2002, 2003, 2006).

As for Dubrovnik, a city that was for many centuries a trading centre of the wealthy maritime Republic of Ragusa, we expected highly diversificated mtDNA haplogroups and the possible presence of some non-European lineages, as a result of an influx from the Near East or North Africa through trading paths. The observed mtDNA haplogroup and haplotype diversity in the mainland sample was indeed high, with 32 (sub)haplogroup and 89 different haplotypes. Concerning non-European markers, they are more likely to be found on the Y chromosome, since trading and sailing were mostly male occupations.

An observed lack of certain haplogroups in our insular populations (N1 subclades and X hg in both samples, together with K hg on Lastovo) could be explained as a consequence of founder effect or as a gradual loss resulting from random genetic drift. Bottlenecks caused by emigration waves, especially in recent times, should also be taken into account. Namely, in the 19th and early 20th century a depopulation trend was initiated on the Croatian islands—over 100 000 people emigrated from Dalmatia overseas to the US or Australia. The main reason for the onset of this trend was the economic crisis caused by the 'wine clause' (Nejašmić 1991), which disabled the export of Dalmatian wines. This crisis, together with the arrival of *Phylloxera*, affected some of the smaller island's economies (such as Mljet and Lastovo) greatly, since they were traditionally based on vine-growing. Soon after that, the second massive emigration wave followed during the first World War. Such recent bottleneck effects could have caused a substantial loss of certain (sub)haplogroups from the original population, together with a general depopulation trend that has been constant since the early 19th century in eastern Adriatic islands, and it is still an ongoing process today.

Haplotype sharing analysis showed that Mljet and Lastovo have considerably less haplotypes in common than expected. The haplotype composition on Mljet indicates a strong and clear influence of evolutionary forces, while the rare haplogroups and haplotypes present in the Lastovo sample could be explained by the island's open-sea position and possible contacts with sailors from different regions of the world. Also, Mljet's extremely low gene and nucleotide diversity and a small portion of haplotypes shared with Dubrovnik were rather unexpected results in comparison to Lastovo, since Mljet is situated relatively close to the mainland, while Lastovo's location is a 50 km distance from the coast. An island's proximity to the mainland usually ensures a relatively constant gene flow, but it seems the influx of women was higher in the population of the more distant island. It seems possible that the aforementioned historical circumstances in the demographic history of Mljet played an important role in the island's past and were responsible for its stigmatization, making it rather closed to newcomers and an undesirable place to live.

However, it has to be taken into account that our results represent only the maternal point of view and additional Y chromosome analyses would be needed to get a complete genetic picture of these Croatian populations and to be able to draw some far-reaching conclusions.

CONCLUSION

Complex patterns of genetic diversity in modern populations are a product of many layers of demographic and evolutionary events acting on different timescales, including colonization, migrations, population expansions, mutations and genetic drift. All the aforementioned shows that by analysing mtDNA of populations and by defining their levels of genetic diversity, we can gain insight into the significant influence evolutionary forces have on the genetic structure of populations. In line with prior research that has showed a connection between reproductive isolation, consanguinity (and, hence, low heterozygosity level) and presence of certain genetic hereditary diseases, the main contributions of our study are: the possibility of witnessing the effect of evolutionary forces at work and the possibility of detecting genetically extremely homogenous populations, suitable for further genetic-epidemiological research. The power of isolates for genetic research lies in pedigrees descended from a small number of founders, living in environmental homogeneity and restricted geographical distribution, with inbreeding as a norm. Although a powerful tool for population genetic and epidemiological studies, such communities are most likely to disappear in the near future. The depopulation process and population ageing are the main factors of Croatian insular demography and some island communities already find themselves on the verge of extinction. That is why we see our results as a valuable contribution to the wider genetic picture of Croatia and Europe and, hopefully, to further research in genetic epidemiology.

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