
Keywords: 7HR/8IT/Canis lupus/colonization/genetic structure/genetics/landscape genetics/Malme/population expansion/wolf

Abstract: After centuries of range contraction and demographic declines wolves are now expanding in Europe, colonizing regions from where they have been absent for centuries. Wolf colonizing the western Alps originate by the expansion of the Italian population. Vagrant wolves of Italian and Dinaric-Balkan origins have been recently observed in the Eastern Alps. In this study we compared the genetic structure of wolf populations in Italy and Croatia, aiming to identify the sources of the ongoing recolonization of the Eastern Alps. DNA samples, extracted from 282 Italian and 152 Croatian wolves, were genotyped at 12 autosomal microsatellites (STR), four Y-linked STR and at the hypervariable part of the mitochondrial DNA control-region (mtDNA CR1). Wolves in Croatia and Italy underwent recent demographic bottlenecks, but they differ in genetic diversity and population structure. Wolves in Croatia were more variable at STR loci ($N_A=7.4$, $H_D=0.66$, $H_E=0.72$; n=152) than wolves in Italy ($N_A=5.3$, $H_D=0.57$, $H_E=0.58$; n=282). We found four mitochondrial DNA (mtDNA CR1) and 11 Y-STR haplotypes in Croatian wolves, but only one mtDNA CR1 and three Y-STR haplotypes in Italy. Wolves in Croatia were subdivided into three genetically distinct subpopulations (in Dalmatia, Gorski kotar and Lika regions), while Italian wolves were not sub-structured. Assignment testing shows that the eastern and central Alps are recolonized by wolves dispersing from both the Italian and Dinaric populations. The recolonization of the Alps will predictably continue in the future and the new population will be genetically admixed and very variable with greater opportunities for local adaptations and survival.
Original Investigation

Genetic structure of expanding wolf (Canis lupus) populations in Italy and Croatia, and the early steps of the recolonization of the Eastern Alps

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A B S T R A C T

After centuries of range contraction and demographic declines wolves are now expanding in Europe, colonizing regions from where they have been absent for centuries. Wolf colonizing the western Alps originate by the expansion of the Italian population. Vagrant wolves of Italian and Dinaric-Balkan origins have recently been observed in the Eastern Alps. In this study we compared the genetic structure of wolf populations in Italy and Croatia, aiming to identify the sources of the ongoing recolonization of the Eastern Alps. DNA samples, extracted from 282 Italian and 152 Croatian wolves, were genotyped at 12 autosomal microsatellites (STR), four Y-linked STR and at the hypervariable part of the mitochondrial DNA control-region (mtDNA CR1). Wolves in Croatia and Italy underwent recent demographic bottlenecks, but they differ in genetic diversity and population structure. Wolves in Croatia were more variable at STR loci (N_a = 7.4, H_a = 0.66, H_t = 0.72; n = 152) than wolves in Italy (N_a = 5.3, H_a = 0.57, H_t = 0.58; n = 282). We found four mitochondrial DNA (mtDNA CR1) and 11 Y-STR haplotypes in Croatian wolves, but only one mtDNA CR1 and three Y-STR haplotypes in Italy. Wolves in Croatia were subdivided into three genetically distinct subpopulations (in Dalmatia, Gorski kotar and Lika regions), while Italian wolves were not sub-structured. Assignment testing shows that the eastern and central Alps are recolonized by wolves dispersing from both the Italian and Dinaric populations. The recolonization of the Alps will predictably continue in the future and the new population will be genetically admixed and very variable with greater opportunities for local adaptations and survival.

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Introduction

During the last few decades, populations of ungulates and carnivores, which declined in the past because of Pleistocene climate changes (Sommer and Benecke 2005; Sommer and Nadachowski 2006; Hofreiter and Stewart 2009) or anthropogenic pressures (Rosser and Sue 2002), began to expand, recolonizing parts of their historical ranges. Expanding carnivore populations often met and admix in contact zones (Hailer and Leonard 2008). The outcomes of hybridization are variable, depending on the extent of genetic divergence and origin of the parental populations. Hybridization and subsequent introgression might disrupt coadapted gene pools, threatening the integrity of local populations or entire species and leading to genetic extinctions (Allendorf et al. 2001). In other cases, admixture zones might generate novel gene arrays that are filtered by natural selection, eventually increasing the fitness of admixing populations (Arnold and Martin 2009; Kays et al. 2010). Moreover, gene flow might help rescuing genetically depleted and isolated inbred populations (Tallmon et al. 2004). Wolves (Canis lupus) in Europe strongly declined in the last centuries due to human persecution and decline of their natural prey (Breitenmoser 1998). Isolated populations survived in Iberia and southern Italy, while larger wolf populations remained in Eastern Europe, particularly in the Carpathian and Dinaric-Balkan mountain ranges (Linnell et al. 2008). Wolves in Italy and Croatia shared partially similar demographic histories. Wolves in Italy were isolated south of the Alps at least for one century, but probably for the past few thousands years (Lucchini et al. 2004), and are genetically differentiated from all the other wolf populations in the world (vonHoldt et al. 2010; their Fig. 3). At demographic bottleneck in the 70’s wolves in Italy survived split in two fragmented subpopulations of less than 100 individuals.

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confined to remote mountain areas of the central and southern Apennines (Boitani 2003). Thereafter, beginning from the ‘80s, wolves expanded rapidly recolonizing the Apennines and moving towards the Italian and French Western Alps where they established a stable transborder population (Fabbri et al. 2007; Marucco et al. 2012). Now the Western Alpine and Italian Apennine wolf population might number c. 800–1000 individuals (Linnell and Boitani 2012).

Wolves were widely distributed across all Croatia until the end of the 19th century (Friković and Huber 1992). Hunting and eradication programmes in the 20th century wiped them out from most of the country. Wolves declined to 30–50 individuals in the 1980s, confined to Croatian mountains in the Gorski kotar and Lika regions. This population always remained and still is connected with the neighbouring small Dinaric wolf population in Slovenia (Adamič 1992), and with a larger one (about 400 individuals) in Bosnia and Herzegovina (Kusak 2010). By the early 1990th, wolves in Croatia started to grow to a current estimated size of 175–240 individuals (Kusak and Huber 2010a). During the last 20 years wolves spread from the Gorski kotar and Lika regions to neighbouring Dalmatia, Banija and Župa regions, reconstructing a single larger and continuous population (Kusak and Huber 2010b).

The central and eastern sectors of the Alps are currently explored by young male wolves in dispersion, which did not yet establish stable reproductive packs (Fig. 1) (Boitani 2000; Weber and Fattebert 2008). Wolves of Italian and eastern European origins have been recurrently detected in Switzerland, Austria and in the eastern Italian Alps (Salvatori and Linnell 2005). A GPS-collared male, named “Slavc”, born in Slovenia in 2010 left the Dinaric population in 2011 reaching first Trentino and then the Veneto regions in Italy in 2012 (Written by Krofel; http://www.volkovi.si/en/blog/233-gibanje-z-ovratnico-opremjeneh-volkov-v-2012; LIFE project: SloWolf http://www.volkovi.si/). Slavc met a first female of Italian origin (named V1302F in this study) that was soon found poisoned in August 2012 (as reported by the local press). In the same area another female of Italian origin (TN2F), travelling with Slavc, was genetically identified from scat samples collected along snow tracks in Autumn 2012. The first successful reproduction between these two individuals (Slavc and TN2F) was reported in August 2013 by the local press (http://www.larena.it/stories/Home/550274_si_primo_brano_di_lupidele_alpi_orientali/SloWolf: http://www.volkovi.si/en/archive/284-slavc-in-2013-materiale-new-and-confirm-by-managers-of-the-regional-Natural-park-of-Lessinia-pers.comm.). Wolf recolonization is characterized by early and sporadic episodes of long-distance dispersal, followed by settings of stable reproductive packs (Valière et al. 2003). Habitat suitability analyses suggest that wolf expansion in the Alps might continue in the future (Marucco and Mclntire 2010). Consequently, wolves of Italian and Dinaric-Balkan origins have chances to continue to breed in the Alps, generating new genotypes and increasing genetic diversity through gene flow, with novel opportunities for local adaptations and evolution (Hedrick and Fredrickson 2010).

This study was planned to describe the genetic structure of the expanding Dinaric and Italian wolf populations at the very early stages of their expansion towards the central and eastern Alps and before the beginning of their predicted admixture process. Aims of the study are to: (1) estimate and compare the genetic variability of the two populations; (2) describe the genetic differentiation between them; (3) to explore local population structuring; and (4) identify the origin of wolves colonizing the central and eastern Italian Alps. These results are being used to build a baseline population genetic dataset, which will help to monitor the current and predict the future dynamics of the wolf populations in the Alps.
Table 1
Population, country and region of origin of wolf individuals used in this study (see Fig. 1). F: females; M: males; U: sex unknown.

<table>
<thead>
<tr>
<th>Population</th>
<th>Country</th>
<th>Region</th>
<th>F</th>
<th>M</th>
<th>U</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dinaric</td>
<td>Croatia</td>
<td>Gorski kotar (GK)</td>
<td>21</td>
<td>19</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>Balkan</td>
<td></td>
<td>Lika (LI)</td>
<td>16</td>
<td>24</td>
<td>1</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dalmatia (DA)</td>
<td>25</td>
<td>31</td>
<td>3</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>Bosnia &amp; Herzegovina</td>
<td>Banovina and Pannonia</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Apennine</td>
<td>Italy</td>
<td>Western Alps (ALP)</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Northern Apennines (NAP)</td>
<td>51</td>
<td>59</td>
<td>0</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Central Apennines (CAP)</td>
<td>65</td>
<td>74</td>
<td>1</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Southern Apennines (SAP)</td>
<td>10</td>
<td>14</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>Alps (dispersers)</td>
<td>Italy</td>
<td>Eastern Alps</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

Material and methods

Sample collection

From 1996 to 2011 we collected 152 wolf samples in Gorski kotar (GK; n = 40), Lika (LI; n = 41) and Dalmatia (DA; n = 59) regions in Croatia (Table 1). Three additional samples from Banovina and Pannonia were added to the Gorski kotar group; nine samples from Bosnia and Herzegovina, very close to the Croatian border, were added to the Dalmatian group. Wolves in Italy (n = 282) were collected from 1990 to 2009 in the Western Alps (ALP; n = 7), Northern Apennines (NAP; n = 110), Central Apennines (CAP; n = 140) and Southern Apennines (SAP; n = 25). All samples were obtained from wolves killed by traffic accidents, legal hunting, poaching, disease, or live-captured for radio-tracking (Kusak et al. 2005). We analyzed DNA extracted from 19 non-invasive samples (scats, hairs and urine) collected during field monitoring programmes in the Central and Eastern Italian Alps and corresponding to five different individuals. Wolves showing phenotypic anomalies (in body dimension, coat colour, etc.), or signals of genetic admixture with dogs (Caniglia et al. 2013a) were discarded and used in a companion study on wolf × dog hybridization (in prep.).

DNA extraction and analysis

DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, USA; Croatian wolves) or the DNEasy Blood and Tissue Kit (Qiagen, USA; Italian wolves). Samples were genotyped at 12 autosomal microsatellites (STR, Supplementary Table S1), and at four Y-linked STRs (MSY34A, MSY34B, MSY41A, MSY41B; Sundqvist et al. 2001). Amplifications were carried out in PCR volumes of 8 μl containing 1 μl of DNA solution, corresponding to c. 20–50 ng of DNA from tissue or blood samples, or 2 μl for extractions obtained from non-invasive samples for which it is difficult to estimate wolf DNA concentrations, and multiplexed using a Qiagen Multiplex PCR Kit (Qiagen, Germany). Amplicons were analyzed in an 3130xl ABI Genetic Analyzer with software GENEMAPPER 4.0. (Life Technologies, Applied Biosystems). The power of markers to identify each unique genotype were calculated by probability-of-identity values (PID and PIDSibs; Waits et al. 2001) in GENALEX 6.41 (Peakall and Smouse 2006). Individual genotypes were matched to exclude duplicates. The non-invasive samples were analyzed using a multi-tube protocol to avoid genotyping errors and obtain reliable genotypes (Caniglia et al. 2013b). About 10% of the tissue samples were randomly selected and independently replicated twice to assess the occurrence of allelic dropout and false alleles. Allele binning and presence of null alleles were computed in MICROCHECKER (Van Oosterhout et al. 2004) with an adjusted p value corresponding to α = 0.05 after Bonferroni correction (Rice 1989). Partial sequences (280 bp) of the hypervariable part of the mitochondrial DNA control-region (mtDNA CR1) were obtained using PCR primers CR1 and CR2R (Palomares et al. 2002) and a protocol described in Gomerˇcic et al. (2010). The sequences were manually edited and aligned in BioEdit (Hall 1999). Identical haplotypes were collapsed using DNASP 5 (Librado and Rozas 2009) and matched in GenBank.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mambio.2013.10.002.

Estimates of genetic variability and population structure

The mtDNA CR1 alignment was analyzed in DnaSP to estimate haplotype (DH) and nucleotide (Pi) diversity, nucleotide differences (k) and Tamura and Nei’s (1993; TN93) genetic distances among populations. Multilocus STR genotypes were analyzed in GENALEX to estimate: allele frequency by locus and population, observed (H0) and expected unbiased (UH) heterozygosity, mean number of alleles per locus (Nk), and the number of private alleles per population (Np), that is the number of alleles unique to a single population. Allelic richness (Na), the number of alleles independent on sample size, was estimated by Hurlbert’s (1971) rarefaction index in FSTAT 2.9.3.2 (Goudet 1995). GENALEX was used also to estimate Weir and Cockerman (1984)’s F-statistics. The Wright’s inbreeding estimator FIS and tests for departure from Hardy–Weinberg equilibrium (HWE) were computed over loci using GENETIX 4.03 (Belkhir et al. 1996-2004). Linkage equilibrium (LE) for each pair of loci were computed in the populations by exact test and a Markov chain method in GENEPOP 4.0 (Rousset 2008).

Population structure was inferred by model-based Bayesian clustering (assuming HWE and LE in the genetic clusters) implemented in STRUCTURE 2.3 (Pritchard et al. 2000; Falush et al. 2003) and GENELAND 4.0.2 (Guillot et al. 2005), and by model-independent multivariate analyses in ADegenet (Jombart et al. 2008). Structure infers population structure and simultaneously assigns individuals to populations, independent of any prior non genetic information. The number of populations was estimated for K = 1–10 with the admixture model. The independent allele frequencies model (f-model) was used to infer the global structure in the sample; the correlated allele frequencies model (F-model) was used to infer the fine-scale structure within Croatian and Italian wolf populations. We used also sampling locations as prior information applying the locprior model (Hubisz et al. 2009). The optimal K values were identified by ΔK, the major rate of increase in the posterior probability of the data (Ln P(D)) between each successive K (Evanno et al. 2005). We performed five independent runs for each K with 200,000 MCMC discarding the first 50,000 burn-ins.

GENELAND uses samples’ spatial coordinates and genotypes to map the range of genetic clusters in the space and to assign individuals to each subpopulation. The optimal K were evaluated from 1 to 10, with 200,000 MCMC iterations. Uncertainty on sampling coordinates were accounted for a delta parameter value = 0.15, corresponding to 10 km, the distance that a wolf can daily travel during
feeding activities or in dispersion (Ciucci et al. 1997; Kusak et al. 2005). A discriminant analysis of principal components (DAPC) in ADegenet was used to plot the maximum separation between groups minimizing the within-group variance, independent of any population genetic model. Multivariate analyses of spatial genetic patterns (sPCA in ADegenet) were used to find the individual scores that maximize the product of variance and spatial autocorrelation (Jombart et al. 2008).

Assignment testing and gene flow

The Rannala and Mountain’s (1997) method in GeneClass 2 (Piry et al. 2004) was used to estimate rates of contemporary gene flow among wolf subpopulations in Italy and Croatia. Dispersing wolves in the Alps were assigned to their source populations with the Paetkau et al.’s (2004) frequency-based model and the leave one out option in Genealex, and using STRUCTURE with the no-admixture model and option useupinfo activated. In this way, the Italian and Croatian wolves were a priori assigned to their predefined clusters (PopFlag = 1), while the unflagged individuals (PopFlag = 0) were identified as immigrants and assigned to their respective populations of origin. STRUCTURE results were compared with an assignment test in GeneClass2, computed using Rannala and Mountain (1997) Bayesian method with 10,000 simulated individuals.

To explore how genetic variability might change in a future admixed wolf population in the Eastern Alps we have used the software HYBRIDLAB 1.0 (Nielsen et al. 2006) to sample 5, 10, and 15 migrants from the Italian (north Apennines and Alps) and Croatian (Gorski kotar and Lika) source populations and generate 20 first generation (F1) and 20 second generation (F2) hybrid genotypes. The genetic variability (number of alleles and heterozygosity) in the simulated populations was compared with the variability in Croatian and Italian wolf population.

Table 3

<table>
<thead>
<tr>
<th>Country</th>
<th>Region (n)</th>
<th>Y-STR</th>
<th>mtDNA CR1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nh</td>
<td>Dh</td>
<td>Uh</td>
</tr>
<tr>
<td>Croatia</td>
<td>Total HR (70)</td>
<td>11</td>
<td>0.51 (0.18)</td>
</tr>
<tr>
<td></td>
<td>GK (17)</td>
<td>6</td>
<td>0.45 (0.16)</td>
</tr>
<tr>
<td></td>
<td>LI (24)</td>
<td>9</td>
<td>0.49 (0.17)</td>
</tr>
<tr>
<td></td>
<td>DA (29)</td>
<td>7</td>
<td>0.5 (0.18)</td>
</tr>
<tr>
<td>Italy</td>
<td>Total IT (145)</td>
<td>3</td>
<td>0.16 (0.08)</td>
</tr>
<tr>
<td></td>
<td>ALP (2)</td>
<td>1</td>
<td>0.00 (0.00)</td>
</tr>
<tr>
<td></td>
<td>NAP (57)</td>
<td>2</td>
<td>0.10 (0.06)</td>
</tr>
<tr>
<td></td>
<td>CAP (72)</td>
<td>3</td>
<td>0.21 (0.10)</td>
</tr>
<tr>
<td></td>
<td>SAP (14)</td>
<td>2</td>
<td>0.11 (0.06)</td>
</tr>
</tbody>
</table>

Results

Genetic variation in Croatian and Italian wolves

All 12 microsatellites were polymorphic in the two populations, showing from three to 14 alleles per locus (Supplementary Table S1). Only locus CPH2 in Dalmatian samples showed evidence of null alleles; no other PCR error (allelic dropout, false alleles) was detected, and all loci were used to analyze the data set. Wolves in Croatia showed larger genetic variation than in Italy. Heterozygosity in the Croatian wolves (Hd = 0.66, Uh = 0.72) was 15–20% larger than in the Italian wolves (Hd = 0.57, Uh = 0.58; Table 2). Croatian wolves had c. 2 more STR alleles per locus than the Italian wolves, on average. The distribution of genetic variability (heterozygosity and allele numbers) was even in the three Croatian wolf subpopulations, although wolves from Gorski kotar showed slightly reduced, heterozygosity (Table 2; pairwise t-tests of H0 by locus between GK and LI: t-value = −2.53, p-value = 0.03, and between GK and DA: t-value = −0.75, p-value = 0.47). Wolves in the Western Italian Alps were less variable than wolves in the three sectors of the Apennines but not significantly (Table 2; pairwise t-tests of H0 per locus between ALP and NAP: t-value = −1.21, p-value = 0.251, between ALP and CAP: t-value = −1.38, p-value = 0.197; between ALP and SAP: t-value = −1.60, p-value = 0.138). The number of low frequency alleles (at threshold f<0.05) was Nk(0.05) = 4.7 and 3.3 on average per locus in Croatian and Italian wolves, respectively. Allelic richness for the smallest sample size n = 133 was significantly lower in Italian (NAk = 5.0) than in Croatian wolves (NAk = 7.2) (paired t-tests of NAk per locus between IT and HR populations: t-value = −43, p-value = 0.015). Wolves in Croatia showed 37 private alleles (3.1 per locus, on average), which were not found in the Italian wolves, who instead showed 14 private alleles (1.2 per locus, on average). Among the Croatian subpopulations, wolves in Dalmatia showed...
the highest number of private alleles (Np = 13). In Italy, wolves from southern Apennines and the Alps did not show private alleles. With values of PID = 2.5 × 10^{-12}, PIDsb = 2.8 × 10^{-5} in Croatian wolves; PID = 6.3 × 10^{-15}, PIDsb = 3.1 × 10^{-4} in Italian wolves, there were no identical genotypes. Overall, the Croatian wolves were significantly out of HWE (average Fst = 0.09, p = 0.000; two loci were not in HWE) and LE (16 pairwise comparisons were not in LE). The three Croatian wolf subpopulations were not in genetic equilibrium (p = 0.005). The four Italian subpopulations were not significantly out of HWE (p = 0.055; Table 2) and LE (three pairwise comparisons were not in LE), but globally the Italian population was not in HWE at α > 0.01 (average Fst = 0.03, p = 0.002).

Three Y-STR (MSY34A, MSY34B and MSY41B) were polymorphic in Croatian and Italian wolves, while MSY41A was monomorphic in both populations. The Croatian wolves showed 11 distinct Y-STR haplotypes; the Italian wolves showed only three haplotypes (Table 3). Haplotype diversity was more than three times larger in the Croatian (Dh = 0.51) than in the Italian wolves (Dh = 0.16). The average number of alleles per haplotype was higher in Croatia (N = 3.7) than in Italy (N = 2.2). Both N and Dh were evenly distributed in the three sampled regions in Croatia, while they were different in Italy: wolves in the Western Alps were monomorphic (Dh = 0.0); only wolves in the central Apennines showed all three haplotypes detected so far in Italy (Dh = 0.21). Wolves in Croatia and in Italy did not share any Y-STR haplotype (Table 4).

The samples showed more extreme differences in the distribution of the mtDNA diversity (Table 4): the Italian wolves showed a unique mtDNA haplotype W14 (Randi et al. 2000; Pilot et al. 2010); the Croatian wolves showed four haplotypes, distributed in the three sampled regions, with one different haplotype over 30 individuals on average (Dh = 0.70; Standard Deviation SD = 0.02). Haplotype diversity was lower in the Gorski kotar (Dh = 0.36; SD = 0.09) than in Lika (Dh = 0.63; SD = 0.05) and Dalmatia (Dh = 0.72; SD = 0.03). Haplotype divergence was small and the average genetic distance was TN93 = 0.021. The nucleotide diversity Pi was low, ranging from 0.01 to 0.02 (Table 3).

Population genetic structure

Wolves from Croatia and Italy plot separately in a DAPC (Fig. 2) and were significantly differentiated (Fst = 0.20; p = 0.01). DAPC and AMOVA results also indicated that the three subpopulations in Croatia were more differentiated (Fst = 0.05; p = 0.01) than the four subpopulations in Italy (Fst = 0.01; p = 0.01). The assignment test in GENALEX showed that 73% of the Croatian wolves were assigned to their population of origin (Table 5). Wolves in Dalmatia were the most differentiated, with 83% genotypes assigned to their population of origin. In contrast, only 55% wolves from Italy were assigned to their population of origin.

STRUCTURE (with the admixture and I models) indicated an optimal K = 2 that split wolves from Croatia (q_Croatia = 0.99) and Italy (q_Italy = 0.98) into two distinct clusters (Fig. 3a) without admixture. Genotypes were assigned to the two clusters with individual proportion of admixture ranging from 0.05 to 0.98, and from 0.83 to 0.99. Croatian wolves were sub-structured in K = 2 (admixture and F models) or K = 3 (locprior and F models) subpopulations, both indicating that wolves from Dalmatia were the most different and showed the smallest admixture (Fig. 3b). Although the optimal clustering of Italian wolves was obtained at K = 2 (with or without the locprior model), individuals were not split in two distinct clusters, but all samples showed strong signal of admixture, with the partial exception of wolves in the southern Apennines, which were less admixed than the other subpopulations (Fig. 3c).

GENELAND identified three clusters in wolves from Croatia (Fig. 4a). Cluster 1 included all wolves from Dalmatia, plus two individuals sampled from Gorski kotar; wolves from Gorski kotar and
Lika were assigned to cluster 2; cluster 3 included some individuals sampled in Bosnia and Dalmatia. The Italian wolves showed no detectable genetic structuring (data not shown). The sPCA analysis gave similar results (Fig. 4b). Croatian wolves were split in two groups: Gorski kotar plus Lika, and Dalmatia. The Italian wolves did not show detectable signals of spatial structuring.

**Gene flow and assignment of vagrant wolves in the Alps**

Rates of historical gene flow estimated from $F_{ST}$ values ranged between 1.5 and 7.1 individuals per generation among sub-populations in Croatia, and between 1.9 and 22.1 among sub-populations in Italy. The lowest rates of gene flow ($Nm = 1.9–3.3$)

---

**Table 5**

<table>
<thead>
<tr>
<th>Population</th>
<th>Assignment test (GeneClass)</th>
<th>First generation migrants (GeneClass)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>From/to</td>
<td>ALP</td>
</tr>
<tr>
<td>GK (43)</td>
<td>GK</td>
<td>31</td>
</tr>
<tr>
<td>LI (41)</td>
<td>LI</td>
<td>11</td>
</tr>
<tr>
<td>DA (68)</td>
<td>DA</td>
<td>5</td>
</tr>
</tbody>
</table>

Number (proportion in percentage) of putative wolf migrants among three areas in Croatia and four areas in Italy estimated using: (1) Paetkau et al.'s (2004) assignment test, with option “leave one out” active, in GeneClass ([Peakall and Smouse 2006]); and (2) Rannala and Mountain's (1997) method in GeneClass2 ([Piry et al. 2004]). In diagonals: the number of individuals that were assigned to the population where they were sampled. In columns: number of individuals which originated in a region and that were assigned to or migrated from another region (rows).
were between wolves in the Apennines and the Alps; the largest one (Nm = 22.7) was between wolves in Central and Northern Apennines. The Bayesian estimates of contemporary gene flow (Table 5) yield 37 first generation migrants among sub-populations in Croatia (n = 20) and Italy (n = 17). Eight wolves sampled in Gorski kotar were likely migrants from Lika (n = 6) and Dalmatia (n = 2). Six wolves sampled in Lika likely migrated from Gorski kotar (n = 2) and Dalmatia (n = 4). Six wolves sampled in Dalmatia were from Gorski kotar (n = 3) and Lika (n = 3) subpopulations. Thus, recent migration rates among the three sub-regions in Croatia ranged from 9% to 19%. Recent migration rates in Italy ranged from 4% to 20%, and migration towards the Alps (14%) and southern Apennines (20%) was apparently larger than between northern and central Apennines (4.5% on average).

Three of the five solitary wolves sampled in the Eastern Italian Alps were assigned by STRUCTURE to the Croatian wolf cluster with q_c = 0.98–0.99 and narrow 90% CI (Table 6). The other two genotypes, wolves Slavc and TN2M, were assigned to the Croatian population, although with lower q_c = 0.95 and wider 90% CI. Results from GenCLASS confirmed the assignment of wolf Slavc to the Croatian population (p = 0.81), whereas TN2M was not assigned to any population at the pre-defined threshold p = 0.05. All the three wolves of presumed Italian origin were assigned to the Italian wolf population although with different p values (Table 6). These three wolves showed the Italian mtDNA CR1 haplotype W14 and male TN1M showed the Y-STR Italian wolf haplotype U that was also found in wolves in the Alps (Table 4). Thus, genetic evidences concordantly support an origin of these three wolves from the western Alpine population. The two non-Italian wolves showed mtDNA CR1 and Y-STR haplotypes widespread in the Croatian wolves, but absent in the Italian wolves (Table 6). A search in GenBank showed that mtDNA CR1 haplotype WCR01, found in these two wolves, was found only in Croatia by Pilot et al. (2010); in this study the haplotype was named W23) and it was not found in any other wolf population worldwide.

Simulated admixed populations in the Alps, originated by crossing 5, 10 or 15 simulated colonizers from each of the two source populations (Croatia and Italy), showed higher heterozygosity than in both the source populations, while the number of alleles was intermediate (Table S1).

**Discussion**

Wolves in Italy and Croatia have different levels of genetic diversity and geographical structure, suggesting distinct demographic histories. Diversity at autosomal STR, mtDNA CR1 and Y-STR haplotypes is lower in Italian than in Croatian wolves. Low diversity in Italian wolves was ascertainment also at the genomic level by genotyping large numbers of SNPs (40–60 K) in DNA microarray experiments (vonHoldt et al. 2011; Stronen et al. 2012). Hence, the consequences of the demographic bottlenecks have been stronger in Italian wolves, or their isolation from other populations have been deeper (Lucchini et al. 2004, vonHoldt et al. 2011). Croatian wolves maintained high genetic variation, perhaps due to permanent gene flow with wolves from Slovenia, Bosnia & Herzegovina (Gomerčić et al. 2010) that continued despite recent habitat changes and infrastructure development (Kusak et al. 2005). In contrast, the Italian wolves are still genetically isolated from any other wolf populations. Nevertheless, both populations are showing great potential to increase and expand their ranges. Their genetic makeup is sharply different in allele frequency and number of private alleles (average FST = 0.20). None of the mtDNA CR1 and Y-STR haplotypes are shared between them.
all the individuals from the two populations are congreuntly split into two genetic clusters with 100% confidence, either using multivariate or Bayesian procedures. Genomic data further indicated that Dinaric-Balkan wolves (Croatia, Greece and Bulgaria) clustered separately from other eastern European populations, and, among them, the Croatian wolves are distinct (Stronen et al. 2012). The concomitant expansion of the Italian Apennine and Dinaric-Balkan wolves into the Central and Eastern Alps will predictably originate a new population with higher genetic diversity than in both source populations.

Wolf expansion apparently follows a propagule model (Ibrahim et al. 1996), characterized by the early long-range dispersal of single solitary individuals, mainly young males in search of new suitable territories and by the subsequent settlement of stable reproductive packs (Valière et al. 2003; Fabbri et al. 2007). This model seems confirmed by empirical data.

Table 6
Assignment of dispersing wolves sampled in the eastern Italian Alps. The columns indicate: Individual ID = the sample IDs include: the individual identification number as it is shown in Fig. 1, the sampling areas (TN: Trentino; V: Veneto); wolf Slavc was sampled in Trentino and in Veneto), and sex (M: male; F: female); N: the number of successfully genotyped non-invasive samples; Years: first and last year the genotypes were detected (3-SlavcM and 5-TN2F are presumably still alive in summer 2013; see Results); the mtDNA CR1 and Y-STR haplotypes; STRUqHR: the individual proportion of membership to the Croatian wolf population; STRUqIT: the individual proportion of membership to the Italian wolf population as inferred by STRUCTURE run with the no-admixture and usepopinfo models (90% credibility intervals in parenthesis); p to HR and p to IT: probability that individual multilocus genotypes to be encountered in a given reference wolf population: Croatia (HR) or Italy (IT) (Piry et al. 2004), as computed by GENEClass2.

<table>
<thead>
<tr>
<th>Individual ID</th>
<th>N</th>
<th>Years</th>
<th>mtDNA CR1</th>
<th>Y-STR</th>
<th>STRUqHR</th>
<th>STRUqIT</th>
<th>p to HR</th>
<th>p to IT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-TN2M</td>
<td>1</td>
<td>2009–2009</td>
<td>WCR01</td>
<td>T</td>
<td>0.95 (0.74–1.00)</td>
<td>0.05 (0.00–0.26)</td>
<td>0.02</td>
<td>0.00</td>
</tr>
<tr>
<td>2-TN1M</td>
<td>9</td>
<td>2010–2013</td>
<td>W14</td>
<td>U</td>
<td>0.01 (0.00–0.06)</td>
<td>0.99 (0.94–1.00)</td>
<td>0.00</td>
<td>0.30</td>
</tr>
<tr>
<td>3-SlavcM</td>
<td>7</td>
<td>2011–2012</td>
<td>WCR01</td>
<td>YH1</td>
<td>0.95 (0.75–1.00)</td>
<td>0.05 (0.00–0.25)</td>
<td>0.81</td>
<td>0.00</td>
</tr>
<tr>
<td>4-W1302F</td>
<td>1</td>
<td>2012–2012</td>
<td>W14</td>
<td>–</td>
<td>0.02 (0.00–0.13)</td>
<td>0.98 (0.88–1.00)</td>
<td>0.00</td>
<td>0.07</td>
</tr>
<tr>
<td>5-TN2F</td>
<td>1</td>
<td>2012–2012</td>
<td>W14</td>
<td>–</td>
<td>0.01 (0.00–0.07)</td>
<td>0.99 (0.93–1.00)</td>
<td>0.00</td>
<td>0.82</td>
</tr>
</tbody>
</table>

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Long-range dispersal events were reported in radio-collared wolves in North America (Linnell et al. 2005; their Fig. 3 and references therein). Straight-line distances >500 km were not uncommon (10/298 records = 3%; maximum 800 km), and in some cases wolves travelled rapidly (maximum c. 20 km/day). Long-distance disperser (>300 km) were mainly males (75%). Boxall local press: http://earthfirstnews.wordpress.com/2012/01/03/lone-grey-wolf-wanders-into-california-first-since-1924/ documented a GPS tracked trip of a lone wolf that travelled more than 1224 km from Oregon to North California. Dispersal distances travelled by wolves in Finland were shorter than 100 km, on average (median = 99 km, range 35–445 km; Kojola et al. 2006), but two males travelled more than 400 km, corresponding to more than 1000 km actual distance (Wabakken et al. 2001). The ongoing recolonization of eastern Germany is being sustained by wolves expanding from north-eastern Poland (Nowak et al. 2011; Czarnomska et al. 2013). A GPS-collared male wolf of German origin travelled in the opposite direction, from Germany to the east across the whole Poland, running 1200 km in 86 days (Schede et al. 2010). In December 2012, after 200 years a wolf of German origin has been recovered in Denmark (Andersen et al. in prep.).

The Western Italian, French and Swiss Alps were colonized by a few long-range dispersers originating from the northern Italian Apennine population (Lucchini et al. 2002; Vallière et al. 2003; Fabbri et al. 2007). Wolves returned in the Eastern Italian Alps in 2006, after 150 years. A found-dead wolf, genetically assigned to the Dinaric-Balkan source population, was collected in 2008 (Lapini et al. 2010). Three eastern European wolves were genetically identified in 2009 in Austria (Schäfer 2012); in 2009–2010 two wolves of eastern and Italian origin were identified in the Eastern Italian Alps (Groff and Pedrini 2009; Fig. 1). Genetic and GPS data documented with details the long-distance dispersal to the Western Alps of a wolf born in the northern Apennines in Italy (Ciucci et al. 2009). In 10 months (March 2004–January 2005) this wolf travelled a minimum distance of 958 km, corresponding to an estimate of more than 1200 km. During the last weeks of his life he joined a female of Italian origin (genetically identified), but it was found-dead before any documented reproduction event. Results in this study confirm that the recolonization of the Eastern Alps is characterized by initial long-range explorative dispersals. Wolf Slav originated in the Slovenian wolf population, very close to the Croatian border and probably in connection with the Croatian wolves. Slav left the Dinaric population in 2011, travelled 1076 km linear distance (about 2000 km on the ground; H. Potočnik, pers. comm.), reached Veneto in 2012 where subsequently met two females of Italian origin, and successfully reproduced in August 2013 (http://www.volko.si/).

The genetic structure of wolf populations in Italy and in Croatia is sharply different. Wolves in Italy are not geographically differentiated, because of the rapid expansion along the Apennine ridge. Wolves in Croatia are significantly sub-structured, the Dalmatian sub-population being sharply different from neighbouring wolves in Gorski kotar and Lika. Wolves in Dalmatia might have origins in distinct refugial areas. Historic data indicate that at the end of 19th and the beginning of 20th century both wolves and jackals existed in Dalmatia (Schwenk 1985). However, in 20th century jackals increased (Kryštufek and Tvrtník 1990), while wolves were apparently extirpated (Frtković and Huber 1992). After the WW II Dalmatia was recolonized by wolves likely dispersing from neighbouring Lika and/or Bosnia & Herzegovina (Kusk and Huber 2010a). However, if Lika wolves were the source population, Dalmatian wolves should be not genetically different. In contrast, wolves in Bosnia & Herzegovina, Serbia and Montenegro were recognized as a distinct subspecies (Canis lupus jurkaj, Describe by Bollay in 1925), and might be genetically differentiated. Milenković et al. (2010) described significant differences between skulls from the Dinaric-Balkan and Carpathian wolf populations. However, the nine wolves from Bosnia and Herzegovina analyzed in this study were not different from other wolves from Dalmatia. It is also possible that wolves were never completely extirpated from Dalmatia, but have survived in low numbers and have recovered.

Ecological and behavioural factors, such as prey selection, could contribute to shape wolf genetic structure, as reported in north America (Carmichael et al. 2001; Gefen et al. 2004) and Europe (Pilot et al. 2006, 2012; Jedrzejewski et al. 2012). The variety of wild ungulate species, with prevalence of larger species (e.g. moose Alces alces) in northern Europe (Wawrzyniak et al. 2010), and smaller species (wild boar Sus scrofa, roe deer Capreolus capreolus; Kusk and Krapince 2010) in southern Europe, may limit the gene flow among wolf sub-populations also in the absence of barriers to dispersal (Pilot et al. 2006; Czarnomska et al. 2013). Differences in body size and skull shape may influence the ability to capture and handle large prey (MacNulty et al. 2009). Costal Adriatic and lowland Dalmatian regions have dramatic climate and habitat differences from neighbouring higher Dinaric-Balkan mountain chains. Dinaric-Balkan wolves were reported to have smaller and differently shaped skulls than individuals from the Serbian portion of the Carpathian Mountains (Milenković et al. 2010), and may be advantaged in capturing smaller and swifter prey. The distribution of wolf prey species is well matching the described wolf population structure in Croatia. Red deer is abundant in Gorski kotar, rare in Lika and absent in Dalmatia (Kusk and Krapince 2010). Wild boars (plus domestic sheep and goats) are available in Dalmatia. Shifting in prey composition has been found in the diet of wolves from different regions of Croatia (Kusk 2002). Thus, differential selection associated with prey defence mechanisms and the traits required to overcome these (e.g. size versus speed) might influence the differentiation observed between wolves preying on different prey assemblages.

The genetic consequences of population expansion and colonizion are of great interest to both evolutionary and practical conservation biology. Genetic data sets obtained from monitoring programmes help to identify the origin of colonizers, to rapidly detect the onset of population admixtures, which is of special concern when feral populations are involved (Randi 2008), to estimate the extent of genetic diversity in newly founded colonies and forecast its evolutionary dynamics in isolated small populations (vonHoldt et al. 2011). The ongoing recolonization of the Alps is an interesting natural experiment that might provide empirical data and deeper insights about patterns of wolf dispersal, founder effects, gene flow rates between source and colonies, consequences of genetic admixture on gene introgression and genomic reorganization, natural selection in the wild and eventual rescue effects. The future wolf populations in Alps will be genetically more variable than both parental populations and adaptable for long-term survival.

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