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The common tern (*Sterna hirundo*) is a migratory seabird experiencing a decline in breeding pairs across several European populations due to various threats, including habitat destruction and human disturbance. This study investigates the population genetic structure and diversity of common terns sampled extensively across three European areas—Northern, Southern Inland and Southern Marine—during the breeding seasons, using 18 microsatellite markers and a mitochondrial DNA control region fragment. High genetic diversity was found in both types of markers, with the Southern Marine group showing the lowest overall diversity, although signals of possible population bottlenecks were detected in all groups. Various analyses indicated that population genetic structure was weak or absent, suggesting high gene flow among groups. The low genetic differentiation is likely influenced by distinct migration patterns, particularly between Southern Inland and Marine groups. Our results suggest that geographical distance between breeding colonies had minimal effect on population genetic structure. Further studies with tracking devices are needed to clarify how migration dynamics impacts genetic structure in common terns, while conservation efforts should focus on securing multiple breeding sites and currently unoccupied areas to increase options for habitat selection, supporting the species' genetic diversity and long-term resilience.

Keywords Genetic diversity, Population genetic structure, Microsatellite, Mitochondrial DNA

The common tern (*Sterna hirundo*) is a migratory seabird within the gull family (Laridae) that breeds in North America, Europe, and the temperate and subarctic regions of Asia and winters in South America, Africa, the tropical and subtropical regions of Asia, and the Australian coast[1]. The European population is estimated at around 881,000 to 1,430,000 individuals. The population trend is currently described as unknown, as many countries have reported only incomplete population size surveys and expert estimates with limited data. The breeding population sizes in individual countries reported in the European Red List of Birds range from a few pairs to over 50,000, with most of the larger populations found in northern and eastern Europe[2]. Common terns nest in both marine and freshwater environments in a variety of habitats but generally prefer those with scarce or no vegetation. Freshwater colonies are situated on riverbanks and gravel islands in rivers and lakes. Marine colonies nest in various types of estuarine and marine habitats, such as lagoons, salt marshes, sandy, rocky, or

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other low, flat areas. They also readily accept artificial sites, such as breeding rafts and platforms[3]. Although the common tern is listed as "least concern" on the global and European IUCN list, its populations have declined in several European countries due to various threats, such as habitat degradation, predation, competition with gulls, human disturbance and, more recently, avian influenza[3,4]. Delayed sexual maturation and low annual fecundity also contribute to their vulnerability. Inland colonies are particularly at risk as many of their natural breeding sites are threatened by flooding caused either by natural events or by hydropeaking from hydropower plants[5]. As many parts of the European continent are becoming less suitable for common terns, they are confronted with a disjunct distribution of breeding areas[3]. Most studies on common terns in Europe have focused on marine colonies, such as those on the Baltic coast and in the international Wadden Sea[6–10]. Studies of the Mediterranean colonies mostly focused on two locations: the Venice Lagoon[11,12], and the river Po Delta[13,14]. Inland colonies have been less thoroughly explored, but several studies have been published in recent years, focusing on area use[15,16], population dynamics[12], migration routes[17], and prospecting[18,19]. Until recently, it was assumed that all European common terns used the western migration route along the globally important East Atlantic Flyway to their wintering grounds in western and southern Africa, as has been found for German terns on the North Sea coast[20] or Italian terns[21]. However, recent studies using geolocators have shown that some European terns use the eastern migration route along the east coast of Africa. For example, it was found that around 70% of the birds from a colony in northern Germany used the western route and wintered on the west African coast, the Gulf of Guinea, or the South African coast. The remaining birds used the eastern route, which follows the east Mediterranean coast, with some birds making a detour via the Black Sea before continuing south along the east African coast settling in the Mozambique channel, or on the South African coast where they meet with birds using the western route[22]. Similarly, while Croatian marine colonies use the western route with wintering areas between Mauritania and Nigeria[23], Croatian inland and Hungarian terns migrate via the eastern route and winter between Tanzania and the southern Mozambique Channel[24]. Because migration routes guide birds to specific wintering areas, these geographic choices determine their nonbreeding distribution. This nonbreeding distribution, in turn, is one of the factors that potentially influence the population genetic structure of seabirds, as many species with multiple nonbreeding areas exhibit phylogeographic structure[25].

Ecological studies can detect current patterns of animal movements such as migration routes and the levels of breeding site fidelity. However, genetic markers can bring additional insights into population demographics by providing important information on population boundaries and connectivity patterns. They also provide estimates of genetic diversity, as low genetic diversity in isolated populations can have detrimental consequences, potentially leading to their collapse and disappearance. The population genetic structure and diversity of the common terns have been investigated in several studies, all of which found some level of genetic differentiation. One of the first studies used mitochondrial DNA (mtDNA) restriction fragment profiles and indicated limited genetic differentiation across the Bering Strait[26]. A later study examined five tern colonies in Lithuania using allozyme analysis and found that genetic differentiation between colonies increased with geographic distance[27]. Further investigation analysed seven Lithuanian common tern colonies using 11 microsatellite markers and found quite low genetic diversity and significant differentiation between subpopulations on the country's two largest rivers[28]. The first and only large-scale study of the population structure of the species was carried out on European and North American common tern colonies using seven microsatellite markers. This study identified four genetic clusters that correlated with sampling locations, while genetic diversity was found to be slightly higher than in other tern species[29]. The next study used six microsatellite loci and the mitochondrial cytochrome b gene fragment to assess the population structure of 12 colonies in eastern North America[30] and found relatively high genetic diversity and some evidence of hierarchical population structure.

High philopatry, which is well established in many seabird species[31], may limit gene flow and thus be one of the causes of strong population structure. Several studies on common terns have indicated high levels of site fidelity, including natal and breeding site fidelity[6,8,32]. In addition, there are differences between the sexes, i.e. male common terns, like the males of many other bird species[33], tend to be more philopatric than females[34].

Therefore, despite the absence of physical barriers to dispersal, population divergence of European populations of common terns can be expected for several reasons. Firstly, genetic divergence between geographically distant colonies has already been identified in previous studies. Secondly, the habitat choice of southern European colonies (marine versus inland) reinforced by their different respective migration routes (western versus eastern) could promote population differentiation. Lastly, high levels of philopatry suggested by ecological studies may also have caused some level of genetic differentiation among tern populations in Europe. The aim of this study is therefore to assess the population genetic structure of common tern populations sampled from twelve breeding areas across Europe, categorised into three groups (Fig. 1, Supplementary Table S1), using mitochondrial and nuclear markers, which will indirectly reveal levels of gene flow among them. Additionally, this study aims to estimate the genetic diversity of these populations, as their evolutionary potential and connectivity could be important for survival in a rapidly changing environment.

Results

Microsatellite loci

We successfully obtained multilocus genotypes of 219 common terns at 18 polymorphic microsatellite loci. Microchecker detected a low frequency of null alleles at several loci (average frequency of 3.6%), but there was no evidence of genotyping errors due to scoring of stutter peaks or large allele dropout. Genepop showed no statistically significant evidence of null alleles in the entire data set or within the groups. Linkage disequilibrium was not detected at any pair of loci after the multiple test correction, implying independent segregation of the loci used. All loci conformed to the Hardy–Weinberg equilibrium (HWE) expectations when testing the groups and the entire dataset for deviation from HWE (Supplementary Table S2).

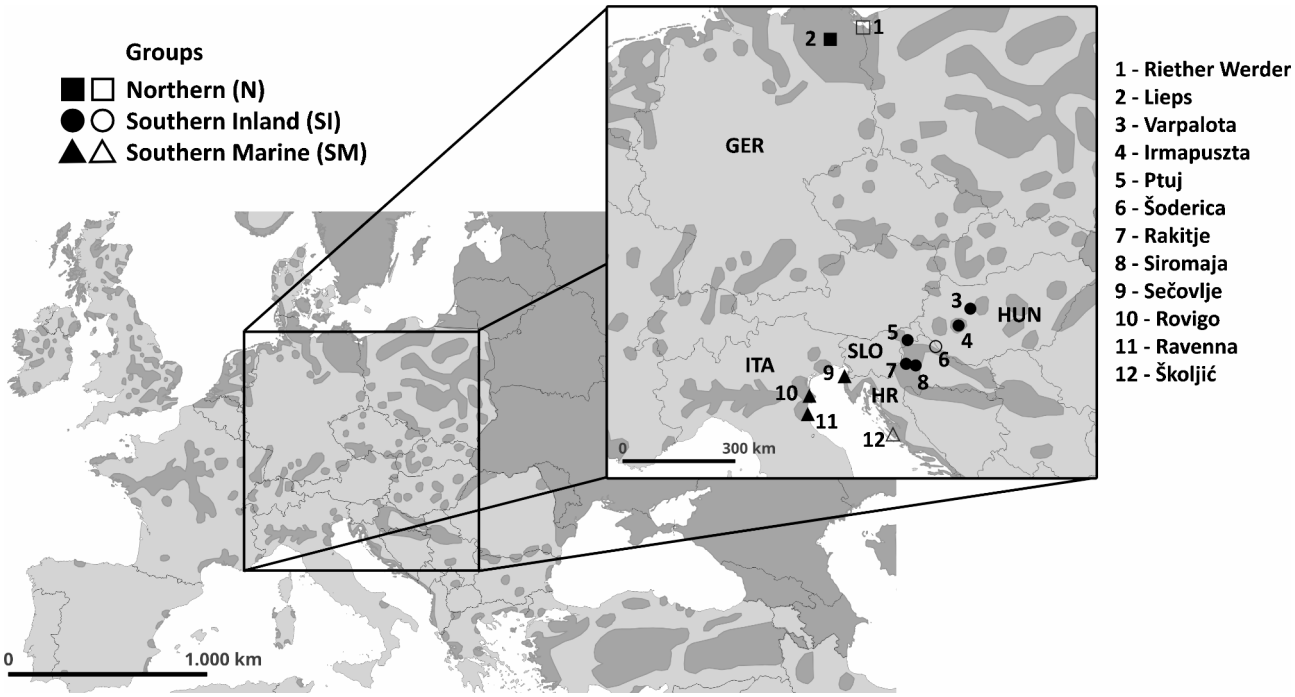

Fig. 1. Breeding distribution of the common tern in Europe (dark-shaded areas) with the sampling locations marked with corresponding numbers. Different symbols designate different groups (see Materials and methods). Filled black symbols indicate sampling locations analysed for both microsatellites and mitochondrial DNA, while transparent symbols indicate sampling locations analysed for mtDNA only. The map was created using QGIS v 3.34.3. (http://www.qgis.org) and distribution data downloaded from the IUCN Red List of Threatened Species.

Group	N	Na (S.D.)	Ar	Pa	Ho (S.D.)	He (S.D.)	Fis	Wilcoxon
Northern	65	7.889 (2.763)	7.748	11	0.705 (0.145)	0.715 (0.150)	0.009	**0.00008**
Southern Inland	95	7.944 (2.900)	7.600	6	0.709 (0.140)	0.722 (0.132)	0.015	**0.00006**
Southern Marine	59	7.222 (2.415)	7.170	4	0.671 (0.139)	0.688 (0.145)	0.019	**0.00008**
All samples	219	8.833 (3.552)	7.768	–	0.698 (0.132)	0.715 (0.140)	0.014	**0.00021**

Table 1. Microsatellite diversity indices per group and for the whole dataset with the mean number of alleles per locus (Na), mean allelic richness for a sample size of 56 individuals (Ar), the number of private alleles (Pa), mean observed (Ho) and expected (He) heterozygosity, inbreeding coefficient (F_{IS}) and probabilities for Wilcoxon's one-tailed signed rank test for heterozygosity excess, with standard deviation shown in brackets where applicable. Significant values are shown in bold ($p < 0.05$).

The number of alleles found in the entire dataset and across 18 loci was 159 and ranged from four to 17 (Supplementary Table S2), with the mean number of alleles per locus being 8.83 (Table 1). Allelic diversity was similar between the Northern (N) and Southern Inland (SI) and lower in the Southern Marine (SM) group. A similar pattern was observed when correcting for the different sample sizes of the groups (i.e. mean allelic richness). Private alleles were observed in each group (21 in total). The N group had the highest number, while the SM group the lowest. The overall observed heterozygosity was 0.70, and the expected heterozygosity was 0.71. Again, these values were similar and higher in the N and SI groups compared to the SM group. F_{IS} values were low ($F_{IS} < 0.02$) and non-significant for each group as well as overall (Table 1).

Our results suggested recent bottlenecks, as Wilcoxon signed rank tests of heterozygosity excess were significant for all groups and the entire dataset (Table 1). Additionally, the qualitative graphical method showed deviations from the normal L-shaped distribution of allele frequencies in all three groups, but not in the entire dataset (Supplementary Fig. S1).

The global F_{ST} value for the entire dataset was low but statistically significant (0.0083, $p < 0.001$). Although statistically significant, the pairwise F_{ST} values among groups were very low for each pair of groups (all $F_{ST} < 0.011$) (Table 2).

F_{ST} between sampling sites were somewhat higher than those between groups, but still very low even for pairs that had significant values (the highest F_{ST} value was 0.036, between the SI site of Rakitje and SM site of Rovigo). No regular pattern in the distribution of F_{ST} values with respect to sampling sites was evident (Supplementary

Group	Northern	Southern Inland	Southern Marine
Northern		**0.0168** (0.009)/0.0079 (0.078)	0.0110 (0.068)/**0.0211** (0.019)
Southern Inland	**0.0053**		**0.0157** (0.011)/**0.0212** (0.002)
Southern Marine	**0.0099**	**0.0105**	

Table 2. Pairwise F_{ST} and Φ_{ST} values between groups: microsatellite F_{ST} values (below diagonal), and mtDNA F_{ST} and Φ_{ST} values (above diagonal), separated by a slash. *p* values are presented as either < 0.001 or in brackets. Significant values are shown in bold ($p < 0.05$).

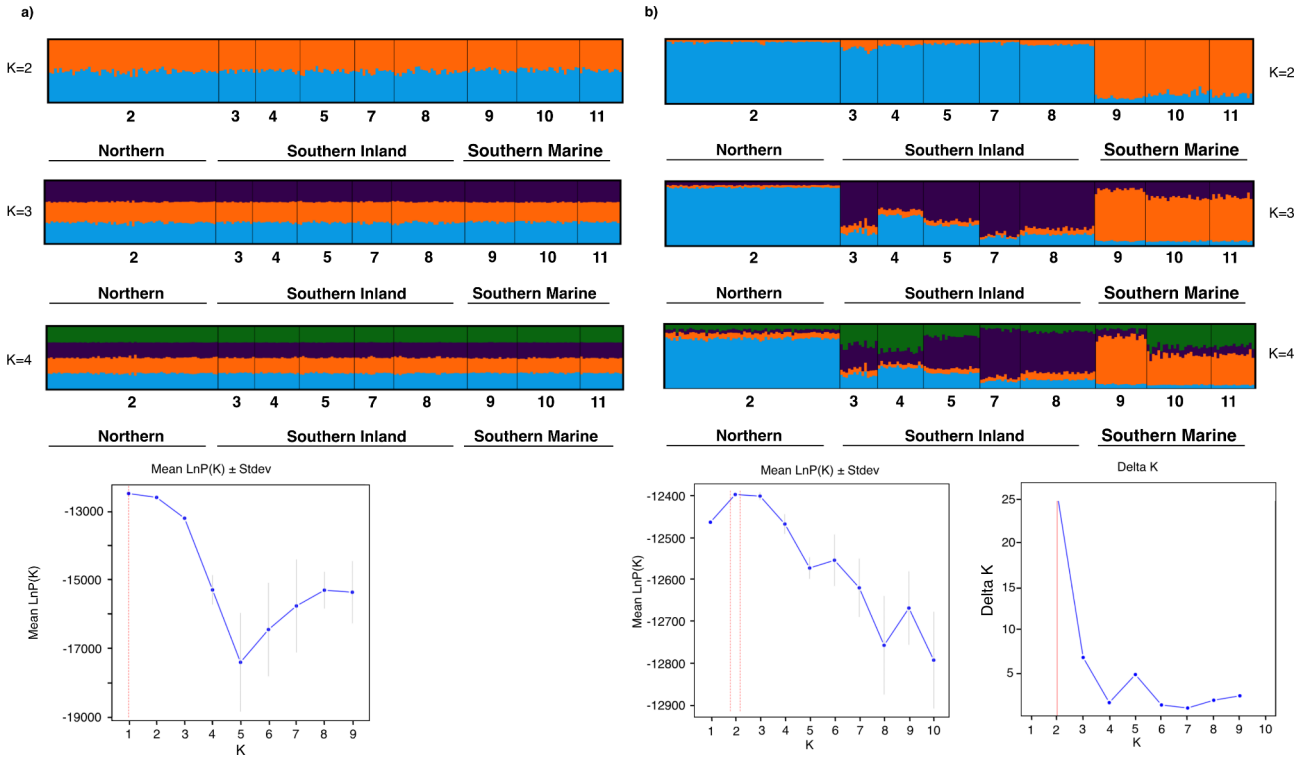

Fig. 2. Genetic clustering of common terns inferred by STRUCTURE analyses for K = 2 to K = 4. Sampling locations are represented on the x-axis and each individual is represented by a column partitioned into K segments that represent its membership fractions in K clusters. (**a**) Results obtained without localities as prior. (**b**) Results obtained using LOCPRIOR model with 9 sampling locations used as prior. Sampling location numbers correspond to those depicted in Fig. 1. Groups were not used as priors; group labels are included solely to enhance clarity and distinguish sampling locations among groups. Mean log likelihood values and the rate of change in the log probability of data between successive values of K (Delta K) are shown under their respective plots. Delta K is reported only for (**b**) as it cannot find the best K if K = 1.

Table S3). Analysis of Molecular Variance (AMOVA) indicated that only 0.83% of the genetic variance was partitioned among groups, while most of the genetic variance in the dataset occurred within groups (99.17%). The Mantel test showed no positive correlation between the genetic and geographic distance of sampling sites ($r = -0.01$, $p = 0.51$). Principal Component Analysis (PCA) analysis revealed no differentiation between the three groups (Supplementary Fig. S2) or the sampling locations (Supplementary Fig. S3).

The STRUCTURE analysis revealed no clustering in the absence of sampling location data, as the most likely number of clusters identified was K = 1 (Fig. 2a). However, re-analyses of the data using the LOCPRIOR model that incorporated information on nine sampling locations identified K = 2, followed by K = 3, as the most likely numbers of clusters, because the average log likelihood values (mean LnP(K)) were very similar for K = 2 and K = 3 with slightly more support for K = 2. The rate of change in the log probability of data between successive values of K (Delta K) showed the highest support for K = 2 (Fig. 2b). Notably, at K = 2, the sampling locations that clustered together and emerged as differentiated from the rest were exactly three locations that we had a priori categorised as the SM group (Sečovlje, Rovigo, and Ravenna). At K = 3, the clustering of five locations into the SI group and their differentiation from the N group again supported our a priori categorisation of the groups.

Mitochondrial DNA control region

We analysed 319 control region sequences, each 709 or 710 base pairs in length, and identified a total 40 haplotypes, 19 of which were novel (GenBank accession numbers PP964749-PP964767). Nine haplotypes were found in the SM group, 18 in the N group, while the largest number of haplotypes, 32, was found in the SI group (20 in a subsample of the SI group) (Table 3). We found 23 variable nucleotide sites across all samples. Two haplotypes in the N group and one in the SI group had an inserted thymine at position 39 (Stehi27, Stehi28, and Stehi35, respectively), while the remaining haplotypes had no indels (Supplementary Tables S4 and S5). Haplotype Stehi03 was found in 40.13% of all samples, while the second most frequent haplotype (Stehi01) was found in 9.72% of samples.

Overall haplotype diversity was estimated at 0.814 and nucleotide diversity at 0.0023. When comparing individual groups, the SI group and its subsample exhibited the highest diversity indices, while the N group was comparable to the SM group for most values (Table 3). Although haplotype sharing occurred between groups, several private haplotypes were detected: 6 in the N group and 22 in the SI group (Supplementary Table S5). No private haplotypes were found in the SM group. Tajima's D and Fu's F_S neutrality tests yielded predominantly negative values and were statistically significant for the entire dataset.

Pairwise F_{ST} values were statistically significant but very low for the N–SI pair (0.0168) and SI–SM pair (0.0157), and the global F_{ST} value was similar to pairwise values, 0.01526 ($p < 0.01$) (Table 2.). Global Φ_{ST} value was almost identical (0.01578, $p < 0.01$), but pairwise values were slightly higher (N–SM pair 0.0211, SI–SM 0.0212; Table 2.). After subsampling the SI group and re-analysing pairwise mtDNA F_{ST} and Φ_{ST}, the values were still below 0.035 for F_{ST} and below 0.038 for Φ_{ST}, which suggests that unequal group size did not substantially influence our results (Supplementary Table S6). Like the microsatellite results, additional analysis by 12 sampling locations did not reveal any meaningful pattern of differentiation based on geographic location (Supplementary Table S7). Significant pairwise F_{ST} ranged from 0.02533 to 0.0971, while pairwise Φ_{ST} values were overall higher and ranged from 0.04081 to 0.15177.

No significant correlation was found between geographic and genetic distances (Mantel test $r = 0.08$, $p = 0.33$). Spatial analysis of molecular variance (SAMOVA) results did not reveal any biologically meaningful grouping. The TCS haplotype network displayed a web-like topology, with the most common haplotype, Stehi03, being ancestral to some, but not all haplotypes (Fig. 3). The network did not show any obvious differentiation into haplotype lineages.

Discussion

The initial results of this investigation suggested an almost complete absence of population genetic structure in our dataset. Not only were classical estimators of population differentiation very low (global and pairwise F_{ST} values among groups were below 0.011 for microsatellite data and below 0.017 for mtDNA data, with Φ_{ST} values for mtDNA data below 0.021), but other analyses, including SAMOVA, PCA and Mantel tests, also did not reveal any clustering or correlation between genetic and geographic distances for either microsatellite or mtDNA data. Even the Bayesian clustering method, when run without LOCPRIOR option, supported these results by revealing homogenous distributions of individual microsatellite genotypes (Fig. 2a). That finding generally contrasts with previous studies on common terns based on microsatellite analyses. For instance, the Mantel test conducted on seven common tern colonies in Lithuania found a positive correlation between genetic and geographic distances and an F_{ST} value of 0.1463[28], while we found no correlation between these distances in our dataset and a global F_{ST} of only 0.0083. This is further contrasted by the geographic distances between colonies in each investigation as the maximum distance between Lithuanian colonies is about 300 km, whereas the distance between our Northern group and two Southern groups exceeds 800 km. Further, evidence of strong population differentiation in common terns in the North Atlantic region and two European colonies was found[29] with their global F_{ST} value (0.16) similar to that found in Lithuania[28] and again, much higher than in our research. This is less surprising as the geographic distances between colonies in that investigation are much higher, some measuring more than 5000 km. In the research conducted in eastern North America[30] on a somewhat smaller spatial scale (around 2700 km), the global F_{ST} value of 0.013 is only slightly higher than in our investigation. Taken together, this suggests that the mere spatial distance between breeding colonies has a weak influence on the extent of population genetic structure. This is consistent with the fact that the common tern is a highly mobile species, traveling thousands of kilometres from its wintering to breeding areas. Therefore, the distance between breeding colonies might only impact their differentiation over a larger geographic extent, presumably on an intercontinental scale. Similar to our initial findings, and contrary to expectations, no genetic structure

Group	N	H	Hd	π	k	S	P	Tajima's D	Fu's F_S
Northern	85	18	0.738	0.0018	1.296	11	6	−1.188	**−13.717**
Southern Inland	153	32	0.862	0.0027	1.914	20	22	−1.298	**−27.386**
Southern Inland (subsample)	81	20	0.880	0.0028	1.969	16	10	−1.12332	**−12.42021**
Southern Marine	81	9	0.773	0.0018	1.296	6	0	0.030	−2.388
All samples	319	40	0.814	0.0023	1.610	23	–	**−1.471**	**−28.718**

Table 3. Mitochondrial DNA genetic diversity indices per group and for all samples. N, number of samples; H, number of haplotypes; Hd, haplotype diversity; π, nucleotide diversity; k, mean number of pairwise differences; S, number of segregating sites; P, number of private haplotypes. Significant values are shown in bold ($p < 0.05$).

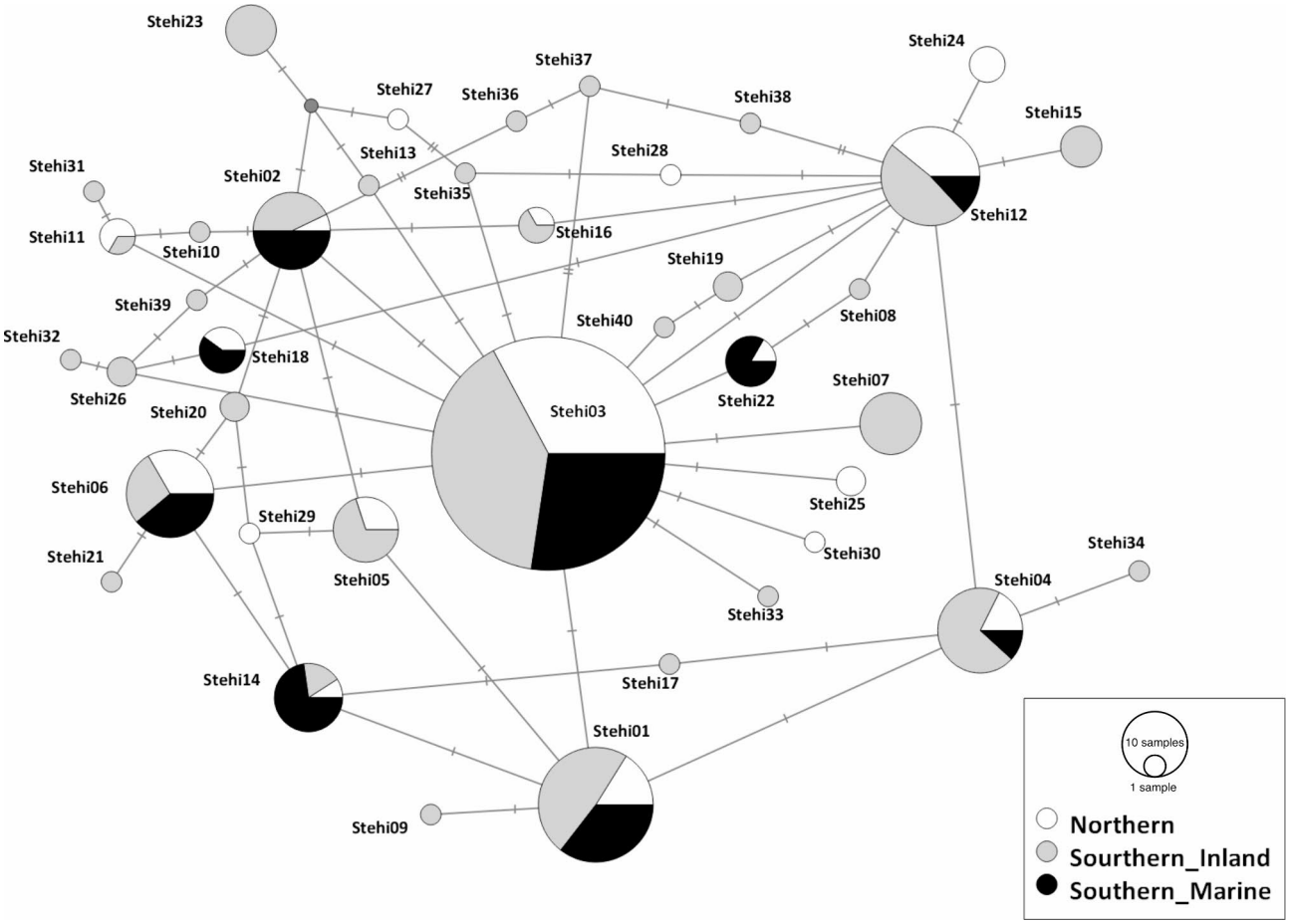

Fig. 3. TCS network showing phylogenetic relationships among European common tern mtDNA control region haplotypes. Different shades represent different groups (N, SI, SM). The size of the circles represents the haplotype frequency. The mutations between the haplotypes are indicated by hash marks.

was detected in Antarctic prions (*Pachyptila desolata*) sampled from two geographically distant breeding areas, whose wintering sites are over 5000 km apart[35]. The authors suggested that the absence of population structure in that pelagic seabird may be due to the broad dispersal of young birds who often spend several years roaming across the oceans before settling down to breed. In addition, extensive movements during the nonbreeding season might counteract the geographical isolation of the breeding colonies.

On the other hand, after applying the LocPrior model—which can detect structure at lower divergence levels by incorporating sampling location data[36]—our microsatellite results indicated weak differentiation among groups. We observed meaningful and expected patterns of population genetic clustering, while classical estimators, although very low, were statistically significant (Table 2). Specifically, Bayesian clustering, which utilised information on 9 sampling locations as a prior, revealed that at K = 2, the three Southern Marine sampling locations cluster together and differentiate from the rest of the colonies, while at K = 3 additional clustering of five Southern Inland locations and their differentiation from Northern location (i.e. the Northern group) is evident (Fig. 2b). This was anticipated, and aligned with our categorisation of sampling locations into a priori set groups, although differentiation between groups was much weaker than expected. Differentiation between the Southern Marine and Southern Inland groups could be at least partially explained by presumed distinct migration patterns and nonbreeding distributions. Fortunately, more information is emerging on connections between breeding and wintering populations. Recent data from geolocator loggers[23] reinforced previous findings[19] that south European inland birds follow the east African migration route and winter along east Africa. They further demonstrated that Croatian marine birds, similar to Italian ones[21], migrate via the western route and winter in west Africa. These findings are in line with our results of differentiation between the SM and SI groups. On the other hand, the weak differentiation we observed indicates high gene flow between these groups, which could suggest that there is no strong migratory divide and that only some birds follow their presumed respective migratory routes, or that immature birds from the two migratory routes intermix during the winter or the second calendar year when they stay away from the breeding area. This is confirmed by the ringing recoveries of the birds from the SI group in NW Africa and along the western African coast[24]. Because the current migratory data relies on a relatively small number of loggers retrieved from tagged birds, namely four[24] and nine[23], more comprehensive geolocator studies are necessary to better understand the migratory

dynamics of common terns in southern Europe. The weak separation of the N group from the rest of the investigated colonies cannot be attributed to distinct migration routes. Common terns from northern Europe predominantly use the west African migration route (i.e. East Atlantic Flyway), with around 20 to 30% opting for east African route. Their wintering areas vary as well: terns using the eastern route often winter in east Africa, while those on the western route typically winter in west Africa. However, some terns from either route choose to winter in south Africa[9,20,22,23]. Thus, north European terns' migration patterns and nonbreeding distributions seem more intricate. Wintering grounds common to different breeding colonies suggest intermixing within them, potentially facilitating social interactions between individuals from widely separated breeding locations. This could contribute to the homogenisation of genetic diversity, explaining the weak genetic structure that we found. A high degree of intermixing within wintering sites was documented among colonies breeding in inland North America[37]. Furthermore, the shared wintering grounds have been previously proposed as a reason for the absence of strong population differentiation between geographically distant breeding colonies[29]. Specifically, the breeding colonies from the Azores and North American Bird Island, which exhibit very low differentiation ($F_{ST}=0.02$), both winter together in South America. Similarly, the weak genetic structure between central and eastern North American black tern (*Chlidonias niger*) breeding populations was explained by shared nonbreeding areas between them[38]. Contrary to that, microsatellite data suggested significant differentiation among Eurasian black tern breeding colonies that were segregated at staging sites[39]. Building on an earlier point, the weak separation of the N group suggests that geographic isolation of the breeding colonies still remains an important, though quite minor, factor generating population differentiation. Geographic distance between colonies was recognised as a factor influencing population genetic structure in seabirds, along with others such as nonbreeding distribution and colony dispersion[25].

Seabirds, including common terns, are generally assumed to exhibit high breeding site fidelity, which favours population differentiation by restricting gene flow. For example, as many as 86% of tagged common terns returned to the breeding colony in a year following tagging[20]. However, our results of a very weak population structure contradict this prevailing view by suggesting that common terns are not as philopatric as some investigations indicated. That notion has already been highlighted[31]—previous studies might have overestimated philopatry, as it might be easier to detect philopatric individuals than those that disperse to other locations. Furthermore, we did not find evidence of female-biased dispersal as both microsatellites and mtDNA revealed very low differentiation, while the general view is that female is the more frequently dispersing sex in most seabirds[40], including common terns[34].

As mentioned, mtDNA analyses (SAMOVA or Mantel test) did not reveal biologically meaningful clustering of colonies into groups or a correlation between genetic and geographic distances. Furthermore, the mtDNA haplotype network did not reveal a phylogeographic structure, as no clear relationships between the haplotype lineages and the geographic locations of the common tern groups were apparent (Fig. 3). This also suggests low divergence and high gene flow between groups.

Although surprising for the common tern, the findings of weak population genetic structure are not entirely unexpected, as similar patterns have been observed in other waterbird species, such as in three subspecies of the least tern (*Sternula antillarum*)[41] and in the black tern[38] in North America, based on both microsatellite and mtDNA analyses. However, contrary to our findings, different isolation-by-distance patterns based on microsatellite and mtDNA data were observed in least terns[41] that indicated that male dispersal in least terns is more limited than female dispersal, which aligns with the prevailing view that males are the more philopatric sex in seabirds.

Our results reveal high genetic diversity of the European common tern, although caution is needed when comparing genetic diversity metrics between studies with different markers, sample sizes and species. The mean expected heterozygosity estimated at 0.715 and ranging from 0.69 to 0.72 (Table 1) is higher than that of common terns from the North Atlantic region (0.47–0.71), the Netherlands (0.66), Ukraine (0.65)[29] and Lithuania (0.31–0.38)[28] and corresponds to that of common terns from eastern North America (range 0.66 to 0.76, mean 0.71)[30]. Compared to related species, expected heterozygosity values are similar to those of black terns (0.67–0.75)[39], but much higher than reported in least terns (0.45–0.56)[41]. Similarly, the overall mean number of alleles per locus of 8.83 in our study (Table 1) is much higher than that found in other populations of common terns. Namely, 3.6 alleles per locus were found in Lithuanian[28], 2.9–5.7 in eastern North Atlantic[29], and 4.8–6.6 in North American terns[30], but this estimate is strongly influenced by sample size as the last study had a sample size comparable to ours, while the other two were much lower. MtDNA control region haplotype diversity is also quite high with an overall value of 0.81, which is comparable to the value previously reported for samples from Slovenia and Croatia (0.86)[42]. This is higher than the haplotype diversity of mitochondrial cytochrome-b in common terns, where values range from 0.21 to 0.77[30], but a little lower than reported for the mitochondrial control region in related species—the least tern (mean haplotype diversity of 0.915)[41] or sooty tern (*Sterna fuscata*) (Hd above 0.94)[43]. Genetic variation at the nucleotide level is moderate with the nucleotide diversity estimated at 0.0023, which is also similar to that previously reported for common terns (0.025)[42], least terns (values ranging from 0.0010 to 0.0069)[41] or sooty terns (0.018 to 0.029)[43].

Analyses of microsatellite diversity patterns revealed the Southern Marine group consistently had the lowest diversity indices, whereas the other two groups displayed similar levels of diversity (Table 1, Supplementary Table S2). Although some of these indices may be influenced by uneven sample sizes, rarefied allelic richness, which accounts for sample size, was also lowest for the Southern Marine group indicating overall lower genetic diversity in this group. Additionally, the SM group exhibited the lowest mtDNA control region diversity, with only nine haplotypes detected, none of which were private for that group (Table 3). Despite similar sample sizes analysed for mtDNA between the Northern and Southern Marine groups (85 and 81, respectively), the N group had a much higher number of haplotypes (18), including six that were private. Most mtDNA diversity indices for the SM group were either the lowest or similar to those of N group. The low genetic diversity in the SM

group, especially at the microsatellite markers, could suggest a recent establishment of the colonies in this group, smaller historical populations, a more severe population bottleneck, and/or lower dispersal compared to the other two groups. The highest mtDNA diversity indices and the largest number of private haplotypes (22) were found in the Southern Inland group, which could be partially attributed to the higher sampling intensity in this group (153 samples analysed). However, subsampling of the Southern Inland group showed that this result was not due to the large sample size; all diversity indices, including the number of private haplotypes, remained the highest in this group (Table 3).

Signals of a population bottleneck were detected in all groups both by the Wilcoxon signed rank test of heterozygosity excess and by a qualitative graphical method for detecting distortion in the distribution of allele frequencies. However, the results for the entire dataset were somewhat ambiguous: Wilcoxon's test indicated a bottleneck, but the graphical test for mode shift did not (Supplementary Figure S1). Occasionally, a mode-shifted distribution of allele frequencies may go undetected, even if a population has recently undergone a bottleneck. Alternatively, the absence of a mode shift may indicate that the bottleneck was not recent[44]. In conjunction with the results of Tajima's and Fu's neutrality tests (Table 3), which indicate possible population expansion following a recent bottleneck, the findings of this study strongly suggest that European common terns have experienced a population bottleneck but have maintained high genetic diversity. This preservation of genetic diversity may be due to high connectivity among tern colonies, potentially compensating for a loss of diversity.

In conclusion, our study demonstrates the high genetic diversity and weak population structure of the common terns in three European breeding areas. Despite the high mobility of this species, which likely facilitates gene flow, we found differentiation, albeit weak, between groups, especially between Southern Marine and Southern Inland groups, which is probably influenced to some extent by relatively distinct migration patterns. Our results suggest that geographic distance between breeding colonies alone does not greatly affect population genetic structure, emphasizing the importance of considering both breeding and nonbreeding distributions. Further comprehensive studies, especially those involving more extensive use of tracking devices, are essential to deepen our understanding of migration dynamics and their effect on genetic structure. Low differentiation, which indicates high dispersal, can support the establishment of new colonies but may also increase the risk of abandonment of small, existing colonies. This risk is particularly relevant for Southern Inland and Southern Marine populations, which breed in fragmented habitats susceptible to flooding, habitat loss, human disturbance, and predation. Conservation measures should focus on the protection of multiple breeding sites so that the destruction of one or a few sites does not cause the loss of the population. They should also ensure the identification and protection of suitable areas that are currently unoccupied. In this way, terns would have more options for habitat selection, which could prove valuable for maintaining their breeding in the future. This approach will likely support the continued genetic diversity and resilience of common tern populations, ensuring the long-term viability of the species in Europe.

Materials and methods

Study locations, sampling, and DNA extraction

We used 260 blood and feather samples of common terns collected during breeding seasons from 2017 to 2022. In addition, we used already published 59 mtDNA control region sequences[42], GenBank accession no. MN337406-MN337426), adding up to 319 control region sequences. We included only 59 sequences instead of the reported 60, as two samples were recognised as duplicates based on their microsatellite profiles. The samples were a priori categorised into groups according to their geographic origin (northern/southern), while southern colonies were further categorized according to the habitat type (freshwater/marine) combined with migration route (eastern/western). Therefore, the Northern group (N) comprised the samples from freshwater habitats in Germany (Riether Werder, Lieps); Southern Inland group (SI) comprised the samples collected from freshwater habitats in Hungary (Irmapuszta, Varpalota), Slovenia (Ptuj) and Croatia (Siromaja, Rakitje, Šoderica); and Southern Marine group (SM) comprised the samples from marine habitat in Italy (Ravenna, Rovigo), Slovenia (Sečovlje) and Croatia (Školjić) (Fig. 1). Around 50 µl of blood was collected from the metatarsal vein and stored on bloodstain cards (Nucleocard, Machery Nagel). When blood sampling was not possible, 10–20 feathers per individual were stored in paper bags at room temperature. For DNA extraction we used DNeasy Blood and Tissue kit for bloodstain storage cards and QIAamp DNA Micro Kit for feathers (Qiagen, Hilden Germany) following the manufacturer's protocol. After DNA extraction, the samples were stored at −20 °C. Subsequent microsatellite analyses included 219 samples from 9 sampling locations (Northern group N = 65, Southern Inland N = 95, Southern Marine N = 59), while mtDNA control region analyses included 319 samples from 12 locations (Northern group N = 85, Southern Inland N = 153, Southern Marine N = 81) (Supplementary Table S1).

Microsatellite analyses

PCR amplification of 18 autosomal microsatellite loci was conducted using primers from Given et al.[45] (loci RBG18, RBG28, RBG29, RBG27 and RBG13), Tirard et al.[46](locus K32), and Janowski et al.[47](loci MsSh21, MsSh23, MsSh07, MsSh31, MsSh10, MsSh20, MsSh18, MsSh09, MsSh08, MsSh03, MsSh48 and MsSh37). Each reaction was performed in 10 µl volume containing 1 × of GoTaq® G2 Colorless Master Mix (Promega Corporation), 0.05 µM forward primer, 0.2 µM reverse primer, 0.2 µM M13-tagged [48] fluorescent dyes (6-FAM, HEX, NED), and 1 µl of unquantified genomic DNA. PCR products from three loci were pooled and sent to Macrogen Europe (Amsterdam, the Netherlands) for fragment analysis on ABI 3730XL Genetic Analyzer (Applied Biosystems). Allele calling was performed using Geneious Prime 2023.0.4 (www.geneious.com).

We checked for genotyping errors using Microchecker 2.2.3[49]. We additionally used Genepop 4.7 (Web version)[50,51] to check for null alleles. The same software was used to test for deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium for each pair of loci using 10,000 dememorizations, 1000 batches and 1000 iterations per batch. We used Arlequin v.3.5.2[52] to estimate the number of alleles per locus, observed

(H_o) and expected (H_e) heterozygosity, allelic size range and frequencies. FSTAT 2.9.4.[53] was used to calculate allelic richness per locus and group. All tests were performed for each group and overall, i.e. for the whole dataset. We calculated the number of private alleles in each group using GenAlEx 6.5[54].

We used the software Bottleneck v.1.2.02[55] to check for signals of recent population bottlenecks, separately for each group and the whole dataset. A one-tailed Wilcoxon sign rank test of heterozygosity excess was used under the two-phase model of mutation (TPM with 95% of single-step mutations and 5% of multiple-step mutations) and variance among multiple steps of 12 parameters recommended for microsatellite data[55] with 10,000 iterations. In Bottleneck software, we additionally tested the distribution of allele frequencies, which should be normally L-shaped in a stable population under mutation-drift equilibrium or shifted if a recent bottleneck has occurred (i.e., the low-frequency alleles are less frequent than intermediate-frequency alleles[44]).

We used Arlequin to calculate population-specific F_{IS}, pairwise and global F_{ST} values, and perform a hierarchical analysis of molecular variance (AMOVA[56]). We tested the dataset for isolation-by-distance using the Mantel test in Arlequin by assessing the correlation between genetic distances and Euclidean geographic distances across all colonies with 10,000 permutations and pairwise F_{ST} values for the genetic distance matrix. *P*-values for multiple tests were adjusted using the false discovery rate[57] with alpha set to 0.05. We used the R package ADEGENET[58] to carry out a Principal Component Analysis. We explored the population genetic structure using STRUCTURE v2.3.4. software[59] which infers the number of genetic clusters (K) in a population and assigns individuals into clusters using a Bayesian clustering algorithm. We ran STRUCTURE analyses for K ranging from 1 to 4 with five iterations per K. We implemented a burn-in of 100,000 generations and Markov chain Monte Carlo (MCMC) run length of 1,000,000 generations for each iteration with correlated allele frequencies[60] and the admixture model. We further re-analysed the data in the same way using the LOCPRIOR model that incorporates information on sampling locations and can detect structure at lower levels of divergence[36]. We used 9 sampling locations as prior (Fig. 1) and ran analyses for K ranging from 1 to 10. We uploaded the results to StructureSelector web server[61], which plots the log probability of the data (LnP(K))[59] and Evanno ΔK[62] to determine the optimal K value, as well as CLUMPAK[63] to visualize the results from multiple runs of each K value.

Mitochondrial DNA amplification and sequencing

We amplified a fragment of mitochondrial DNA control region using PCR primers and protocols described by Svetličić et al.[42]. Sanger sequencing was performed by Macrogen Europe (Amsterdam, Netherlands) in both directions. Sequences were aligned, checked, and shortened to 709 or 710 bp using Geneious Prime. The same program was used to assign previously known haplotypes. MEGA version 11[64] was used to calculate the best nucleotide substitution model according to the Bayesian Information Criterion (BIC), which was then applied to calculate diversity indices and pairwise Φ_{ST} in Arlequin. The model with the lowest BIC score was the Kimura 2-parameter with Gamma distributed rates of 0.05 and a proportion of invariable sites of 0.48. Arlequin was used for calculating relative haplotype frequencies, diversity indices such as haplotype diversity (*Hd*)[65], nucleotide diversity (π)[66], the mean number of nucleotide differences among haplotypes—*k*, as well as pairwise and global F_{ST}, Φ_{ST} and AMOVA[56]. Neutrality tests of Tajima's D[67] and Fu's F_S[68] were also calculated in Arlequin with 10,000 simulated samples. These analyses were also performed on randomly subsampled portion of the largest group (Southern Inland, N = 153) matched to the sample size of the smallest group (Southern Marine, N = 81), to minimize the effects of unequal sample sizes in estimates of diversity indices and differentiation between groups. The Mantel test was performed for mtDNA haplotypes with the same parameters as microsatellite data. Spatial analysis of molecular variance was conducted using SAMOVA 2.0[69]. This method defines geographically homogeneous groups of populations that are also maximally differentiated from each other. We ran the software with K (number of assumed groups) ranging from 2 to 4 using the default settings. We constructed a TCS haplotype network[70] in PopArt[71] to visualize relationships among haplotypes.

Data availability

MtDNA haplotypes have been deposited in GenBank (PP964749-PP964767). Microsatellite genotypes and remaining data are included in the Supplementary information files.

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Author contributions

A.G. and J.K. designed the study. J.K., Ž.P., L.J., S.P., C.H., I.Š., D.T., G.K., B.P., P.S. and S.V. organised and performed field sampling. V.L., I.S. and M.G. handled the laboratory work. V.L., A.G. and A.V.S. analysed the data with help from S.S and E.B. V.L. and A.G. wrote the first version of the manuscript with input from J.K., Ž.P., A.V.S. and E.B. All authors contributed to the manuscript and gave final approval for publication.

Declarations

Competing interests

The authors declare no competing interests.

Ethics declarations

All methods and procedures in this study were carried out in accordance with relevant guidelines and regulations, including ARRIVE guidelines and regulations. Research was conducted with the approval of the Ethical Committee of the University of Zagreb, Faculty of Science, Croatia. Sampling permissions were obtained from each country's relevant institutions. This includes Germany's Landkreis Vorpommern-Greifswald (No. 60.5/Br, VG-19-028), and Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern (No. 7221.3-2-007/19); Hungary's Pest County Government Office, Department of Environmental Protection and Nature Conservation (No. PE-KTFO/1672-6/2019); Slovenia's Environmental Agency (No. 35601-8/2015 - 7), and the Ministry of the Environment, Climate and Energy (No. 35601-37/2013-14); Croatia's Ministry of Environment and Energy (No. 517-07-1-1-1-18-4), and The Ministry of Economy and Sustainable Development (Nos 517-05-1-1-21-4 and 517-10-1-2-23-4); and the Italian Institute for Environmental Protection and Research (ISPRA), under the authorisation of Law 157/1992 [Art. 4(1) and Art. 7(5)].

Additional information

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