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1 Genetic origins, singularity and heterogeneity of Basques

2 André Flores-Bello,¹ Frédéric Bauduer,² Jasone Salaberria,³ Bernard Oyharçabal,³ Francesc
3 Calafell,¹ Jaume Bertranpetit,¹ Lluís Quintana-Murci,^{4,5} David Comas^{1*}

4
5 ¹Departament de Ciències de la Salut i de la Vida, Institut de Biologia Evolutiva (CSIC-UPF),
6 Universitat Pompeu Fabra, Barcelona, 08003, Spain; ²Laboratoire PACEA UMR 5199 CNRS,
7 Université de Bordeaux, Pessac, 33615, France; ³Centre de Recherche sur la Langue et les
8 Textes Basques, Centre National de la Recherche Scientifique, UMR5478 IKER, 64100,
9 Bayonne, France; ⁴Human Evolutionary Genetics Unit, Institut Pasteur, Centre National de la
10 Recherche Scientifique, UMR 2000, Paris, 75015, France; ⁵Human Genomics and Evolution,
11 Collège de France, Paris, France.

12 *Correspondence: david.comas@upf.edu

14 Summary

15 Basques have historically lived along the Western Pyrenees, in the Franco-Cantabrian region,
16 straddling the current Spanish and French territories. Over the last decades, they have been the
17 focus of intense research due to their singular cultural and biological traits that, with high
18 controversy, placed them as a heterogeneous, isolated, and unique population. Their non-Indo-
19 European language, Euskara, is thought to be a major factor shaping the genetic landscape of
20 the Basques. Yet, there is still a lively debate about their history and assumed singularity due to
21 the limitations of previous studies. Here, we analyze genome-wide data of Basque and
22 surrounding groups that do not speak Euskara at a micro-geographical level. A total of
23 ~629,000 genome-wide variants were analyzed in 1,970 modern and ancient samples, including
24 190 new individuals from 18 sampling locations in the Basque area. For the first time, local- and
25 wide-scale analyses from genome-wide data have been performed covering the whole Franco-
26 Cantabrian region, combining allele frequency and haplotype-based methods. Our results show
27 a clear differentiation of Basques from the surrounding populations, with the non-Euskara-
28 speaking Franco-Cantabrians located in an intermediate position. Moreover, a sharp genetic
29 heterogeneity within Basques is observed with significant correlation with geography. Finally,

30 the detected Basque differentiation cannot be attributed to an external origin compared to other
31 Iberian and surrounding populations. Instead, we show that such differentiation results from
32 genetic continuity since the Iron Age, characterized by periods of isolation and lack of recent
33 gene flow that might have been reinforced by the language barrier.

34

35 **Keywords**

36 Basques, Euskara, Franco-Cantabrian, population structure, isolated population, demographic
37 history, genome-wide data

38

39 **Introduction**

40 The Franco-Cantabrian region, which includes the western part of the actual border of Spain
41 and France through the Pyrenees, has drawn the attention of several disciplines due to its
42 relevant role in European human history. This region was one of the most densely populated
43 glacial refugia in Europe during the Last Glacial Maximum (LGM), and it is related to pivotal
44 archaeological discoveries, especially the oldest known European cave paintings¹. One of the
45 most interesting features of the region is the presence of the Basques. They have been
46 historically distributed along the western edge of the Pyrenees, spanning across the present
47 Spanish and the French territories currently organized in seven provinces: Gipuzkoa, Bizkaia,
48 Araba and Nafarroa in the southern side of the Pyrenees; and Zuberoa, Lapurdi, and Nafarroa
49 Beherea located in the northern side. Basques have presumably stood out due to their
50 historical, anthropological, and biological traits that define their singularity and isolation within
51 the European context. A remarkable feature is Euskara, with its five main dialects (Figures 1, S1
52 and Table 1), which is a non-Indo-European language isolate with no close relationship to any
53 other extant language^{2,3}. Beyond its current distribution, Euskara was historically spoken in the
54 seven present provinces, before its geographic regression due to the pressures of Romance
55 languages⁴. Moreover, an archaic Euskara-related language is suggested to have been spoken
56 in a much wider area in earlier periods. This area would include the neighboring Northern
57 Spanish areas and the Southern half of Aquitaine in France⁵ (Figure S1). Whereas Euskara has
58 been pointed as a possible cultural barrier between Basques and neighboring populations, its

59 dialects might have also acted as an internal barrier⁶. They show reduced interintelligibility and
60 a standard language (*batua* in Euskara) was not established until 1968 and was widely used
61 only since the 1980s.

62

63 Although numerous studies have focused on the genetics of Basques, a lively debate on their
64 population history is still ongoing (see, for instance, Laayouni et al.⁷ and Rodríguez-Ezpeleta et
65 al.⁸). Such an interest in the genetics of Basques started with the remarkable observation of a
66 high frequency of the Rh-negative blood group⁹, a genetic variant associated with the hemolytic
67 disease of the newborn, which was confirmed in following studies¹⁰. Subsequent studies using
68 more genetic markers revealed that Basques were particularly differentiated within the
69 European genetic context^{11,12}. These results, together with archaeological, cultural, and
70 linguistic data, were interpreted as Basques descending from an ancient population that
71 remained isolated in the region¹³. However, other studies have not provided evidence of a
72 genetic distinctiveness for the Basque population, suggesting genetic homogeneity through
73 Europe¹⁴.

74

75 The origins of Basques have also been controversial. Some studies based on uniparentally-
76 inherited markers have proposed that Basques represent isolated Pre-Neolithic European
77 groups that stayed in the region after the resettlement of Europe from the glacial refugia in the
78 post-LGM periods^{15,16}. Conversely, other studies have shown the influence of the Neolithic
79 migrations in the Basque area, refuting the Paleolithic genetic continuity¹⁷. Exhaustive analysis
80 of uniparental genomes showed a continuity since Pre-Neolithic times and a pre-Roman genetic
81 structure in Basques^{6,18}. In support of this, ancient DNA data has suggested that Basques can
82 actually be explained as a common Iberian Iron Age population, with an important genetic
83 influence of the post-Neolithic Steppe pastoralists ancestries, but lacking admixture from
84 subsequent incoming populations such as Romans or North Africans¹⁹.

85

86 The controversies about the Basques have not only been focused on their distinctiveness and
87 their origins, but also on their internal genetic heterogeneity. Genome-wide data in Basque
88 groups have shown contradictory results, with some studies suggesting that French Basques

89 are markedly different from Spanish Basques, being the latter similar to other Iberian
90 populations ⁷; whereas other data were interpreted as showing internal homogeneity within
91 Basques and marked genetic differentiation from non-Basque groups⁸.

92

93 These remarkable contradictory results might be explained by a limited methodology and
94 resolution. The low number of samples used in these analyses to represent the Basque groups
95 and their neighboring areas has supposed the major limiting factor. Furthermore, they have
96 been based on the allele frequencies of classical genetic markers, the phylogeny of uniparental
97 genomes, or a reduced number of sample and markers¹³. To overcome the limitations in the
98 previous studies, we adopt a robust genome-wide study design that enable us to unravel the
99 controversial population history of Basques, including their uniqueness, their origins, and their
100 genetic structure. The unique and exhaustive dataset of the whole Franco-Cantabrian region
101 presented here (Figure 1, S1 and Table 1) limit any possible sampling biases affecting the
102 previous studies and affords an exhaustive analysis at micro-geographical and wide-scale level.
103 Moreover, the ethno-linguistic information considered in our sampling allowed us to interpret the
104 results beyond the genetic data, considering how cultural factors could be involved in shaping
105 the genetics of the Basques and surrounding populations. Finally, we leverage the more precise
106 haplotype-based methods to uncover fine-grained genetic structure and admixture patterns^{20,21}.

107

108 **Results**

109 ***Basques display marked differentiation in the European/Mediterranean landscape***

110 We first studied Basques in a wide-scale context to assess their genetic variability within a large
111 and diverse population panel, which includes a complete dataset of West Eurasia and North
112 African samples. In a Principal Component Analysis (PCA) the Basque samples fall in the
113 opposite edge of the North African samples and in the periphery of Europe, similarly to
114 Sardinians, with the Peri-Basque groups (surrounding traditionally Gascon- and Spanish-
115 speaking areas; see sample collection in STAR★METHODS) being in an intermediate position
116 (Figure 2A). Using admixture analyses, considering the global genetic ancestry components of
117 these populations (Figure 2B), a differential genetic pattern is observed in Basques. In K=6,

118 Basques present mainly two components: a major component (green), which is also present in
119 European samples and it is found at low frequencies in the Middle East/Caucasus and North
120 Africa; plus, a minor component (pink) found at high frequencies in Central/Eastern Europe. The
121 other components found in the rest of European groups are not present in Basques
122 (frequencies <1%). The Peri-Basque samples show a similar pattern to Basques but with low
123 frequencies of other external components absent in Basques. From K=7 onwards a new specific
124 component appears, maximized in Basques and with frequencies over 50% in Peri-Basques.
125 This component is also observed in Spanish and French samples, while it is virtually absent in
126 the other external European samples.

127

128 Using the haplotype-based analyses implemented in fineSTRUCTURE, we detected marked
129 levels of differentiation in Basques (Figures 3, S2 and Table S1). First, the Basque groups
130 cluster together within the large European branch, but in a differentiated external cluster
131 (Figures 3A and S2). This result points to low haplotype sharing between this cluster and the
132 rest of European groups, showing a clear internal and specific genetic profile of Basques.
133 Second, the Peri-Basque groups also exhibit a differentiation from the other external
134 populations, clustering internally with Europeans, but forming a specific branch with the
135 exception of the Cantabrian samples (gCAN), which cluster with Spanish samples (Figures 3A
136 and S2). To discard putative artifacts due to the overrepresentation of Franco-Cantabrian
137 samples in the fineSTRUCTURE analysis, we performed a random sampling of the Franco-
138 Cantabrian region (50 samples including Basques and Peri-Basques), and obtained similar
139 results (data not shown). Furthermore, the ancestry profile calculated in the Non-negative least
140 squares (NNLS) analysis mirrored the results above (Figure 3B). Basques share haplotypes
141 exclusively with the internal groups in the Franco-Cantabrian region. Peri-Basques mainly share
142 haplotypes internally with the groups in the region but also with the non-Franco-Cantabrian
143 Spanish and French groups, acting as a buffer zone between Basques and the surrounding
144 external populations. The intermediate ancestry profile observed in Peri-Basques suggests
145 gene flow between the Franco-Cantabrian region and the external groups. Therefore, potential
146 admixture events were tested in Peri-Basques by using GLOBETROTTER, considering
147 Basques and all the external clusters inferred by fineSTRUCTURE as surrogates (Figures S2D,

148 S2E and S2F). Single admixture events involving two sources were detected for all Peri-Basque
149 targets, with close dates between the 11th and 16th centuries. Similar sources were described in
150 each target cluster: a major source represented by mainly Basque and Spanish ancestries; and
151 a minor source that is predominantly represented by Spanish ancestry. Moreover, the
152 confidence intervals for the dates estimated from bootstrapping resulted in overlapped and quite
153 large ranges in each Peri-Basque target, especially in Bigorre (gBIG) (Figures S2E and S2F).
154 This might evoke a single, but continuous, pulse of admixture in the Peri-Basques, and a
155 general large-scale demographic event that affected the area in recent historical times, between
156 the 11th and 16th centuries.

157

158 To explore further the genetic differentiation of Basques, we performed an analysis of runs of
159 homozygosity (ROHs). Basques show the overall highest total number (NROH) and total length
160 (SROH) of ROHs, even higher than Sardinians, which are reported to carry long ROHs²² and
161 show ROH values slightly above the European average²³, and followed by the Peri-Basque
162 groups (Figures 3C and S3A). In the intermediate ROH categories, the total proportion of
163 samples represented in the external populations is very small (Figure S3A). This shows that
164 these categories are more common in the isolated groups, Basques and Sardinians, as well as
165 in the Peri-Basques, while in the external groups the values observed could be more related to
166 cryptically inbred outliers²⁴. These results are in agreement with the exploration of the
167 proportion of identity-by-descent sharing between samples (PI_HAT) within the groups (Figure
168 S3B). Moreover, the estimation of the effective population size (N_e) over time showed Basques
169 with low and stable values, whereas external groups (i.e., Spanish and French) show a dramatic
170 increase around one thousand generations ago (Figure S3C). These results suggest a pattern
171 of isolation together with inbreeding in Basques, and to a lower extent in Peri-Basques. Such
172 patterns of isolation, which by all other evidence is much more recent (see Discussion), seems
173 to have been deep enough to erase the traces of an apparent N_e Paleolithic growth in the
174 surrounding populations.

175

176 ***Post-Iron Age demographic processes explain the genetic uniqueness of Basques***

177 Then, analyses including ancient samples were performed to throw light on the origin of the
178 genetic singularity of Basques. The PCA projection of ancient samples shows Basques closer to
179 Pre-Neolithic hunter-gatherers and European Neolithic farmers, but also to some post-Neolithic
180 Steppe herders associated to the Pontic-Caspian Steppe Yamnaya ancestry (Figure S4A).
181 ADMIXTURE analysis (K=4, lowest cross-validation error, Figure S4B) show Basques and Peri-
182 Basques with the lowest proportions of a Levant- and Iran-related Neolithic components,
183 together with a slightly higher proportion of the Anatolian/European farmer component
184 compared to other European populations. When testing for the shared drift with ancient
185 samples, outgroup f3-statistics show high shared drift between Basques and the three major
186 ancient components in Europe (i.e., Paleolithic hunter-gatherers, Neolithic farmers, and post-
187 Neolithic Steppe herders related to the Yamnaya ancestry) (Figure S4C). We then modeled
188 Franco-Cantabrian groups and other European populations with these ancient samples by using
189 qpGraph (Figure S4D). The model fit each tested European population, with Z-scores close to 0
190 for all the 100 permutations performed. The inferred admixture proportions for the three ancient
191 components do not show differences of Basques compared to the general European context
192 (Figure S4E), following the Northern-Southern European expected cline of these ancient
193 components²⁵. Moreover, no internal differences regarding the proportions of these ancient
194 components were observed when modeling the Franco-Cantabrian groups individually (Figure
195 S4F). This suggests that the genetic singularity of Basques might rely on demographic
196 processes after the Iron Age, such as recent historical influences in Roman and Islamic periods.
197 Therefore, qpAdm analysis was performed to formally test plausible post-Iron Age admixture
198 models that might explain the singularity of Basques. This analysis shows that Basques can be
199 mostly explained with the Iron Age samples from the Iberian Peninsula¹⁹, with very limited
200 influence of Roman Empire samples²⁶ in some groups next to Peri-Basques (Figure 4 and Table
201 S2). These Roman-related proportions increase as farther the target populations are from the
202 Franco-Cantabrian region. Moreover, no significant results were observed for any model
203 considering North African samples²⁷ in the region (Figure 4 and Table S2). Overall, these
204 results suggest that Basques might have received limited gene flow during recent historical
205 events in the Iberian Peninsula.
206

207 ***Franco-Cantabrians show a heterogeneous genetic landscape correlated to geography***

208 Since one of the major controversies regarding Basques is their internal genetic heterogeneity,
209 we focused our analyses on the Franco-Cantabrian region to explore the internal genetic
210 diversity in the region. A first PC separates all Franco-Cantabrian groups through a genetic
211 cline, with all Basques in one extreme, the Spanish and French non-Franco-Cantabrians in the
212 opposite one, and the Peri-Basques in an intermediate position (Figure 5A). The second PC, in
213 turn, separates the regions from the western and eastern areas of the Franco-Cantabrian
214 region. The PCA shows a remarkable micro-geographical genetic structure and clustering of the
215 Basque groups. To obtain an external reference of similar size and sampling density at a micro-
216 geographical scale such as the present study, the Catalan samples from Biagini et al.²⁸ were
217 compared to our dataset. The PCA of Catalans does not show any geographical structure
218 comparable to that of Basques and Peri-Basques (Figure S5), which may imply that the
219 clustering observed in Basques is specific to them and unrelated to the sample strategy. To
220 quantify and compare the genetic differentiation among the Franco-Cantabrian and Catalan
221 groups, F_{ST} distances for each pairwise combination were estimated and shown in a MDS plot
222 (Figure S6). Again, Franco-Cantabrians show a clear internal differentiation with distances in a
223 range of 10^{-2} , whereas Catalans showed no evidence of genetic structure or extreme internal
224 differentiation with distances in the range of 10^{-3} . Moreover, heterogeneity was tested within the
225 region by performing an analysis of molecular variance (AMOVA) analyses at different strata in
226 the geography. Though the explained genetic variance was small, all the results were
227 statistically significant, pointing to an internal differentiation of the region and especially in the
228 Basque groups (Figure S7). In fact, the same analysis in Catalonia shows lower explained
229 variance in all comparisons (Figure S7D).

230

231 The analysis of genetic components performed in the region with ADMIXTURE mirrors the
232 results described above (Figure 5B). At $K=2$ (best K with the lowest cross-validation error),
233 Basques present a main component (green) that is also present at substantial proportions in the
234 Peri-Basque groups and marginally found in the external samples. At $K=3$ and $K=4$, internal
235 different components appear within Basques. In $K=3$, the Basque-related component splits in
236 two specific components: a Western component (blue) and an Eastern component (green).

237 These components are barely presented in the non-Franco-Cantabrian samples. Finally, at K=4
238 another component arises, maximized in Araba and the surrounding groups (pink). Thus, these
239 four components could be summarized in: non-Franco-Cantabrian (orange), Eastern-related
240 Basque (green), central-related Basque (blue), and Western-related Basque (pink) components.
241 The distribution of these components among the samples evidences the correlation between
242 genetics and geography in the region. To formally test for this correlation, we performed an
243 isolation by distance (IBD) analysis. A Mantel test was applied between the F_{ST} values and the
244 geographic distances, resulting in a positive and clear statistically significant result ($R^2 = 0.242$,
245 p -value = 0.0163) (Figure S5B). Then, the spatially explicit statistical method, EEMS, showed a
246 well-defined internal pattern of barriers (Figure S5A), both between the Basque and Peri-
247 Basque area, as well as within them. In fact, the pattern of the corridors with higher migration
248 rate mirrors the observed relationships in the ADMIXTURE analysis and the PCA, between
249 groups with overlapping standard deviations (Figures 5 and S5C). The same analyses were
250 performed for the Catalan samples and despite being a geographical region of similar size, the
251 results show a non-significant and negative trend for the Mantel test ($R^2 = -0.151$, p -value =
252 0.749), and the absence of barriers in the EEMS analysis (Figures S5A and S5B).

253

254 Finally, to refine the relationships between the populations within the area, we applied
255 haplotype-based methods, and similar patterns of internal heterogeneity were observed
256 (Figures 3, 6 and Table S1). Besides the differentiation of the Basque cluster in the
257 fineSTRUCTURE dendrogram (Figures 3A, 3B and S2), several internal clusters in the region
258 can be defined mostly related to geography and language. On the one hand, three clusters are
259 shown in the Basque branch (Figure 6A right): one that encompasses the Central Basques, plus
260 an Eastern Basque and a Western Basque cluster. On the other hand, two clusters are shown
261 in the Peri-Basque branch (Figure 6A left): a Western and an Eastern Peri-Basque clusters,
262 besides the Cantabrian samples (gCAN) that fall within the external Spanish cluster. Similar
263 clusters are observed when performing the analysis reducing the dataset (Figures S2B and
264 S2C). The differences found in Basques are also shown in the ancestry profiles calculated in
265 the NNLS analysis (Figures 3B and 6B). Basque clusters are formed by exclusively Basque or
266 Peri-Basque components, whereas Peri-Basques show also external ancestries. Focusing on

267 Basques, the Central group is defined exclusively by Basque ancestry, whereas Eastern and
268 Western Basques present ~25% of Peri-Basque ancestry. Despite some traces of Spanish and
269 French ancestries in the Western Basque group, other external ancestries are absent in
270 Basques, suggesting haplotype sharing within the region without external contributions.

271

272 **Discussion**

273 Our results show a clear genetic distinctiveness of the Basques within the European landscape
274 in all the analyses performed, with evidence of continuous inbreeding reflected in their small *N_e*
275 values, the large number and length of ROHs and PI_HAT values (Figures 3C and S3), which
276 suggests a pattern of genetic isolation in Basques in their recent history. In contrast, Peri-
277 Basques show a transition between the Basques and the external Spanish and French
278 populations (Figures 3, 5, 6 and S6A), as an example of intermediate area between open and
279 isolated populations²⁹. Indeed, Basques do not show evidence of recent admixture events with
280 non-Franco-Cantabrian groups, whereas Peri-Basques show gene flow at least since Medieval
281 times (Figures 3B, 6B, S2E and S2F). The inferred admixture events in these groups, involving
282 potential Franco-Cantabrian-related and Spanish-related sources, mainly dated back to the so-
283 called period of the Reconquista and the immediate next centuries. This was a long period
284 characterized by the conflicts between the Islamic troops and the Christian kingdoms to
285 maintain their territories in the Iberian Peninsula, expanding from the alleged Battle of
286 Covadonga in 718 until the end of the Iberian Islamic rule with the fall of the Nasrid Kingdom of
287 Granada in 1492³⁰. It led to a complex political and administrative situation in the history of the
288 Iberian Peninsula, so these results might be echoing the consequences of the recurrent
289 reorganization of the territory during these centuries along the Franco-Cantabrian region and
290 specially the Peri-Basque area.

291

292 Nevertheless, our analyses support the notion that the genetic uniqueness of Basques cannot
293 be attributed to a different origin relative to other Iberian populations, but instead to a reduced
294 and irregular external gene flow since the Iron Age as suggested by Olalde et al.¹⁹. The
295 observed clines of post-Iron Age gene flow in the region suggest that the specific genetic profile
296 of Basques might be explained by the lack of recent gene flow received. Our analyses confirm

297 that Basques were influenced by the major migration waves in Europe until the Iron Age, in a
298 similar pattern as their surrounding populations. At that time, Basques experienced a process of
299 isolation, characterized by an extremely low admixture with the posterior population movements
300 that affected the Iberian Peninsula, such as the Romanization or the Islamic rule, as observed in
301 the present genetic landscape (Figures 3B, 4, S4 and Table S2). This does not exclude
302 plausible previous periods of isolation as attested by the presence of short ROHs and small *Ne*
303 values (Figure S3) that support signals of ancient inbreeding in the region, even higher than in
304 Sardinia, which is suggested to be isolated after Neolithic times. Thus, the increase of the *Ne*
305 observed only in the external groups about 1000 generations ago might be potentially linked to
306 the role of the Franco-Cantabrian region as glacial refugium during Last Glacial Maximum
307 periods and the subsequent expansion³¹. Although our results support the genetic continuity
308 from the Iron Age¹⁹ in most of the present-day Basques, those located in the periphery of the
309 Basque core area show signals of contacts compatible with the Roman Empire presence in the
310 Iberian Peninsula (Figure 4 and Table S2). These results are in agreement with archaeological
311 and historical records. An important presence of the Roman Empire has been reported in the
312 whole Franco-Cantabrian region, but the scholars suggest a much higher impact in the
313 peripheral areas of the southern side, specially Nafarroa and Araba³². Otherwise, North African
314 influence only fit the models where southern and northwestern Iberians are included (Figure 4
315 and Table S2). This confirms the reduced gene flow between the eastern and northern areas of
316 the Iberian Peninsula with the North African incomers during the Islamic rule, as already
317 reported by using uniparental markers³³ and more recently through genome-wide data and
318 haplotype-based methods³⁴.

319

320 Language might play a major role in the demographic processes of the populations, and in the
321 present study, given the extraordinary traits of Euskara, the ethno-linguistic scenario of the
322 region should be considered in the interpretation of the analyses performed. Our results are
323 compatible with the Euskara as one of the main factors preventing major gene flow after the
324 Iron Age and shaping the genetic panorama of the Basque region. The genetic continuity of
325 Basques since the Iron Age also supports the hypothesis that the expansion of the Steppe
326 ancestry did not completely erase Pre-Indo-European languages in Western Europe, as

327 previously suggested in other studies¹⁹. Before the arrival of the Romans to the Iberian
328 Peninsula, the Euskara coexisted with other pre-Indo-European languages, such as Iberian.
329 The contact with the Romans, and thus with Latin, was earlier and stronger in the
330 Mediterranean watershed of the Iberian Peninsula followed by their expansion towards the
331 Atlantic coast, with a later and lower impact in the Franco-Cantabrian area³⁵ as confirmed in our
332 analyses (Figure 4 and Table S2). Thus, it is expected that Latin had a deeper effect in the
333 areas with stronger Roman rule, speeding the language replacement, whereas Euskara was
334 scarcely affected. Once Latin was established in most of the Iberian Peninsula, Euskara might
335 have acted as a cultural barrier to gene flow, leading to a genetic differentiation of the Basques
336 and a low influence of the linguistic Romance substratum in Euskara^{4,5}.

337

338 Together with a positive strong correlation with geography (Figure S5), our results confirm a
339 clear internal heterogeneity in Basques and Peri-Basques, where East-West genetic clusters
340 are evident along the Franco-Cantabrian region, showing a genetic cline from the core to the
341 external areas with higher gene flow among the closest groups. This genetic substructure is
342 more complex than the Northern-Southern orographic and administrative limits between the
343 present-day Spanish and French territories, separated by the Pyrenees Mountains. Instead,
344 haplotype-based methods enabled to accurately define a central Basque cluster, plus a
345 Western and an Eastern Basque and Peri-Basque genetic clusters (Figures 3B, 5 and 6);
346 clarifying previous results that barely suggested this pattern in the Franco-Cantabrian region by
347 using classical markers^{36,37}. Whereas the Eastern and the Central Basques do not show
348 relevant gene flow with external sources, the groups from the Western Basque cluster present
349 small levels of gene flow with them (Figures 3B, 4, 5 and 6). This genetic substructure reflects
350 the historical and linguistic context between the Basques and the neighboring areas. Those
351 from the Central region are the most differentiated, due to the smaller influence from external
352 sources along history regarding its farthest location^{37,38}. Moreover, the linguistic, political and
353 administrative situation in the Central and the Eastern areas have been quite stable along
354 history³⁹. However, the Western areas are characterized by a complex scenario, related to the
355 reorganization of the administrative limits and the regression of Euskara due to the strong
356 influence of surrounding Romance languages and mainly Spanish^{32,39,40}.

357

358 Finally, the role of the Euskara dialects on the present-day Basque genetic substructure is more
359 difficult to assess. Most linguist scholars agree on a Western-Eastern dialectal discontinuity with
360 small distances among the closest dialects^{41,42}, resembling the genetic clusters observed in the
361 present analysis. The most accepted hypothesis about their origin is that they emerged during
362 the medieval period^{4,42,43}. Yet, our results reveal a clearly defined genetic structure within
363 Basques that might have been formed in earlier times. Indeed, a pre-Roman genetic
364 substructure has been already suggested based on uniparental markers⁶. Therefore, the
365 diversification of the dialects and the genetic heterogeneity within the Basques might have in
366 common their correlation to geography and it might be worth to reconsider an earlier origin of
367 the Euskara dialects in linguistic studies.

368

369 In this study, we disentangle long-standing controversies about the genetic uniqueness of
370 Basques from a new perspective, providing finer and more reliable conclusions that help to
371 resolve the debate on the demographic history of Basques. This contributes to demystify the so
372 often assumed genetic characteristics of Basques, even though little clear evidence was
373 present to support their position as some sort of Proto-Europeans. Moreover, some new lines of
374 evidence are presented to the Archaeology and Anthropology fields that can be further pursued.
375 The genetic results presented here give support to the anthropological and chronological
376 connections with the ancient remains, as well as the historical and linguistic relationships
377 around the Euskara and its dialects. It is pivotal to emphasize the importance of a
378 multidisciplinary approach when studying the evolution and demographic history of populations
379 to contextualize the study, test hypotheses and interpret the results. In this sense, it is crucial to
380 encourage the integration and cooperative research among the different areas of knowledge
381 with the aim of boosting complete, contrasted, and reliable studies.

382

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397

398 **Author Contributions**

399 A.F-B. and D.C. designed and performed research; F.B., J.S., B.O. collected and provided the
400 biological and ethno-linguistic data; A.F-B. processed and analyzed the data; A.F-B., F.C., J.B.,
401 L. Q-M. and D.C. contributed to the interpretation of results. A.F-B. and D.C. wrote the paper; all
402 authors revised the final version of the manuscript.

403

404 **Declaration of Interests**

405 The authors declare no competing interests.

406

407 **Figures and Table Legends**

408 **Table 1. Information of the Franco-Cantabrian samples reported in this study.**

409 Our dataset includes a total of 190 samples from 18 areas in the present-day Spanish and
410 French territories of the Franco-Cantabrian region. Both Euskara and surrounding Romance
411 speaking groups were considered. All four grandparents of the individuals were born in the
412 same geographical region of the collected sample. N represents the number of individuals
413 genotyped for each region. See also Figures 1 and S1.

414

415 **Figure 1. Geographic distribution of the Franco-Cantabrian region and the areas included**
416 **in the study.**

417 ALA, Araba; BBA, Bizkaia; BOC, Western Bizkaia; GUI, Central Gipuzkoa; GSO, Southwestern
418 Gipuzkoa; NNO, Northwestern Nafarroa; NCO, Central Western Nafarroa; ZMX,
419 Lapurdi/Baztan; NLA, Lapurdi Nafarroa; SOU, Zuberoa; RON, Roncal; CAN, Cantabria; BUR,
420 Northern Burgos; RIO, Rioja; NAR, Northern Aragon; CHA, Chalosse; BEA, Béarn; BIG,
421 Bigorre. The dark shaded area represents the current extension of the main Euskara-speaking
422 zone. The colors represent the ethno-linguistic information of the sample set: in green, the
423 sampled areas where the Euskara was spoken in historical times and at least until the 19th
424 century; here referred as Basques. In pink and blue, the sampled areas where Spanish and
425 Gascon/French are spoken today, respectively; referred as Spanish (the former) and French
426 (the latter) Peri-Basques in this study. See sample collection in STAR★METHODS for detailed
427 information. Km, Kilometers. See also Table 1 and Figure S1.

428

429 **Figure 2. Contextualizing the Franco-Cantabrian region.**

430 (A) Principal component analysis including all modern samples analyzed in the present study.
431 (B) ADMIXTURE results with similar and lowest cross-validation errors ($K=6$ and $K=7$). ★,
432 Spanish Basque and Peri-Basque; ●, French Basque and Peri-Basque. In the large
433 Caucasus/Middle East section, the two populations with a maximized component are Bedouin
434 (light green) and Druze (gold). In the North African section, the purple component is maximized
435 in Mozabite. See also Figure S2.

436

437 **Figure 3. Haplotype-based analyses.**

438 (A) European branch of the fineSTRUCTURE dendrogram inferred for the whole modern
439 dataset. The complete dendrogram is shown in Figure S2A. The Franco-Cantabrian, other
440 Spanish, and other French clusters are highlighted in color. (B) Inferred proportions of shared
441 ancestry among genetic clusters using the NNLS method from GLOBETROTTER. Ancestries
442 that are neither Spanish nor French were pooled and labeled “external”. (C) Whisker plots
443 representing the individual information of number (NROH, left) and length (SROH, right) of Runs
444 of Homozygosity for Basques, Peri-Basques, French and Spanish. Sardinian, Central Western

445 Europeans (CEU), and sub-Saharan Africans (YRI) were also included as reference populations
446 with well-known demographic histories. Mb, Megabases. For the sake of clarity, the name of
447 those genetically homogeneous clusters inferred from fineSTRUCTURE are referred with “g”.
448 See also Figures S2, S3 and Table S1.

449

450 **Figure 4. Modelling potential post-Iron Age gene flow in the Iberian Peninsula.**

451 (A) Geographic distribution of the inferred proportions in the map. Shadings for proportions are
452 scaled according to the maximum and minimum proportions of each source. (B) Representation
453 of the admixture proportions in each target population based on the statistically significant
454 models obtained with qpAdm. See also Figure S4 and Table S2. Km, Kilometers.

455

456 **Figure 5. Population stratification of the Franco-Cantabrian region.**

457 (A) PCA using the samples from the Iberian Peninsula and France. The PCA average and
458 standard deviation values of the different geographic groups were plotted. (B) ADMIXTURE
459 results from K=2 to K=4. The lowest cross-validation error in the analysis was K=2. The
460 asterisks represent “Spanish_Pais_Vasco_IBS” and “French_Bearn”. PB, Peri-Basque. See
461 also Figures S5, S6 and S7.

462

463 **Figure 6. Haplotype distribution and sharing within the Franco-Cantabrian region.**

464 (A) Internal Franco-Cantabrian clusters inferred with fineSTRUCTURE. Left and right
465 dendrograms represent a zoom of the Peri-Basque and Basque specific branches, respectively,
466 in the complete dendrogram from Figure 3. The central map shows the geographic distribution
467 of the haplotypes inferred in the fineSTRUCTURE dendrogram. Each pie chart represents the
468 proportion of the clusters in the corresponding region. (B) Inferred proportions of shared
469 ancestry among the Franco-Cantabrian groups. This plot is in continuity with the Figure 3B.
470 Here, the internal proportions of haplotype sharing in the Franco-Cantabrian region are
471 dissected. Wavy lines represent external proportions. See also Figures S2, S5, S6, S7, and
472 Table S1.

473 **STAR★METHODS**

474 **KEY RESOURCES TABLE**

475

476 **RESOURCES AVAILABILITY**

477 ***Lead Contact***

478 Further information and requests for resources should be directed to and will be fulfilled by the

479 Lead Contact, David Comas (david.comas@upf.edu).

480 ***Materials Availability***

481 This study did not generate new unique reagents.

482 ***Data and Code Availability***

483 The original dataset generated during this study is available for download at Figshare:

484 <https://figshare.com/s/61a54472b63fd0101859>

485

486

487 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

488 ***Sample Collection***

489 A total of 190 individual samples were collected from 18 microgeographical areas in the

490 present-day Spanish and French territories of the Franco-Cantabrian region, including Basque

491 and Romance (Spanish, French, Aragonese and Gascon) speaking groups (Table 1 and Figure

492 1). Written informed consent was obtained from the participants, who were interviewed to

493 determine their local dialect and to confirm that their four grandparents were born in the same

494 microgeographical area (Figures 1, S1 and Table 1). The collection procedures were approved

495 by the CCPPRB (Comité Consultatif de Protection des Personnes dans la Recherche

496 Biomédicale d'Aquitaine) and the IRBs at Universitat Pompeu Fabra, Institut Pasteur and

497 Université Michel de Montaigne Bordeaux 3. The dataset includes seven areas where Euskara

498 is spoken at present^{6,18} (Central Gipuzkoa, Southwestern Gipuzkoa, Bizkaia, and Northwestern

499 Nafarroa in the southern side of the Pyrenees; the zone of Lapurdi/Baztan, Lapurdi/Nafarroa

500 Beherea, and Zuberoa in the northern side), besides three regions where it was spoken at least

501 until the end of the 19th century^{39,44} (Central Western Nafarroa, Roncal, and Araba). For the

502 sake of clarity, the previous ten regions where Euskara was spoken in historical times will be

503 referred as “Basque” groups. The dataset also includes i) five surrounding Spanish-speaking
504 areas (Cantabria, Northern Burgos, La Rioja, Northern Aragon and Western Bizkaia; in the case
505 of Northern Aragon, a relict local language, Aragonese, is still spoken) and ii) three traditionally
506 Gascon-speaking areas (Bigorre, Béarn and Chalosse)^{4,39-41}. In the latter, influences of
507 Euskara-related archaic languages have been suggested⁵ (Figure S1), but records of spoken
508 Euskara are absent. Moreover, Gascon has been replaced extensively by French, with less
509 than half of the population in Béarn being fluent in Gascon. We refer to these two groups of
510 populations as Spanish and French Peri-Basques, respectively.

511

512 **METHOD DETAILS**

513 ***Sample Genotyping***

514 DNA was extracted from blood samples using standard methods, and genotyped with the
515 Axiom™ Genome-Wide Human Origins Array (~629,443 variants)⁴⁵. Genotype calling was
516 performed by using the software Axiom™ Analysis Suite 3.1.51.0 through the Affymetrix Best
517 Practices Workflow. All samples passed the genotype calling process with an average quality
518 control call rate of 99.8%. After exporting those variants that properly passed the recommended
519 thresholds, 597,638 single-nucleotide polymorphisms (SNPs) remained in the dataset.

520

521 ***Data Quality Control***

522 PLINK 1.9⁴⁶ was used to apply the quality control filters detailed below, after excluding
523 uniparental markers and the X chromosome. A filter for more than 10% missing SNPs per
524 sample resulted in no sample exclusion. A total of 434,664 SNPs remained after removing
525 SNPs that were missing in more than 5% of the samples, with an extreme deviation from Hardy-
526 Weinberg equilibrium ($p < 10^{-5}$), and a minor allele-frequency (MAF) below 0.05. For those
527 analyses that required linkage equilibrium between SNPs, linkage disequilibrium (LD) pruning
528 was performed using a window size of 200 SNPs, shifting by 25 SNPs and a maximum pairwise
529 LD threshold (r^2) of 0.5. A total of 171,275 SNPs remained after LD pruning. Two individual
530 samples (one from the Lapurdi/Baztan zone and one from Zuberoa) were discarded from the
531 dataset due to their high genetic relatedness to other samples in the study ($PI_HAT > 0.125$).

532

533 ***Merging Data***

534 Our Franco-Cantabrian samples were merged with public available data genotyped with the
535 same array: samples from Catalonia, Valencia, and Balearic Islands²⁸; plus modern and ancient
536 North African and Western Eurasian samples^{19,26,27,47}. Modern Basque samples from Lazaridis
537 et al.²⁷ were included in the analyses in the Basque category as general “Spanish” and “French”
538 Basques due to the lack of information about their microgeographical origin. Samples from
539 Eivissa (within the Balearic Islands dataset) were removed from most of the analyses since it
540 has been reported to be an isolated population differentiated from the Iberian mainland
541 context²⁸. Thus, the final dataset contained 1,970 samples, as well as 434,664 SNPs in the
542 dataset used for haplotype-based methods (modern data) and 171,275 SNPs after linkage
543 disequilibrium pruning in the dataset used for allele frequency methods (both modern and
544 ancient data).

545

546 **QUANTIFICATION AND STATISTICAL ANALYSIS**

547 ***Allele frequency methods***

548 Principal Component Analysis (PCA) eigenvectors were computed by using SmartPCA v13050
549 from EIGENSOFT v6.0.1⁴⁸. For the PCA including ancient samples, these were projected to the
550 PCA space obtained with the modern samples.

551

552 In order to explore the population structure in the region, Weir and Cockerham’s pairwise F_{ST}
553 indexes were calculated between the different groups by using the R package stAMPP⁴⁹, with
554 confidence intervals after 1,000 bootstrap repetitions, and represented in a multidimensional
555 scaling (MDS) plot. Then, an AMOVA was performed with the R package poppr⁵⁰. For that,
556 1,000 permutations as described by Excoffier et al.⁵¹ were carried out with ade4 R package⁵², in
557 order to obtain an empirical distribution under the null hypothesis.

558

559 To test the correlation between the genetic and geographic distances among groups, an
560 isolation by distance (IBD) analysis was performed. Geographic Distance Matrix Generator
561 v1.2.3 software⁵³ was used to calculate a geographic distance matrix between the groups

562 included in the analysis. Then, a Mantel test was performed between the genetic and
563 geographic distance matrices with the mantel.rtest function in the ade4 R package⁵².

564

565 Effective migration and diversity rates were modeled in the Franco-Cantabrian region dataset
566 using the estimated effective migration surfaces (EEMS) software⁵⁴. The number of nDemes
567 was set to 300, and four simultaneous EEMS analyses were run with four different random
568 seeds. Next, the MCMC chain that converged with the highest final log-posterior value was
569 continued with four new runs. This process was repeated three more times, taking the best
570 chain after the previous set of runs in order to be confident that the MCMC chain had converged
571 with the optimal log-posterior value. For every set of four runs, a total of 10 million iterations
572 each were performed, with 5 million discarded as burn-in and sampling every 50,000 iterations.

573

574 To explore patterns of population structure, Model-based individual ancestries were defined by
575 using ADMIXTURE v1.3.0⁵⁵. The unsupervised method was run for 10 independent iterations,
576 both for the present-day and ancient samples analyses, and with K ancestral clusters ranging
577 from 2 to 12. Then, PONG v1.4.7⁵⁶ was used to combine the result of the 10 different iterations,
578 obtain the major modes of the ADMIXTURE results and plot them in a consensus graph.

579

580 AdmixTools package⁴⁵ was used to study the relationship between modern and ancient
581 samples. Outgroup f3-statistics were performed with the qp3Pop program to calculate the
582 shared drift between each potential ancient group and all the modern groups in the dataset. The
583 analysis was performed in the form f3(Mbuti; Ancient, Modern), with Mbuti set as outgroup.
584 Then, the qpGraph program was used to model the modern European samples to estimate the
585 three major ancestral proportions in Europe as in Haak et al²⁵: Western Pre-Neolithic hunter-
586 gatherer, European Neolithic farmer, and Yamnaya from Post-Neolithic Pontic-Caspian Steppe.
587 Based on previous studies^{25,27,57} and our results, WHG, Europe_EN and Steppe_EMBA
588 samples from Lazaridis et al.²⁷ were used as proxies, respectively. A total of 100 replicates were
589 performed for each target modern population, and the average and the 95% confidence
590 intervals of the replicates were calculated. Furthermore, the qpAdm program was run to
591 explicitly test potential admixture models including representatives of the main post-Iron Age

592 migrations that might have affected the Iberian Peninsula, i.e., the influence of the Roman
593 Empire and North African expansions. 1-way, 2-way and 3-way admixture models were tested
594 by considering Iberian Iron Age¹⁹, Imperial/Late Antiquity Roman²⁶ and North African²⁷ samples
595 as proxies (See Table S2 for further details about the modeling process).

596

597 ***Haplotype-based analyses***

598 Runs of Homozygosity (ROHs) were estimated with PLINK 1.9⁴⁶, including some external
599 populations, in order to test for inbreeding. The ROH analysis was performed in sliding windows
600 of 50 SNPs across the genome, allowing for 1 heterozygous call and 5 missing calls per
601 window. The minimum hit rate of all scanning windows containing the SNP to be included in a
602 ROH was set to 0.05. Only ROHs with a minimum length of 500 kilobases (kb) and containing at
603 least 50 SNPs were included in the analysis. One SNP per 50 kb was set as the minimum
604 density threshold and a maximum gap of 100 kb was allowed between two consecutive SNPs in
605 a tract. Due to their isolated nature, the Canary and Balearic Islands²⁷ samples were removed
606 from the dataset for this analysis. In addition, internal genetic relationships were explored within
607 populations. To that, the PI_HAT values (i.e., proportion of segments shared between two
608 individuals due to identity-by-descent) were inferred with PLINK 1.9⁴⁶.

609

610 SHAPEIT v2⁵⁸ was used to phase the data, with the population-averaged genetic map from
611 HapMap phase III⁵⁹ and the available 1000 genomes data as reference panel⁶⁰. The data was
612 first aligned to the reference and the mismatched SNPs were removed, then the proper phase
613 inference was performed.

614

615 Haplotype sharing between individual modern samples was estimated with ChromoPainter⁶¹. It
616 depicts individually the haplotypes of each “recipient” sample, without population specification,
617 as a haplotype combination of all the other samples, treated as “donor”. Thus, it estimates the
618 total number and length of haplotype fragments (chunks) in the recipient’s genome that shares
619 the most common recent ancestor with every donor sample. First, ChromoPainter was run to
620 estimate the global mutation probability and the switch rate parameters. Thus, the expected-
621 maximization (EM) algorithm implemented in ChromoPainter was used over chromosomes 1, 4,

622 17 and 20 for all individuals, with 10 iterations and parameters -in -iM. Then, the inferred values
623 were averaged across the four chromosomes and individuals, weighting by the number of SNPs
624 per chromosome. It resulted in 0.000661 and 222.54421 for the global mutation (M) and switch
625 rates (n), respectively. Finally, ChromoPainter was run for all chromosomes and individuals
626 fixing the previously estimated parameters to obtain the final count and length sharing
627 coancestry matrices. Then ChromoCombine was used to sum the matrices across
628 chromosomes and obtain the copying profile for each individual, as well as the C parameter
629 (C=0.281698702) needed for running fineSTRUCTURE.

630

631 FineSTRUCTURE v2.1.0⁶¹ was run to cluster the data provided by ChromoPainter into
632 homogeneous genetic groups following Leslie et al.⁶². The chunkcount coancestry matrix of the
633 total number of chunks copied among individuals was used as input. A total of 2 million MCMC
634 iterations were performed, with 1 million burn-in iterations and sampling values from the
635 posterior probability every 10,000 iterations. The FineSTRUCTURE dendrogram was built with
636 the default parameters -m T. The analysis was repeated for three different seeds to check
637 consistency among the dendrograms. Major differences were not observed among them, except
638 the relocation of a few individuals to other genetically close clusters. When confusion between
639 sampling and genetic definitions of the groups might exist, the homogeneous genetic clusters
640 inferred with fineSTRUCTURE were explicitly named by adding a "g" to the group label (as
641 show in Table S1).

642

643 GLOBETROTTER⁶³ was used following the recommended protocol to estimate the ancestry
644 profile of all the modern samples in the dataset, and test for admixture events in the target
645 populations that showed interesting patterns of admixture. To perform this analysis,
646 ChromoPainter was run first, but classifying the individuals based on the previous clustering
647 results from fineSTRUCTURE. All of them were included in the analysis as donors and
648 recipients. Next, the non-negative least squares (NNLS) method included in GLOBETROTTER
649 was applied to infer the haplotype sharing proportions between the different clusters. Thus,
650 prop.ind parameter was set to 1, null.ind to 0 and num.mixing.iterations to 0. A different NNLS
651 analysis was performed for each cluster as recipient and the others as donors, disallowing self-

652 copying from the recipient itself. Then, GLOBETROTTER was run to check if some admixture
653 events could be detected in our target clusters, using the other clusters as surrogates. The
654 copying vectors and the painting samples from the second run of ChromoPainter were used to
655 run this analysis. As recommended, null.ind parameter was set to 1 when testing for plausible
656 admixture events. This accounts for uncommon patterns in the linkage disequilibrium decay that
657 could show false signals of admixture. Then, null.ind parameter was set to 0 when estimating
658 admixture proportions, dates, and sources. The confidence intervals for the date estimates were
659 inferred by performing 100 bootstrap iterations, for one-date and two-date admixture models.

660

661 Finally, in order to show how population dynamics can explain the genetic diversity observed in
662 our analyses, effective population size (N_e) was estimated for the Basques, Peri-Basques,
663 Spanish and French groups. The *NeON* R package⁶⁴ was used to calculate the effective
664 population size across generations in the target groups, by considering the relationship between
665 N_e and the population-specific patterns of linkage disequilibrium from genome-wide SNP data.
666 The recommended guidelines were followed to perform the analysis and interpret the results⁶⁴.
667 First, linkage was estimated between markers considering sliding windows of 500 kb and a
668 maximum of 10,000 confrontations in each by using the *NeLD* function. Then, N_e was estimated
669 with the function *Nestimate*, setting a minimum and maximum r^2 values to 0.001 and 0.999 in
670 each comparison, respectively. Quantiles of the distribution and median for the long-term N_e
671 (5th, 50th and 95th percentiles) were estimated by using the functions *Ne_CI* and *Ne_Med*.

672

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847 **Table 1**

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Label	Location	Territory	Main language/dialect	N
BIG	Bigorre	France	French	10
BEA	Béarn	France	French	10
CHA	Chalosse	France	French	10
ZMX	Lapurdi/Baztan	France	Euskara - Upper Navarrese/Lapurdian	11
NLA	Lapurdi Nafarroa	France	Euskara - Lower Navarrese	11
SOU	Zuberoa	France	Euskara - Zuberoan	11
RON	Roncal	Spain	Euskara - Roncalese (now Spanish)	11
NCO	Central Western Nafarroa	Spain	Euskara - Upper Navarrese (now Spanish)	11
NNO	Northwestern Nafarroa	Spain	Euskara - Upper Navarrese	11
GUI	Central Gipuzkoa	Spain	Euskara - Gipuzkoan	11
GSO	Southwestern Gipuzkoa	Spain	Euskara - Biscayan	11
ALA	Araba	Spain	Euskara - Biscayan (now Spanish)	11
BBA	Bizkaia	Spain	Euskara - Biscayan	11
BOC	Western Bizkaia	Spain	Spanish	10
CAN	Cantabria	Spain	Spanish	10
BUR	Northern Burgos	Spain	Spanish	10
RIO	Rioja	Spain	Spanish	10
NAR	Northern Aragon	Spain	Spanish	10

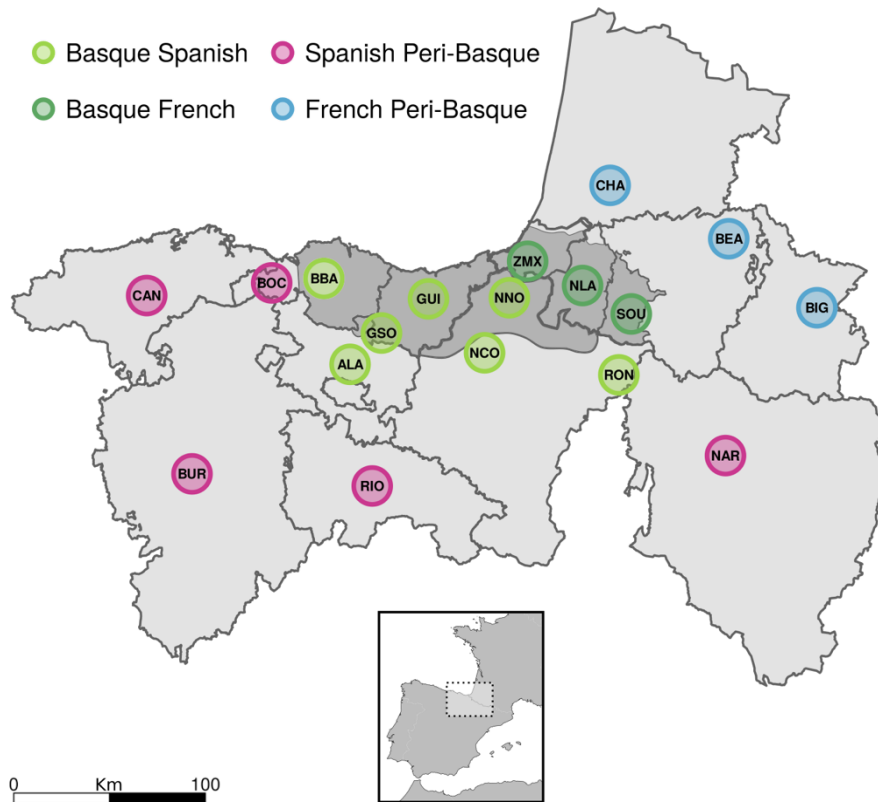
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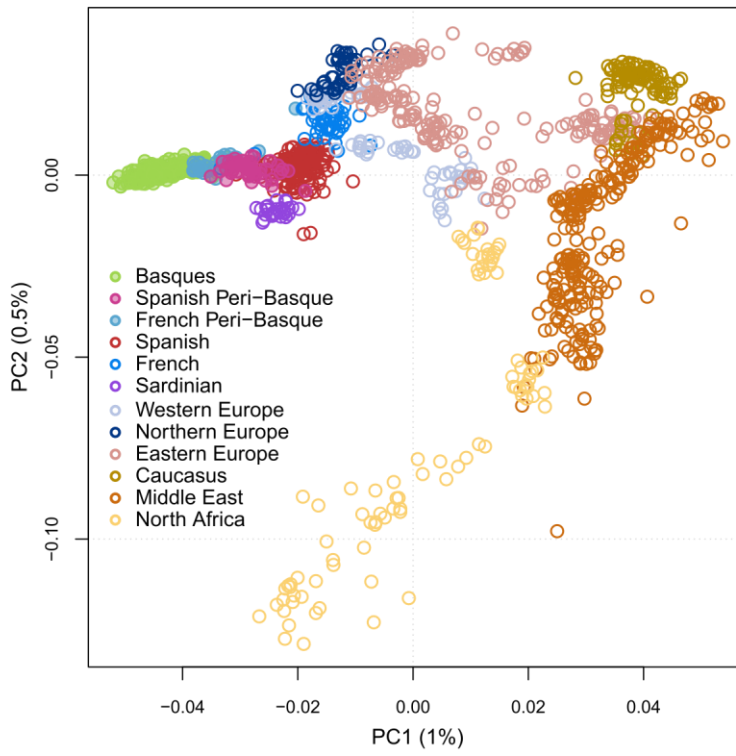
853 **Figure 1**
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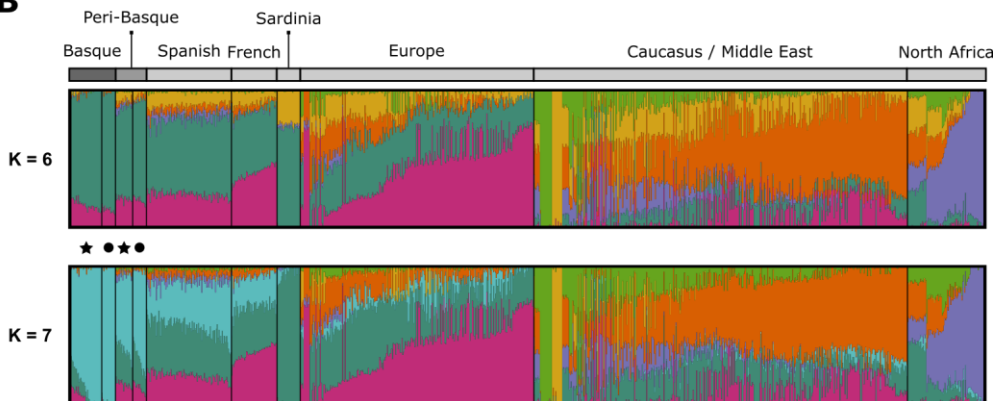
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858 **Figure 2**
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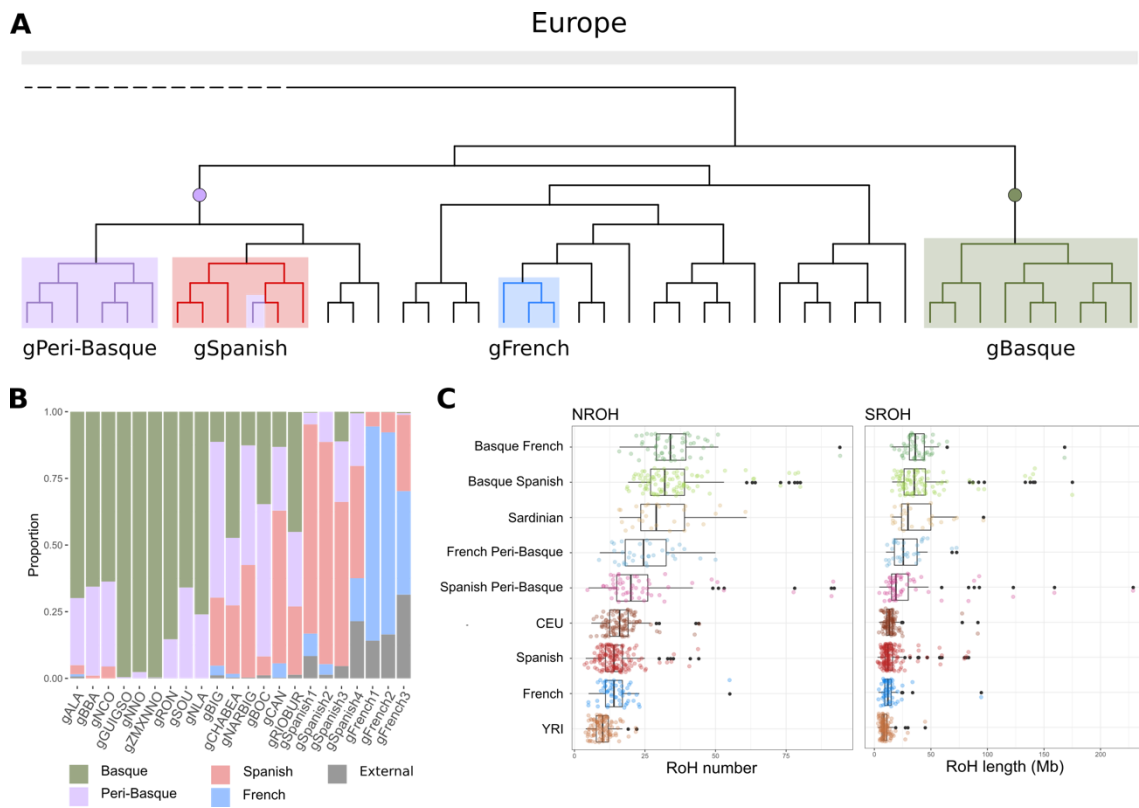


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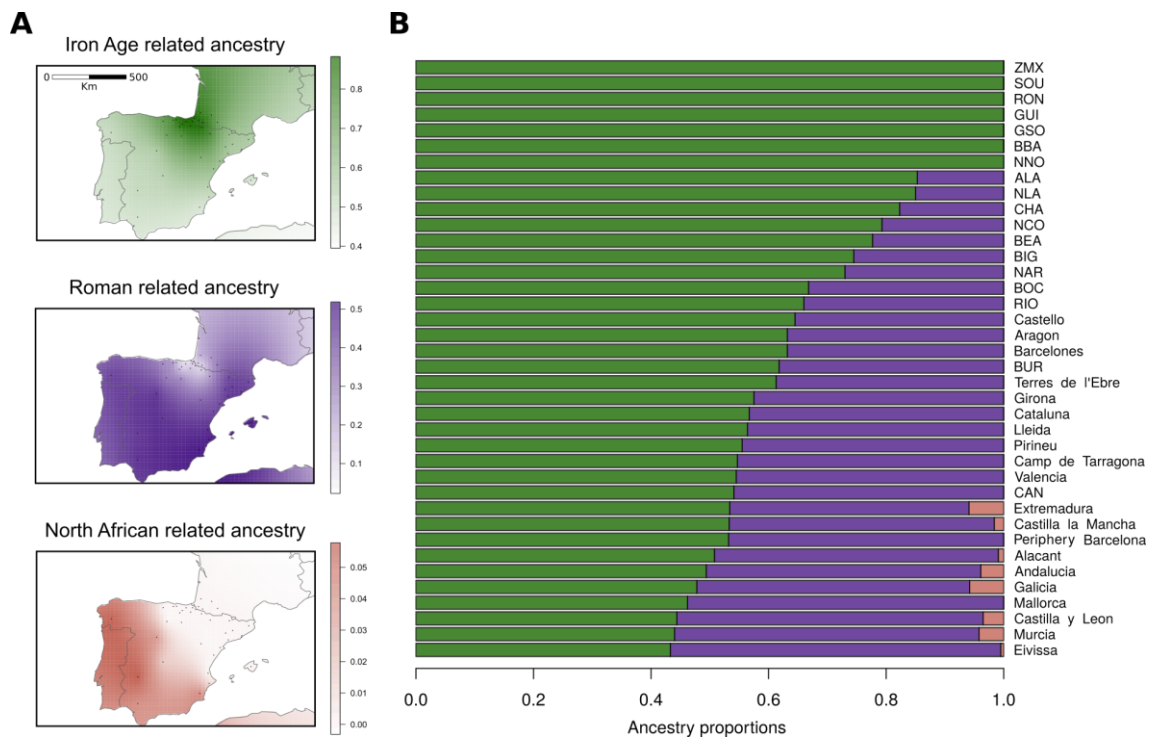


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864 **Figure 3**
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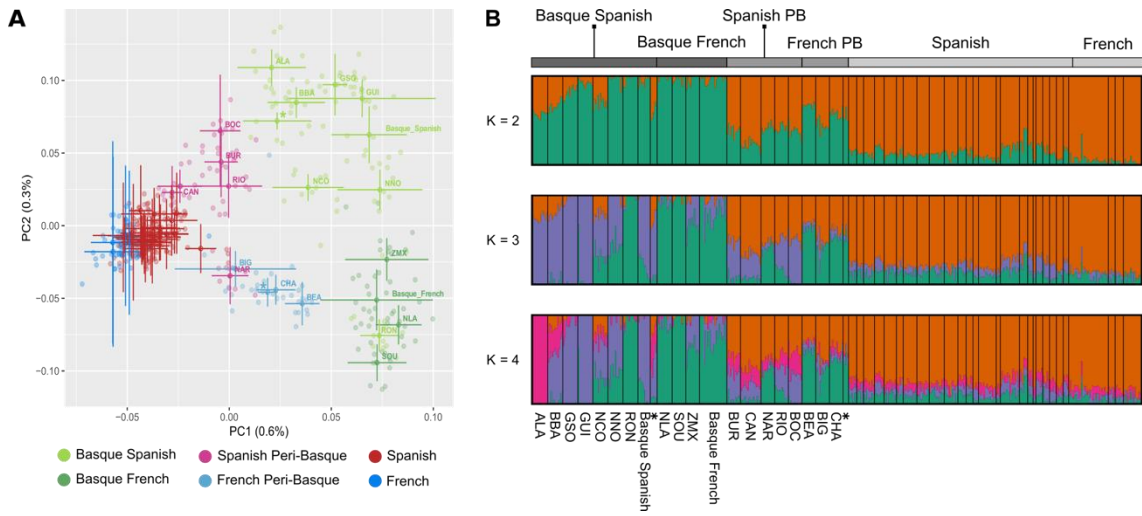
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 869 **Figure 4**
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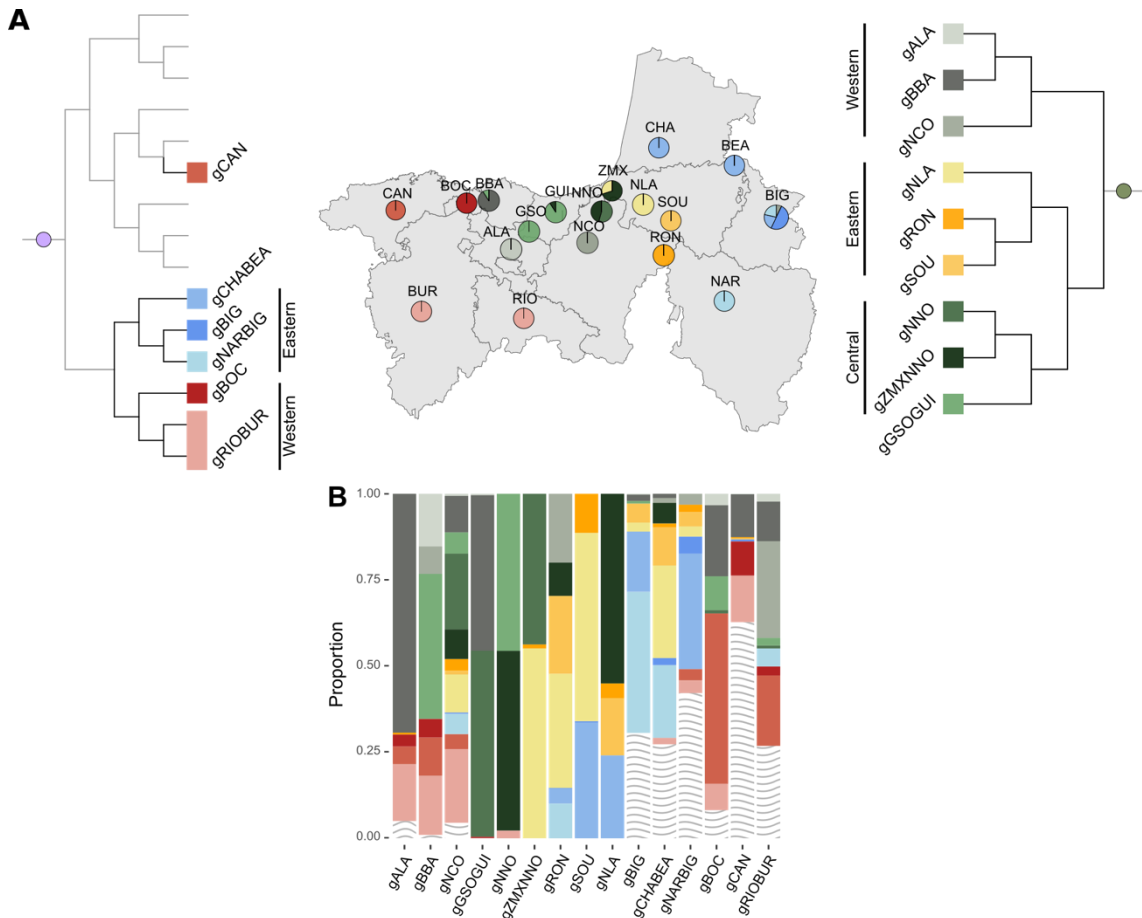
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Figure 5



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Figure 6



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