Naturalia Cantabricae 11(3): 41-52
ISSN 1577-1814 (versión impresa)
ISSN 1577-2756 (versión en línea)

Naturalia Cantabricae
http://www.indurot.uniovi.es/

Contribution to the knowledge of the distribution of *Alnus* species in southern Europe based on cpDNA

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Received: 23 October 2023 / Accepted: 31 October 2023 / Published online: 7 November, 2023

Sanna, M., González-Toral, C., Nava, H.S., Cuesta, C., Loidi, J., Herrera, M., Rodríguez-Guitián, M.A., Bueno, Á, Fernández Prieto, J.A. & Cires, E. 2023. Contribution to the knowledge of the distribution of *Alnus* species in southern Europe based on cpDNA. *Naturalia Cantabricae 11*(3): 41-52

ABSTRACT: In the Iberian Peninsula occur two species previously systematized such as *Alnus glutinosa*: *Alnus glutinosa* s.s. (diploid) in the North-East and *A. lusitanica* (tetraploid) in the West. Other studies based on the analysis of cpDNA, reveal haplotypes characteristic of diploids and tetraploids species, turning these markers into a good tool for discriminating *Alnus* species. However, neither all the northern territories of the Iberian Peninsula, where *A. glutinosa* s.s. or *A. lusitanica* grow, nor the *Alnus* populations of Sardinia Island (Italy), were considered in these studies. Our aims are a first genetic characterization of Sardinian alders and a detailed overview of the Iberian *Alnus* species distribution using the *ndhf-rpl32* plastid region. The *Alnus lusitanica* holotype from Tormes River, (Salamanca, Spain) shared the same haplotype detected in all the Western Iberian samples until Asturias, Cantabria and the Ebro River. Between Asturias and Cantabria regions, into the distribution area of *A. lusitanica* were found samples with the characteristic haplotype of *Alnus glutinosa* from Central and North Europe. The Sardinian black alder sample exhibited a variation of *A. glutinosa* haplotype, previously detected in North Central Africa (Algeria and Tunisia) and Corsica island (France), suggesting some genetic relationships throughout these populations.

KEYWORDS: Alders, cpDNA, Haplotypes, ndhF-rpl32, Phylogeography, Plant systematics

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RESUMEN: En la Península Ibérica medran dos especies previamente sistematizadas como *Alnus glutinosa*: *Alnus glutinosa* s.s. (diploide) en el Noreste y *Alnus lusitanica* (tetraploide) en el Oeste. Otros estudios basados en el análisis del cpDNA, revelan haplotipos característicos de las especies diploides y tetraploides, convirtiendo estos marcadores en una buena herramienta para discriminar las especies de *Alnus*. Sin embargo, ni todos los territorios del norte de la Península Ibérica, donde crecen *A. glutinosa* s.s. o *A. lusitanica*, ni las poblaciones de *Alnus* de la isla de Cerdeña (Italia), fueron considerados en estos estudios. Por lo tanto, nuestros objetivos son una primera caracterización genética de los alisos de Cerdeña y una visión detallada de la distribución de las especies ibéricas de *Alnus* utilizando la región plastidial *ndhf-rpl32*. El holotipo de *Alnus lusitanica* del río Tormes, (Salamanca, España) comparte el mismo haplotipo detectado en todas las muestras del oeste ibérico hasta Asturias, Cantabria y el río Ebro. Entre Asturias y Cantabria, dentro del área de distribución de Alnus lusitanica, se encontraron muestras con el haplotipo característico de *Alnus*

glutinosa del centro y norte de Europa. La muestra de aliso de Cerdeña presenta una variación del haplotipo de *A. glutinosa*, detectada previamente en el norte de África Central (Argelia y Túnez) y en la isla de Córcega (Francia), lo que sugiere ciertas relaciones genéticas entre estas poblaciones.

PALABRAS CLAVE: Alisos. cpDNA. Haplotipos. ndhF-rpl32. Filogeografía. Sistemática de plantas.

1. INTRODUCTION

Alnus Mill. is a genus of the Betulaceae family which includes 41 species distributed throughout the temperate regions of the Northern Hemisphere and only in the Andes in the Southern Hemisphere (POWO 2023). Currently, six native *Alnus* accepted species are present in European territories sensu Brummit (2001): *Alnus cordata* (Loisel.) Duby, present in the SE; *Alnus viridis* (Chaix) DC., which occurs in C and NE Europe; *Alnus glutinosa* (L.) Gaertn., which is present in almost the whole of Europe and N of Africa (except SW of the Iberian Peninsula and Morocco); *Alnus incana* (L.) Moench, native to N and C Europe; *Alnus lusitanica* Vít, Douda & MandáK, which occurs in Spain, Portugal and Morocco; and *Alnus rohlenae* Vít, Douda & Mandák, found in the western part of the Balkan Peninsula (Euro+Med 2006-2022; Vít *et al.* 2017).

Alnus glutinosa s.l., commonly known as black alder, is distributed from the Scandinavian peninsula and Russia in the North, to the Mediterranean countries and parts of North Africa in the South, however, recent cytological and genetic studies have revealed the existence of diploid and tetraploids populations across this species (Lepais *et al.* 2013; Mandák *et al.* 2016; Vít *et al.* 2017). Consequently, two new geographically well-defined tetraploid species derived from *A. glutinosa* s.l. populations were described by Vít *et al.* (2017). The first one is *A. lusitanica* Vít, Douda & Mandák, which occurs frequently in flooded ash-alder riparian, seepage, and wetland forests of the West Iberian Peninsula and Morocco, and the second one is *A. rohlenae* Vít, Douda & Mandák, found in the Balkan Peninsula. (Vít *et al.* 2017). On the other hand, the diploid *A. glutinosa* sensu Vít *et al.* (2017) would occur in Central Europe, with the Spanish Basque Country as its South-West limit.

Havrdová *et al.* (2015) studied the genetic diversity of *A. glutinosa* s.l. across European territories, using the *ndhF-rpl32*, *psbJ-petA,* and *3'rps16-5'trnK* intergenic spacers plastid markers, and nuclear microsatellites identifing the characteristic cpDNA haplotypes of diploids and tetraploids (Fig. 1). These molecular markers proved to be a good tool to discriminate *Alnus* species. More recently, Šmíd *et al.* (2020) first, uncover the presence of triploid individuals using plastid markers and microsatellites in the area where the diploid *A. glutinosa* s.s. is sympatric with the Balkan tetraploid *A. rohlenae,* and than (Šmíd *et al.* 2020) used cytometry to define the the new triploid distribution using flow cytometry and morphometrics analysis. In the Iberian Peninsula there is a lack of information about the distribution of *A. glutinosa* and *A. lusitanica* in the Iberian northern territories, where populations come into contact with each other. This area, between Spanish Basque Country and Galician territories, was proposed as a temperate refugia by Tzedakis *et al.* (2013). This study is a key point to understand the origin and the recolonization process of the two Iberian *Alnus* species. However, although various genetic studies about the intraspecific and specific variability of European and North African alders have been conducted (Havrdová *et al.* 2015; Vít *et al.* 2017; Mandák *et al.* 2016; King and Ferris 1998), some Southern European territories as the North, South and East of the Iberian Peninsula or Sardinia have not been studied properly.

In this work, a genetic study of Iberian populations of *Alnus* spp. from areas not included in previous works was carried out. The study area was defined to complete the Havrdová *et al.* (2015) sampling in the Iberian Peninsula, including southern, central and northern territories. In addiction, samples from the Mediterranean island of Sardinia where included in the study, because not genetic studies were done in *Alnus* spp. from these territories and this is an opportunity to fill a gap in the knowledge of the Mediterranean alders. Havrdová *et al.* (2015) detect the same haplogroup in the Corsican and in the Central North of Africa but not samples were studied from Sardinia that is geographically positioned between these territories.

The aim of this study is a genetic characterization, with plastid DNA secuences, of the new studied populations of *Alnus* species of southern europe for a better knowledge of their chorology. From this work, it will be possible to evaluate which are the most interesting areas to continue studying the alder populations in the Iberian Peninsula and to apply techniques of flow cytometry to detect ploidy levels in sympatric populations, if detected.

In the Iberian conservational context, the detection of overlapping territories of *A. glutinosa* and *A. lusitanica* will be very useful for the management of riparian habitat 91E0* "Alluvial forests with *Alnus glutinosa* and *Fraxinus excelsior* (*Alno-Padion*, *Alnion incanae*,

Salicion albae)" of Natura 2000 network, included as a priority habitat in Annex I of Council Directive 92/43/EEC. In habitat restouration the knowledge about genetic diversity of species used for plantation, is a key point to choose the sources of plant material and avoid the mistakes of new introductions.

In this context, we aim to genetically characterize *Alnus* spp. samples from the Iberian Peninsula and Sardinia, using the chloroplastic *ndhF-rpl32* intergenic spacer region to: i) contribute to the knowledge of the chorology of *A. lusitanica* and *A. glutinosa* s.s. in the Iberian Peninsula, ii) discover the contact zones between the two species present in the Iberian Peninsula and iii) reveal for the first time the haplotype of an *A. glutinosa* Sardinian population.

2. MATERIAL AND METHODS

2.1. Plant material and sampling

We collected foliar tissue from 52 black alder individuals sampled throughout a total of 50 localities (Figure 1). These localities comprised the North Atlantic region of the Iberian Peninsula (from Galicia to Basque Country, Spain), the Central Iberian Peninsula (Salamanca (CW Spain)), the South East and South West Iberian Peninsula (Sierra Nevada and Huelva, respectively) and Sardinia (Italy) (Annex 1). Leaves were dehydrated and conserved in silica gel for further molecular study. Additionally, a voucher for herbarium specimens for each population was held in the Herbarium FCO (University of Oviedo, Spain) (Annex 1).

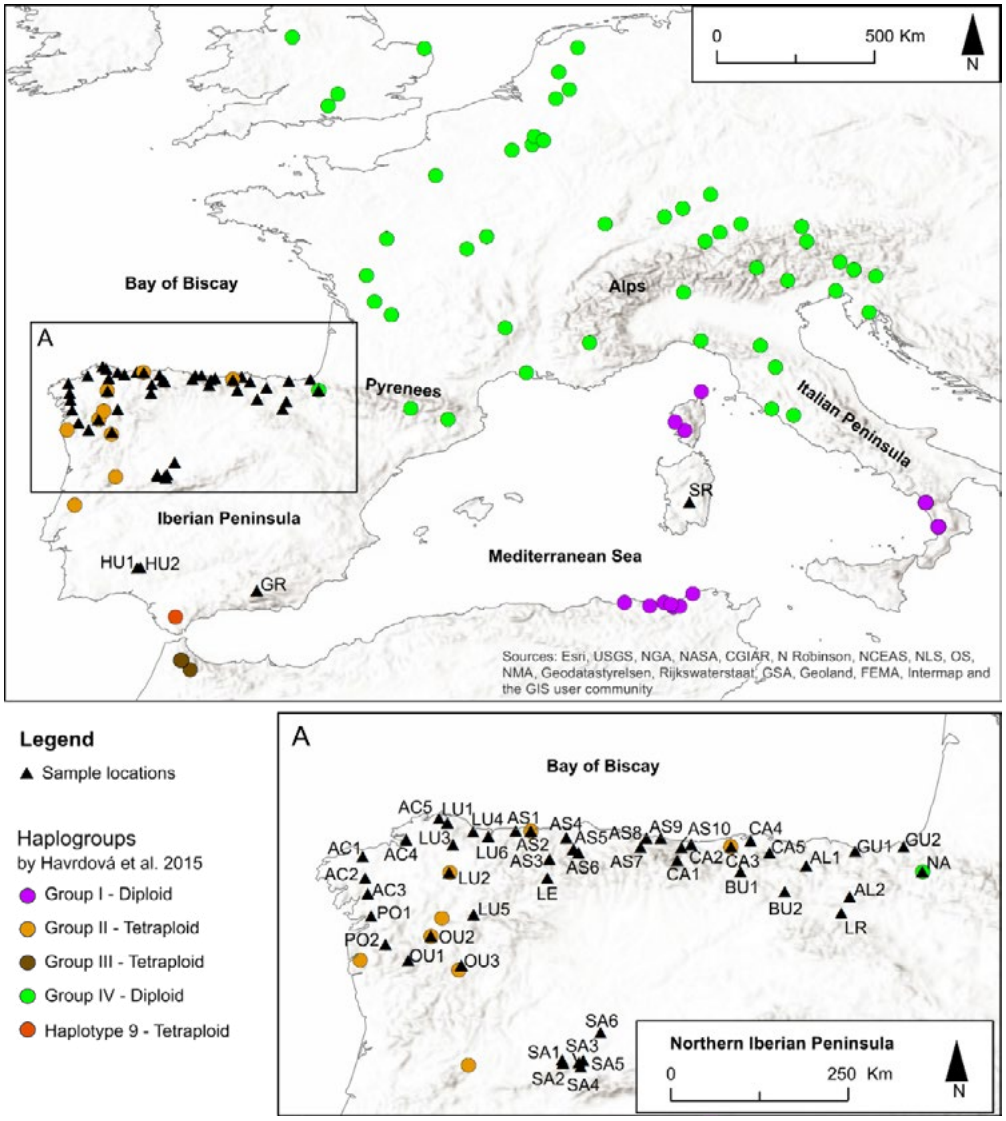

Figure 1. Locations of *Alnus* s.l. populations sampled for this study (black triangles). Circles of diferent colors represent the haplogroups and haplotypes detected by Havrdová *et al.* (2015).

Figura 1. *Localidades de las poblaciones de* Alnus *s.l. muestreadas para este estudio (triángulos negros). Los círculos de diferentes colores representan los haplogrupos y haplotipos detectados por Havrdová* et al. *(2015).*

2.2. DNA extraction, amplification and sequencing

The total genomic DNA of each sample was extracted from 20 mg of dried tissue using DNeasy® Plant Mini Kit (Qiagen, Germany), following the protocol modifications of Sanna *et al.* (2019). DNA concentration and quality were estimated by horizontal electrophoresis in 1 % agarose gels and 0.5 µg mL^{-1} ethidium bromide, using digested lambda DNA (New England Biolabs) as a marker. We amplified the *ndhF-rpl32* intergenic spacers region using Shaw *et al.* (2007) and following the amplification protocol of Havrdová *et al.* (2015). PCR product sequencing was performed at the DNA Synthesis and Sequencing Facility Macrogen (Madrid, Spain) using 'Big Dye Terminator' cycle sequenced and analysed on an ABI 3730XL automated sequencer (Perkin-Elmer; Foster City, CA, USA).

2.3. Phylogenetic and network analysis

All the newly generated 52 *ndhF-rpl32* sequences plus the 42 haplotype sequences from Havrdová *et al.* (2015) were assembled and edited using the software MEGA X (Kumar 2018). The sequences were automatically aligned by MUSCLE method (Edgar 2004), as implemented in MEGA X, and manually edited. A sequence of *Alnus alnobetula* retrieved from GenBank (KP244652) was used as outgroup in the phylogenetic analyses. The Bayesian Markov Chain Monte Carlo (MCMC) method was used to construct a Bayesian Inference (BI) phylogenetic tree, using MrBayes v3.2.6 (Huelsenbeck & Ronquist 2001; Ronquist *et al.* 2012) as implemented in XSEDE of CIPRES Scientific Gateway (Miller *et al.* 2010). The analysis consisted of two independent runs of four chains, running for 1,600,000 generations with a sampling frequency of 1,000 and a burnin fraction of 0.25. The topology of a 50% Majority Rule consensus tree was used to represent the evolutionary relationships among the samples and Posterior Probability (PP) was used as a method to estimate the statistical branch support. In addition, to ensure the robustness of the phylogenetic estimates, a Maximum Parsimony (MP) and a Maximum Likelihood (ML) analysis were conducted. The MP analysis was performed in MEGA X (Kumar, 2018) and consisted of 10,000 bootstrap replications obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with a MP search level of three. The ML phylogeny tree was generated using the stochastic algorithm of IQ-TREE (Nguyen *et al.* 2015) implemented in the online phylogenetic tool W-IQ-Tree (Trifinopoulos *et al.* 2016) and 1000 bootstrap replicates. Furthermore, an unrooted haplotype network was constructed by statistical parsimony network estimation implemented in the .

3. RESULTS

3.1. Sequences characteristics

The alignment of the 94 sequences of *ndhF-rpl32* region (52 own sequences + 42 sequences from Havrdová *et al.* (2015)) was 926 positions length. The shortest sequences were 911 bp long and the longest were 922 bp. There were 896 conserved sites, 19 variables sites (defined as sites containing at least two types of nucleotides), 12 parsimony informative sites (defined as sites containing at least two types of nucleotides, having at least two of them a minimum frequency of two) and 7 singletons (defined as sites which contain at least two types of nucleotides with, at most, one occurring multiple times). Across the alignment we identified four microindels (sensu Gonzalez *et al.* 2007) of 34, 11, 10 and 10 positions.

3.2. Phylogenetic and network analysis

The phylogenetic tree based on the plastid *ndhF-rpl32* region, inferred by Bayesian Inference (BI) was used to define the topology of the tree of Figure 2. With a cut-off of 0.7 Posterior Probability (PP), the condensed tree shows ten clades, however, not all the nodes are supported by bootstrap values (BS) greater than 50%, retrieved by the Maximum Parsimony (MP) and Maximum Likelihood (ML) analysis.

The basal clade is composed of two subclades, the Bulgarian and Balkan Peninsula (*A. rohlenae*) groups. This clade shows a high value of PP (0.95), but it is not supported by the BS of MP and ML analysis. The rest of the haplotypes from Havrdová *et al.* (2015) are all included in a heterogeneous clade together with all the sequences used in this work and supported by a PP of 0.86 and a ML of 66, but not supported by the bootstraps of MP. The sample from Sardinia (SR) is included in the diploid Mediterranean Group I, with characteristics haplotypes from CN Africa and Corsica island but not with southern Italy Haplotypes H14 and H15, as in Havrdová *et al.* (2015) in this case they form a subclade of the Group II.

The samples from the N of the Iberian Peninsula are included in three clades: Group II, III, and IV. Group II comprises all the samples of NW of Iberian Peninsula and the haplotypes H10 and H11 found by Havrdová *et al.* (2015), considered *A. lusitanica* tetraploids *sensu* Vít *et al.* (2017). Group II also comprises six samples from Tormes River (SA1–SA6), the location of the holotype of *A. lusitanica*, close to Salamanca. The eastern geographical limit of this group is defined by samples LR and BU2 from La Rioja and Burgos respectively, both from

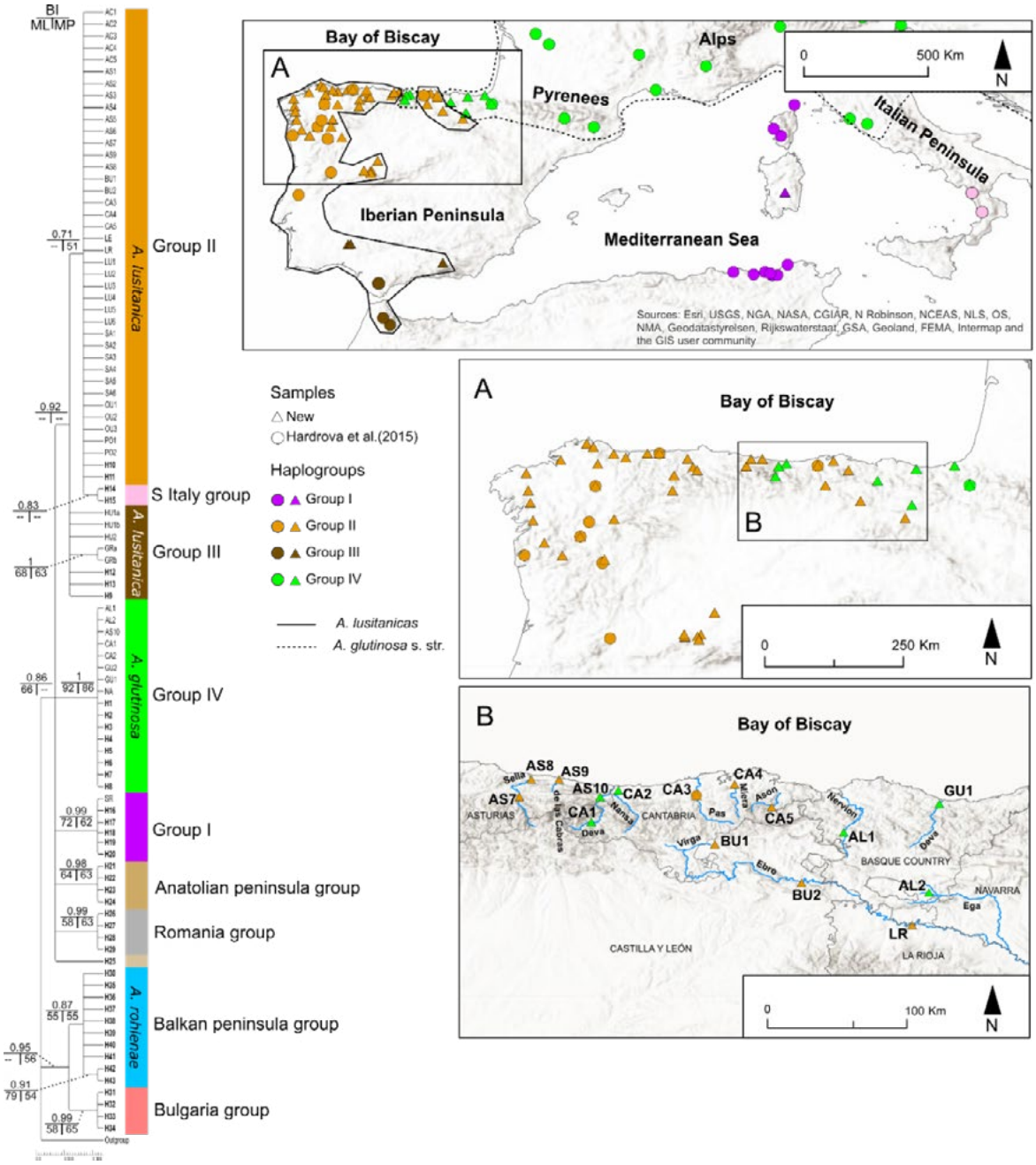

Figure 2**.** Bayesian Inference tree of European *Alnus* populations resulting from MrBayes phylogenetic analysis based on *ndhF-rpl32* plastid marker. The numbers depicted in the tree represent the support values obtained during the phylogenetic analyses. Specifically, the numbers above the branches indicate the Posterior Probability (PP) from the Bayesian Inference (BI) analysis, while the bootstrap values are derived from the Maximum Likelihood (ML) and Maximum Parsimony (MP) analyses. On the right side of the tree, each color corresponds to a specific haplogroup, as depicted on the maps. It is worth noting that the Anatolian Peninsula group, Romania group, Haplotype H25, Balkan Peninsula group, and Bulgaria group are not represented in the maps.

Figura 2. Árbol de inferencia bayesiana de las poblaciones europeas de Alnus *spp. resultante del análisis filogenético MrBayes basado en el marcador plastidial* ndhF-rpl32*. Los números incorporados en el árbol representan los valores de soporte obtenidos durante los análisis filogenéticos. En concreto, los números sobre las ramas indican la Probabilidad Posterior (PP) del análisis de Inferencia Bayesiana (BI), mientras que los valores bootstrap se derivan de los análisis de Máxima Verosimilitud (ML) y Máxima Parsimonia (MP). En la parte derecha del árbol, cada haplogrupo se identifica con el mismo color representado en los mapas. Cabe señalar que el grupo de la península de Anatolia, el grupo de Rumanía, el haplotipo H25, el grupo de la península de los Balcanes y el grupo de Bulgaria no están representados en los mapas.*

the Ebro River (Figure 2 B). BU1 sample from Virga River, a tributary of Ebro River is part of group II as all the samples CA3–CA5 from Cantabria, belonging all of them to three different rivers that flow to the Bay of Biscay.

The second Iberian haplogroup is the Group III comprises all the southern samples, including haplotypes H9 from the populations of Jerez de la Frontera, haplotypes H12 and H13 from Morocco studied by Havrdová *et al.* (2015), and the samples from Sierra Nevada (GR) and Huelva (HU1 and HU2).

Thanks to these results, we can define in a better way the chorology of the tetraploid *A. lusitanica,* including two new genetic subgroups. The Southern subgroup (Group III) is from Morocco to Huelva and Sierra Nevada and the Northern (Group II) is from the North of Districto de Santarem (Portugal) to the Rio Ebro, close to Logroño (Spain) but its distribution is not continuous across these territories.

Populations showing the characteristic haplogroup of diploid *A. glutinosa* (Group IV) sensu Havrdová *et al.* (2015) were detected in the Deva (14 and 15) and Nansa (13) Rivers, across the frontier between Principality of Asturias and Cantabria. These *A. glutinosa* populations are inside the distribution area of *A. lusitanica* and the closest *Alnus glutinosa* populations are from the Nervión River (Álava, Basque Country) at more than 120 km of distance. From there to the east, only populations of *Alnus glutinosa* (Group IV) has been detected in the Basque country and Navarra.

A total of 20 haplotypes are recognised and graphically represented in the haplotype network of Figure 3, constructed with the software TCS. Haplogroups were defined using the clades of the phylogeny tree (Figure 2). Haplogroups I–IV are well defined, being all connected to haplotype H25, a rare haplotype from a population of the Balkans. The Romania group, the S Italy group and the Alnus groups from Eastern Europe are also connected to haplotype H25 from Balkan peninsula. The diploid Mediterranean Group I, shows 4 haplotypes and the sequence of sample SR from central Sardinia is identical to the haplotypes H16 and H20 of Havrdová *et al.* (2015), found in populations of Corsica and CN of Africa.

The samples of *A. lusitanica* are all included in Group II and III with two and four haplotypes, respectively. Group III includes new haplotypes from Sierra Nevada (GR), and Huelva (HU1 and HU2) populations, differing from Morocco and Jeréz de la Frontera ones, being, however, in the same haplogroup. Group IV comprises only one haplotype. Finally, the Anatolian Peninsula Group is more genetically similar to the western haplotypes, compared with the Balkan Peninsula and the Bulgaria group. It should be noted that group IV of *A. glutinosa* is less diverse than the other haplogroups, although it is represented by a much larger number of samples.

2. DISCUSSION

The use of *ndhF-rpl32* intergenic spacer region as a molecular marker effectively discriminated the intraspecific and specific groups of European alders. The variability of *ndhF-rpl32* region is not enough to form the same groups as in Havrdová *et al.* (2015), who used more plastid markers. Nevertheless, its sole use discriminated the samples that formed the same haplogroups except for the Southern Italy haplotypes. The results of the *ndhF-rpl32* analysis help us to define the distribution area of Iberian *Alnus* species, in that regions not considered in previous works.

First of all, the Salamanca populations (Tormes River), holotype of *A. lusitanica,* shows the same haplotype as all the individuals from western Iberian populations studied by Havrdová *et al.* (2015). This information is useful to establish the characteristic haplotypes, until the contrary is proven, of teraploid group II, and the variant of group III, as *A. lusitanica* specific and assume that all the individuals with that haplotype are *A. lusitanica* tetraploids. On the other hand, all the individuals with a haplotype belonging to haplogroup IV, including *A. glutinosa* sensu Havrdová *et al.* (2015), are diploids and, therefore, *A. glutinosa*.

With the results of this study, it has been possible to define the *A. lusitanica* and *A. glutinosa* distribution areas that intersect in the central area of N of the Iberian Peninsula, from the Atlantic region between the E of Asturias to the W of the Basque Country. According to our studies, the two species have not been found in the same river, however, a more exhaustive study of this region is necessary to confirm the existence of mixed populations. In this sense, a new ploidy study focused on individuals from these areas would be fundamental to unveiling the level of admixture between diploid and tetraploid species and uncovering the existence of triploid individuals as in the case of the diploid *A. glutinosa* s.s. and tetraploid *A. rohlenae* co-occur in the Balkans. In this sense, we found individuals of tetraploid species *A. lusitanica* (Group II) in the three samples from the Ebro River, however, individuals of the diploid *A. glutinosa* s.s. (Group IV) were detected in the tributary Ega River. These results suggest that the downstream areas of the Ega River, where it flows to the Ebro River (in the northern region of Navarre) should be analysed as it seems likely to be a contact area between the diploids and the tetraploids. The alders from Deva and Nansa river have been for the first time, genetically characterized as *A. gluti-*

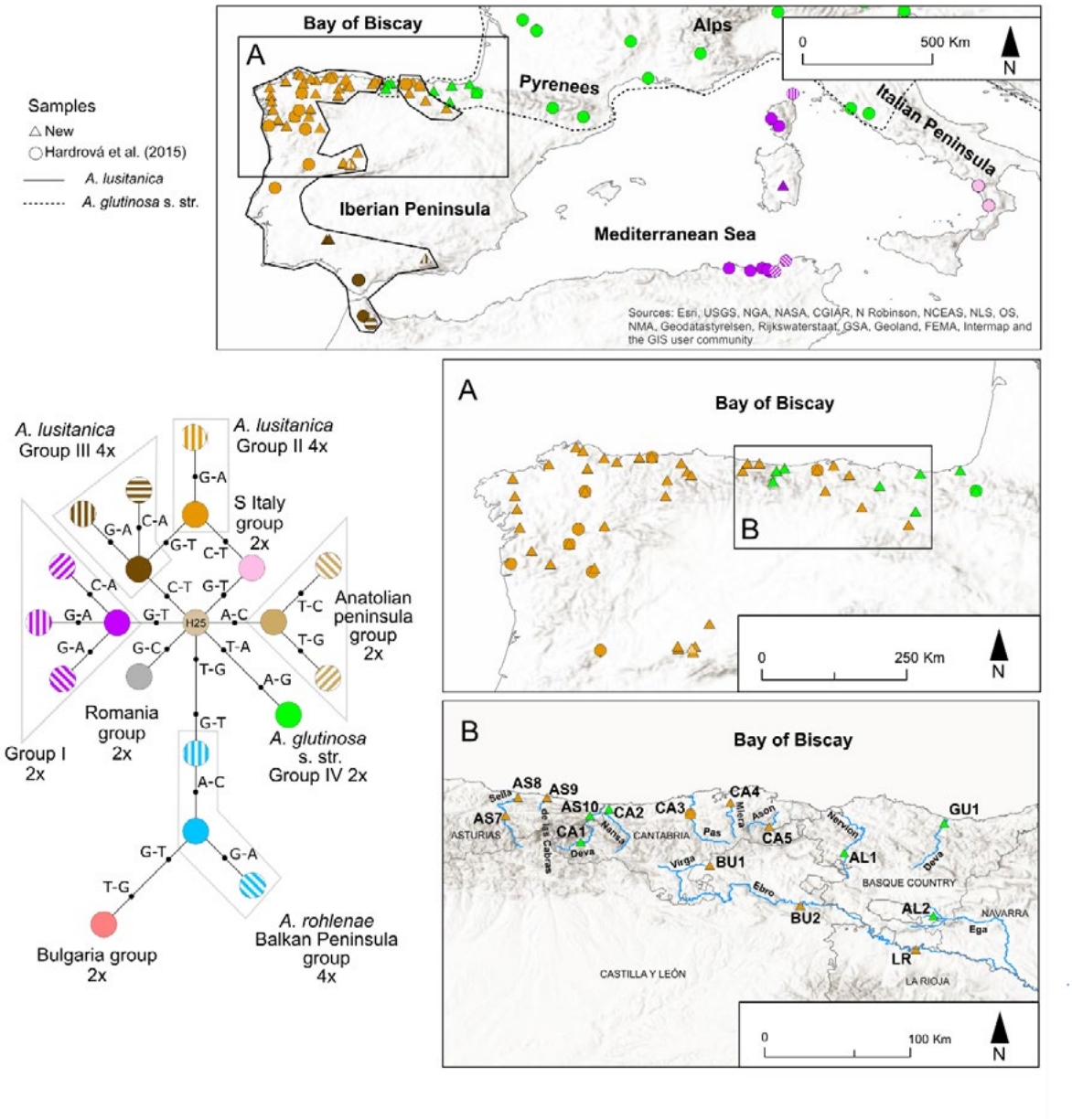

Figure 3. The TCS Haplotype network is constructed using the ndhF-rpl32 plastid marker. In this network, gaps are treated as missing data. Each haplogroup has been assigned ploidy levels based on information provided by Havrdová *et al.* (2015). The colors and patterns used in the map correspond to those in the haplotype network.

Figura 3. La red de haplotipos construida con el software TCS se creó utilizando el marcador de plástidos ndhF-rpl32. *En esta red, los gaps se tratan como datos faltantes. A cada haplogrupo se le han asignado niveles de ploidía basados en la información proporcionada por Havrdová* et al. *(2015). Los colores y patrones utilizados en el mapa corresponden a los de la red de haplotipos.*

nosa (Haplogroup II) but are completely surrounded by *A. lusitanica* populations. Maybe these rivers are the ancient area of origin of actual *A. glutinosa* European population and deserve to be studied in more detail. The Iberian peninsula is recognised as a southern European refugia supported and northern of Spain is the more western temperate tree refugia in agreement with the pollen and macrofossil data (Tzedakis *et al.* 2013). Similar postglacial migrations from Iberian peninsula has been studied by Palmé (2002) in *Corylus Avellana,* another riparian forest tree species that has probably had a similar historical course. On the other hand tetraploid relict populations have high ecological and evolutionary values and deserve a high priority conservation status (Lepais *et al.* 2013).

In light of the current state of knowledge, we can postulate an autopolyploid origin from the diploid *A. glutinosa* s.s. populations from North of the Iberian Peninsula or the diploids of *A. glutinosa* populations of Northern Africa (Group I). that first, origins of *A. lusitanica* tetraploid populations of Morocco and south of Iberian Peninsula of basal Group III and then the tetraploids of the more recent group II (Figure 2). On the other hand, an allopolyploid origin of haplogroups II and III is possible if we consider the existence of an ancestral *A. glutinosa* diploid haplogroup in the south of *A. lusitanica* distribution area, maybe occupying the North African diploid group I actual distribution area. Regarding the diploid *A. glutinosa* of Group I and IV, an allopolyploid origin is less probable, as we found no evidence suggesting the geographical convergence of these two groups and the areas where the group I could theoretically grow (Mediterranean part of the Iberian Peninsula) are poor in *Alnus* populations. These deductions should be confirmed by flow cytometry analysis, also considering that in other studies *Alnus* species with different ploidy, share the same haplotypes (Šmíd *et al.* 2020).

Interestingly, the genetic data of the Sardinian sample give further support to the continuity of Group I, also found in Corsica and the North of Africa. These Mediterranean populations could represent a variety or a subspecies of diploid *Alnus glutinosa* s.s. ecologically adapted to the central Mediterranean climate and geographically isolated from northern European populations. Antonio Bertoloni, who studied the alders at the specific level, described the alders from Sardinia as very branched, with small leaves and catkins (Bertoloni 1854). Bertoloni taxonomized this plant as *Alnus morisiana* Bertol., Fl. Ital. [Bertoloni] x. 163, treatment which could be reconsidered valid, however, a more comprehensive study of genetic and morphological traits of Sardinian alders is needed. Our results also indicate that Southern Italy group is a subclade of Group II, nevertheless, we believe that this relationship should not be taken into account as it is incongruent with the results of Havrdová *et al.* (2015); who, using additional cpDNA markers, found this population to be more genetically linked with Mediterranean haplotypes of group I than the group II.

The tree layer of habitat 91E0* (Alluvial forests with *Alnus glutinosa* and *Fraxinus excelsior* (*Alno-Padion*, *Alnion incanae*, *Salicion albae*)) is dominated, among other riparian species, by *Alnus glutinosa s. l.* and in the last decades it is suffering fast dieback, called alder disease, caused by the oomycete *Phytophthora alni* complex (Bjelke *et al.* 2016). Projects aimed at the conservation of this habitat has been developed, such as the LIFE Alnus project (Restoration, conservation and governance of the *Alnus* alluvial forests in the Mediterranean Region, https://lifealnus.eu) improving the conservation status, structure and associated biodiversity of pre-existing alluvial forest. In this context, the knowledge of the genetic diversity of European *Alnus* species, both at large and small scales, is an important tool for riparian habitat conservation and restoration actions, because of a phytopathological situation that worsens. Species with smaller distribution areas are the ones that would be most compromised in the event of a massive expansion of the pathogen since they would have fewer source areas for repopulation. Furthermore, genetic phylogeographical data will be of relevance in the study of the spread of alder disease in the correlation of each different haplotypes groups and maybe it will be possible the detection of some resistant populations. Defining with molecular markers the populations that could be used for habitat restoration, the results of this work are a practical tool for alluvial forest restoration projects, especially for the regional governments, but also at Iberian Peninsula level, better defining which are the populations that could be used as plant material source for habitat restoration.

3. CONCLUSIONS

The tetraploid *Alnus lusitanica* spreads from Morocco to NE of Iberian Peninsula, only interrupted by diploid *Alnus glutinosa* s.s. populations from W of principality of Asturias and Cantabria. The NE geographical limit of *A. lusitanica* is the Ebro River, which separates this species from the more widespread *A. glutinosa* s.s. Triploid individuals are most likely to appear in the contact zones between *Alnus lusitanica* and *A. glutinosa* range from the E part of Ebro river basin and from de E of Principality of Asturias to W and S of Basque Country. The Sardinian alder studied in this work, shares the same haplotype with the Corsican and CN African diploid black alders, unreveling their genetic relationship.

Despite the importance of geography and niche to the diversification in southern Europe demonstrated by our analyses, these results would be strengthened by the inclusion of additional data, especially in sympatrics areas between *A. glutinosa* and *A. lusitanica*.

ACKNOWLEDGEMENTS:

We would like to thank all people who collected samples of *Alnus* species or helped us with the manuscript, especially Javier Amigo Vázquez and Jesús Rojo Úbeda. This study was was partially supported by the research program (PAPI-19-GR-2016-0010) funded by the University of Oviedo.

DECLARATIONS:

Funding: This research was partially supported by the research program (PAPI-19-GR-2016-0010) funded by the University of Oviedo.

Availability of data and material: All cpDNA sequences were deposited in GenBank under Accession OR756478-OR756529

Authors' contributions: Conceptualization: JAFP, HSN, EC. Sampling: JAFP, HSN, EC, CC, JL, MH, MARG, AB. Methodology: MS, JAFP, CGT, EC. Formal analysis and investigation: MS, JAFP, EC. Writing-original draft preparation: MS. Writing-review and editing: all authors. Funding acquisition: JAFP, EC. Resources: JAFP, EC. Supervision: JAFP, EC.

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ANNEXES

Annex 1. Populations of *Alnus* spp. sampled for molecular analysis and GenBank accession numbers

Anexo 1. Poblaciones de Alnus *spp. muestreadas para análisis moleculares y números de acceso de GenBank*

Code	Locality, Province, Country, Altitude (Alt.) and UTM coordinates	Collectors	Voucher	Genbank numbers
AC1	Anllóns River, Carballo, A Coruña, Spain. Alt. 99 m, 29T 525164 4784153	J. Amigo	FCO-38281	OR756478
AC2	Tambre River, Vilouta, A Coruña, Spain. Alt. 157 m, 29T 527929 4753207	J. Amigo	FCO-38280	OR756479
AC3	Ulla River, Rego da Manga, A Coruña, Spain. Alt. 10 m, 29T 530419 4731063	J. Amigo	FCO-38279	OR756480
AC4	Eume River, Chao de Ombre, A Coruña, Spain. Alt. 5 m, 29T 570108 4808417	J. Amigo, J.A. Fernández Prieto, A. Bueno	SANT-75602	OR756481
AC5	Sor River, Por da Tronca, A Coruña, Spain. Alt. 78 m, 29T 603735 4837966	M.A. Rodríguez Guitián	FCO-41012	OR756482
AL1	Nervión River, Luiaondo, Álava, Spain. Alt. 174 m, 30T 499746 4770895	N. Villar	BIO-51422	OR756483
AL2	Izki River, Corres, Álava, Spain. Alt. 692 m, 30T 545540 4727084	D. Liendo, D. García-Magro	BIO-52222	OR756484
AS1	Navia estuary, Navia, Asturias, Spain. Alt. 3 m, 29T 683367 4822607	J.A. Fernández Prieto, H. Nava	FCO-41013	OR756485
AS2	Negro River, Almuña, Asturias, Spain. Alt. 12 m, 29T 699219 4822600	J.A. Fernández Prieto, E. Cires, C. Cuesta	FCO-41014	OR756487
AS3	Somiedo River, Aguasmestas, Asturias, Spain. Alt. 393 m, 29T 719582 4784054	J.A. Fernández Prieto, E. Cires, C. Cuesta	FCO-41015	OR756488
AS4	Nalón River, Ferreras, Asturias, Spain. Alt. 26 m, 29T 736237 4814704	J.A. Fernández Prieto, E. Cires, C. Cuesta	FCO-41016	OR756489

Code	Locality, Province, Country, Altitude (Alt.) and UTM coordinates	Collectors	Voucher	Genbank numbers
AS5	Trubia River, San Andrés, Asturias, Spain. Alt. 129 m, 30T 257754 4799315	J.A. Fernández Prieto, E. Cires, C. Cuesta	FCO-41017	OR756490
AS6	Morcín River, La Carbayosa, Asturias, Spain. Alt. 572 m, 30T 262560 4793394	J.A. Fernández Prieto, E. Cires	FCO-41018	OR756491
AS7	Sella River, El Barredo, Asturias, Spain. Alt. 91 m, 30T 327225 4799064	J.A. Fernández Prieto	FCO-41019	OR756492
AS8	de Llovio stream, Llovio, Asturias, Spain. Alt. 8 m, 30T 333846 4811594	J.A. Fernández Prieto	FCO-41020	OR756493
AS9	de las Cabras River, San Antolín, Asturias, Spain. Alt. 9 m, 30T 348592 4811248	J.A. Fernández Prieto	FCO-41021	OR756494
AS10	Cares River, La Barquesa, Asturias, Spain. Alt. 23 m, 30T 370121 4797940	J.A. Fernández Prieto, M.J. Buñuelos, R. Arbesú	FCO-41022	OR756486
BU1	de Puntillera stream, Momañin, Burgos, Spain. Alt. 847 m, 30T 430925 4762575	J.A. Fernández Prieto	FCO-41023	OR756495
BU2	Ebro River, Frías, Burgos, Spain. Alt. 516 m, 30T 477151 4734098	I. García-Mijangos, N. Villar	BIO-51792	OR756496
CA1	Deva River, Congarna, Cantabria, Spain. Alt. 340 m, 30T 365153 4779683	J.A. Fernández Prieto, M.J. Buñuelos, R. Arbesú	FCO-41024	OR756497
CA2	Nansa River, El Salín, Cantabria, Spain. Alt. 8 m, 30T 379931 4802632	J.A. Fernández Prieto, M.J. Buñuelos, R. Arbesú	FCO-41025	OR756498
CA3	Pas River, Carandía, Cantabria, Spain. Alt. 37 m, 30T 421411 4798227	J.A. Fernández Prieto, M.J. Buñuelos, R. Arbesú	FCO-41026	OR756499
CA4	Miera River, Puente Agüero, Cantabria, Spain. Alt. 14 m, 30T 441812 4806194	M. Herrera	BIO-51419	OR756500
CA5	Asón River, Ramales de la Victoria, Cantabria, Spain. Alt. 79 m, 30T 461472 4789361	M. Herrera	BIO-51418	OR756501
GR	de Espique stream, La Peza, Granada, Spain. Alt. 1002 m, 30S 474402 4125148	J.A. Fernández Prieto, E. Cires	FCO-41027	GRa OR756502 GRb OR756503
GU1	Deba River, Astigarribia, Guipúzcoa, Spain. Alt. 18 m, 30T 550753 4791674	M. Herrera, N. Villar	BIO-51417	OR756504
GU2	Bidasoa River, Irún, Guipúzcoa, Spain. Alt. 9 m, 30T 601091 4798583	I. Salcedo	BIO-51421	OR756505
HU1	Múrtiga River, Galaroza, Huelva, Spain. Alt. 525 m, 29S 700516 4200095	H. Nava	FCO-41028	HU1a OR756506 HU1b OR756507
HU2	Affluent of de Guijarra stream, Cortelazor, Huelva, Spain. Alt. 501 m, 29S 708593 4202121	H. Nava	FCO-41029	OR756508

Code	Locality, Province, Country, Altitude (Alt.) and UTM coordinates	Collectors	Voucher	Genbank numbers
LE	Sil River, Villablino, León, Spain. Alt. 957 m, 29T 718257 4756578	J.A. Fernández Prieto	FCO-41030	OR756509
LR	Ebro River, Fuenmayor, La Rioja, Spain. Alt. 393 m, 30T 536680 4703519	M.J. Buñuelos	FCO-41031	OR756510
LU1	Landro River, Río Pedroso, Lugo, Spain. Alt. 5 m, 29T 612650 4832430	M.A. Rodríguez Guitián	FCO-41032	OR756511
LU2	Miño River, Lugo, Lugo, Spain. Alt. 370 m, 29T 616216 4762253	M.A. Rodríguez Guitián	FCO-41033	OR756512
LU3	de Sever stream, A Arnela, Lugo, Spain. Alt. 463 m, 29T 618799 4801568	M.A. Rodríguez Guitián	FCO-41034	OR756513
LU4	Masma River, O Barral, Lugo, Spain. Alt. 4 m, 29T 639363 4820305	M.A. Rodríguez Guitián	FCO-41035	OR756514
LU5	Sil River, Quiroga, Lugo, Spain. Alt. 239 m, 29T 642205 4703164	M.A. Rodríguez Guitián	FCO-41036	OR756515
LU6	Eo estuary, Ribadeo, Lugo, Spain. Alt. 5 m, 29T 655113 4813967	M.A. Rodríguez Guitián	FCO-41037	OR756516
NA	Arga River, Casa Olazar, Navarre, Spain Alt. 740 m, 30T 621359 4763478	M. Herrera, N. Villar	BIO-51420	OR756517
OU1	de Vilameá River, A Devesa, Ourense, Spain. Alt. 374 m, 29T 574047 4636291	M.A. Rodríguez Guitián	FCO-41038	OR756518
OU2	Arnoia River, Allariz, Ourense, Spain. Alt. 437 m, 29T 597598 4671073	M.A. Rodríguez Guitián, C. Real	FCO-41039	OR756519
OU3	Tamega River, Feces de Abaixo, Ourense, Spain. Alt. 360 m, 29T 630467 4630046	M.A. Rodríguez Guitián	FCO-41040	OR756520
PO1	Lérez River, As Pontes, Pontevedra, Spain. Alt. 21 m, 29T 534259 4699455	J. Amigo	FCO-38278	OR756521
PO2	Termes River, Sucarreira, Pontevedra, Spain. Alt. 27 m, 29T 549791 4659125	J. Amigo	FCO-38277	OR756522
SA1	Yeltes River, Zarzoso, Salamanca, Spain. Alt. 899 m, 29T 742354 4498373	J.A. Fernández Prieto	FCO-41041	OR756523
SA2	de la Barranca stream, El Cabaco, Salamanca, Spain. Alt. 942 m, 29T 743414 4494478	J.A. Fernández Prieto	FCO-41042	OR756524
SA3	del Cántaro source, Valdecarros, Salamanca, Spain. Alt. 985 m, 30T 253151 4494794	J.A. Fernández Prieto	FCO-41043	OR756525
SA4	Alagón River, San Esteban de la Sierra, Salamanca, Spain. Alt. 638 m, 30T 254494 4488786	J.A. Fernández Prieto	FCO-41044	OR756526
SA5	Riofrío stream, Santo Domingo de Herguijuela, Salamanca, Spain. Alt. 873 m, 30T 257832 4497588	J.A. Fernández Prieto	FCO-41045	OR756527
SA6	Tormes River, Salamanca, Salamanca, Spain. Alt. 777 m, 30T 277785 4537016	J.A. Fernández Prieto	FCO-41046	OR756528
SR	Sarcidano River, Craddaxoleddu, Sardinia, Italy. Alt. 571 m, 32S 510041 4407493	F. Mascia	FCO-41047	OR756529