Changes in genome size in a fragmented distribution area: the case of *Artemisia crithmifolia* L. (Asteraceae, Anthemideae).

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Abstract — *Artemisia crithmifolia* is a hexaploid shrub which inhabits the coastal Atlantic sand dunes of Western Europe, from the Southern Iberian Peninsula to the Netherlands, reaching the British Isles. Genome size data of 45 populations of *A. crithmifolia*, covering its entire distribution area, were obtained using the flow cytometry method. The 2C nuclear DNA content in this species ranged from 14.27 to 15.72 pg, the mean value being 14.98 pg. A negative correlation between nuclear DNA amount and latitude has been found, and statistically significant differences between two groups resulting from the fragmentation of the distribution area were evidenced.

Key words: 2C value, Compositae, dunes, flow cytometry, nuclear DNA amount.

INTRODUCTION

The genus *Artemisia* L. is one of the largest of the Asteraceae, with more than 500 species (Oberprieler *et al.* 2007). Different taxonomic rearrangements, based on morphological traits, have been carried out, and five large subgenera are considered at present (*Absinthium* DC., *Artemisia*, *Dracunculus* Besser, *Seriphidium* Besser and *Tridentatae* (Rydb.) McArthur). The genus is widely dispersed across the Northern Hemisphere although a few species are distributed in the Southern Hemisphere (Ling 1982; Vallès and Garnatje 2005).

Artemisia crithmifolia L. is a hexaploid shrub distributed over the Western coast of Europe, from the Southern Iberian Peninsula to the Northern Netherlands, reaching the British Isles. Two important disjunctions have been detected along its range, which are coincidental with the Northern coasts of both the Iberian Peninsula [where it seems to be extinct with the exception of one population (Aedo *et al.* 1990)] and France. This species occupies the maritime sands, principally at the back of the dunes in process of stabilization on the Northern Atlantic beaches, being part of two associations, *Corynephoretum atlanticum* and *Roseto-Ephedretum* (Kuhnzholtz-Lordat 1927), which are closely related (Vanden 1958).

Artemisia crithmifolia, a species from subgenus *Dracunculus*, presents capitula with glabrous receptacles, the outer florets female and the remaining ones functionally male, as commonly reported in the subgenus. The panicle branches are not sticky and the leaf lobes are short, fleshy, convex but not keeled beneath, according to Tutin *et al.* (1976). This species is closely related to *A. campestris* L. at morphological and molecular levels (Torrell *et al.* 1999). Its taxonomical consideration has been variable, as morphological features have been used to describe not only the species as an independent one, but also some subspecific entities subordinated to *A. campestris*. For this reason, different synonyms related to *A. crithmifolia* can be found in the literature [e.g. *A. campestris* L. subsp. *maritima* Arcangeli; *A. campestris* L. subsp. *lloydii* (Rouy) Cout., *A. gayana* Besser., *A. lloydii* (Rouy) A.W. Hill]. All these taxonomic considerations could be related to the fact that the species presents a certain degree of morphological

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variability, maybe due to different environmental conditions where it grows. This taxon shows morphological and ecological affinities with another member of the *A. campestris* complex, *A. campestris* subsp. *sericea* (Fr.) Lemke et Roth., growing in Baltic coastal sand dunes, and also with some related Asian taxa, such as *A. jordanica* Danin and *A. monosperma* Delile.

BAKKER (1976), in his study on phytogeographical aspects of the vegetation in the Atlantic province of Europe, postulated the relationship between the presence of *A. crithmifolia* (cited as *A. lloydii*) and other chamaephytes and the maintenance of a warm refuge during the glacial periods in the Southern part of this province. This author rules out that this presence can be due to lime content in the soils. In their work on general threats to plant species growing in the coastal habitats, VAN DER MAAREL & VAN DER MAAREL-VERSLUYS (1996) present *A. crithmifolia* in an uncertain status of conservation but needing attention. We agree with these authors that the development of urban-industrial-recreational facilities, especially in dunes, has caused the loss of the extensive areas where these plants used to grow. We have already experienced this fact during the sampling for the present work.

The DNA C-value for a species is the amount of nuclear DNA in the unreplicated haploid genome of a gamete (SWIFT 1950). It seems that C-value tends to be characteristic of a taxon, and quite constant within a species, the C accounting for constant. Since the C-value term was coined to date, many studies concerning the relationships between genome size and biological and ecological traits, involving aspects like polyploidy, cell physiology, biogeographic distribution, genetic plasticity or breeding system, have been carried out (e.g., BENNETT 1972; JASIENSKI and BAZZAZ 1995; MACGILLIVRAY and GRIME 1995; GRIME 1996; VINOGRADOV 2003; LEITCH and BENNETT 2004; CHASE *et al.* 2005; PRICE *et al*. 2005; BEAULIEU *et al.* 2007; GARCIA *et al.* 2008), suggesting that genome size does in fact have an evolutionary effect or actually its size is a consequence of evolution. Reports on infraspecific variation of genome size have been conducted, and the idea of a relative constancy of genome size within a species has been evidenced (BARANYI and GREILHUBER 1996; BENNETT *et al.* 2000).

The aims of the present work are: i) to contribute to the enlargement of genome size knowledge in plants, especially in the Asteraceae and in the genus *Artemisia*, on which many of our works are focused; ii) to study the constancy of the C-value parameter along the geographical distribution range in a given species; iii) to evaluate whether some morphological differences observed between populations are also reflected at genome size level; and iv) to study possible changes of genome size related to insularity.

MATERIAL AND METHODS

Table 1 shows the provenance of all the populations investigated with collectors, dates and voucher information. In order to confirm the presence or not of the studied species, different transects covering the Atlantic coast along its range of distribution were explored. In all cases, fresh young leaves were collected for flow cytometric measurements. Herbarium vouchers have been deposited in the Herbarium BCN (Centre de Documentació de Biodiversitat Vegetal, Universitat de Barcelona). The internal standard used, *Petunia hybrida* Vilm. 'PxPc6' (2C = 2.85 pg; MARIE and BROWN 1993), was cultivated in the greenhouses of the Facultat de Farmàcia (Universitat de Barcelona) and the Institut Botànic de Barcelona (CSIC-ICUB). It was provided by the Institut des Sciences du Végétal, Gif-sur-Yvette (France).

DNA content assessment - Fresh young leaves from the plants studied were co-chopped using a razor blade in a plastic Petri dish with an internal standard in 600 µl of Galbraith's isolation buffer (GALBRAITH *et al.* 1983) and supplemented with 100 µl/ml ribonuclease A (RNase A, Boehringer, Meylan, France). A sample containing only the standard was first prepared and analysed to determine its peak position. Nuclei were filtered through a 30-mm nylon filter in order to eliminate cell debris before adding 36 µl of propidium iodide (Sigma-Aldrich Química, Alcobendas, Madrid, Spain) in a final concentration of 60 µg/ml. Samples were kept on ice for 20 min before measurement. Five individuals per population were analysed. Two samples from each individual were extracted and measured independently. Fluorescence analysis was carried out using an Epics XL flow cytometer (Coulter Corporation, Hialeah, Florida). The total nuclear DNA content was calculated by multiplying the known DNA content of the standard by the quotient between the 2C peak positions of the target species and the standard in the histogram of fluorescence intensities, under the assumption that there is a linear correlation between the fluorescent signals from stained nuclei of the unknown specimen, the known internal standard, and DNA

content. We also calculated the mean half peak coefficient of variation (HPCV) corresponding to ten samples.

Statistical analyses - One-way ANOVA has been carried out to test differences between the datasets. We have indeed performed analyses either considering two groups (populations growing in the Northern part of the distribution area, including France, Belgium and the Netherlands, and those at the Spanish-Portuguese coasts at the South) or three groups (those of the Iberian Peninsula, the French group and the Belgian-Dutch one). We have done it both ways because, although we think that there is a real fragmentation in the distribution area of *A. crithmifolia* between the Iberian Peninsula and France, on the Cantabrian coast (so that two groups should only be considered), we cannot be sure that another fragmentation has not occurred between the French and Belgian-Dutch populations (hence, three groups should be considered). The 2C value corresponding to Loredo (on the Cantabrian coast) was excluded, because it is isolated in the Cantabrian territory (North coast of Spain), marking the border between the Iberian and the French datasets. Also, populations from the British Isles have been excluded as they were not numerous enough to perform a single group for the statistical analysis. Correlation between 2C values and the latitude of populations was evaluated using the non-parametrical Spearman correlation test.

RESULTS AND DISCUSSION

Nuclear genome size was assessed for 45 populations of *Artemisia crithmifolia* covering its entire natural range (Table 1, Fig. 1). The mean 2C value is 14.98 pg (equivalent to 14,650.44 Mbp according to DOLEŽEL *et al.* 2003), with a standard deviation of 0.41.

Variation in DNA amount within the species - The minimum value (14.27 pg = 13,956.06 Mbp) was obtained for the only population from Belgium and the maximum (15.72 = 15,374.16 Mbp) for the population from Tróia, in Portugal, the largest interpopulational difference being of 1.45 pg, equivalent to 9.22%, that is 1.1-fold. The data presented fit well with the only previous record of DNA content in *A. crithmifolia*, from a Portuguese population (Esposende, 2C = 15.60 pg, SD = 0.27; TORRELL and VALLÈS 2001), but it is to be remarked that the nuclear DNA amount currently assessed in plants of the same population is clearly lower [14.80 pg (SD = 0.38); Table 1] than the one calculated in the precedent work. In any case, the 2C values reflect the constancy of the hexaploid level, which is the only one reported in the taxon studied ($2n = 6x = 54$; KAWATANI and OHNO 1964; PASTOR 1992; OLIVA and VALLÈS 1994; TORRELL *et al.* 2001; and references therein). These values indicate that a certain interpopulational variability exists in this species along its distribution area, even if this intraspecific variation can be considered as not very high, according to the results reported for several plant groups (DOLEŽEL and BARTOŠ 2005; GARCIA *et al.* 2006; 2008 and references therein).

Numerous data reporting intraspecific genome size variations can be found in the literature (BENNETT and LEITCH 1997; RAYBURN *et al.* 2004) but this phenomenon is controversial (GREILHUBER 1997; 1998; 2005; OBERMAYER and GREILHUBER 1999). AUCKLAND *et al.* (2001) have found constancy in the genome size of disjunct populations in *Abies fraseri* (Pursh) Noir, whereas SUDA *et al.* (2005) postulated that the variability in *Hieracium* subgenus *Pilosella*, could indicate hybridogenous lineages. It is known that ecogeographical conditions could cause intraspecific variation in plant genome size (GASMANOVÁ *et al.* 2007). The constancy of the chromosome number makes it difficult to think of hybridogenic processes within *A. crithmifolia* (although an allopolyploid origin cannot be discarded for the taxon itself). The ecological conditions in which this plant grows are rather constant. In addition, the low HPCV value (0.99) suggests the absence of secondary metabolites which could be responsible for variation in DNA content. Taking into account these arguments, we consider that the differences in genome size in *A. crithmifolia* reflect a true populational or individual specific variability.

Differences in DNA amount between groups of populations - In order to detect significant changes, at genome size level, we have carried out different tests in concordance with the distribution of the species. The one-way ANOVA indicates a statistically significant difference ($F = 8.62$, $P = 0.0054$) between the 2C value means of the populations of the Southern (Iberian Peninsula and Portugal) and the Northern (remaining) areas, 14.82 pg being the mean of the populations of the Northern part and 15.15 pg for the Southern ones. If we take into account the geographic disjunctions, three main groups of distribution can be observed, so we also performed the analysis considering these three groups and results are comparable. The 2C values still remain significantly different ($F=7.19$, $P=0.0022$), but the populations from France (mean

TABLE 1 — Origin, DNA nuclear amount and latitude of the studied populations of *Artemisia crithmifolia* L. - [1]Nuclear DNA content: mean (standard deviation) of five individuals. - [2]1 pg = 978 Mbp (DOLEŽEL *et al.* 2003).

Code	Locality, collectors and herbarium voucher	Latitude (°)	2C(SD)(pg)[1]	2C (Mbp)[2]
PI1	Spain: Huelva, Playa de Mazagón, Garnatje & Pellicer. 18.02.2008. GR-272 (BCN)	37.14	15.36(0.27)	15022.08
PI2	Spain: Huelva, Playa de la Antilla, Garnatje & Pellicer. 18.02.2008. GR-271 (BCN)	37.21	15.67(0.51)	15325.26
PI3	Portugal: Algarve, Praia do Anção, Garnatje & Pellicer. 17.02.2008. GR-270 (BCN)	37.03	15.58(0.35)	15237.24
PI4	Portugal: Algarve, Alvor, Garnatje & Pellicer. 17.02.2008. GR-269 (BCN)	37.12	15.61(0.42)	15266.58
PI5	Portugal: Leiria, Foz de Arelho, Garnatje & Pellicer. 16.02.2008. GR-258 (BCN)	39.43	15.06 (0.30)	14728.68
PI6	Portugal: Setúbal, Sines, Garnatje & Pellicer. 17.02.2008. GR-266 (BCN)	37.95	15.31 (0.41)	14973.18
PI7	Portugal: Setúbal, Tróia, Garnatje & Pellicer. 17.02.2008. GR-264 (BCN)	38.49	15.72 (0.21)	15374.16
PI8	Portugal: Setúbal, Praia da Mata, Garnatje & Pellicer. 17.02.2008. GR-259 (BCN)	38.62	15.17 (0.17)	14836.26
PI9	Portugal: Leiria, Agua de Madeiros, Garnatje & Pellicer. 15.02.2008. GR-255 (BCN)	39.74	14.92 (0.18)	14591.76
PI10	Portugal: Coimbra, Praia da Mira, Garnatje & Pellicer. 15.02.2008. GR-250 (BCN)	40.46	14.78 (0.46)	14454.84
PI11	Portugal: Aveiro, Furadouro, Garnatje & Pellicer. 15.02.2008. GR-249 (BCN)	40.88	15.09 (0.50)	14758.02
PI12	Portugal: Porto, Vila do Conde, Garnatje & Pellicer. 15.02.2008. GR-248 (BCN)	41.35	14.61 (0.53)	14288.58
PI13	Portugal: Braga, Esposende, Garnatje & Pellicer. 15.02.2008. GR-247 (BCN)	41.53	14.80 (0.38)	14474.40
PI14	Spain: Pontevedra, Praia de Armona, Garnatje & Pellicer. 14.02.2008. GR-246 (BCN)	41.87	14.92 (0.18)	14591.76
PI15	Spain: Pontevedra, Praia de Areamilla, Garnatje & Pellicer. 14.02.2008. GR-245 (BCN)	42.25	15.08 (0.25)	14748.24
PI16	Spain: Pontevedra, Praia de Lanzada, Garnatje & Pellicer. 13.02.2008. GR-244 (BCN)	42.43	15.33 (0.33)	14992.74
PI17	Spain: Pontevedra, Praia Aguieira. Garnatje & Pellicer. 13.02.2008. GR-243 (BCN)	42.47	14.79 (0.53)	14464.62
PI18	Spain: Pontevedra, Praia do Rostro. Garnatje & Pellicer. 13.02.2008. GR-242 (BCN)	42.96	15.45 (0.42)	15110.10
PI19	Spain: A Coruña, Praia de Soesto. Garnatje & Pellicer. 12.02.2008. GR-241 (BCN)	43.20	14.55 (0.40)	14229.90
PI20	Spain: Santander, Ribamontán al Mar, Loredo. Garnatje & Vallès. 30-12-2007. (BCN)	43.46	15.62 (0.26)	15276.36
F1	France: Pyrénées Atlantiques, Anglet, plage des dunes. Pellicer & Vallès. 27.02.2008. JP-1 (BCN)	43.53	14.97 (0.14)	14640.66
F2	France: Landes, Capbreton, plage de Savanne. Pellicer & Vallès. 27.02.2008. JP-2 (BCN)	43.65	14.33 (0.39)	14014.74
F3	France: Landes, Vieux-Boucau-les-Bains. Pellicer & Vallès. 27.02.2008. JP-3 (BCN)	43.79	14.66 (0.45)	14337.48
F4	France: Landes, Contis-Plage. Pellicer & Vallès. 27.02.2008. JP-4 (BCN)	44.09	14.60 (0.36)	14278.80
F5	France: Landes, Biscarrose-Plage. Pellicer & Vallès. 27.02.2008. JP-5 (BCN)	44.45	14.51 (0.35)	14190.78
F6	France: Gironde, Pyla-sur-Mer, plage de la Corniche. Pellicer & Vallès. 27.02.2008. JP-6 (BCN)	44.63	14.93 (0.36)	14601.54

Code	Locality, collectors and herbarium voucher	Latitude (º)	2C(SD)(pg)1	2C (Mbp)2
F7	France: Gironde, Le Porge, Le Porge-Océan. Pellicer & Vallès. 28.02.2008. JP-7 (BCN)	44.89	15.07 (0.32)	14738.46
F8	France: Gironde, Hourtin, Hourtin-Plage. Pellicer & Vallès. 28.02.2008. JP-9 (BCN)	45.22	14.45 (0.53)	14132.10
F9	France: Gironde, Soulac-sur-Mer, plage d'Amélie-sur-Mer. Pellicer & Vallès. 28.02.2008. JP-10 (BCN)	44.52	15.20 (0.43)	14865.60
F10	France: Charente Maritime, La Palmyre, Phare de la Coubre. Pellicer & Vallès. 28.02.2008. JP-11 (BCN)	45.70	14.91 (0.23)	14581.98
F11	France: Charente Maritime, Île d'Oléron, Saint-Denis d'Oléron, plage des Boiries. Pellicer & Vallès. 28.02.2008. JP-12 (BCN)	46.03	14.63 (0.40)	14308.14
F12	France: Charente Maritime, Île d'Oléron, Saint-Pierre d'Oléron, plage de Vert-Bois. Pellicer & Vallès. 28.02.2008. JP-13 (BCN)	45.87	14.45 (0.37)	14132.10
F13	France: Charente Maritime, plage de Saint Jean des Sables, between Rochefort and La Rochelle. Pellicer & Vallès. 29.02.2008. JP-14 (BCN)	46.10	14.69 (0.40)	14366.82
F14	France: Charente Maritime, Île de Ré, Rivedoux-Plage. Pellicer & Vallès. 29.02.2008. JP-15 (BCN)	46.16	14.39 (0.52)	14073.42
F15	France: Vendée, Bretignolles-sur-Mer, plage des dunes. Pellicer & Vallès. 29.02.2008. JP-17 (BCN)	46.61	15.03 (0.18)	14699.34
F16	France: Loire Atlantique, dunes du Collet. Pellicer & Vallès. 29.02.2008. JP-18 (BCN)	47.04	14.79 (0.58)	14464.62
F17	France: Loire Atlantique, La Turballe, way to Pen-Bron. Pellicer & Vallès. 01.03.2008. JP-19 (BCN)	47.35	14.97 (0.15)	14640.66
F18	France: Morbihan, Quiberon. Pellicer & Vallès. 01.03.2008. JP-20 (BCN)	47.48	15.19 (0.25)	14855.82
B1	Belgium: De Panne Plage, Dunnes de Perruquet. Garcia & Ibarria. 16.03.2008. SC-1 (BCN)	51.23	14.27 (0.20)	13956.06
H1	Netherlands: Veere, Koudekerke. Garcia & Ibarria. 18.03.2008. SC-3 (BCN)	51.49	15.24 (0.33)	14904.72
H2	Netherlands: Katwijk, Zuidduinen. Garcia & Ibarria. 19.03.2008. SC-5 (BCN)	52.19	14.28 (0.20)	13965.84
H3	Netherlands: Zandvoort. Garcia & Ibarria. 20.03.2008. SC-7 (BCN)	52.37	14.93 (0.34)	14601.54
H4	Netherlands: Ijmuiden. Garcia & Ibarria. 22.03.2008. SC-8 (BCN)	52.46	14.85 (0.28)	14523.30
UK1	United Kingdom, England: Crosby. Smith, Twibell, Vallès & Wilcox. 20.08.2008. (BCN)	53.62	15.52 (0.29)	15178.56
UK2	United Kingdom, Wales: Swansea/Abertawe, Crymlyn Burrows. Guest, Jones, Twibell, Vallès & Woodman. 19.08.2008. (BCN)	51.62	15.63 (0.32)	15286.14

2C value = 14.76 pg) and Belgium-Netherlands (mean 2C value = 14.71 pg) appear as homogeneous groups with non-significant differences. We excluded from the analysis the population from the Cantabrian coast (because of its intermediate, isolated geographic location), which has a mean genome size of 15.62 pg, and also the British ones because of the scarce number of representatives.

It is very likely that the distribution area of the species could have been continuous along the Atlantic coast from the Iberian Peninsula to Netherlands, but fragmentations of this area may have occurred, judging by the presence of the Loredo population on the coast of the Cantabrian sea (Northern Spain). The records of some probably recently disappeared populations from Sestao and Desierto de Bilbao (GREDILLA 1914) referenced in the South Basque Country Flora (ASEGINOLAZA *et al.* 1984), and also others, such as the one from El Sardinero, at Santander (testified at the beginning of the 20th century by herbarium vouchers and not found from the 1980's on), point to this fragmentation too. Contrary to that, neither bibliographic nor herbarium testimonies have been found concerning the existence of the species along the north coast of France to Belgium. In any case, the present results indicate that differences between the two groups created by this recent

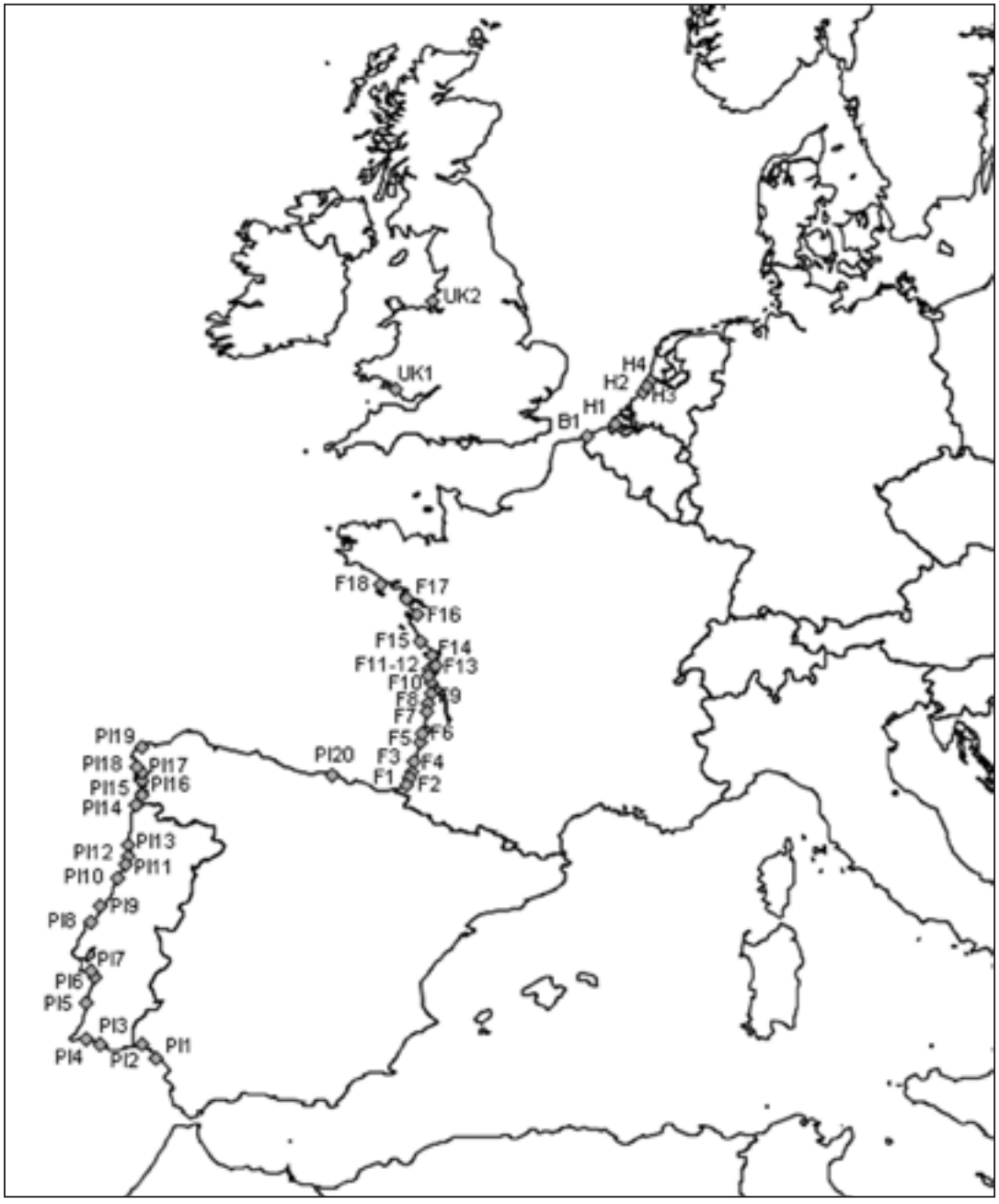

Fig. 1 — Distribution of the populations of *Artemisia crithmifolia* studied. The codes are the same as in Table 1.

fragmentation currently exist. These differences could be related to the lime-content in the soil, which is low in the southern and northern part of the Atlantic province (stretching from Portugal to Norway), with higher figures in the central part, the transitions being especially abrupt near the Gironde (France), but no pattern was found (BAKKER 1976). According to this author, one of the factors that could explain the changes in the vegetation is the number of days with frost, but no relationship has been found in our research. However, maybe the fact that some regions of the Southern part acted as refuges during the glacial periods could possibly have had some effect on the genome size of these populations.

A slightly negative correlation exists between genome size and latitude in *A. crithmifolia* (r=0.32, P=0.03). We have also found a correlation between both variables in *Cheirolophus intybaceus* (GARNATJE *et al.* in press) and closely related taxa

distributed along the Mediterranean coast of Spain and France, but in that case the correlation was positive. We postulated that genome size was smaller in drier and warmer regions of the Southern part of the Iberian Peninsula, but this pattern is not valid for the Atlantic region judging from the results which indicate a higher DNA amount in the warm and dry areas of the South. PRICE *et al.* (1981a;b; 1986) and CASTRO-JIMÉNEZ *et al.* (1989) suggested that plants growing in mesic habitats have larger C-values than those growing in drier ones and OHRI and KHOSHOO (1986) suggested the existence of a positive relationship between genome size and latitude, but conflicting reports about this topic appear in the literature. Pines have been thoroughly investigated from this point of view (see BOGUNIC *et al.* 2007, and references therein) although their results are not conclusive but our findings agree with those of GROTKOPP *et al.* (2004) who found that a Northern latitudinal limit was negatively correlated with genome size in *Pinus*.

We have observed a certain degree of variation in the indumentum in and within several populations. These differences do not have a clear geographical basis and also we did not observe any correlation between glabrescence or pilosity and genome size.

Genome size and insularity - Artemisia crithmifolia also occurs on some small isles, which are dispersed along the Atlantic littoral, close to the coast. In fact, some of them are connected to the continent by bridges (e.g. Île de Ré and Île d'Oléron), or 10-20 km distant such as the Cíes Islands (Galicia, North-West Spain) and Belle Île or Île d'Yeu (France). As expected, the results obtained in three populations of Île d'Oléron and Île de Ré do not show discordant 2C values compared to those of continental ones, indicating that apparently no genome size changes occur on islands located close to the continent.

Genome size of the two British populations is among the highest in all accessions studied, their 2C values being clearly larger than the mean (Table 1). Although no statistical tests can be performed due to the scarce number of insular populations, their relatively high nuclear DNA content is to be pointed out, as well as the fact that the number of individuals found is very small and apparently in recession (A. Jones, Ph. Smith, J.D. Twibell, pers. comm.). Studies including analyses of genome size variation at interpopulational level are scarce (GARCIA *et al.* 2006; GARNATJE *et al.* in press), and a common trend towards an increase or decrease of genome size between continental and insular populations is not detected. Despite the lack of a general trend, our results seem to be in concordance with those found in *Artemisia arborescens*, where increased 2C values were found in insular populations (GARCIA *et al.* 2006).

The origin of these two British populations is uncertain. Authors have hypothesized, at least for the population from Crosby, on the arrival of seed mixtures from the mounds of the adjacent pumping station (SMITH 2005). More studies addressed to elucidate the phylogeographic history of the species will be interesting for a better understanding of the genome size changes, and to confirm the direction towards genome expansion, if any, after island colonization.

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