Genetic and phenotypic patterns of variabilities in Arenaria grandiflora L. species complex (Caryophyllaceae) : new elements for taxonomy and conservation

Marwa Daoud

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New elements for taxonomy and conservation

Sous la direction de **: MACHON Nathalie et SILJAK-YAKOVLEV Sonja**

Composition du jury :

Nathalie MACHON	Professeur, Muséum National d'Histoire Naturelle	Directrice de Thèse
Sonja SILJAK-YAKOVLEV	Directrice de Recherche, CNRS	Directrice de Thèse
Magda BOU DAGHER-KHARRAT	Professeur, Université Saint-Joseph de Beyrouth	Rapporteur
Joan VALLES	Professeur, Université de Barcelone	Rapporteur
Errol VELA	Maître de Conférences, Université de Montpellier	Examinateur

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Dedicated to:

My country (Syria)

My parents (Hayat and Louay)

My husband (Moutasem)

And my cute daughter (Ame)

My brother (Nebras)

My sisters (Mhar and Oshine)

. . . for their unconditional support, their wishes, their fortitude, their understanding, their help and guidance in every walk of life. . .

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Many inspiring people have been involved in the work leading to my PhD thesis. I will like to acknowledge everyone for their contributions to the studies conducted during my time as a PhD student and I am especially thankful to:

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Preface

Five years ago, I started this project as a thesis project, but it turned out to be long battle against a large-flowered Sandwort from Caryophyllaceae (*Arenaria grandiflora* L.). This thesis is the report of this long process, but it cannot express the long days spent in both the field and the lab, battling shoulder to shoulder with my fellow scientists. It cannot also express the stress, the sadness and the tiredness because of the war and the victims in my country (Syria). The first year was only just unsuccessful attempts to detect both the nuclear microsatellite markers and the chloroplast markers (we used 4 pairs of universal primers (MatK 390F / 1440R, TrnK-MatK 3914F / 1110R, TrnL-trnF e / f and Trnt-L La / Lb), in dry and old herbarium' specimens (Paris' herbarium, Montpellier' herbarium, Geneva' herbarium and Barcelona' herbarium). A new and different scheme to search was established in the second year.

This work is to the best of my knowledge original except where references are made to previous work. This thesis has been writing based on experiments conducted in the scientific group of both two laboratories Center for Ecology and Conservation Sciences (CESCO; MNHN) and Ecology, Systematics and Evolution (ESE, CNRS, University of Paris-Sud Saclay).

I would like to thank my supervisors **Nathalie MACHON** and **Sonja SILJAK-YAKOVLEV** for their excellent guidance and support during this process.

It is great to have finally completed my thesis. Within, this interesting subject, I have gained much knowledge and discovered a domain, where I like to work in the future.

At the end, thanks to you, reader, if you are reading this line after the others, you at least read one page of my thesis.

Thank You

Summary of the thesis

Population-level conservation is being extremely required to restrain the biodiversity loss within a species. So, the assessment of the variability within the species complex is being renowned as an important first step to well implement the future conservation settings for threatened species. The species complex of *Arenaria grandiflora* is a short-lived perennial herbaceous and a threatened taxon in certain of sites of its distribution areas in Europe, with unresolved genetics and taxonomy, which lead to potential problems in the conservation and utilization of the resource. A differentiation among populations of the species complex of *A. grandiflora* is presented in this study based on the genetic, cytogenetic and phenotypic patterns. Intraspecific ploidy level variation is an important aspect of numerous species, so, the present study explores this phenomenon within the *A. grandiflora* species complex in some type of populations (27 natural populations). To infer the intraspecific genetic and cytogenetic patterns of variability among the studied natural populations of the investigated species complex (*A. grandiflora*), three methods were used: nuclear microsatellite markers, cytogenetic and flow cytometry approaches. Moreover, the phenotypic patterns of variation among both the stock of seeds and the herbarium materials of *A. grandiflora* were defined. These patterns were detected using three methods of seed germination (*in vitro* culture, filter papers and potting soil) and morphometric approaches. A significant differentiation among populations' patterns of molecular, cytogenetic and phenotypic variation was detected within the *A. grandiflora* species complex. Presence of two closely related cytotypes (diploids 2n=2x=22 and tetraploids 2n=4x=44) was detected using both classical and more recent methods (chromosome number count and flow cytometry respectively). The species complex of *A. grandiflora* exhibits high variation in 2C-DNA value, the genome size ranges from 2.11±0.74 pg to 2.70±0.11 pg for the diploid populations and from 4.30±1.51 pg to 5.27±0.14 pg for the tetraploid populations. Moreover, the seeds of tetraploids germinate well and in high proportion than the seeds of the diploid ones. In addition, both acicular and linear leaves from the diploid populations differ significantly within the diploids and with the lanceolate leaves of the tetraploid ones. New protocol of seed germination for the tetraploids by *in vitro* culture after scarifying was described for the first time. The affected factors on seed germination percentages were determined by an explanatory model of six predictors (altitude, longitude, latitude, ploidy levels, both period and condition of seed storage).

Consequently, all these findings are fundamental for the determination of the evolutionarily significant units (ESUs) within *A. grandiflora* species complex and thus the definition of efficient restoration plans in the future. This study would consider as the preliminary signal for necessary revision for the intraspecific taxonomic keys problematic for this species complex.

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1. Chapter I:

General introduction

Population-level conservation is recognized as an important requirement to prevent biodiversity loss within species (Purvis & Hector 2000), but the first necessity is to determine both number and distribution of populations and then differentiation among populations, that is needed to define population-based units for conservation purposes. Presently, there are three population-based approaches for identifying populations to be protected; as the use of evolutionarily significant units (ESUs) advocated by Ryder (1986). This approach was expanded by Moritz (1994) and (Waples 1998) to the concepts of management units (MUs) and distinct population segments (DPS), respectively, for species that had undergone distribution range fragmentation. Furthermore, it is a difficult task to identify the ESUs within a species, it requires the use of natural history information on populations, i.e. its ranges and distribution data, morphometric analysis, as well as genetic and cytogenetic analysis… etc. (Ryder 1986). Setting conservation priorities within species (i.e. at the population level) may be a challenge in species complex for which ranges become fragmented, such as a case of *Arenaria grandiflora* species complex.

Little is known about the biology of this widespread species complex through Western Europe and its taxonomic status is even controversial and problematic. Seven or five geographic subspecies, few varieties, and forms have been described until now. While the presence of intraspecific variability was documented within *A. grandiflora* species complex, no research has addressed how the assessment of differentiation among populations, could be important to take the best conservation measure for preserving this species complex that is distributed in small and disjunctive populations. The genetic variability within *A. grandiflora* species complex, is still little known, especially, the polyploid nature of this species complex, that has been neglected in a previous conservation program in the Fontainebleau forest in Paris.

This manuscript will present in five successive chapters the results of analyses carried out to explore the genetic and phenotypic patterns of variation. Before showing the results of analyses, this manuscript starts with a general introduction about the polyploidy in plants, the history of the evolutionarily significant units (ESUs) and the biology of the genre *Arenaria* L., with a little available information about our interesting species complex of *A. grandiflora*. The second chapter describes genetic and cytogenetic studies. For that purpose, we used nuclear microsatellite markers for the study of genetic

variability, a classical karyological method for chromosome number count and flow cytometry for assessment of DNA content variability. The third chapter used seed germination protocols to determine the percentage of germination for different populations of *A. grandiflora* species complex. The fourth chapter presents the morphometric variability observed in the leaf shape among specimens of herbarium' Paris. It is followed by a general discussion and conclusion.

1.1 Polyploidy in plants – a brief history

The widespread occurrence of polyploidy in plants is well-known because it has attracted much of scientific studies for almost a century. The polyploids have multiple (more than two) sets of chromosomes in their cells. A series of estimates of the frequency of whole genome duplication (WGD; polyploidy) in angiosperms have been provided during the past years by plant biologists (70% by (Masterson 1994); 50 % by (Müntzing 1936)**.** Many reviews have uncovered that most of the angiosperm

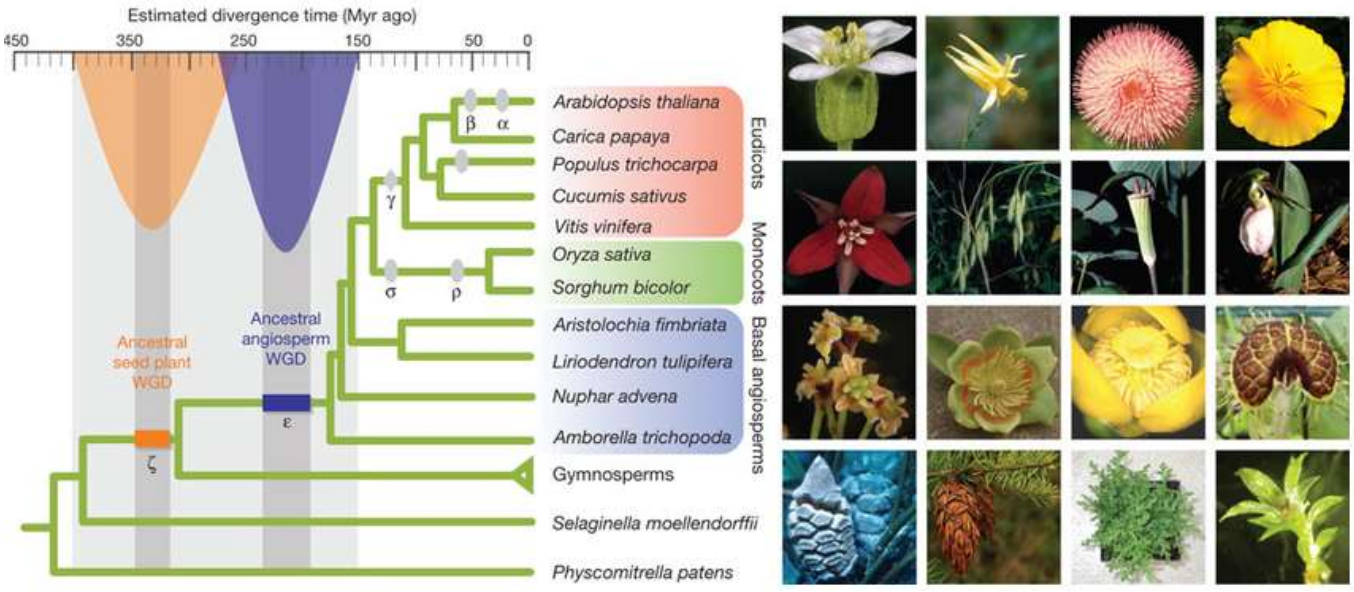

Fig. 1 Ancestral polyploidy events in seed plants and angiosperms; from (Jiao et al. 2011)

have a polyploid origin (Leitch & Bennett 1997); (Soltis & Soltis 1999); (Mable 2003); (Soltis et al. 2004); (Soltis et al. 2014). Recently, Jiao et al. (2011) have elucidated that there are two WGDs in ancestral lineages before the diversification of extant seed plants and extant angiosperms, (see Fig.1).

Since its discovery in 1907, polyploidy has been considered as an important dynamic process in the evolutionary success (Otto & Whitton 2000), but the direct effect of polyploidy on the evolutionary

trajectory of a species is still unclear (Madlung 2013). The abundance of polyploids refers to multiple sets of genomes and offers an evolutionary advantage, probably through a broader phenotypic and genotypic range than that of their progenitors and because of the increased fitness afforded by fixed heterozygosity (Hegarty & Hiscock 2007); (Fawcett & Van de Peer 2010). For example, the genetic, epigenetic and transcriptional changes associated with polyploidy in plants, like the greater pool of genes and alleles available for selection, might contribute to the evolutionary success of polyploid plants (Hegarty & Hiscock 2008, 2007)

The whole-genome duplication has provided an important pathway for diversification and speciation in plants (Vamosi & Dickinson 2006); (Rieseberg & Willis 2007). However, the complexity of understanding the role of genome duplication in species diversification is still disputed. It has been established that 15% of angiosperm speciation events involve a change in genome duplication (Wood et al. 2009). Moreover, (Laport et al. 2016) reported a part of this complexity in the *Larrea tridentata* (DC.) Coville polyploid complex, that its cytotypes have different environmental distributions, phenological isolation, and varied genetic structure. In another hand, the polyploidization events are responsible for the increased species richness in *Rosaceae* (Vamosi & Dickinson 2006). The complexity of polyploids has been underlined by several different genetic, ecological, physiological and morphological aspects that have been associated with polyploidy. The interactions among these various possible factors can give a large part of the phenotypic, genomic, ecological and evolutionary consequences of polyploidy (reviewed in (Balao et al. 2011); (Yang et al. 2011); (Birchler 2012); (Moghe & Shiu 2014); (Ramsey & Ramsey 2014)).

1.2 Evolutionarily significant units (ESUs) – a confusing history

The general concept of the evolutionarily significant units (ESUs) has evolved over time. The question is: how precisely identifying this basic unit of conservation? In this question which seems even more complicated is defining the criteria for ESUs recognition within species that composes of many subspecies and ecotypes (Ryder 1986). Historically, the ESUs are defined as subsets of the more inclusive entity species, which possess genetic attributes significant for the present and future generations of the species in question (Ryder 1986). Then, the ESUs concept was developed by (Waples 1998), this novel concept recognizes the ESUs as a population or group of populations that: (i) is

substantially reproductively isolated from other conspecific population units; and (ii) represents an important component of the evolutionary legacy of the species.

In the middle of the 1990s, (Moritz 1994) focused on the evolutionary past of the taxa and thought that "ESUs should be reciprocally monophyletic for mtDNA alleles (the divergence in mtDNA reflects long-term restriction of gene flow) and show a significant divergence of allele frequencies at nuclear loci". However, (Crandall et al. 2000) thought that this concept of ESUs was not adequate for determining appropriate conservation actions. In their paper, they suggested a broader categorization of population distinctiveness based on concepts of ecological and genetic exchangeability. For example, the populations within a species that show adaptive genetic differentiation may justify separate units.

In this thesis, we used different technics to better explore the genetic and phenotypic variation (1) to improve the knowledge of the species complex of *Arenaria grandiflora* (2) to examine if Moritz (1994) and Crandall et al. (2000) points of view are compatible for this case of *A. grandiflora* species complex.

1.3 Arenaria L. – a complex history

The biology of the genus *Arenaria* has been studied through several points:

1.4.1 The complex taxonomic history

Over the years, the taxonomy of the genus *Arenaria* and its allied genera has been controversial and considered unsatisfactory. For the first time, Linnaeus (Linnaeus 1737) defined this genus in the thirties of the eighteenth century. Many detailed studies have focused on the circumscription of the generic and infra-generic boundaries in *Arenaria*, that consistently has a confusing history. Certainly, these studies included two concepts: one held by Fernald, a wide point of view, maintaining the genus *Arenaria* as a whole unit. Fernald and several American botanists did not consider the different characters (valves of the capsule, foliage, ovary, inflorescence and seeds) sufficiently to demonstrate the segregated genera as distinct from *Arenaria* sensu stricto (Fernald 1919). Whereas the other concept divides the group *Arenaria* into many genera and subgenera, many botanists have adopted this strict generic concept. For example, Bentham and Hooker have recognized 11 subgenera, including the subgenus of *Arenaria* s. str., and proposed up to 130 species in the genus *Arenaria* (Bentham & Hooker 1862). Furthermore, in

the first taxonomic revision of *Arenaria*, Williams defined seven subgenera and 34 sections and subsections, which grouped 168 species (Williams 1898). Likewise, there are more than 300 species of *Arenaria* in the world belonging to ten subgenera and 24 sections, according to (Zhou 1995). Recently, in the last thirteen years, several authors defined a new key for the genus *Arenaria* and new species in Iran and in Turkey (Aytaç & Duman 2004); (Fadaie et al. 2010); (Fadaie 2013).

In addition, Fior and Karis have suggested that the seed strophiole should be considered when determining keys to separate *Moehringi*a L. from *Arenaria* (Fior & Karis 2007). However, McNeill's viewpoint has more clearly outlined by defining the criteria of generic and infra-generic classification of *Arenaria*. Seven genera, and ten subgenera had been distinguished by McNeill, including the subgenus of *Arenaria,* that has also subdivided into several sections and series (McNeill 1962). Despite the difficulties in selecting the characters to determine the limitations, there are exceptions in a limited area, for example, in Europe (Fernald 1919), where the lines separating the *Arenaria* genus complex *(Arenaria, Moehringia* and *Minuartia*) are well defined by McNeill (1962).

Fig.2: Illustration of *Arenaria grandiflora* from Tela Botanica

The section *Grandiflorae* is one of 11 sections created by McNeill (1962). It has closely related to section *Plinthine*. Over and above, it comprises only two species complexes: *Arenaria grandiflora* and *Arenaria valentina Boiss*. Members of section *Grandiflorae* are perennial herbs and native to the Iberian Peninsula, Balearic Islands, Southern, Western and Central Europe and North Africa.

The difficulties in splitting the *Arenaria grandiflora* species complex (Fig. 2) have led to a confusing intraspecific taxonomic history. The so-called *A. grandiflora* species complex has explicated by its polymorphous populations and its variation in the level of ploidy. However, a few micro morphological features in the indumentum can use as key characters for some infraspecific taxa (Sáez et al. 2002). Therefore, the species complex *A. grandiflora* includes only three subspecies as mentioned in Flora Europaea: *Arenaria grandiflora* ssp. *grandiflora*; *A. grandiflora* ssp. *bolosii* and *A. grandiflora* ssp. *incrassata*). Nevertheless, according to the Global Biodiversity Information Facility (GBIF; international open data infrastructure), this species complex involves five accepted subspecies, two accepted varieties and two accepted forms. The five subspecies are:

1) *Arenaria grandiflora* ssp. *grandiflora*, that distributes in Baleares (Mallorca), France, Spain, Andorra, Italy, Yugoslavia, Tunisia, Algeria and Morocco.
2) *Arenaria grandiflora* ssp. *bolosii* (Cañigueral) Küpfer, it is an endemic restricted in Baleares (Mallorca)
3) *Arenaria grandiflora* ssp. *incrassata* (Lange) C. Vicioso ex Rivas Mart, that exists in Baleares and Spain
4) *Arenaria grandiflora* ssp. *valentina* (Boiss.) O. Bolòs and Vig, that occurs in SE of Spain
5) *Arenaria grandiflora* ssp. *gomarica* L. Sáez. J.M. Monts. and Rosselló, that distributes in Baleares (Mallorca) and Morocco

Also, the accepted varieties and forms are as following:

a) *Arenaria grandiflora* var. *stolonifera* Ser, its distribution extends along the Pyrenees Chain and Switzerland (Alpine mountains)

b) *Arenaria grandiflora* var. *grandiflora*

c) *Arenaria grandiflora* f. *longifolia* Maire

d) *Arenaria grandiflora* f. *grandiflora*

Furthermore, according to other databases such as: The Plant List and International Plant Names Index and NUB Generator, seven subspecies have accepted, they are the first five subspecies plus two subspecies which are:

1) *Arenaria grandiflora* ssp. *glabrescens* (Willk.) G. López and Nieto Feliner, that is a Majorcan mountain endemic
2) *Arenaria grandiflora* ssp *pseudoincrassata* Malag.

Moreover, the subspecies which have been reported in the cytogenetic studies are: the subspecies *Arenaria grandiflora* ssp. *grandiflora* that have a basic chromosome number x=11 including two cytotypes, one diploid (2n= 22) throughout the Pyrenean chain and one tetraploid (2n= 44) in the massif of Nevada in Spain Küpfer (1974). The tetraploid cytotype, detected in the Balearic taxa of this complex, is the endemic *Arenaria grandiflora* ssp. *bolosii* that is a tetraploid (2n = 44; (Cardona & Contandriopoulos 1980). The chromosome numbers of *Arenaria grandiflora* ssp. *glabrescens* (Willk.) G. López and Nieto Feliner (2n = 44), indicated that it is tetraploid subspecies (Castro & Rossello 2006). This subspecies *Arenaria grandiflora* ssp. *glabrescens* is closely related to the North African *A. grandiflora* ssp. *gomarica*, which is also tetraploid (Sáez et al., 2002). The *Arenaria grandiflora* ssp. *incrassata* is another subspecies of this complex but it is diploid. Unfortunately, no chromosome counts are available for neither *Arenaria grandiflora* ssp. *valentina* nor *Arenaria grandiflora* ssp. *pseudoincrassata.*

1.4.2 ***The distribution and ecology***

The genus *Arenaria* is geographically widespread, mainly distributed in arctic regions, such as the Euro-Siberian region. An example is the *Arenaria norvegica ssp. anglica* Halliday, which is an arctic-montane endemic that occurs in Iceland, Norway, Sweden, Finland, Shetland and North-West Scotland (Walker 2000). The genus also occurs in northern temperate regions (Eurasian Mountains, Andes., Asia Minor). Likewise, according to Flora Iranica (Rechinger 1988). *Arenaria* genus is present in the Irano-Turanian region (N. W. NW. C of Iran). The Subgenus of *Arenaria* is centered in Asia and in the Mediterranean region, where the section *Plinthine* McNeill extended to the Maritime Alps (S.E. of France and N.W. of Italy). Other sections of the *Arenaria* Subgenus are distributed in Greenland, America (Texas and New Mexico) and Northeastern Africa.

The species complex of *A. grandiflora* has a distribution stretching from France, Spain, Switzerland, Andorra, Italy and ex-Yugoslavia, to Tunisia, Morocco and Algeria (Fig. 3); where it occurs in fragmented areas (disjunctive distributions). However, most members of the *Arenaria*

grandiflora species complex are orophilous plants, aside from those in the lowlands, where it very rarely occurs, such as in the Indre-et-Loire (Les Sablons et Beaumont-en-Véron) and in Seine-et-Marne (Fontainebleau) (Chater & Halliday 1964; Bottin et al. 2007) as well as in the Pavlov Hills in Czech Republic (Danihelka et al. 2008), also, in the low hills of Rocamadour (300m) and the Dordogne Valley (Galinat 1938).

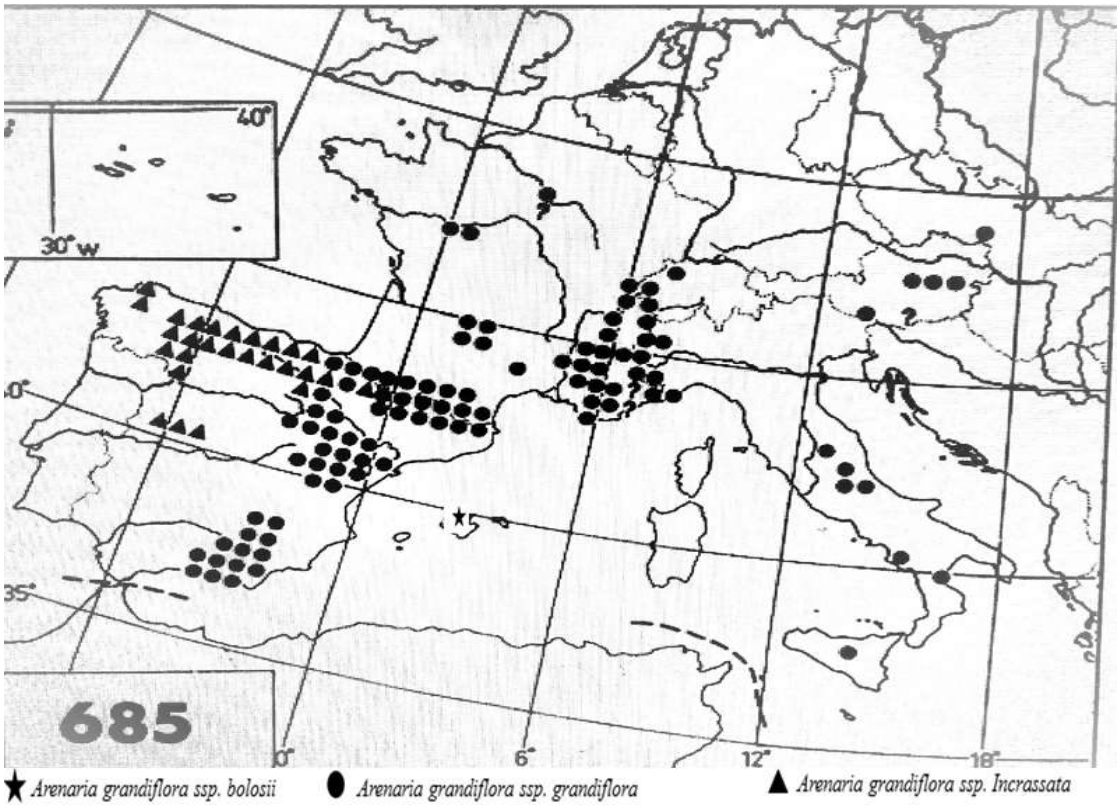

Fig. 3 Distribution of some subspecies of *Arenaria grandiflora* species complex in Europe according to Flora Europaea: *Arenaria grandiflora* ssp *grandiflora; Arenaria grandiflora* ssp. *incrassata; Arenaria grandiflora* ssp. *bolosii*

Moreover, in France, this taxon has distributed from the Hautes-Corbieres to all chain of Pyrenees, with calcicolous taxa occurring on cliffs and bare, limestone rocks. It is native to the Causses in the Cevennes in dolomitic limestone cracks, spreading scree and pastures to the foot of the crags, in supra Mediterranean bio-climate (Massif Central) and to the Vaucluse in Provence-Alps-Côte d'Azur. Therefore, in Switzerland, its distribution is limited to the alpine summits in the Jura region, in rocks or

rock debris and in full sun exposure in very well-drained soil with low humidity (Mt Chasseron, Mt Suchet and Mt Chasseral) (Favarger 1959; Hess et al. 1976).

Indeed, the species complex of *A. grandiflora* has a wide range of distribution in Spain and it has represented by several subspecies, as like as *A. grandiflora* ssp. *incrassata*, *A. grandiflora* ssp. *glabrescens* and *A. grandiflora* ssp. *grandiflora*, these subspecies have been featured by several studies in many provinces (Huesca, Alicante, Galician, Balearic Islands, Soria, Castellón, Sierra Tramuntana of Mallorca and Sierra Pozo in Jaén, Gerona, Lleida, Andalusia, Tarragona and Valencia). However, the *A. grandiflora* ssp *grandiflora* is a widespread subspecies, shows a restricted geographical distribution in dry rocky and stony places in the southern Provence of France such as in the summits and crests above 900 m in Sainte Baume (Youssef et al. 2011). In addition, it is restricted to the highest summits (1600-2150 m) of the mountains of the western Rif in Morocco (Sáez et al. 2002).

1.4.3 *The life forms and cycles*

As we mentioned above, the geographical distributions differ extremely in genus *Arenaria*. Therefore, the members of this genus can be grouped into different life-forms and cycles. In general, the species of genus *Arenaria* are either annual or perennial herbs, sometimes biennial. However, very few detailed studies have focused on plant growth forms for this genus, McNeill has worded its life form spectrum in a way, which was categorized as plant habit. Some of McNeill's categories are small annuals, xerophytes of Mediterranean, densely pulvinate alpines and spiny sub-fruticose (i.e., buds that are protected by dead plant remains) perennials (McNeill 1962).

Raunkiaer (1934) has classified plants according to the place where the growth point is located during the less favorable seasons. The buds of Chamaephytes are on persistent shoots near the ground and the Hemicryptophytes have their dormant buds in the upper crust of the soil, just below the surface (Raunkiaer 1934). The Chamaephytes reach their highest density rates within 1400–2000m (Giménez et al. 2004), thus, we can understand that the *Arenaria tomentosa* Willk. (endemic species in the south of the Iberian Peninsula) is chamaephytes (Fig. 4). Furthermore, *Arenaria polytrichoides* Edgew is a long-lived perennial herb that forms cushion chamaephytes (Yang et al. 2010). A cushion chamaephyte is a life form in harmony with the alpine conditions.

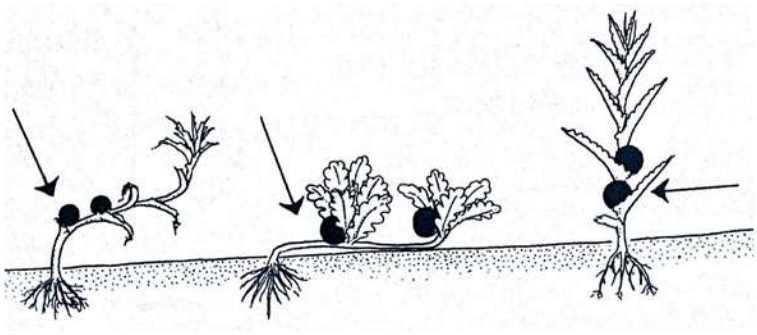

Fig. 4 Chamaephytes (buds near soil) according to Raunkiaer's classification (Beentje and Williamson 2010)

Therefore, the winter annual *Arenaria serpyllifolia* L. is a chamaephyte or therophyte herb (Fig. 5b). However, there are no studies that explain clearly the life forms in *Arenaria grandiflora* species complex, it is a chamaephyte or a hemicryptophyte (Fig. 5a). Under less favorable conditions, the chamaephyte form, as well as the hemicryptophyte form, have been found (Raunkiaer 1934).

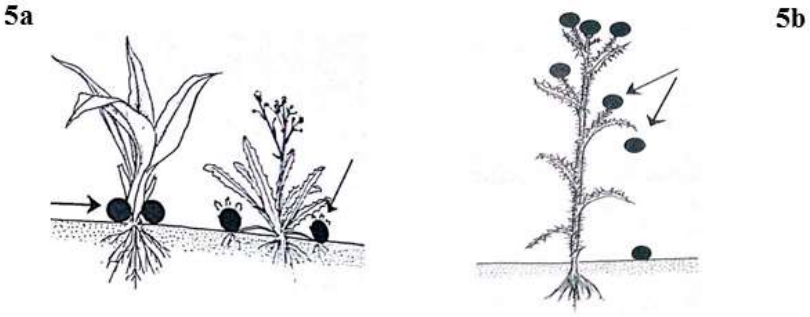

Fig.5 (a-b) 5a: Hemicryptophytes: buds in the upper crust of the soil, **5b:** Therophytes: the growing point surviving in the form of seeds, according to Raunkiaer's classification (Beentje & Williamson 2010).

Dispersal of seeds is an important process within the plant's life cycle. Up to now, dispersal modes at the level of Arenaria species have not been studied in detail. However, Youssef et al (2011) have reported the mode of dispersal occurring in some species of *Arenaria* in southeastern France. The dispersal modes in *Arenaria grandiflora*, *A. aggregata*, *A. cinerea* and *A. provincialis* has been reported to be anemochory (dispersal by wind) or barochory (dispersal by means of gravitation) i.e. without clear morphological adaptations for seed dispersal (Youssef et al. 2011). Indeed, a species has sometimes

dispersed by more than one process. Such cases have called diplo- or polychory, like as *Arenaria lanuginose* (Michaux) (Frantzen & Bouman 1989).

1.4.4 The morphological description

The morphological characteristics of *Arenaria* genus have described in many Floras in the world as Flora of China, Flora Europaea, Flora of North America, Flora Coste, etc. Therefore, the descriptive details have been defined in different artificial keys to subgenera, sections, and species by McNeill (1962), (Zhou 1995) and (Williams 1898). Here, we exhibit a few of characteristics of the genus *Arenaria*. The stems are ascending, simple or branched, often caespitose or pulvinate. Leaves are opposite, mostly sessile, linear or linear-lanceolate, usually elliptic to ovate or rarely orbicular, sometimes sub-succulent or succulent, subulate, setaceous and apex blunt or obtuse to acute, acuminate, margin entire and usually flat. Inflorescences are solitary flowers. Stamens 10 (2–5 or 8 in *A. livermorensis*) and its ovary are with numerous ovules. Styles are often 2 or 3(–5).

Our interesting species complex of *Arenaria grandiflora* has outlined the morphological characteristics in the France Flora (Coste 1937), Flora of the Jurassic Chains (Grenier 1865) and Flora Helvetica (Switzerland) etc. The stem is 5–15 cm tall, branched, ascending and sometimes hairless. The rootstock is woody and many-headed. The simple leaves are opposite and sessile; they are lanceolate or linear-lanceolate, acuminate, thickened at the margin. Actinomorphic flowers with long petals characterize the large flower sandwort, these petals are clearly exceeding the calyx (Figure 2. The sepals are ovate or glandular-pubescent. The petals are oblong-obovate and they are twice as long of the sepals. Inflorescence 1–3(–6)-flowered, with large white flowers in spring. The flowers are with pedicels that measure about 2–6 times as long as sepals and these pedicels are glandular-pubescent. The stamens are 10 and the styles are usually 3. The plants of the species complex of *A. grandiflora* have a short capsule, opening by valves in several equal or doubles that of styles. The fruit is an ovoid capsule (Coste 1937).

Very few of studies reported the diagnostic features of the infraspecific taxa of *A. grandiflora* species complex. For instance, the subspecies of *A. grandiflora* species complex can be differentiated mainly on the base of the leaf shape, pedicels, calyx, seeds and the pubescence on the pedicels and calyx of plants (Sáez et al., 2002).

The seeds

Seed characteristics generally have great contributions in taxonomic studies. In the genus *Arenaria* and its allied genera, they are significant enough to allow taxonomic delimitation of the family Caryophyllaceae at various levels (Kanwal et al. 2012) like the five types of strophioles which have been recognized in *Moehringia* species by (Minuto et al. 2006). Furthermore, the micromorphology studies of the seeds showed a significant variation at the species level in several species of *Minuartia* L. (Mostafavi et al. 2013) and suggested a variation in the exomorphology of the seeds of *Stellaria* L. species (Mahdavi et al. 2012). Interspecific variation in seed morphology of *Arenaria* was noted by Fernald (1919) and (Sadeghian et al. 2014) which found a significant polymorphism in seed coat morphology in certain of *Arenaria* species. Furthermore, an intraspecific variation in seed morphology was analyzed in populations of *A. uniflora* (Walt.) Muhl. by (Wyatt 1984).

According to Central and East-European flora (Bojnanský & Fargašová 2007), seeds of *A. grandiflora* species are reniform and laterally compressed and the margin is dense, the surface is tuberculate, it is ranged from brown to reddish-brown. The seed traits (such as mass and size of seeds, number of seeds) are vital components for distribution of *A. grandiflora* which inhabit dry and rocky habitats because of the correlations between habitats, seed traits and root development (Youssef 2011).

In some species of *Arenaria*, the seed dormancy has been rather questionable. In their studies of *A. glabra* Michx and *A. fontinalis* (Short and Peter) Shinners, Baskin and Baskin (1982, 1987) mentioned that most of the freshly-matured seeds were innately dormant. A physiological dormancy was prevalent in *Arenaria ciliata* L., according to (Schwienbacher et al. 2011).

1.4.5 The threat and protection

The European Red List of Vascular Plants defined the status of many species of *Arenaria* such as *A. nevadensis*, which is Critically Endangered and *A. humifusa*, which is Near Threatened (Bilz et al. 2011) (see Figure 6) Additionally, the status of *Arenaria cinerea* DC., is determined as vulnerable (VU) in Provence-Alps-Côte d'Azur (France) as mentioned in the Red Book of endangered flora in France (Olivier et al. 1995). There are also others national sources, such as the Red List of endangered species in Switzerland (ferns and flowering plants), that mentioned the status of two species of *Arenaria*, one of which is Near Threatened (*A. bernensis*) and the other is Regionally Extinct in Switzerland (OFEV 2011). Although IUCN is the best scientific tool, there are some cases with increasing knowledge of

taxa, where the re-evaluation of its status is necessary, such as *Arenaria provincialis* Chater and Hallyday, which has reevaluated by (Véla et al. 2008). Its new status is "Near Threatened" (NT), instead of the ancient category "Rare" (R).

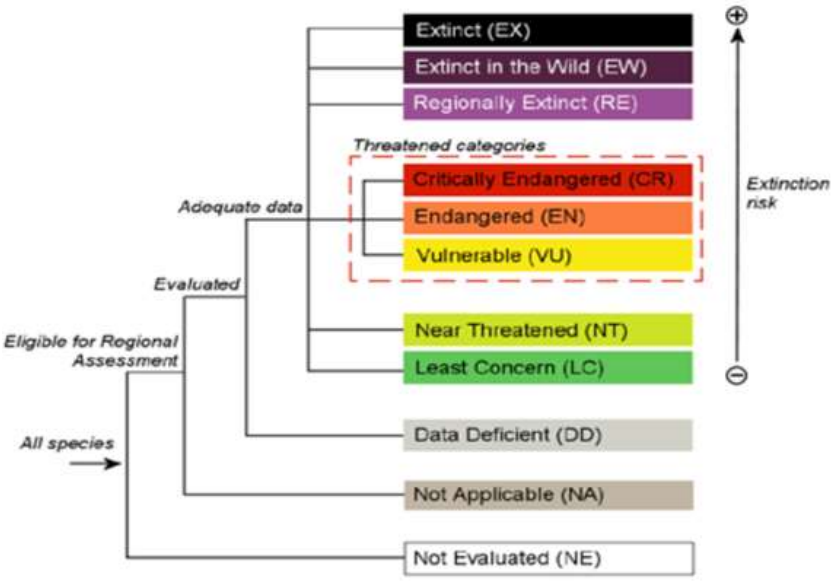

Fig. 6: IUCN Red List Categories at regional scale (Bilz et al. 2011)

Globally, the Red List of the International Union for the Conservation of Nature (IUCN) is an important scientific tool for making decisions for evaluating the species and their conservation. In 1997, *Arenaria grandiflora* was as a vulnerable (VU) in Jura, according to IUCN (list of threatened species) and it has been completely protected since 2005 in Vaud (Switzerland). Furthermore, in Austria, *A. grandiflora* has been characterized as "Rare" and is potentially endangered. Else, this taxon occurs only at a few localities in the Czech Republic are considered as rare species and critically threatened taxa (CR) (Grulich 2012), because of its occurring at 1-5 localities and one out of these five populations is known to have become extinct. In France, according to the Regional Red List of the vascular flora of Ile-de-France, *Arenaria grandiflora* species has nationally characterized as Critically Endangered in areas of occupancy and in areas of occurrence. Its decline is continuing in the quality of habitat, the number of locations or subpopulations, or in the number of mature individuals (Auvert et al. 2011).

In 1991 and 1993, this species has protected by regional texts in Ile de France and Centre. In 1999, a restoration plan was initiated in Fontainebleau Forest in Paris region (Machon et al. 1999). It

consisted of founding 3 populations of the same mixture of plants of Fontainebleau (2/3 of local plants) and plants from the Chinon region (exogenous contribution for 1/3). Seventeen years later, two populations still exist. Transplanted individuals have virtually all disappeared but have been replaced by abundant offspring. The dataset consists of the follow-up of each individual transplanted or born in the reintroduction sites since the implementation of the plan (about 17 000 data), as well as its size and the number of flowers it produced each year.

1.4.6 ***The phylogeny***

The phylogeny has been illustrated *Arenaria* sensu McNeill (1962) by Nepokroeff et al (2001) conducting a DNA survey of the Caryophyllaceae using sequence data from matK and the rps16 intron. This phylogeny mentioned to *Arenaria* is polyphyletic, with two major distinct clades. Recently, the molecular studies have referred to three subfamilies of Caryophyllaceae [Alsinoideae (Burnett.) Caryophylloideae (Arn.) and Paronychioideae *(*A. St)], which are polyphyletic (Fior et al. 2006). It has revealed by using ITS sequence data in combination with the chloroplast marker matK.

Thereafter, Harbaugh et al. (2010) found that the traditional three-subfamily subdivision of the Caryophyllaceae has not reflected and the morphological characters used to delimit them are unreliable due to the extensive convergent evolution. According to their study, large genera, such as *Arenaria* are polyphyletic and their species have scattered among three separated clades corresponding to several subgenera in McNeill (1962) system and have described as a new tribe; *Eremogoneae* Rabeler and W. L. Wagner (Harbaugh et al. 2010). With only a few exceptions, these results of tribal classification have supported by Greenberg et al (2011) via inferring a larger, more equally sampled, phylogenetic tree for Caryophyllaceae to more obviously resolve relationships within this clade.

Nevertheless, the phylogenetic analysis of the western Mediterranean *Arenaria* section *Plinthine* (Valcárcel et al. 2006) distinguishes between genus *Moehringia* and *Arenaria.* and has not been without controversy. *Moehringia* is paraphyletic to *Arenaria* with Iberian taxa, according to the phylogeny of the genus *Moehringia*, that was investigated by Fior and Karis (2007) by analyzing nuclear (ITS) and chloroplast (matK) sequence data in combination with morphological characters. However, the phylogenetic knowledge of the species *Arenaria* in southern Provence of France, *A. provincialis*, *A. modesta, A. serpyllifolia, A. aggregata, A. grandiflora, A. cinerea DC., A. ermarginata, A.*

conimbricensis Brot. and *A. hispida* has obtained by Youssef et al. (2011). The molecular studies, specifically of the genus *Arenaria* s.str., are limited to the findings of Sheidai et al. (2011).

Finally, in 2007, 13 microsatellite markers were developed for the species *A. grandiflora* (Zavodna et al., 2009). They allowed to genotype the founding individuals of the populations (which always existed in ex-situ collection) and to determine the gene's share from Chinon and Fontainebleau in the progenies that lived in 2007 and 2010.

1.4 Conclusion of the introduction

It is apparent from this introduction that there is generally high complexity in the genus *Arenaria*, but for this thesis, we are interested in the species complex of *Arenaria grandiflora*, currently composed of two cytotypes (diploid and tetraploid), a few subspecies (3, 5 or 7 accepted subspecies according different authors) and varieties (2 accepted varieties), especially, the subspecies *A. grandiflora* ssp. *grandiflora* composed also of two cytotypes (diploid and tetraploid) and many widespread populations that show disjunctive and declining distributions and encountered in many different types of habitats in Western Europe. Disjunctive populations are often particularly important to study, because most of them are small (less than 100 individuals) and thus threatened and because they also often contain original traits. We use the term of species complex to reflect the complicated nomenclatural and taxonomical history of this taxon. Despite of the previous studies on *A. grandiflora*, there are still major gaps in the knowledge of this species complex. To implement coherent and effective conservation plans, it is necessary to clearly identify the protected species. Especially, it is necessary to well delineate evolutionary significant units (ESUs) for its conservation. In this thesis, we used the opportunity of the work on both the natural population and the herbarium' specimens of *Arenaria grandiflora* species complex to bring new elements to the definition of ESUs for the future conservation plans. Thus, the scientific question of this work is: on what should we base to define conservation units?

2. Chapter II:

Genetic variation within the species complex of *Arenaria grandiflora* L.

2.1 Introduction to Chapter II

As mentioned earlier in the preface, there had been difficulties to reveal nuclear microsatellite loci on ancient (e.g. >200 years) herbarium' specimens within the *Arenaria section Grandiflorae,* collected from different herbaria (Montpellier, Geneva, Barcelona and Paris herbaria). The DNA was perhaps too degraded. That is why we used the same technic but with fresh specimens of the species complex of *Arenaria grandiflora*. Thus, in the next year, samples of natural populations have been collected from its disjunctive populations in Western Europe. These fresh specimens have been studied to reveal the nuclear microsatellite loci at the intraspecific level at the Molecular Systematics Service (SSM) in the French National Museum of Natural History. Because we found for some specimens (in some populations) more than two alleles per locus, we supposed that there could be tetraploids within our fresh samples which were collected from the natural populations?

To confirm this result, cytogenetic analyses had been carried out at the Ecology and Systematics Evolution (ESE) in University of Paris-Sud Saclay to detect the chromosome numbers and flow cytometry approach had been used to reveal the intraspecific genome size variability and thus better define the ploidy levels within this species complex.

2.2 Paper 1:

Intraspecific DNA Content Variability: Consequences for Conservation

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Keywords: chromosome number, evolutionarily significant units, flow cytometry, intraspecific ploidy variation, microsatellite genotyping, monoploid genome size

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Intraspecific DNA Content Variability: Consequences for Conservation

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ABSTRACT

Background and aims-In the context of the implementation of management plans for threatened species, the phenomenon of intraspecific polyploidy must be considered because crosses between some populations with different ploidy levels may be impossible or lead to sterile offspring. Here, we studied natural populations of *Arenaria grandiflora* L. (Caryophyllaceae), which is a species complex that is in regression in some of its distribution ranges in Western Europe.

Methods-We examined the genetic patterns of individuals using nuclear microsatellite markers, determined their chromosome number via a cytogenetic approach and assessed genome size using flow cytometry.

Key results-The results indicated a clear and interesting intraspecific variation in genome size and ploidy level among the studied natural populations. Two ploidy levels were detected (diploids 2n=2x=22 and tetraploids 2n=4x=44). The mean DNA 2C value ranged from 2.11±0.74 pg to 2.70±0.11 pg for the diploid populations and from 4.30±1.51 pg to 5.27±0.14 pg for the tetraploid populations.

Conclusions-These results are essential for the delimitation of evolutionarily significant units within *A. grandiflora* species complex and thus the definition of efficient restoration plans.

KEYWORDS: Chromosome number, evolutionarily significant units, flow cytometry, intraspecific ploidy variation, microsatellite genotyping, monoploid genome size

INTRODUCTION

The doubling of complete chromosome sets (polyploidization) occurs in angiosperms at several levels (Madlung 2013; Weiss-Schneeweiss et al. 2013), including the intraspecific level (Schönswetter et al. 2007; Trávnícek et al. 2011). In the context of the implementation of management's plans for threatened species, and the definition of evolutionarily significant units (ESUs: a group of organisms that has been isolated from other groups within the same species for a sufficient period of time to exhibit significant genetic divergence (Ryder 1986; Moritz 1994)), the phenomenon of intraspecific polyploidy must be taken into account (Brown and Young 2000; Severns and Liston 2008). Indeed, because crosses between some populations with different ploidy levels may be impossible or lead to sterile offspring, it is necessary to document the spatial distribution of such populations to define the best management plans. In this framework, the natural populations of *Arenaria grandiflora* species complex from the carnation family (Caryophyllaceae) in Western Europe represent an interesting study case.

The distribution of the *A. grandiflora* species complex stretches from France, Spain, Switzerland, Andorra, Italy and Yugoslavia to Tunisia, Algeria and Morocco, with populations occurring in fragmented areas; (Sáez et al. 2002; Stanisci et al. 2005; Gavilán et al. 2012). All members of the *A. grandiflora* species complex, are orophilous plants but, they can grow in lowlands, though this habitat is rare. In France, they have been found in the Loire region near the city of Chinon (Chater and Halliday 1964), in the low hills (300 m) of Rocamadour (Lot) in the Dordogne Valley (Galinat 1938) and in the Fontainebleau forest 50 km south of Paris (Arnal 1999) and in the Pavlov Hills in the Czech Republic (Danihelka et al. 2008). Moreover, this species complex of *A. grandiflora* grows in small isolated populations almost everywhere it is found (Bottin et al.2007; obs. perso.)According to several regional and national Red Lists, the species complex of *A. grandiflora* is considered as a threatened taxon in certain locations in Europe, such as in the Czech Republic, it is classified as a critically threatened taxon, while it is vulnerable and protected in Switzerland, and it is defined as a rare taxon in Austria. In addition, *A. grandiflora* species complex is currently extinct in two regions of France: on the French side of Mt Jura and in the Fontainebleau forest (Prunier 2001 and personal observation). Furthermore, it is now generally a rare species in southeastern France (Youssef et al. 2011). This species complex is only abundant in the Pyrenees Mountains and in Spain at present (Machon et al. 1991). In the particular case of the Fontainebleau forest, the *A. grandiflora* populations [Mont Merle

and Mt Chauvet] rapidly declined during the last century and probably became extinct in 2014 (Bottin et al. 2007 and personal observation). In 1999, before the complete extinction of *A. grandiflora*, a restoration programme was undertaken at three natural sites in the Fontainebleau forest (Maurice et al. 2013) through the creation of three populations, with 450 clones of both local (Fontainebleau) and non-local (Chinon) individuals being mixed in each population (Zavodna et al. 2015). The programme was implemented as an emergency measure, ignoring the potential intraspecific variation of polyploidy in the *A. grandiflora* species complex. However, the restoration project was relatively successful (Zavodna et al. 2015): two of the three populations still exist 15 years after their creation, although a slow decline in the size of the remaining populations has been observed since 1999 (personal observation in 2016).

Based on the necessity of documenting the evolutionarily significant units (ESUs) within *A. grandiflora* species complex to better define future management plans, we first assessed the neutral genetic variation in their natural populations using nuclear microsatellite (SSR) loci. Then, we counted the chromosome numbers of some of these natural populations using cytogenetic approaches and quantified the genome size of individuals from the same natural populations using flow cytometry (FCM). According to the available literature, the only previous study reporting the genetic diversity of *A. grandiflora* species complex based on microsatellite markers (Zavodna et al. 2015), conducted in two populations (Chinon and Fontainebleau) and their hybrids, considered the plants to be diploids. The most frequent cytotypes within the *A. grandiflora* species complex in Europe are 2n=20; 2n=22 and 2n=44 (Favarger 1959). Despite the increasing use of flow cytometry techniques to estimate genome size and ploidy levels in plants due to the rapidity and sensitivity of these methods, genome size has been studied in only three species of *Arenaria* L. (*A. serpyllifolia* L.; *A. gracilis* Waldst. and Kit. and *A. deflexa* Dec.) (Siljak-Yakovlev et al. 2010; Bou Dagher-Kharrat et al. 2013).

The present study assessed a combination of cytogenetic and multi-allelic molecular markers (microsatellites) in many wild populations to characterize the genomes of the *A. grandiflora* species complex. The main objectives of this study were to a) perform molecular profiling using microsatellite markers; b) determine the chromosome number in each population c) assess genome size; d) detect any cytogenetic differences between the investigated populations; and e) define potential the evolutionarily significant units (ESUs) for restoration plans.

MATERIALS AND METHODS

Plant material

The large-flowered sandwort (*A. grandiflora*) is a perennial plant that is cushion-shaped with woody and ascending stems that can reach a height of 15 centimetres. This small plant of Caryophyllaceae is a Pyrenean-Alpine species that grows on calcareous and sedimentary rocks, xerophytic and oro-basophils lawns and schist screes (Küpfer 1974). It blooms from May to August, *A. grandiflora* is insect pollinated but is also able to self-reproduce in the absence of pollinators (Machon et al. 1999, 2001). The mating system of *A. grandiflora* is probably predominantly outcrossing, although vegetative reproduction is also possible (Bottin et al. 2007).

Seed and leaf samples were collected from natural populations in different eco-geographic sites in Western Europe (table 1). Some of the collected seeds were dried and stored in paper or plastic bags under ambient laboratory conditions (approximately 20°C) and some of the collected leaves were stored in silica gel. The geographical origins of the investigated populations are indicated in table 1. The leaves were generally collected from natural populations, but for BBV (the population from Chinon), the leaves were obtained from the clones cultivated *in vitro* by the National Botanical Conservatory for the Parisian Basin (CBNBP). The root meristem was also sampled from *in vitro* plants. A protocol was implemented to sterilize the seeds and to cultivate them *in vitro,* as reported in another article (M. Daoud et al., National Museum of Natural History, France, unpubl. res.).

Microsatellite genotyping

DNA extraction and PCR conditions--Leaf samples were stored in silica gel and were subsequently used to identify SSR loci. To purify *A. grandiflora* DNA, the leaves were processed using the DNeasy Plant Mini Kit (QIAGEN). Firstly, 20 mg of dry leaves from each individual was weighed and then homogenized using a TissueRuptor homogenizer to obtain a fine powder. The cells were then lysed via the addition of lysis buffer, followed by incubation. After the addition of buffer P3 and incubation of the finale lysate on ice, the proteins and polysaccharides were precipitated. Finally, the lysate was cleared to promote binding of the DNA to the DNeasy membrane. Optimal results were obtained by eluting the samples twice, the elution volume was reduced to 2 x 50 µL.

The samples were genotyped using the ten specific microsatellite markers for *A. grandiflora* (Zavodna et al. 2009). All reactions were carried out in a 10 µL final volume containing 2 ng/µL of template DNA; 0.20 µ/L Taq pol; 0.30 mM dNTPs; 1.00 mM $MgCL^2$; 0.25 µM Primer R; 0.10 µM Primer fluo F; 5.5 µL H_2O and 1.00 µL of 10X TP. Optimization of the PCR conditions was performed for each PCR simplex. After this optimization step, all the loci that exhibited non-overlapping size ranges, similar primer annealing temperatures and similar cycles in the same reaction were co-amplified. Finally, all the microsatellite loci were combined into three multiplexes for simultaneous amplification and analysis using the Qiagen Multiplex PCR Kit. The composition of the multiplexes was as follows: AGA3--98; AGA5--123 and AGA2--74 in Multiplex 1; AG9--49; AG3--36 and AGA5--109 in Multiplex 2 and AG3--2; AGA1--150; AGC5--93 and AGA2--73 in Multiplex 3.

The cycling conditions were as follows: 94°C for 5 min; 30 or 32 cycles of 94°C for 1 min (30 for Multiplexes: 1 and 3; and 32 for Multiplex 2), the specific annealing temperature (55°C for Multiplex 1; 50°C for Multiplex 2; and 53°C for Multiplex 3) for 1 min and 72°C for 1 min; with a final extension step at 72°C for 10 minutes. Then, 1 µL of the PCR product was added to 8.8 µL of formamide and 0.20 µL of the marker standard, followed by evaluation via electrophoresis on an automated sequencer (ABI Genetic Analyzer). Gene Mapper software (Applied Biosystems) was used to determine the size of alleles. In addition, the size of the amplification product was followed through visual verification. For each locus, the alleles were recorded based on their size in base pairs and scored, after which a matrix of allelic phenotypes was constructed.

Determination of chromosome numbers

Root tips obtained from seedlings germinated in *in vitro* culture were pre-treated with 0.002 M 8-hydroxyquinoline (Merck. Prolabo), for 3 h at 16°C or in a 0.05% solution of colchicine (Sigma), and for 45 minutes at room temperature. Fixation was performed in 3:1 (v/v) ethanol-acetic acid at 4°C for at least 24–48 h. The fixed root tips were stored for several days in the first fixative or for several months in ethanol (70%) at 4°C. Root tip meristems were then hydrolysed in 1 N HCl for 5 minutes at 60°C or in 5 N HCL for 6 minutes at room temperature and stained with acetic orceine. Squashing was performed using a drop of acetic carmine. Chromosome numbers were determined from at least ten individuals per population and from several well spread metaphases per root tip. Chromosome plates were observed under a Ziess Axiophot

microscope and analysed using a CCD camera (RETIGA 2000R. Princeton Instruments Evry. France) in association with an image analyser (Metavue; version 7).

Genome size estimation via flow cytometry and statistical analysis

Cotyledons, fresh leaves, stored seeds and dried leaves (stored in silica gel) were used to obtain nuclear suspensions for genome size evaluation. The quantity of DNA (2C DNA value) was determined via flow cytometry following the technique of (Marie & Brown 1993). Leaves of the investigated *Arenaria* specimens and an internal standard [*Petunia hybrida* (Hook) Vilm. cv.' PxPC6', 2.85/2C pg or *Solanum lycopersicum* L. cv. Montfavet 63-5. 2C=1.99 pg (Lepers-Andrzejewski et al. 2011)] were chopped together with a razor blade in 600 µL of cold Gif Nuclear Buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 60 mM 4—morpholine propane sulfonate pH 7. 0.1% (w/v) Triton X--100. 1% polyvinylpyrrolidone (~10.000 M_r. Sigma P6755), 5 mM sodium meta-bisulfite and 10 µg/ml RNase; Sigma Aldrich, Saint Quentin, France). The nuclear suspension was then filtered through nylon mesh (pore size 30 µm) and kept at 4°C. The nuclei were stained with a specific DNA-intercalating fluorochrome dye (30 µg/ml propidium iodide; Sigma Chemical Co. St. Louis, USA) after RNase treatment (2.5 units/ml; Roche). In total, 58 samples were analysed using a flow cytometer (CyFlow SL3, Partec, Munster, Germany). At least 2--5 individuals per population and 5.000 to 10.000 nuclei per individual were analysed to obtain the mean DNA content. The 2C DNA value was calculated based on the linear relationship between the fluorescent signals from the stained nuclei of *A. grandiflora* specimens and the internal standard.

ANOVA with the Welch test for unequal sampled population sizes was performed, followed by a Games--Howell test using IBM--SPSS--statistics version 24--win32 (Field 2013). ANOVA with the Welch test was employed to determine whether there were any significant differences between the mean monoploid genome sizes. A multiple comparisons test (Games-Howell test) was conducted to demonstrate all pairwise comparisons. Because of the unequal number of samples, a nonparametric measure (Spearman's Correlation Coefficient) was employed to investigate the relationships between altitude and both 2C DNA values and the 1C*x* monoploid genome size.

RESULTS

The main results of the molecular assessment using microsatellite markers are summarized in table 2; those of the cytogenetic analysis (chromosome counts and ploidy levels) in table 3; and those for the intrapopulation genome size variability over all spatial scales in fig.1.

Nuclear microsatellite analysis

Ten microsatellite loci were amplified in seven populations. The allelic phenotypes of the analysed samples are presented in table 2. The ploidy level was deduced from the allelic phenotypes at the ten microsatellite loci, and a total of 258 alleles were revealed for the 10 loci (between 4 and 15 alleles per locus over all seven investigated populations). A maximum of four alleles per locus per individual were detected at most loci in the individuals from Mt Chasseral population in the Swiss Jura, Mt Lure population in the Alps and from Gabas population in the Spanish Pyrenees (fig. 2). Individuals from the populations in Andalusia (Mt Sagra), Rocamadour, Chinon (Bauemont en Veron) and the French Pyrenees (Gèdre) displayed a maximum of two alleles per locus per individual that are sometimes recognizable heterozygosity (table 2).

Chromosome number and ploidy level

Two chromosome numbers corresponding to two ploidy levels, diploid ($2n=2x=22$; figs 3 a & b) and tetraploid ($2n=4x=44$; figs 3c & d), were detected in the *A. grandiflora* species complex (table 3). The metaphase chromosomes were relatively small (1 µm to 2 µm), morphologically similar and difficult to spread. Diploid populations were frequent in populations of Chinon (Bauemont en Veron), the Spanish Pyrenees (Plan) and Andalusia (Mt Sagra), whereas the tetraploid populations were found in both alpine and pre-alpine populations (Mt Lure, Mt Raton) also in and the Spanish Pyrenees (Ceresa).

Genome size data

The holoploid genome size (2C value) ranged from (2.08 pg) to (2.98 pg) for diploid populations and from 4.28 pg to 5.62 pg for tetraploid populations. The monoploid (1C*x*) genome size ranged from 1.015.85 to 1.455.58 Mbp. The average quantity of nuclear DNA for each population is given in table 3. Based on the flow cytometric analysis of isolated nuclei, histograms of the relative nuclear DNA content were obtained, which showed two dominant peaks corresponding to both the internal standard and the species complex of *A. grandiflora* for all studied diploid and tetraploid populations (figs 4 a & b).

Statistical analyses (ANOVA) of genome size--ANOVA with the Welch test for monoploid genome sizes (1C*x*) revealed significant differences between the studied populations (F=32.394. p=0.000). To indicate pairwise differences in the populations, we applied Games-Howell analysis (see Supplementary 1). There was an interesting significant difference between the diploid population of Sainte Baume in the southeast of France (Pas de l'Ai) and all other diploid populations (fig. 5), except for the population of the Vilas del Turbon and Mt Sagra in Spain (Supplementary 1). Another interesting difference was found between diploid populations one from the Spanish Pyrenees (Vilas del turbon) and others from the French Pyrenees (Lake Gloriettes, Coumely Plateau and Saint Sauveur) (Supplementary 1). Additionally, there was a significant difference among the alpine population of Mt Cheval Blanc and the two pre-alpine populations of the Chaudière and Mt Raton (fig. 5). Indeed, our results also showed a significant difference (p=0.03) between the tetraploid populations in the Spanish Pyrenees (Ceresa and Gabas). Furthermore, a significant difference was detected between the tetraploid population of Mt Chasseral in Swiss Jura and the tetraploid pre-alpine populations of the Chaudière and Mt Raton.

In general, a strong positive correlation was found between altitude and the 2C DNA value (r=0.69. P=0.000). A strong positive correlation was also detected between altitude and the 1C*x* monoploid genome size (r=0.58. P=.000). In the diploid populations, a strong positive correlation (r=0.59; P=0.000) was detected between altitude and both the 2C DNA value and 1C*x* monoploid genome size, while in the tetraploid populations, there was no statistically significant correlation (r=- 0.24. P=0.224)

DISCUSSION

Microsatellite genotyping

The analysis of 10 polymorphic microsatellite loci detected 258 alleles in natural populations of *A. grandiflora* species complex and the number of alleles per sample (2–4) corresponded to the intraspecific variation in ploidy observed via flow cytometry or chromosome counting. For the individuals from Swiss Jura (Mt Chasseral) and the Spanish Pyrenees (Gabas) that displayed up to four alleles per locus (fig. 2), so, the number of alleles could be an indicator of the ploidy level. Here, the observed microsatellite patterns suggested that these populations are tetraploid and the count chromosomes affirmed these findings.

Furthermore, because all ten loci that we examined using microsatellite markers were the same for the diploid and tetraploid plants and tetraploids exhibited up to 4 alleles, we can conclude that the tetraploids

that occurred in some of the *A. grandiflora* populations could have resulted from spontaneous genome duplication or from the joining of unreduced gametes. Polyploids that form via genome doubling within a species have been well described by Soltis et al. (2007) in many species, such as *Tolmiea menziesii* (Pursh) Torr. & Gray., *Galax urceolata* (Poir.) Brummit., and *Chamerion angustifolium* (L.) Holub.

Chromosome number

Our results indicated a basic chromosome number of x=11, including two cytotypes, one diploid (2n=22) and one tetraploid (2n=44). These results basically agreed with those reported by Küpfer (1974) but differed from the x=10 chromosome number observed for *A. grandiflora* subsp. *grandiflora* in the Sierra de Baza in the Province of Granada (Luque & Lifante 1991), which we were unable to find during our collection session. We did not detect any cases of mixed ploidy, as found in other species complex (e.g., *Festuca pallens* (Smarda & Bures 2006)).

To our knowledge and according to the available literature, our identification of the chromosome number (2n=22) is novel for the population of Chinon from the Loire Valley. Furthermore, a 2n=22 chromosome number was also observed in population from the Spanish Pyrenees (Huesca. Plan), which is in agreement with the chromosome counts previously reported in populations of *A. grandiflora subsp. grandiflora* throughout the Pyrenean chain (Küpfer 1974). While our results indicated that the population from Mt Sagra in Andalusia was a diploid population (2n=22), Küpfer observed two populations on the other massif (Sierra Nevada), among which one was described as tetraploid (Küpfer 1974). On the other hand, this result agrees with the counts previously detected in east-central Spain (Teruel) and the Province of Cordoue in Andalusia (Peniarroya) (Küpfer 1974).

Our chromosome count of 2n=44 for populations from the Spanish Pyrenees (Huesca; Ceresa altitude: 1711 m) also strongly supported an earlier report on *A. grandiflora ssp grandiflora* (Favarger 1959. 1962) in the Spanish Pyrenees, also located in Huesca, at an altitude of 1.600 m. Furthermore, the population from both the Alps and the pre-Alps (Mt Lure, Mt Raton) showed 2n=44 as well, which is in accordance with another tetraploid population from Mont Ventoux in the same massif (Monts de Vaucluse -Alps) (Küpfer 1974). Our identification of the chromosome number (2n=44) is unprecedented for all the investigated populations from both the Alps and the pre-Alps.

Because most tetraploid individuals that we identified grew at altitude, we can assume that polyploidy has occurred and spread in regions with extreme climatic conditions, such as alpine zones, as polyploidy may increase adaptability to unfavourable habitats (Flovik 1940).

In accordance with an earlier report on the chromosome counts (2n=44) and sizes of Majorcan mountain-endemic *A. grandiflora* subsp. *glabrescens* in the Balearic Islands (Castro & Rosselló 2006). The metaphase chromosomes of the populations investigated in the present study were relatively small (1–2 μm) and morphologically similar.

Intraspecific variation in genome size

To our knowledge and according to the available databases, our results regarding 2C DNA values are novel for the *A. grandiflora* species complex in Western Europe. This study identified intraspecific variation in genome size among 21 populations of the species complex of *A. grandiflora* from its natural distribution ranges for the first time. The obtained 2C DNA values ranged from 2.08 pg to 5.62 pg (i.e. 1.015.85 to 1.455.58 Mbp for monoploid genome size) and were higher than the values estimated previously for tetraploid *A. serpyllifolia* (1.60 pg and 391 Mbp for 1Cx. pers. comm. Brand SR. 1984 in Bennett & Smith 1991), for diploid *A. gracilis* (1.19 pg and 582 Mbp. Siljak-Yakovlev et al. 2010) and for diploid *A. deflexa* (2.04 pg and 993 Mbp. Bou Dagher-Kharrat et al. 2013).

Results of ANOVA--In general, the variation of 1C*x* values may be explained by the potential for adaptation to different environmental conditions, including environmental extremes at high latitudes and altitudes and harsh climatic conditions (Comai 2005; Ramsey 2011; Brochmann et al. 2004). In the samples of the species complex of *A. grandiflora*, the higher ploidy levels showed a positive correlation with altitude. This correlation was noted previously by Löve & Löve (1943) and Sharma & Sharma (1958). The findings showed that the genome size of the Mt Cheval Blanc population (the highest population) was significantly different from those found at Beaumont-en-Véron near the city of Chinon (the lowest population, at 35 m) and Mt Raton (an intermediate population, at 1.473 m). These results suggested that local adaptation may be related to genome enlargement and duplication (Ramsey 2011; García-Fernández et al. 2012). However, genome downsizing is a widespread phenomenon in polyploids (Leitch et al. 2008) and our findings highlighted that this phenomenon is not observed in the tetraploid populations in the Pre-Alps (Chaudière 1.560 m and Mt Raton 1.473 m) or Spanish Pyrenees (Gabas 1830 m). The largest monoploid genome size

was found in the diploid population from the southeast of France (Pas de l'Ai) growing at 1.034 m. According to Youssef et al. (2011), this population exhibits a narrower and more marginal niche than other species of *Arenaria*. Moreover, the Sainte Baume mountain chain has a Mediterranean climate. In this case, it might be assumed that the increased monoploid genome size could be correlated with the climatic conditions of Mediterranean mountains, which often present stressful environments (high temperatures or serious drought conditions in summer (Grime & Mowforth 1982; Giménez-Benavides et al. 2008; 2007)). However, two diploid populations were also located at high altitudes of the Mt Sagra in Andalusia (2279 m) and the Spanish Pyrenees (the Vilas del Turbon 1720 m) and displayed larger monoploid genome sizes compared with other diploid populations. It was also found that the population from Plan in the Spanish Pyrenees (1720 m) presented a large monoploid genome size compared with other diploid populations from plains and small hills. At the genomic level, the intraspecific variation in genome size is usually caused by differences in the content of noncoding repetitive DNA, such as transposable elements (e.g., via the activity of retrotransposons) (Smarda et al. 2008), satellite DNA, introns, and pseudogenes (Bennett & Leitch 2005; Smarda & Bures 2010)

Intraspecific polyploidy and conservation

The study of intraspecific genome size variation can reveal inter- and even intra-populational variations in ploidy (Siljak-Yakovlev et al. 2010). The phenomenon of intraspecific ploidy variation is prevalent (Keeler 2004; Suda et al. 2007; Duchoslav et al. 2010; Realini et al. 2015), and its frequency has recently been estimated to be 12–13% for angiospermes (Wood et al. 2009). However, less attention has been paid to this phenomenon when conservation priorities are being set and it remains unaddressed in plant conservation programmes. For example, two-thirds of threatened or endangered plants in the USA are known to exhibit intraspecific chromosome number variation. yet this has been taken into account for very few species in conservation plans to our knowledge (Severns & Liston 2008; Delaney & Baack 2012). To avoid the risk of omitting information on ploidy levels in restoration measures, it would be desirable to first determine the existence of potential cytotypes. Our study identified different ploidy levels in native populations of threatened and endangered species and highlighted the importance of this form of genetic variation in plant conservation. In programmes aimed at reinforcement of populations, mixing of individuals with different ploidy levels should be stringently avoided. Here, we might consider all the populations with different ploidy

levels, such as the alpine and pre-alpine populations (Mt Lure, Mt Cheval Blanc, the Mt Chaudière and Mt Raton) also, the Mt Chasseral population in the Swiss Jura, as distinct ESUs. Moreover, we could define the diploid populations presenting intraspecific variation in monoploid genome size from Pas de l'Ai in the Mt Sainte Baume chain in France and the Mt Sagra in Andalusia as distinct ESUs.

CONCLUSION

In this report, we show that different techniques can be used to estimate ploidy levels in threatened and rare species complex of *A. grandiflora* and can contribute to improved definition of restoration plans. Among these techniques, flow cytometry can be a useful tool. Small amounts of leaf tissue, cotyledons of germinated seedlings, dried seeds, or stored leaves in silica gel are sufficient for the estimation of ploidy level and nuclear genome size. Because of the difficulty of applying classical karyological techniques in the *A. grandiflora* species complex due to seed dormancy, crystals of oxalate of calcium that occur in the roots or the presence of endophytic fungi in seeds, the technique of flow cytometry was found to be very helpful for the rapid estimation of ploidy levels in this study and may be a powerful alternative karyological approach for threatened species. The use of microsatellite markers provided an additional opportunity to obtain information on genetic diversity, which can also aid in the definition of conservation priorities (Loeschcke et al. 1994).

The co-occurrence of diploid-tetraploid populations of *A. grandiflora* species complex in the Spanish Pyrenees leads to many questions regarding the origins of polyploid populations. The most interesting finding emerging from this study was the presence of populations with different ploidy levels, even within the same subspecies of the species complex of *A. grandiflora*.

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Figure Captions

Figure 1--Map of the distribution of the sampled populations of *Arenaria grandiflora* species complex from Western Europe, indicating ploidy levels (2x and 4x); the map was created using ArcGIS online.

Figure 2--The four alleles of three individuals from two tetraploid populations of *Arenaria grandiflora* species complex in both Gabas in the Spanish Pyrenees and Mt Chasseral in the Swiss Jura, as revealed through genotyping microsatellite markers (AG9-49, AGA5-109 and AG3-36). The sizes of the alleles were determined with Gene Mapper software (Applied Biosystems).

Figures 3A, B, C & D--Metaphase chromosome plates of *Arenaria grandiflora* species complex, that were investigated from diploid (**A**. Chinon and **B.** Spanish Pyrenees) and tetraploid (**C.** Mt Lure and **D**. Spanish Pyrenees) populations. Scale bar=10 μm.

Figures 4A & B--Histograms of relative DNA contents after flow cytometric analysis of propidium iodide-stained nuclei of *Arenaria grandiflora* species complex and *Petunia hybrid,* which were isolated, stained and analysed simultaneously; (A) Nuclei isolated from young leaves of the diploid population (Rocamadour, Castle) and (B) nuclei isolated from dried and stored seeds of the tetraploid population (Mt Raton), using *Petunia hybrida* as an internal standard.

Figure 5--Histogram showing the genome size (2C in pg) for each population of *Arenaria grandiflora* species complex, with error bars.

Table 1-- Geographical origins of the studied materials.

No	Population	Latitude	Longitude	Altitude (m)	Type of Tissue/Date	Region
1	Baumont-en-Véron*	47°11'42.93"N	0°11'9.87"E	35	leaves/2015	Loire Valley
2	Rocamadour.Casetl	44°47'53.03"N	1°36'53.69"E	331	leaves /2014	Languedoc-Roussillon Midi Pyrenees
3	Rocamadour.roadside	44°47'53.46"N	1°37'5.25"E	260	leaves /2014	Languedoc-Roussillon Midi Pyrenees
4	Lake Gloriettes	42°45'24.29"N	0° 3'19.89"E	1690	leaves /2013	Gèdre-Hautes-Pyrenees
5	Coumely Plateau	42°43'0.54"N	0° 1'17.42"W	1600	leaves /2013	Gavarnie-Hautes-Pyrenees
6	Plan	42°33'13.5"N	0°26'9.80"E	1720	seeds + leaves /2015	Huesca-Pyrenees
7	Mt Sagra	37°57'13.20"N	2°33'34.90"O	2279	leaves/2014 + seeds /2015	Granada-Andalusia
8	Queue de vache East**	48°23'17.21"N	2°36'24.81"E	131	leaves /2016	Fontainebleau forest
9	Queue de vache West**	48°23'11.12"N	2°36'17.75"E	129	leaves /2016	Fontainebleau forest
10	Mont Chauvet	48°25'43.36"N	2°40'59.83"E	87	leaves /2013	Fontainebleau forest
11	Mt Lure	44° 7'26.93"N	5°46'23.16"E	1718	seeds /2013	Monts de Vaucluse-Alps
12	Mt Chasseral	47° 7'50.43"N	7° 3'42.98"E	1 502	leaves /2013	Mt Jura -Vaud
13	Ceresa	42°29'24.90"N	0°12'34.60"E	1711	seeds + leaves /2015	Huesca-Pyrenees
14	Gabas	42°27'48.9"N	0°28'00.5"E	1830	leaves/2014	Huesca-Pyrenees
15	Mt Cheval Blanc	44° 07′ 39″ N	6° 25′ 26″ E	2300	seeds /1990	Alps de Haute Provence
16	Pas de l'Ai	43° 34′ 180″ N	5° 81′ 854″ E	1034	leaves/2016	Sainte Baume-Provence
17	Mt Raton	44°23'53.71"N	5°27'47.25"E	1473	seeds /2008	Prealps
18	Saint Sauveur	42°51'35.1"N	0°00'44.0"O	899	leaves/2016	Languedoc-Roussillon Midi Pyrenees
19	Butte de Sers	42°53'16.0"N	0°02'28.7"O	1185	leaves/2016	Languedoc-Roussillon Midi Pyrenees

20	The Vilas del Turbon	42°24'14.20"N	0°31'35.40"E	1500	seeds + leaves/2015	Huesca-Pyrenees
21	The Chaudière	44° 37′ 57″ N	5° 13′ 02″ E	1560	seeds /2007	Prealps

*_In vitro_-cultured clones from the Paris Basin Conservatory

**Mixed population introduced in Fontainebleau forest in 1999

No: Studied population number

Table 2 -- The allelic phenotypes at the ten microsatellite loci for some analysed individuals. Allele size is given in bp. Number of alleles per individual for each locus: (Aind); number of alleles per locus for each individual: (ALoc).

Individus	**Sagra**	**Sagra**	**B. en V.**	**B. en V.**	**Lure**	**Gabas**	**Gabas**	**Lake Glo.**	**Lake Glo.**	**Roca.**	**Roca.**	**Chass.**	**Chass.**	**Chass.**	**= 14 Ind**
Aind	15	15	14	15	23	26	24	16	17	16	13	24	20	20	**= 258**
Ploidy Levels	2*x*	2*x*	2*x*	2*x*	4*x*	4*x*	4*x*	2*x*	2*x*	2 *x*	2 *x*	4*x*	4*x*	4*x*	**A**Loc
Loci / Allele	184	178	181	181	181	178	181	178	178	196	181	184	184	184	
AGA3-98	184	178	181	181	181	181 184 190	184 190	184	184	196	181	184	184	184	**4**
AG3-2	148 179	143 167	177 179	161 179	146 146 159 163	146 146	146 146	173 173	167 170	148 152	140 181	155 155 159 181	155 181 181 181	159 159 159 163	**14**
AG3-36	138 142	138 138	150 170	150 150	- - - -	136 136 138 160	136 136 138 160	162 170	148 148	160 182	170 182	128 130 138 154	138 144 148 154	128 138 138 154	**15**
AG9-49	267 267	265 267	287 287	263 263	259 259 259 265	259 259 267 271	281 287	265 281	263 267	263 273	263 263	263 263 265 267	267 267 267 277	267 267 277 267	**9**
AGA2-74	209 209	209 209	209 215	209 209	203 209 209	209 215 227 227	209 215 227 227	215 221	209 209	215 215	209 215	209 209	209 209	209 209	**5**
AGC5-93	147 151	145 151	147 147	143 145	- - -	143 145 147	145 145 149	145 151	145 147	153 159	153 153	149 149 153	149 151 155	149 149 149	**8**

					-	149	159					153	155	151	
AGA5-109	174	180	177	177	171	168	174	171	190	174	174	168	168	190	**15**
	202	180	180	211	177	174	180	180	202	186	174	168	168	199	
					183	180	196					190	168	217	
					193	196	199					211	190	217	
AGA2-139	182	185	185	185	173	176	176	185	173	173	185	173	173	182	**8**
	182	188	188	188	185	191	176	185	185	185	185	176	173	182	
					188	191	191					176	185	182	
					191	191	194					182	185	182	
AGA1-150	168	168	174	168	160	163	163	168	168	168	168	171	171	171	**5**
	168	168	174	168	168	168	168	168	168	168	168	174	174	174	
					171	174	168					174	174	174	
					174	174	174					174	174	174	
AGA5-123	174	174	180	174	174	174	174	180	174	174	174	176	176	178	**7**
	178	184	180	184	180	174	174	180	180	180	180	180	192	180	
					184	174	174					184	192	184	
					190	184	184					184	192	184	

Aind: N° of alleles per individual. ALoc: N° of alleles per locus. Ind: individus Sagra: Mt Sagra; B. en V. : Bauemont en Veron; Lure: Mt Lure; Lake Glo.: Lake Gloriettes; Roca: Rocamadour.Casetl; Chass.: Mt Chasseral.

Table 3 -- The results of cytogenetic analyses including: Chromosome number (2*n*), ploidy level and genome size for the investigated *Arenaria grandiflora* populations.

Population	**2*n***	**Ploidy level**	**Mean 2C value in pg ±SD**	**1C*x* in Mpb**	**Number of studied individuals/population**
Bauemont en Veron	22	2*x*	2.13±0.78	1039.17	10
Plan	22	2x	2.19±0.77	1071.89	2
Mt Sagra	22	2x	2.44±0.96	1195.12	4
Mont Chauvet		2x	2.11±0.74	1033.01	2
Queue de vache East		2x	2.11±0.74	1033.9	5
Queue de vache West		2x	2.13±0.74	1039.8	5
Rocamadour. roadside		2x	2.17±0.76	1064.85	5
Rocamadour. Castle		2x	2.17±0.76	1062.04	5
Gloriettes Lack		2x	2.17±0.76	1059.37	5
Coumely Plateau		2x	2.16±0.76	1054.77	5
Mt Lure	44	4x	4.64±1.63	1134.11	4
Ceresa	44	4x	4.30±1.51	1051.47	2
Mt Chasseral	44*	4x	4.39±1.54	1072.7	3
Gabas		4x	4.81±0.12	1 177.03	5
Mt Cheval Blanc		4x	4.56±0.03	1 115.40	5
Pas de l'Ai		2x	2.70±0.11	1 321.90	5
Mt Raton	44	4x	4.98±0.05	1 218.32	3
Saint Sauveur		2x	2.21±0.09	1 079.09	5
Butte de Sers		2x	2.23±0.03	1 090.08	5
The Vilas del Turbon		2x	2.39±0.01	1147.10	2
The Chaudière		4x	5.27±0.14	1 288.75	5

***** From Favarger (1959. 1962), Küpfer (1974); N – number of investigated individuals
1 pg=978 Mbp according (Doležel et al. 2003)

Figure 1--Map of the distribution of the sampled populations from Western Europe, indicating ploidy levels (2x and 4x); the map was created using ArcGIS online.

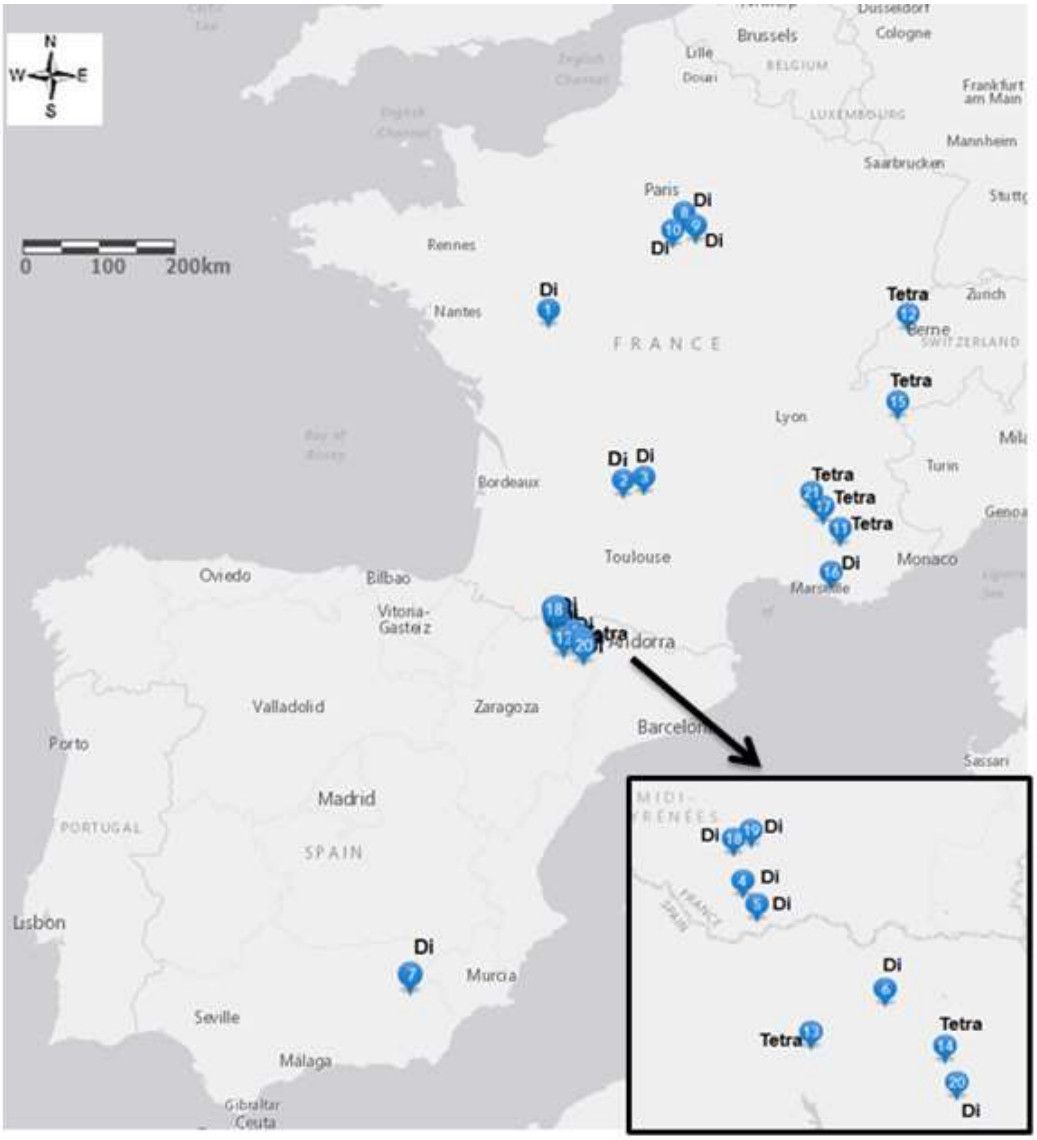
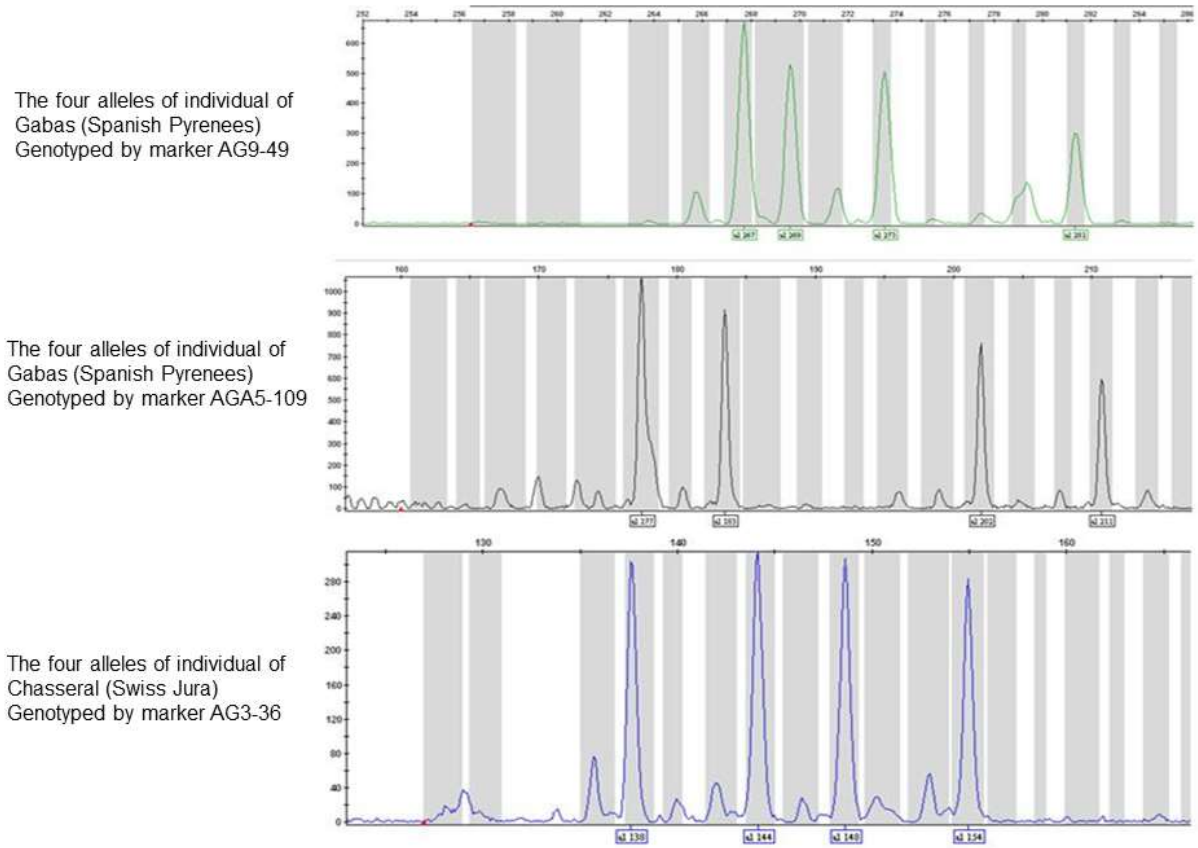
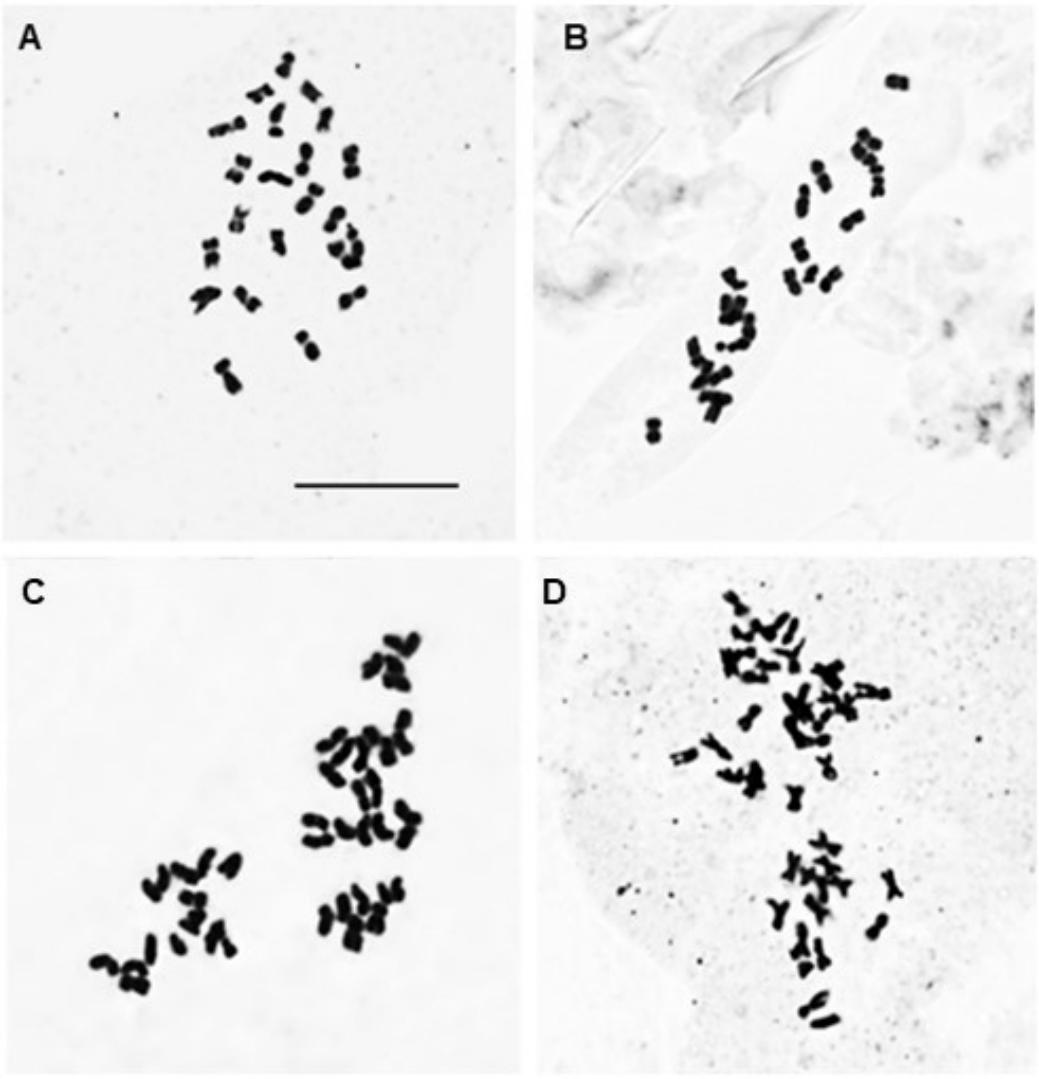


Figure 2--The four alleles of three individuals from two tetraploid populations: Gabas in the Spanish Pyrenees and Mt Chasseral in the Swiss Jura, as revealed through genotyping microsatellite markers (AG9-49, AGA5-109 and AG3-36). The sizes of the alleles were determined with Gene Mapper software (Applied Biosystems).



Figures 3A, B, C & D--Metaphase chromosome plates of *Arenaria grandiflora* species complex, that were investigated from diploid (**A**. Chinon and **B.** Spanish Pyrenees) and tetraploid (**C.** Mt Lure and **D**. Spanish Pyrenees) populations. Scale bar=10 μm.



Figures 4A & B--Histograms of relative DNA contents after flow cytometric analysis of propidium iodide-stained nuclei of *Arenaria grandiflora* species complex and *Petunia hybrid,* which were isolated, stained and analysed simultaneously; (A) Nuclei isolated from young leaves of the diploid population (Rocamadour, Castle) and (B) nuclei isolated from dried and stored seeds of the tetraploid population (Mt Raton), using *Petunia hybrida* as an internal standard

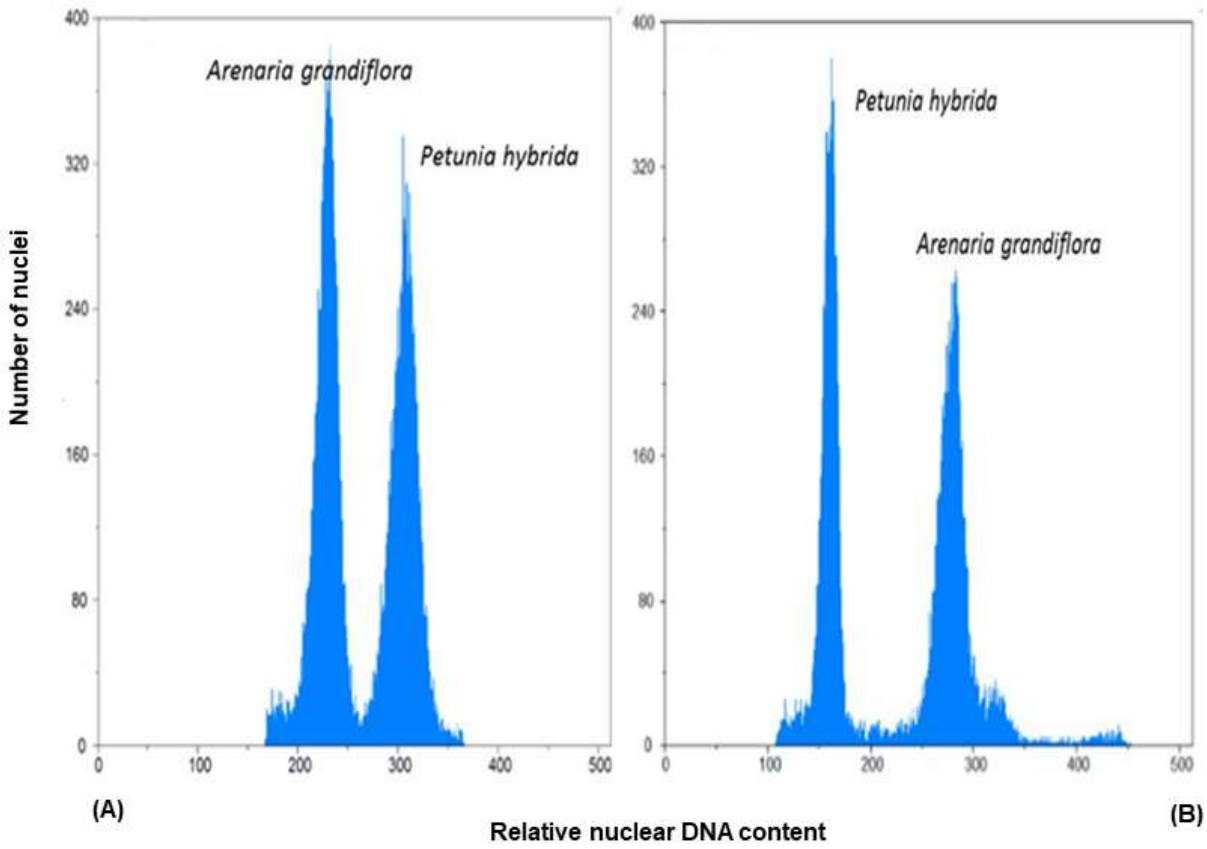
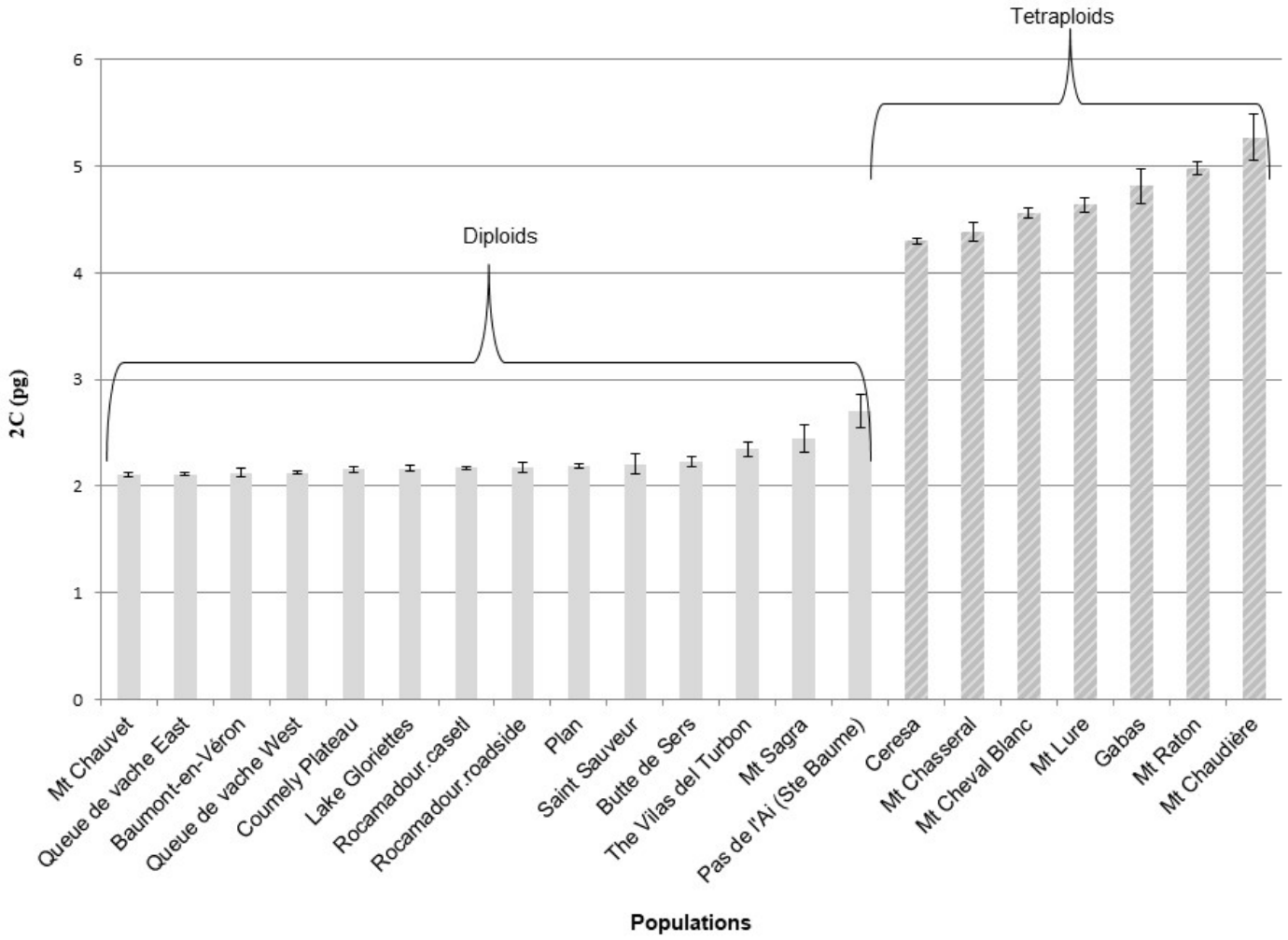


Figure 5--Histogram showing the genome size (2C in pg), for each population of *Arenaria grandiflora* species complex, with error bars



Electronic appendix 1 to:

Marwa Daoud Sonja Siljak-Yakovlev & Nathalie Machon (2017):

Intraspecific DNA content variability: consequences for conservation

Plant Ecology and Evolution

Appendix 1 – Multiple Comparisons (Dependent Variable: Mbp [1C*x*]), with the average difference between the two populations and standard error

(I) Population	(J) Population	Average Difference (I-J)	Standard Error	P value
Beaumont-en-Véron	Rocamadour.Casetl	-21.9561	6.7339	0.274
	Rocamadour.roadside	-25.6725	12.7352	0.838
	Lake Gloriettes	-20.1957	8.8920	0.740
	Coumely Plateau	-15.5991	8.8791	0.941
	Plan	-32.7141	9.3462	0.366
	Mt Sagra	-155.9421	31.8956	0.167
	Queue de vache East	5.2716	7.6070	1.000
	Queue de vache West	-.6250	7.4023	1.000
	Mt Chauvet	6.1614	9.5267	1.000
	Mt Lure	-94.9394*	10.4953	**0.002**
	Mt Chasseral	-33.5291	13.3947	0.644
	Ceresa	-12.2983	7.5338	0.954
	Gabas	-137.8601*	18.8675	**0.019**
	Mt Cheval Blanc	-76.2291*	8.3754	**0.000**
	Pas de l'Ai	-282.7289*	34.8047	**0.020**
	Mt Raton	-179.1459*	11.3165	**0.002**
	Saint Sauveur	-39.9135	21.0962	0.868
	Butte de Sers	-50.9046	11.4878	0.089
	Vilas del Turbon	-130.3436*	6.3626	**0.000**

	Chaudière	-249.5782*	24.9161	**0.007**
Rocamadour.Casetl	Beaumont-en-Véron	21.9561	6.7339	0.274
	Rocamadour.roadside	-3.7164	11.2501	1.000
	Lake Gloriettes	1.7604	6.5914	1.000
	Coumely Plateau	6.3570	6.5740	0.999
	Plan	-10.7580	7.1923	0.916
	Mt Sagra	-133.9860	31.3323	0.246
	Queue de vache East	27.2277*	4.7166	**0.035**
	Queue de vache West	21.3311	4.3788	0.070
	Mt Chauvet	28.1175	7.4255	0.483
	Mt Lure	-72.9833*	8.6331	**0.031**
	Mt Chasseral	-11.5730	11.9916	0.996
	Ceresa	9.6578	4.5975	0.777
	Gabas	-115.9040	17.8987	0.050
	Mt Cheval Blanc	-54.2730*	5.8759	**0.006**
	Pas de l'Ai	-260.7728*	34.2892	**.030**
	Mt Raton	-157.1898*	9.6147	**.020**
	Saint Sauveur	-17.9574	20.2343	1.000
	Butte de Sers	-28.9485	9.8158	0.473
	Vilas del Turbon	-108.3875*	2.2050	**0.000**
	Chaudière	-227.6221*	24.1907	**0.013**
Rocamadour.roadside	Beaumont-en-Véron	25.6725	12.7352	0.838
	Rocamadour.Casetl	3.7164	11.2501	1.000
	Lake Gloriettes	5.4768	12.6605	1.000
	Coumely Plateau	10.0734	12.6514	1.000
	Plan	-7.0416	12.9835	1.000
	Mt Sagra	-130.2696	33.1444	0.259

	Queue de vache East	30.9441	11.7935	0.582
	Queue de vache West	25.0475	11.6625	0.776
	Mt Chauvet	31.8339	13.1140	0.663
	Mt Lure	-69.2669	13.8337	0.057
	Mt Chasseral	-7.8566	16.1443	1.000
	Ceresa	13.3742	11.7464	0.996
	Gabas	-112.1876*	20.9094	**0.043**
	Mt Cheval Blanc	-50.5566	12.3031	0.162
	Pas de l'Ai	-257.0564*	35.9526	**0.024**
	Mt Raton	-153.4734*	14.4666	**0.002**
	Saint Sauveur	-14.2410	22.9405	1.000
	Butte de Sers	-25.2321	14.6010	0.936
	Vilas del Turbon	-104.6711*	11.0319	**0.013**
	Chaudière	-223.9057*	26.4959	**0.007**
Lake Gloriettes	Beaumont-en-Véron	20.1957	8.8920	0.740
	Rocamadour.Casetl	-1.7604	6.5914	1.000
	Rocamadour.roadside	-5.4768	12.6605	1.000
	Coumely Plateau	4.5966	8.7715	1.000
	Plan	-12.5184	9.2440	0.971
	Mt Sagra	-135.7464	31.8659	0.236
	Queue de vache East	25.4673	7.4812	0.281
	Queue de vache West	19.5707	7.2730	0.542
	Mt Chauvet	26.3571	9.4265	0.562
	Mt Lure	-74.7437*	10.4045	**0.013**
	Mt Chasseral	-13.3334	13.3237	0.997
	Ceresa	7.8974	7.4067	0.998
	Gabas	-117.6644*	18.8171	**0.039**

	Mt Cheval Blanc	-56.0334*	8.2613	**0.008**
	Pas de l'Ai	-262.5332*	34.7774	**0.026**
	Mt Raton	-158.9502*	11.2323	**0.004**
	Saint Sauveur	-19.7178	21.0511	0.999
	Butte de Sers	-30.7089	11.4049	0.540
	Vilas del Turbon	-110.1479*	6.2116	**0.001**
	Chaudière	-229.3825*	24.8780	**0.010**
Coumely Plateau	Beaumont-en-Véron	15.5991	8.8791	0.941
	Rocamadour.Casetl	-6.3570	6.5740	0.999
	Rocamadour.roadside	-10.0734	12.6514	1.000
	Lake Gloriettes	-4.5966	8.7715	1.000
	Plan	-17.1150	9.2316	0.859
	Mt Sagra	-140.3430	31.8623	0.218
	Queue de vache East	20.8707	7.4658	0.494
	Queue de vache West	14.9741	7.2572	0.819
	Mt Chauvet	21.7605	9.4144	0.712
	Mt Lure	-79.3402*	10.3935	**0.010**
	Mt Chasseral	-17.9300	13.3151	0.974
	Ceresa	3.3008	7.3912	1.000
	Gabas	-122.2610*	18.8110	**0.033**
	Mt Cheval Blanc	-60.6300*	8.2474	**0.004**
	Pas de l'Ai	-267.1298*	34.7741	**0.025**
	Mt Raton	-163.5468*	11.2221	**0.003**
	Saint Sauveur	-24.3144	21.0457	0.996
	Butte de Sers	-35.3055	11.3948	0.379
	Vilas del Turbon	-114.7445*	6.1931	**0.001**
	Chaudière	-233.9791*	24.8733	**0.009**

Plan	Beaumont-en-Véron	32.7141	9.3462	0.366
	Rocamadour.Casetl	10.7580	7.1923	0.916
	Rocamadour.roadside	7.0416	12.9835	1.000
	Lake Gloriettes	12.5184	9.2440	0.971
	Coumely Plateau	17.1150	9.2316	0.859
	Mt Sagra	-123.2280	31.9956	0.296
	Queue de vache East	37.9857	8.0157	0.295
	Queue de vache West	32.0891	7.8217	0.382
	Mt Chauvet	38.8755	9.8561	0.369
	Mt Lure	-62.2253	10.7952	0.095
	Mt Chasseral	-.8150	13.6310	1.000
	Ceresa	20.4158	7.9463	0.659
	Gabas	-105.1460	19.0360	0.067
	Mt Cheval Blanc	-43.5150	8.7483	0.215
	Pas de l'Ai	-250.0148*	34.8963	**0.032**
	Mt Raton	-146.4318*	11.5952	**0.014**
	Saint Sauveur	-7.1994	21.2469	1.000
	Butte de Sers	-18.1905	11.7624	0.954
	Vilas del Turbon	-97.6295	6.8460	0.149
	Chaudière	-216.8641*	25.0439	**0.013**
Mt Sagra	Beaumont-en-Véron	155.9421	31.8956	0.167
	Rocamadour.Casetl	133.9860	31.3323	0.246
	Rocamadour.roadside	130.2696	33.1444	0.259
	Lake Gloriettes	135.7464	31.8659	0.236
	Coumely Plateau	140.3430	31.8623	0.218
	Plan	123.2280	31.9956	0.296
	Queue de vache East	161.2137	31.5315	0.157

	Queue de vache West	155.3171	31.4827	0.173
	Mt Chauvet	162.1035	32.0488	0.151
	Mt Lure	61.0027	32.3499	0.856
	Mt Chasseral	122.4130	33.4034	0.306
	Ceresa	143.6438	31.5139	0.208
	Gabas	18.0820	35.9493	1.000
	Mt Cheval Blanc	79.7130	31.7256	0.641
	Pas de l'Ai	-126.7868	46.3437	0.520
	Mt Raton	-23.2038	32.6256	1.000
	Saint Sauveur	116.0286	37.1674	0.398
	Butte de Sers	105.0375	32.6854	0.419
	Vilas del Turbon	25.5985	31.2546	1.000
	Chaudière	-93.6361	39.4611	0.685
Queue de vache East	Beaumont-en-Véron	-5.2716	7.6070	1.000
	Rocamadour.Casetl	-27.2277*	4.7166	**0.035**
	Rocamadour.roadside	-30.9441	11.7935	0.582
	Lake Gloriettes	-25.4673	7.4812	0.281
	Coumely Plateau	-20.8707	7.4658	0.494
	Plan	-37.9857	8.0157	0.295
	Mt Sagra	-161.2137	31.5315	0.157
	Queue de vache West	-5.8966	5.6299	0.999
	Mt Chauvet	.8898	8.2255	1.000
	Mt Lure	-100.2110*	9.3302	**0.005**
	Mt Chasseral	-38.8007	12.5028	0.490
	Ceresa	-17.5700	5.8016	0.477
	Gabas	-143.1317*	18.2451	**0.020**
	Mt Cheval Blanc	-81.5007*	6.8592	**0.000**

	Pas de l'Ai	-288.0006*	34.4713	**0.020**
	Mt Raton	-184.4176*	10.2452	**0.006**
	Saint Sauveur	-45.1851	20.5414	0.753
	Butte de Sers	-56.1762	10.4341	0.060
	Vilas del Turbon	-135.6152*	4.1694	**0.000**
	Chaudière	-254.8499*	24.4481	**0.008**
Queue de vache West	Beaumont-en-Véron	.6250	7.4023	1.000
	Rocamadour.Casetl	-21.3311	4.3788	0.070
	Rocamadour.roadside	-25.0475	11.6625	0.776
	Lake Gloriettes	-19.5707	7.2730	0.542
	Coumely Plateau	-14.9741	7.2572	0.819
	Plan	-32.0891	7.8217	0.382
	Mt Sagra	-155.3171	31.4827	0.173
	Queue de vache East	5.8966	5.6299	0.999
	Mt Chauvet	6.7864	8.0366	0.998
	Mt Lure	-94.3143*	9.1641	**0.008**
	Mt Chasseral	-32.9041	12.3793	0.610
	Ceresa	-11.6733	5.5305	0.779
	Gabas	-137.2351*	18.1607	**0.025**
	Mt Cheval Blanc	-75.6041*	6.6314	**0.000**
	Pas de l'Ai	-282.1039*	34.4267	**0.022**
	Mt Raton	-178.5209*	10.0941	**0.008**
	Saint Sauveur	-39.2884	20.4665	0.854
	Butte de Sers	-50.2796	10.2859	0.095
	Vilas del Turbon	-129.7186*	3.7831	**0.000**
	Chaudière	-248.9532*	24.3852	**0.009**
Mt Chauvet	Beaumont-en-Véron	-6.1614	9.5267	1.000

	Rocamadour.Casetl	-28.1175	7.4255	0.483
	Rocamadour.roadside	-31.8339	13.1140	0.663
	Lake Gloriettes	-26.3571	9.4265	0.562
	Coumely Plateau	-21.7605	9.4144	0.712
	Plan	-38.8755	9.8561	0.369
	Mt Sagra	-162.1035	32.0488	0.151
	Queue de vache East	-.8898	8.2255	1.000
	Queue de vache West	-6.7864	8.0366	0.998
	Mt Lure	-101.1008*	10.9519	**0.023**
	Mt Chasseral	-39.6905	13.7554	0.530
	Ceresa	-18.4597	8.1578	0.735
	Gabas	-144.0215*	19.1252	**0.019**
	Mt Cheval Blanc	-82.3905	8.9409	.061
	Pas de l'Ai	-288.8903*	34.9451	**.018**
	Mt Raton	-185.3073*	11.7412	**.007**
	Saint Sauveur	-46.0749	21.3270	0.770
	Butte de Sers	-57.0660	11.9064	0.129
	Vilas del Turbon	-136.5050	7.0905	0.110
	Chaudière	-255.7396*	25.1118	**0.006**
Mt Lure	**Beaumont-en-Véron**	94.9394*	10.4953	**0.002**
	Rocamadour.Casetl	72.9833*	8.6331	**0.031**
	Rocamadour.roadside	69.2669	13.8337	0.057
	Lake Gloriettes	74.7437*	10.4045	**0.013**
	Coumely Plateau	79.3402*	10.3935	**0.010**
	Plan	62.2253	10.7952	0.095
	Mt Sagra	-61.0027	32.3499	0.856
	Queue de vache East	100.2110*	9.3302	**0.005**

	Queue de vache West	94.3143*	9.1641	**0.008**
	Mt Chauvet	101.1008*	10.9519	**0.023**
	Mt Chasseral	61.4103	14.4432	0.207
	Ceresa	82.6410*	9.2706	**0.018**
	Gabas	-42.9208	19.6257	0.763
	Mt Cheval Blanc	18.7102	9.9666	0.878
	Pas de l'Ai	-187.7896	35.2215	0.086
	Mt Raton	-84.2066*	12.5399	**0.036**
	Saint Sauveur	55.0259	21.7769	0.620
	Butte de Sers	44.0347	12.6947	0.263
	Vilas del Turbon	-35.4043	8.3468	0.252
	Chaudière	-154.6389*	25.4950	**0.045**
Mt Chasseral	Beaumont-en-Véron	33.5291	13.3947	0.644
	Rocamadour.Casetl	11.5730	11.9916	0.996
	Rocamadour.roadside	7.8566	16.1443	1.000
	Lake Gloriettes	13.3334	13.3237	0.997
	Coumely Plateau	17.9300	13.3151	0.974
	Plan	.8150	13.6310	1.000
	Mt Sagra	-122.4130	33.4034	0.306
	Queue de vache East	38.8007	12.5028	0.490
	Queue de vache West	32.9041	12.3793	0.610
	Mt Chauvet	39.6905	13.7554	0.530
	Mt Lure	-61.4103	14.4432	0.207
	Ceresa	21.2307	12.4584	0.894
	Gabas	-104.3310	21.3175	0.079
	Mt Cheval Blanc	-42.7000	12.9846	0.428
	Pas de l'Ai	-249.1998*	36.1915	**0.027**

	Mt Raton	-145.6168*	15.0505	**0.015**
	Saint Sauveur	-6.3844	23.3131	1.000
	Butte de Sers	-17.3755	15.1797	0.996
	Vilas del Turbon	-96.8145	11.7871	0.103
	Chaudière	-216.0491*	26.8191	**0.009**
Ceresa	Beaumont-en-Véron	12.2983	7.5338	0.954
	Rocamadour.Casetl	-9.6578	4.5975	0.777
	Rocamadour.roadside	-13.3742	11.7464	0.996
	Lake Gloriettes	-7.8974	7.4067	0.998
	Coumely Plateau	-3.3008	7.3912	1.000
	Plan	-20.4158	7.9463	0.659
	Mt Sagra	-143.6438	31.5139	0.208
	Queue de vache East	17.5700	5.8016	0.477
	Queue de vache West	11.6733	5.5305	0.779
	Mt Chauvet	18.4597	8.1578	0.735
	Mt Lure	-82.6410*	9.2706	**0.018**
	Mt Chasseral	-21.2307	12.4584	0.894
	Gabas	-125.5618*	18.2147	**0.035**
	Mt Cheval Blanc	-63.9308*	6.7779	**0.010**
	Pas de l'Ai	-270.4306*	34.4552	**0.025**
	Mt Raton	-166.8476*	10.1909	**0.011**
	Saint Sauveur	-27.6151	20.5144	0.982
	Butte de Sers	-38.6063	10.3809	0.255
	Vilas del Turbon	-118.0453	4.0343	0.073
	Chaudière	-237.2799*	24.4255	**0.010**
Gabas	**Beaumont-en-Véron**	137.8601*	18.8675	**0.019**
	Rocamadour.Casetl	115.9040	17.8987	0.050

	Rocamadour.roadside	112.1876*	20.9094	**0.043**
	Lake Gloriettes	117.6644*	18.8171	**0.039**
	Coumely Plateau	122.2610*	18.8110	**0.033**
	Plan	105.1460	19.0360	0.067
	Mt Sagra	-18.0820	35.9493	1.000
	Queue de vache East	143.1317*	18.2451	**0.020**
	Queue de vache West	137.2351*	18.1607	**0.025**
	Mt Chauvet	144.0215*	19.1252	**0.019**
	Mt Lure	42.9208	19.6257	0.763
	Mt Chasseral	104.3310	21.3175	0.079
	Ceresa	125.5618*	18.2147	**0.035**
	Mt Cheval Blanc	61.6310	18.5786	0.354
	Pas de l'Ai	-144.8688	38.5537	0.218
	Mt Raton	-41.2858	20.0769	0.816
	Saint Sauveur	97.9467	26.8340	0.204
	Butte de Sers	86.9555	20.1739	0.129
	Vilas del Turbon	7.5165	17.7623	1.000
	Chaudière	-111.7181	29.9304	0.196
Mt Cheval Blanc	**Beaumont-en-Véron**	76.2291*	8.3754	**0.000**
	Rocamadour.Casetl	54.2730*	5.8759	**0.006**
	Rocamadour.roadside	50.5566	12.3031	0.162
	Lake Gloriettes	56.0334*	8.2613	**0.008**
	Coumely Plateau	60.6300*	8.2474	**0.004**
	Plan	43.5150	8.7483	0.215
	Mt Sagra	-79.7130	31.7256	0.641
	Queue de vache East	81.5007*	6.8592	**0.000**
	Queue de vache West	75.6041*	6.6314	**0.000**

	Mt Chauvet	82.3905	8.9409	0.061
	Mt Lure	-18.7102	9.9666	0.878
	Mt Chasseral	42.7000	12.9846	0.428
	Ceresa	63.9308*	6.7779	**0.010**
	Gabas	-61.6310	18.5786	0.354
	Pas de l'Ai	-206.4998	34.6489	0.065
	Mt Raton	-102.9168*	10.8280	**0.022**
	Saint Sauveur	36.3156	20.8382	0.911
	Butte de Sers	25.3245	11.0069	0.717
	Vilas del Turbon	-54.1145*	5.4465	**0.011**
	Chaudière	-173.3491*	24.6980	**0.032**
Pas de l'Ai	**Beaumont-en-Véron**	282.7289*	34.8047	**0.020**
	Rocamadour.Casetl	260.7728*	34.2892	**0.030**
	Rocamadour.roadside	257.0564*	35.9526	**0.024**
	Lake Gloriettes	262.5332*	34.7774	**0.026**
	Coumely Plateau	267.1298*	34.7741	**0.025**
	Plan	250.0148*	34.8963	**0.032**
	Mt Sagra	126.7868	46.3437	0.520
	Queue de vache East	288.0006*	34.4713	**0.020**
	Queue de vache West	282.1039*	34.4267	**0.022**
	Mt Chauvet	288.8903*	34.9451	**0.018**
	Mt Lure	187.7896	35.2215	0.086
	Mt Chasseral	249.1998*	36.1915	**0.027**
	Ceresa	270.4306*	34.4552	**0.025**
	Gabas	144.8688	38.5537	0.218
	Mt Cheval Blanc	206.4998	34.6489	0.065
	Mt Raton	103.5830	35.4749	0.480

	Saint Sauveur	242.8155*	39.6919	**0.024**
	Butte de Sers	231.8243*	35.5299	**0.038**
	Vilas del Turbon	152.3853	34.2182	0.176
	Chaudière	33.1507	41.8475	1.000
Mt Raton	**Beaumont-en-Véron**	179.1459*	11.3165	**0.002**
	Rocamadour.Casetl	157.1898*	9.6147	**0.020**
	Rocamadour.roadside	153.4734*	14.4666	**0.002**
	Lake Gloriettes	158.9502*	11.2323	**0.004**
	Coumely Plateau	163.5468*	11.2221	**0.003**
	Plan	146.4318*	11.5952	**0.014**
	Mt Sagra	23.2038	32.6256	1.000
	Queue de vache East	184.4176*	10.2452	**0.006**
	Queue de vache West	178.5209*	10.0941	**0.008**
	Mt Chauvet	185.3073*	11.7412	**0.007**
	Mt Lure	84.2066*	12.5399	**0.036**
	Mt Chasseral	145.6168*	15.0505	**0.015**
	Ceresa	166.8476*	10.1909	**0.011**
	Gabas	41.2858	20.0769	0.816
	Mt Cheval Blanc	102.9168*	10.8280	**0.022**
	Pas de l'Ai	-103.5830	35.4749	0.480
	Saint Sauveur	139.2325*	22.1843	**0.032**
	Butte de Sers	128.2413*	13.3816	**0.004**
	Vilas del Turbon	48.8023	9.3584	0.234
	Chaudière	-70.4323	25.8439	0.542
Saint Sauveur	Beaumont-en-Véron	39.9135	21.0962	0.868
	Rocamadour.Casetl	17.9574	20.2343	1.000
	Rocamadour.roadside	14.2410	22.9405	1.000

	Lake Gloriettes	19.7178	21.0511	0.999
	Coumely Plateau	24.3144	21.0457	0.996
	Plan	7.1994	21.2469	1.000
	Mt Sagra	-116.0286	37.1674	0.398
	Queue de vache East	45.1851	20.5414	0.753
	Queue de vache West	39.2884	20.4665	0.854
	Mt Chauvet	46.0749	21.3270	0.770
	Mt Lure	-55.0259	21.7769	0.620
	Mt Chasseral	6.3844	23.3131	1.000
	Ceresa	27.6151	20.5144	0.982
	Gabas	-97.9467	26.8340	0.204
	Mt Cheval Blanc	-36.3156	20.8382	0.911
	Pas de l'Ai	-242.8155*	39.6919	**0.024**
	Mt Raton	-139.2325*	22.1843	**0.032**
	Butte de Sers	-10.9912	22.2722	1.000
	Vilas del Turbon	-90.4302	20.1138	0.171
	Chaudière	-209.6648*	31.3830	**0.009**
Butte de Sers	Beaumont-en-Véron	50.9046	11.4878	0.089
	Rocamadour.Casetl	28.9485	9.8158	0.473
	Rocamadour.roadside	25.2321	14.6010	0.936
	Lake Gloriettes	30.7089	11.4049	0.540
	Coumely Plateau	35.3055	11.3948	.379
	Plan	18.1905	11.7624	0.954
	Mt Sagra	-105.0375	32.6854	0.419
	Queue de vache East	56.1762	10.4341	0.060
	Queue de vache West	50.2796	10.2859	0.095
	Mt Chauvet	57.0660	11.9064	0.129

	Mt Lure	-44.0347	12.6947	0.263
	Mt Chasseral	17.3755	15.1797	0.996
	Ceresa	38.6063	10.3809	0.255
	Gabas	-86.9555	20.1739	0.129
	Mt Cheval Blanc	-25.3245	11.0069	0.717
	Pas de l'Ai	-231.8243*	35.5299	**0.038**
	Mt Raton	-128.2413*	13.3816	**0.004**
	Saint Sauveur	10.9912	22.2722	1.000
	Vilas del Turbon	-79.4390*	9.5649	**0.022**
	Chaudière	-198.6736*	25.9194	**0.014**
Vilas del Turbon	**Beaumont-en-Véron**	130.3436*	6.3626	**0.000**
	Rocamadour.Casetl	108.3875*	2.2050	**0.000**
	Rocamadour.roadside	104.6711*	11.0319	**0.013**
	Lake Gloriettes	110.1479*	6.2116	**0.001**
	Coumely Plateau	114.7445*	6.1931	**0.001**
	Plan	97.6295	6.8460	0.149
	Mt Sagra	-25.5985	31.2546	1.000
	Queue de vache East	135.6152*	4.1694	**0.000**
	Queue de vache West	129.7186*	3.7831	**0.000**
	Mt Chauvet	136.5050	7.0905	0.110
	Mt Lure	35.4043	8.3468	0.252
	Mt Chasseral	96.8145	11.7871	0.103
	Ceresa	118.0453	4.0343	0.073
	Gabas	-7.5165	17.7623	1.000
	Mt Cheval Blanc	54.1145*	5.4465	**0.011**
	Pas de l'Ai	-152.3853	34.2182	0.176
	Mt Raton	-48.8023	9.3584	0.234

	Saint Sauveur	90.4302	20.1138	0.171
	Butte de Sers	79.4390*	9.5649	**0.022**
	Chaudière	-119.2346	24.0900	0.128
Chaudière	**Beaumont-en-Véron**	249.5782*	24.9161	**0.007**
	Rocamadour.Casetl	227.6221*	24.1907	**0.013**
	Rocamadour.roadside	223.9057*	26.4959	**0.007**
	Lake Gloriettes	229.3825*	24.8780	**0.010**
	Coumely Plateau	233.9791*	24.8733	**0.009**
	Plan	216.8641*	25.0439	**0.013**
	Mt Sagra	93.6361	39.4611	0.685
	Queue de vache East	254.8499*	24.4481	**0.008**
	Queue de vache West	248.9532*	24.3852	**0.009**
	Mt Chauvet	255.7396*	25.1118	**0.006**
	Mt Lure	154.6389*	25.4950	**0.045**
	Mt Chasseral	216.0491*	26.8191	**0.009**
	Ceresa	237.2799*	24.4255	**0.010**
	Gabas	111.7181	29.9304	0.196
	Mt Cheval Blanc	173.3491*	24.6980	**0.032**
	Pas de l'Ai	-33.1507	41.8475	1.000
	Mt Raton	70.4323	25.8439	0.542
	Saint Sauveur	209.6648*	31.3830	**0.009**
	Butte de Sers	198.6736*	25.9194	**0.014**
	Vilas del Turbon	119.2346	24.0900	0.128

3. Chapter III:

Germination patterns within the species complex of *Arenaria grandiflora* L.

3.1 Introduction to Chapter III

As reported in the first paper, we had many difficulties in seed germination of *Arenaria grandiflora* species complex certainly because of seed dormancy and presence of endophytic fungi in the used seeds. In addition, according to the literature, there was a lack of knowledge on seed germination and no adequate or valid protocol to germinate the seeds of *A. grandiflora* complex existed. Furthermore, while implementing our different studies (such as intraspecific DNA content variation) at ESE in University of Paris-Sud Saclay, we had frequently needed young roots and seedlings of *A. grandiflora* species complex. Consequently, there was a challenge to find the most appropriate protocol to overcome difficulties of seed germination of *A. grandiflora* species complex. Therefore, we reviewed many currently available data about seed germination for other species of the genus of *Arenaria* and its close genera as *Minuartia* and *Moehringia* and then, we tried different conditions of storage and germination protocols, on different genotypes collected in natural populations.

Because the results varied greatly from one population to the other but also with the protocols, we took advantage to these experiments to determine the ecological and genetic factors that could have influenced the germination capacity of the seeds.

3.2 Paper 2

Seed germination of *Arenaria grandiflora* L. species complex (Caryophyllaceae)

This study was conducted with Sandrine Fontaine and Christian Raquin from University of Paris-Saclay

This study is still preliminary to be submitted in Seed science research

Seed germination of *Arenaria grandiflora* L. species complex (Caryophyllaceae)

Keywords: dormancy, germination protocol, *in vitro* culture, ploidy levels, scarification, seed provenance

Abstract

The seed germination responses vary greatly within a single species. Here, we studied the seed germination percentages for eleven accessions of *Arenaria grandiflora* species complex representing in two different cytotypes across Western Europe. To investigate both the methodology of seed germination and the variation in germination percentage of diploid and tetraploid populations, seeds from several sites in Western Europe were tested on different substrates (filter paper, pot and agar). A methodology which allows germinating the seeds of *A. grandiflora* was defined, for the first time. A variation in germination percentages among the investigated populations was found. The factors which influenced the germination percentages were determined among six predictors (altitude, longitude, latitude, ploidy levels. both period and condition of seed storage). Our results suggest that the ploidy levels and the maternal environmental effects should be considered when performing seed germination for the species complex of *Arenaria grandiflora.*

Introduction

Ex-situ seed storage is one of the best procedures to maintain large pools of plant genetic resources (Satterthwaite et al. 2007; Coppi et al. 2015). Defining the storage conditions and periods is essential to preserve the seed viability during long periods (Leino & Edqvist 2010). Hay & Probert (2013) presented a review of the recent research in seed conservation of wild plant species. The impacts of various storage conditions and different storage periods on seed germination were reported in *Swertia chirata* (Roxb. ex Fleming) H. Karst., by (Pradhan & Badola 2012), in *Caesaplinia bonduc* (L.) Roxb by (Kazeem Ibrahim et al. 2016) and in *Treculia africana* Decne by (Onyekwelu et al. 2007).

Actually, the seeds of many plant species are extremely tolerant to harsh environmental conditions provided they are in a state of desiccation (Hay and Probert 2013). In this dry state, their metabolic activity is drastically reduced to a very low level (quiescence) while retaining their ability to germinate for considerable periods; e.g. 2000 years for date seeds (*Phoenix dactylifera* L.) recovered from a

Herodian fortress near the Dead Sea (Rajjou & Debeaujon 2008) and 151 years for Acacia Mill. spp. seeds from Egypt stored in Swedish museums (Leino and Edqvist 2010).

The reinforcement of wild populations using stored seed material may be a valuable tool for conserving endemic and threatened species (Giménez-Benavides et al. 2005; Coppi et al. 2015). Likewise, the study of seeds germination of protected flora species is vital for the success of ecological restoration projects (Bischoff et al. 2006). For twenty years, the preservation of a species very rare and threatened in lowlands (for *Arenaria grandiflora* in Fontainebleau forest) was conducted. This plant is more common but progressively disappearing in western mountains of Europe (Bottin et al. 2007). Concerning the germination of this species complex, information is lacking and up to now, no studies were conducted on the seed germination of *A. grandiflora.* For different studies, such as the impact of climatic changes (Fontaine et al., submitted) and intraspecific DNA content variability in species complex of *A. grandiflora* (Daoud et al., submitted), young roots and seedlings are needed. Therefore, while carrying out these studies in our laboratory, we have made many attempts relying on many and previous protocols in the germination of seeds for other species of the genus of *Arenaria* and its close genres as *Minuartia* and *Moehringia*. For example, the seeds of some species of *Arenaria* do not have a light requirement for germination like as *A. meyeri* (Boiss) Edgew and Hook f and *A. serpyllifolia* L., according to King (1975) and Ronnenberg et al. (2007). On the other hand, the germination of other species such as *A. glabra* Michx and *A. fontinalis* (Short and Peter) is controlled by both the temperature and availability of water (Baskin & Baskin 1982, 1987). Several authors showed the impact that period of seed storage had on the germination percentage; for example, Ronnenberg et al. (2007) found that the germination of fresh seeds was significantly higher in *A. meyeri*. Also, (Baskin & Baskin 1987) revealed that seeds of *A. fontinalis* did not germinate after ripening when stored in dry at ambient laboratory conditions.

It is also well-known that dormancy-breaking and germination requirements are specific for each species and depend on geographic distribution, habitat and life cycle (Vandelook et al. 2009; Copete et al. 2011; Zharare 2012). In general, studies can show variation in germination responses between populations of one single species (Lord 1994; Baskin et al. 1998; Hernández Verdugo et al. 2001; Cochrane et al. 2014). The altitude and latitude of the seed source, population size and abiotic

factors like substrate type or climate are generally regarded as important factors that affect seed germination percentages (Menges 1991; Lord 1994; Keller & Kollmann 1999; Vandelook et al. 2009; Wang et al. 2012; Rosbakh & Poschlod 2015).

The species complex of *A. grandiflora* occurs as diploid and tetraploid cytotypes in its native range in Western Europe. The case of polyploidy is also interesting because it can induce the changes in seed characteristics and may have important consequences on their germination response as mentioned by (Tyler et al. 1978; Thomas et al. 1994; Hoya et al. 2007; Hahn et al. 2013).

In this paper, we report germination experiments executed on seeds collected in Spanish and French natural populations of the species complex of *A. grandiflora*. The purposes of this study were: (a) to define the methodology which allows *A. grandiflora* seed germination; b) to assess, if it exists, a variation of germination rates of seeds according to their geographical origin (altitude, latitude and longitude), period and condition (temperature) of seed storage and their ploidy levels.

Material and Methods:

Study Species

The large-flowered sandwort (*A. grandiflora*) is a perennial plant that is cushion-shaped with woody and ascending stems that can reach a height of 15 centimeters. This small Caryophyllaceae is a Pyrenean-Alpine species that grows on calcareous and sedimentary rocks, xerophytic and oro-basophils lawns, and schist screes (Küpfer 1974). In general, it blooms from May to August. The species complex of *A. grandiflora* is insect pollinated but is also able to self-reproduce in the absence of pollinators (Machon et al. 1999. 2001). The mating system of *A. grandiflora* is probably predominantly outcrossing, although vegetative reproduction is also possible (Bottin et al. 2007). The lowland populations of *A. grandiflora* in the Puys of Chinonais and in the Fontainebleau forest have been protected since 1991 when a restoration program was conducted by the Botanical Conservatory in Ile-de-France. According to field observations, the natural populations of the species complex of *A. grandiflora* in the Fontainebleau forest got probably extinct (fully) in 2014.

Origin of material

The seeds used for the experiments of germination were collected from eleven populations of *A. grandiflora* complex in Western Europe. These populations are representing the natural distribution of

the species in Western Europe (Mt Merle, Chinon, Rocamadour, Mt Lure, Mt Raton, Mt Chaudière, Mt Cheval Blanc, Mt Rié, Mt Sagra, Plan and Ceresa). Table (1) shows each studied population with indications of altitude, latitude, longitude, collection year and site, both condition and period of seeds storage and ploidy levels. The size of the collected seed samples was variable depending on the population size Table 2.

Conditions (Temperature) and periods of seed storage

Most of the seed samples were kept in paper bags and stored in a dry place at room temperature, during three months for the populations of Chinon, Plan and Ceresa. However, the seeds from Mt Raton and Mt Chaudière populations that were stored during 84 and 96 months, respectively.

Except for the Mt Merle population, the seeds were kept in paper bags at room temperature (for 2 years), then they were conserved in a refrigerator at (-4 °C) during seven years in CNPMAI (Conservatoire National des Plantes à Parfum, Médicinales, aromatiques et Industrielles). In addition, for the seeds of Mt Cheval Blanc population were kept in paper bags and stored in a cold room at + 4 °C at 40% of humidity in CBNA (Conservatoire botanique national alpin) during 300 months (see Table 1).

Test of germination methods

Three germination methods were used: (i) seed germination in pots, (ii) seed germination on filter paper and (iii) in vitro culture with scarified seeds. These three methods are detailed below.

1) Test of germination in pots

The experiment of germination on potting soil was preliminary attempt to germinate the seeds of *A. grandiflora*, according to a protocol which was inspirited of Meta-analysis for the genre *Arenaria* and its allied genera *Minuartia* and *Moehringia* which were already reported in the literature and in previous studies. Considering the results of this Meta-analysis, we started these experiments with four French populations of *A. grandiflora* (Table 1): Mt Raton, Mt Chaudière, Mt Rié, and Chinon. Seeds were planted on potting soil in terrine at a depth of 0.5cm with a spacing of 2 cm between each seed, 150 seeds per terrine. The number of seeds tested could vary according to the quantity of seed available. The seeds were kept on the moist soil, continuously. The terrines were placed in a seedbed under controlled conditions of temperature and light: 22°C (light 14h) + 15°C (dark 10h). The temperature was measured using Tinytag Temperature Data Logger. Data were recorded every 10 min.

The terrines were examined at 1-day intervals and during each examination, the number of germinated seeds was recorded. Protrusion of the cotyledons was the criterion of germination. The seed germination was observed and recorded every day during 50 days' germination experiments in the pots. We subdivided seeds into two categories: Normal (N) and Deformed (D), because of the number of seeds in *A. grandiflora* capsule ranged from one to eleven but the size of the capsules was roughly the same. In a capsule containing a few seeds, the seeds had more space to grow and therefore grew into a rounded shape. Contrariwise, the capsules with many seeds did not have ample space for the seeds to grow and consequently grow deformed.

We tested the impacts of two seed parameters on germination percentage: (a) the seed sources (provenances of seeds), and (b) the seed type ("Normal" vs "Deformed"). Experiments were carried out in autumn 2007 or 2008.

In view of this first attempt, we tried again by using another methodology, but with less seeds than for the first attempt because of the low available quantity remaining.

2) Test of germination on filter paper

The material which was used for these experiments were seeds of *A. grandiflora* species complex from Mt Merle, Rocamadour, Mt Lure, Mt Cheval Blanc, Mt Raton, Mt Chaudière, Mt Sagra, Plan and Ceresa populations. Three Petri dishes of 9 cm in diameter were used for the seeds of each population under ambient lab conditions (approximately 20 °C). Each dish contained (10-18) seeds. As required, the filter paper was moistened with distilled water at the beginning of the experiment. The seed germination was observed every day. The germination percentage is the fraction of seeds germinated in each Petri-dish after 45 days of incubation. These experiments of germination on filter paper were carried out in autumn 2015.

Therefore, in view of the above (two attempts and their results), we decided to perform the 3rd test, by *in vitro* culture.

3) In vitro germination (with scarification) experiments

Ten out of the eleven populations were sown *in vitro* experiments, except Mt Rié population (French Pyrenees), because of lack of seeds. The experiments of *in vitro* culture were carried out in autumn 2008 or 2016.

Three steps were performed:

(i) Media preparation

We prepared four culture media: two of these media were GMC6 and GMC610 containing the macronutrients of Estelle and Somerville (1987) modified as follow 2.5 mMol KH_2PO_4 were replaced by 2 mM KH_2PO_4 and 0.5 mM K_2HPO_4 $4H_2O$. So, the medium was directly adjusted to pH=6. The mineral nutrient solution contained per liter 5mM KNO3; 1.85 mM MgSO4 $7H_2O$; 1mM $Ca(NO_3)_2$ $4H_2O$; 0.1 mM Fe-EDTA; 1mM NaCl Half concentration of the mineral micronutrients of Murasnige and Skoog (1962) was added 2g/l gelrite and 4g/l of agar was used as gelling agents before autoclaving for 30 minutes at 115°C. 10 g/l of sucrose (about 30mM) was only added to the GMC610 medium. The author two media were prepared one with sugar (GH0) and one without sugar (GH10), contained the macronutrients (MCC2) modified according to (Guillon and Raquin 2002). The modification of the MS micronutrient solution just consisted in the addition of 0.01mM of Na3VO4 (sodium vanadate). The composition of the solution of macronutrients (MCC2) was as the following per liter: 40 mM NH_4NO_3; 100 mM KNO3; 30 mM MgSO4; 10 mM Mg(NO3)2; 30mM Ca(NO3)2. The four media were placed in 55mm diameter Petri dishes and they were left to dry a few hours. They were then stored at room temperature in closed boxes to limit evaporation of the medium. To detect the possible problem of contaminations we waited a few days before using them.

(ii) Surface sterilization

Because there was a possibility of infections caused by fungus or bacteria present over the coating of the collected seeds from the field. The seeds were treated with a solution containing calcium hypochlorite (Ca $(ClO)_2$ 1 2% w/v) and sodium hydroxide (NaOH 0 4% w/v). About 15-20 small seeds were put in each Eppendorf 2ml with about 1.7 ml of the solution of sterilization. They were first centrifuged for 1 minute to eliminate air bubbles on the surface of the seeds then they were kept 20 min in the solution at room temperature. The hypochlorite was removed by a transfer pipette. Because the seeds were fragile they were rinsed with the same solution diluted 1/10. The disinfected seeds were placed on germination media in Petri dishes and were left during six days to ensure that there were no contaminations observed.

(iii) Scarification and seeds germination

Mechanical scarification was done by using a scarifier to cut the seed coat of each seed under the Enlarger. The surface-scarified seeds were placed on germination media in Petri dishes and incubated in a growth chamber of 23°C± 2. According to the availability of the seeds (Table 2), 12-15 Petri dishes containing different types of agar media, for each population, each one including the seeds (7-20 per dish) were used in the seed germination experiments. The seeds were considered germinated when the root tip was visible (protruded 1 mm above the seed coat). In each run, the number of germinated seeds was recorded during one month (30 days), because almost no further germination occurred afterwards. Here, we tested the influences of three factors on germination percentages: (a) the seed source (provenances of seeds), (b) the four media of culture *in vitro* and (c) the ploidy levels.

Statistical analyses

- *In general, for each trial*

The final germination percentage (GP) of sown seeds was defined as the proportion of germinated seeds on the total number. Germination percentage is the fraction of seeds germinated in each Petri-dish after 30 to 50 days of incubation. To test the impact of the different methodologies used on the GP, we used the non-parametric test of Kruskal-Wallis coupled to Wilcoxon tests to explore the differences of germination rates among germination methods.

- *For the germination experiments in pots*

All statistical tests were performed using R version 2 software (R Development Core Team 2016). Germination percentage (GP) in each Petri-dish was calculated after 50 days of incubation. To test the impacts of both the seed sources (seed provenances) and the seed type (“Normal” vs “Deformed”) on GP, we used Chi^2 Pearson test. A Tukey’s honest significant test (HSD), for equal sampled population sizes, was used to explore the differences of germination rates among seed sources (provenances of seeds) with mixed seeds (“Normal” and “Deformed”). The significance level for all statistical tests was p-value <0.05.

- *For the germination experiments in filter paper*

No statistical tests were performed because all the results of the germination percentages were zero.

- *For the germination experiments, in vitro culture (with scarification)*

All statistical tests were performed using SPSS (IBM SPSS Statistics version 24). Germination percentage (GP) in each Petri-dish was calculated after 30 days of incubation. To test the effects of each one of these following factors on the germination percentage: the seed sources (seed provenances), their four media of culture (GH0, GH10, GMC6 and GMC610) *in vitro* and their ploidy levels, we used one-way ANOVAs.

The significant variance in germination percentages among the seed sources (provenances of seeds) was investigated by an ANOVA with Welch test, for unequal sampled population sizes. Also, a test of multiple comparisons (Games-Howell test), for unequal sampled population sizes, was conducted to demonstrate all pairwise comparisons of the means for the seed sources (provenances of seeds). The significance level for all statistical tests was p-value <0.05.

To characterize, as much as possible, the optimal germination conditions, we excluded all GP < 10% from the whole data, because of the probability that the seeds were dead or immature was too high. Then we applied statistical analyses on the new data, like following:

- *For germinated experiments in all trials (GP>10 %)*
  - Impact of some parameters on GP

For the populations having significantly germinated (GP> 10 %) in all the trials, we tested the significant effects of each one of the following factors: the geographical parameters (altitude, latitude and longitude) of the collected seeds provenances, their ploidy levels and the period and temperature of seed storage on the germination percentage, by using an ANOVA with Welch test. To demonstrate all pairwise comparisons of the means of GP, a test of Multiple Comparisons (Games-Howell test) was conducted. The significance level for all statistical tests was p-value <0.05.

  - Modeling (linear regression**)**

To model the relationships among six explanatory variables (predictors) and a response variable of germination (different values of GP), we used two methods: simple and multiple linear regression analyses. The six predictors were latitude, longitude, altitude, ploidy levels and both period and condition of seeds storage. The multiple linear regression analysis was fitted by a linear equation $y = a + b X_{(1-6)}$. To compare the models, a standard error of estimation and both associated r and r^2 and a P

value were given for each suggested model. All regressions were considered significant at $P < 0.05$. A regression analysis was performed also by SPSS (IBM SPSS Statistics version 24).

Results

Methodology

The three methods under which seeds were sown had a significant impact on GP (Kruskal-Wallis test; $p < 0.0001$). For the method of seed germination on filter papers, no germination was obtained and fungal contamination was observed for all the seed provenances. Both the method of seed germination in potting soil and the method of seed germination in scarification in *vitro* culture (with pretreatment by scarification) were significantly better than the method of seed germination in filter papers (Wicoxon tests $p < 0.0001$). Even if, the GP greatly varied according to the tested populations, from 0.4% to 72 % (for method of potting soil) and from 0% to 83 % (for method of *in vitro* culture) (see Table 1). For populations whose seeds were not contaminated with fungi, the method of *in vitro* culture was the one that yielded the best percentages. So, the two populations located in the mountains, Mt Raton and the Mt Chaudière, had have a very low germination rates (1.1% to 4%) with the method of potting soil, but had have a good GP with the method of *in vitro* culture.

Germination in potting soil

In our experiments, the seed GP varied considerably from 0% to around 74 % (see Fig. 1A). The seed provenances had a significant impact on the percentage of germination (Chi^2 Pearson test. $P = 0.023$). On the contrary, seed type ("Normal" vs "Deformed") had no influence on GP (Chi^2 Pearson test. $p > 0$ 05) for the four studied sites (Fig. 1A). So, we used mixed seeds for the rest of the analyses. There was a variation among the seed provenances (with mixed seeds) ($F = 278.234$; $P = 0.000$). In other words, the seeds harvested from the two locations of the alpine mountains, Mt Raton and the Mt Chaudière showed a very low germination rate (1.1% to 4%). Moreover, seeds from Mt Rié (French Pyrenees) had a good germination percentage. 50.3%; but these germination percentages were lower than that of the seeds from the Puy Chinonnais (plains) (71.58%) (see Fig. 1B). The Chinon population differed significantly in the germination percentage compared with both Mt Raton and Mt Chaudière population and Mt Rié population ($P = 0.000$). There were no significant differences between alpine populations (Mt Raton and Mt Chaudière) ($P > 0.05$).

Germination in filter papers

In our preliminary experiments in filter paper, no germination was obtained and fungal contamination was observed.

Germination of scarified seeds in vitro

Means of seed germination percentage (GP) *in vitro* for scarified seeds greatly varied from 0% to 80 % (see Fig.2). However, both their seed provenances and their ploidy levels affected the percentage of germination (ANOVA: P = 0.021; P = 0.000, respectively), but there was no impact of the four media (GH0, GH10, GMC6 and GMC610) of *in vitro* culture (ANOVA: $p > 0.05$). Thus, ANOVA with Welch test was conducted only for the seed provenances and the ploidy levels. Significant differences among both the seed provenances and their ploidy levels were observed ($F=51.232$, $p = 0.000$; $F=71.349$, $p = 0.000$ respectively). The variation in GP among the seed provenances in function of their ploidy levels was showed in Fig. (2).

The pairwise differences of the seed provenances were demonstrated by using Games-Howell analysis (see Supplementary1). We observed that the lowest GP of seeds from Rocamadour populations (GP ± s.d. = 10.76 ± 12.37 %), Chinon and Mt Merle populations (GP = 0.00 %) differed significantly from all other seed provenances that very well germinated *in vitro* as well as Mt Chaudière (GP ± s.d. = 69.57± 11.31%), Ceresa (GP ± s.d. = 68.63± 20.54%), Mt Lure (GP ± s.d. = 68.92± 17.59 %), Mt Raton (GP ± s.d. = 62.03± 14.29 %), Mt Cheval Blanc (GP ± s.d. = 82.85± 7.76%), Plan (GP ± s.d. = 72.48± 16.24 %) and Mt Sagra (GP ± s.d. = 52.86± 22.14 %) ($P < 0.05$). Furthermore, a significant difference in the germination percentage was reported among the Mt Cheval Blanc population (GP ± s.d. = 82.85± 7.76%), in one side, and both Mt Raton population (GP ± s.d. = 62.03± 14.29 %) and Mt Sagra population (GP ± s.d. = 52.86± 22.14 %) on the other side.

Moreover, the seeds from Chinon population (the Puy du Chinonnais) and Mt Merle population in the Fontainebleau forest had not germinated after the scarification. The culture dishes were infected by fungi that are probably of endogenous origin (endo-fungal) in the seeds. Generally, we could affirm that differences in GP among the seed provenances were highly significant for all the germinated seed provenances, in all our experiments, except for the experiments in filter papers where the GP = 0 % (see Table 2).

Germination percentage in all trials (GP > 10 %)

- *Impacts of intrinsic and environmental parameters*

The factors like the geographical parameters (altitude, latitude and longitude) (F=9.306; P= 0.000 for each one out three of these factors) induced significant differences among the populations, as well as the polyploidy seemed to have a significant impact on the germination rate (F=12.434; P=0.001) (see Fig. 3). Significant differences were reported among Rocamadour population that occurs at (331 m; 44.47 N° and 1.36 E°) and all other germinated populations (GP >10 %) as well as Chinon population at (90 m; 47.19 N° and 0.24 E°) and Mt Cheval Blanc population at (2280 m; 44.08 N° and 6.20 E°) but not for Mt Rié population at (900 m; 42.54 N° and 0.40 E°). Moreover, Mt Cheval Blanc population at (2280 m; 44.08 N° and 6.20 E°) differed significantly from Mt Raton population at (1450 m; 44.25 N° and 5.25 E°), on one side and Mt Sagra population on the other side (2279 m; 37.57 N° and 2.33 E°).

- *Impacts of conditions (temperature) and periods of seed storage*

The periods and conditions of seed storage had a significant impact on the GP (F=4.129, P=0.004; F=11.743, P=0.001 respectively). Furthermore, the seeds from Mt Cheval Blanc population which were stored for 300 months germinated differently and significantly compared with each of Mt Raton and Mt Chaudière populations (that were stored for 84 and 96 months respectively). In addition, all the seeds of populations, that were stored three months (Chinon, Plan and Ceresa) germinated differently and significantly compared to seeds of Mt Cheval Blanc population.

- *Modeling*

We tested six predictor variables in the regression analysis. Every value of each one of the independent variables $x_{(1-6)}$ is associated with a value of the dependent variable (GP) y. So, the value of the GP changed when each of the predictor variables (altitude, longitude, latitude, ploidy levels, both period and condition of seed storage) varied, while the others were fixed. We defined the regression line for 6 explanatory variables $x_{(1-6)}$ to be y= (-328.238) + (-4.355) *ploidy levels + (0.041) *altitude + (8.913) *latitude + (-4.431) *longitude+ (0.117) *period of seeds storage + (-32.758) *condition of seeds storage. The best model among all models which had the upper value of r = 0.633 and the lowest value of the standard error of estimation =16.43872, this model that was more explicit by its six predictors (altitude, longitude and latitude, ploidy levels, both period and condition of seed storage) (see Table 3).

There was a good significant impact of both period and condition of seeds storage on the final germination percentage (r = 0.350, P = 0.000; r = 0.326, P = 0.000 respectively). Furthermore, the germination percentages of seeds had been equally and well affected by ploidy levels and altitude (r = 0.334, P = 0.000), the tetraploids germinated better than diploids. However, both altitude and longitude of seed provenances seemed to have influences on the final germination percentage (r = 0.334, P = 0.000; r = 0.208, P = 0.018 respectively), the latitude had no effect on the GP (r = 0.165. p >0.05).

Discussion

Methodology of seed germination of Arenaria grandiflora

While seeds of some species of *Arenaria* germinate easily without any special treatment, others are more difficult. For example, seeds of *A. meyeri* Fenzl and *A. serpyllifolia* L., according to (King 1975) and Ronnenberg *et al.* (2007) do not need light for germination. Reliable technics for achieving *in vitro* seed germination with pre-treatment (scarification) in the complex of *Arenaria grandiflora* is documented in the present study. Our results showed that a scarification is required for a successful *in vitro* seed germination of the tetraploids of *A. grandiflora* species complex. The mechanical scarification allowed water to penetrate the tough outer coating to start the germination process. Indeed, we found that the mechanical scarification produced higher germination percentage for most of the tetraploid populations.

Thus, we could consider that the scarification of the seeds and then the use of *in vitro* culture technics could be a great method for both overcoming the inhibition of germination caused by seed-covering and multiplication the tetraploids of *Arenaria grandiflora* complex. For other species, these technics initiated from seeds of orchids has been widely used at Kew (Muir 1989). Moreover, many carnivorous plants have been propagated *in vitro* from seeds (Vasile et al. 2011; Fay 1992; Holobiuc and Blindu 2007). Furthermore, Ghosh and Maiti (2014) indicated that both of the *in vitro* germination of seeds and the shortening of their dormancy period for the *Abrus precatorius* L. species are possible with the different method of seeds scarification treatment. However, we recommend these technics for the tetraploids of *A. grandiflora* species complex. The diploid populations could better germinate with another method, clearly simple.

Differences among populations

Despite differences in the protocol design, results were congruent, in both cases (potting soil and agar substrates) and showed that germination percentage (GP) could be highly affected by the seed provenances of the species complex of *A.grandiflora*. Our study reported the variability in seed germination rates in growth chambers and in the field (potting soil), for the first time, among the natural populations of *Arenaria grandiflora* species complex.

For example, the GP for the diploid population of Chinon (in plains) differed significantly from others diploid populations as such as Mt Rié population. In addition, the GP of some seed provenances of *A. grandiflora* species complex varied significantly among the tetraploids ones, like seeds of Mt Cheval Blanc population geminated differently and significantly from seeds of Mt Raton population. Also, *in vitro* seed germination percentages were the lowest for the diploid populations like Rocamadour, Chinon and Mt Merle populations and differed significantly from all other seed provenances that germinated very well *in vitro* like the tetraploid populations (Mt Chaudière, Ceresa, Mt Lure, Mt Raton and Mt Cheval Blanc) and the diploid ones (Plan and Mt Sagra). In addition, the GP of seeds from the tetraploid population of Mt Cheval Blanc differed significantly from the GP of seeds of the diploid population of Mt Sagra.

The provenance effect on seed germination is a very well-known phenomenon. For example; it was shown for six provenances of *Faidherbia albida* (Delile) A. Chev in tropical Africa (Fredrick et al. 2016) or, in species of *Tamarindus indica* L. in Ethiopia (Bahru et al. 2014). In addition, seed germination percentages are five Mediterranean species from the family of Lamiaceae greatly within single species from one population to another like for *Jatropha curcas* L. in Central India (Ginwal et al 2005) and the range of germination variability within species of *Pinus densata* Mast., was explored by (Xu et al. 2016). Furthermore, the differentiation among populations in seed germination of five Mediterranean species from the family of *Lamiaceae* had already been reported by (Pérez-García et al. 2003).

Environmental effects and germination

In the present study, the seed collection sites differed widely in elevation (from 87 to 2280 m) and in longitude (from 0.12 to 6.20 E°) and the germination percentage correlated with the altitude and longitude of the seed sources. These findings agreed with the altitudinal and interpopulation variability in high mountain Mediterranean species that had reported by (Giménez-Benavides et al. 2005). These factors are likely to be associated with differentiation among populations because they represent the different environments under which the seeds were produced and matured (Cavieres and Arroyo 2000). The seed germination of populations from different environments may have conducted different responses as it was found for *Banksia* species that were collected from a longitudinal climate gradient in Western Australia (Cochrane et al. 2014). Thus, the differences could arise from inherited environmental effects, "environmental preconditioning" or "maternal environmental effect" (Luzuriaga et al. 2005; Hermesh and Acharya 1992; Galloway 2001). These sources of variation are widespread in plants and could be the product of both the parental genes and the parental environment, or their interaction, and can include contributions that reflect the abiotic, nutritional and other ecological features of a parental environment (He et al. 2014; Rossiter 1996). The findings suggest that variability in germination percentages could reflect a local adaptive strategy to unpredictable environmental conditions (Gutterman 1994).

However, seeds collected at higher altitudes reached higher germination percentage than those from lower elevations (Giménez-Benavides et al. 2005), the results present that the GP of seeds from both similar and higher altitudinal populations significantly differed among themselves. For instance, the GP of seeds from the Mediterranean mountains (Mt Sagra at 2279 m) differed significantly from seeds of the alpine mountains (Mt Cheval Blanc at 2280 m). Furthermore, the type of climate, especially seasonal changes, could be the most obvious pre-harvest factor affecting seed viability and storability, because the different pre-harvest conditions will have caused different amounts of deterioration by the time (Justice and Bass 1978). Thus, we could interpret these differences by the type of climate in which each population grows. According to Köppen classification based on precipitation and temperatures, the climate of Mt Cheval Blanc population (Cfb) is temperate and humid, the precipitation occurs every

month of the year, there is no dry season and the summer is temperate with warmest month temperature <22° C, when the climate of Mt Sagra (Csa) is temperate, but the summer is hot and dry with low precipitation (<40 mm) and the mean temperature of the warmest month is > 22 °C.

The genetic variation and the germination percentage

Another possible source of differentiation among populations is the ploidy level of the individuals. As found in another study (e.g. Daoud et al. submitted), *Arenaria grandiflora* can have tetraploid or diploid populations. Our results showed that the germination rate was generally higher for the tetraploids than for the diploids (at the exception of the population of Plan). A similar phenomenon occurs in *Matricaria perforata* Mérat (Thomas et al. 1994). The whole genome duplication events may provide a subfunctionalization of duplicate genes. So, it has been hypothesized that polyploidy could induce small changes in the characteristics of seeds that may induce a variation in germination ability. Thus, the differences in germination rate (Fig. 2) among populations may have been also attributed to genotypic differences among these populations**.**

Years' seed and conditions storage effects

Our findings demonstrated that there were also significant effects of storage conditions (papers bags in room temperature and +4°C at 40% humidity) and different periods of seed storage on the germination rates. These findings agree with the high effects of the storage conditions (room temperature, +4°C, and −15°C) on seed germination percentage in *Swertia chirata* by (Pradhan & Badola 2012). Our study indicated that the storage at +4°C temperature and 40% humidity (Mt Cheval Blanc population) can retain seed viability for the longer period than the storage at -4°C temperature (Mt Merle population). Also, our results are in agreement with mentioned results by (Andersson and Milberg 1998) which showed that the germination of four species (*Sinapis arvensis* L., *Silene noctiflora* L., *Spergula arvensis* L. and *Thlaspi arvense* L.) differed between seeds collected in different years. However, these results did not agree with results of germination of the perennial widespread *Arenaria meyeri* (Ronnenberg et al. 2007) in the mountain steppes of southern Mongolia where the germination of fresh seeds (100%) was significantly higher than dry seeds (91.5%). In addition, Baskin and Baskin (1987) revealed that seeds did not germinate after ripening when stored in dry at ambient laboratory conditions. Other alpine species of *Minuartia* Loefl which is a genus closed to *Arenaria*. as e.g. *M.*

obtusiloba (Rydb.) House and *M. rubella* (Wahlenb.) Some of the alpine species exhibited a general tendency toward improved germinability with years' seed storage according to (Bonde 1965).

Seed germination and dormancy

Generally, differential seed dormancy mechanisms arise among plant provenances (Andersson and Milberg 1998) due to adaptations to local environmental (Zharare 2012; Huang et al. 2015). Our results could suggest that the seeds of *A. grandiflora* of the mountain populations (alpines and sup-alpines populations) have a physical dormancy. So, the seed coat inhibition is probably the most common cause of alpine seed dormancy as mentioned by (Amen 1966). Moreover, other species of *Arenaria* showed some degrees of dormancy like (*Arenaria serpyllifolia* L.) (Pons 1991) and *Arenaria nevadensis* Boiss. & Reuter (Lorite et al. 2007). The range of suitable conditions for the germination of the dormant seeds is restricted. Seed dormancy in *A. grandiflora* had been a major problem for our previous cytogenetic research. The results of this study showed that the mechanical scarification could remove the physical barriers due to water-impermeable seed coat (Baskin & Baskin 2004; Chaves et al. 2017; Amen 1966) and thus, highlight the benefit of *in vitro* culture technics for species with dormant seeds (Fay 1992). Moreover, the mechanical scarification which induced the highest percentage of germination in our Arenarias is known to break physical dormancy of hard seed coats especially in *Acacia polyacantha* Willd (Missanjo et al. 2014) and in *Faidherbia albida* (Fredrick et al. 2016).

Conclusions

Our results revealed that seed dormancy in some populations of *A. grandiflora* complex is mainly due to the hard seed coat, which can be broken effectively by physical scarification. This study of seed germination of an endangered flora species (*A. grandiflora*) is crucial and vital for its future conservation strategies and provides an overall view and basic information needed for genetic resources conservation. The present study stressed 1) the variability in seed germination in eleven populations collected from different altitudes in Western Europe that were stored in two diverse conditions for various periods.
2) the high seed germination percentages found for the tetraploids of *A. grandiflora* complex pretreated with mechanical scarification and cultivated *in vitro*.

Considering the significant differences in germination rates observed among populations, the question of the provenance of seeds to be reintroduced in situ should be resolved in choosing the seeds

with the best GP. However, the local seed provenance is often used and recommended in restoration because seeds are expected to be better adapted to local habitat conditions, and no effect of outbreeding depression is expected. The use of non-local seed provenance might be also interesting when the aim is to introduce both diversity and seeds with better germination capacities. For example, introducing Chinon' individuals whose seeds well germinate, in the Fontainebleau sites where plants hardly germinated was certainly one of the factors that led to the success of the restoration plan (Maurice et al. 2013).

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Fig. Captions

Fig. 1 The seed germination percentages in the potting experiments for four populations of Arenaria grandiflora in function of three factors: (A) type of seeds (normal and deformed); (B) geographical origin with mixed seeds; N = normal seeds; D = deformed seeds; NS= no significance; *** levels of Pearson's tests are represented by asterisk: p<0.000

Fig. 2 Histogram shows the means of germination percentage (GP) for cultivated seed provenances in vitro (scarification) with error bars in function of the ploidy levels for each population. An ANOVA with welch test for both of seed provenances and their ploidy levels are represented by (F=44.002. p = 0.000; F=54.009. p = 0.000 respectively).

Fig. 3 Histogram shows the means of germination percentage (GP) for good germinated populations of *A. grandiflora* species complex in all trials (without GP< 10 %) with error bars in function of both of periods and conditions of seeds storage for each population. An ANOVA with welch tests are represented by (F=4.129. P=0.004; F=11.743. P=0.001 respectively)

Table 1 : Seed provenances, ploidy levels, year of seed collection, both period and temperature of seeds storage and geographical parameters (latitude, longitude, and altitude) of investigated populations

No	Population	Latitude (°N)	Longitude (°E)	Altitude (m)	Year collection	Period of seed storage (month)	Temperature of seed storage	Ploidy levels	Collection site (Provenance)
1	Mt Raton	44.25	5.25	1450	2007/2008	3/84/96	room temperature	4	The pre-Alps
2	Mt Sagra	37.57	2.33	2279	2015	3	room temperature	2	Mt Mediterranean
3	Plan	42.33	0.26	1720	2015	3	room temperature	2	Spanish Pyrenees
4	Ceresa	42.29	0.12	1711	2015	3	room temperature	4	Spanish Pyrenees
5	Rocamadour	44.47	1.36	331	2015	3	room temperature	2	French Pyrenees
6	Mt Lure	44.7	5.46	1718	2013	24	room temperature	4	The pre-Alps
7	Mt Rié	42.54	0.40	900	2007	3	room temperature	2	French Pyrenees
8	Mt Chaudière	44.37	5.13	1560	2007	96	room temperature	4	The pre-Alps
9	Chinon	47.19	0.24	90	2007/2008	3	room temperature	2	Loire Valley
10	Mt Merle	48.25	2.40	87	2006	108	room temperature (for 2 years) then at (-4 °C) for 7 years	2	Fontainebleau Forest
11	Mt Cheval Blanc	44.08	6.20	2280	1990	300	+ 4 °C and 40% of Humidity	4	The Alps

Table 2 The mean values ± standard deviation of germination percentages for all the trials with the substrate of the germination and number of the cultivated seeds for each trial

Populations	**Means of GP (± s.d.) In Filtre Papers**	**Means of GP (± s.d.) In Potting Soil**	**Means of GP (± s.d.) *In vitro* with scarification**	**Nb cultivated seeds**
Plan	0.00			36
Plan			72.48± 16.24	134
Ceresa	0.00			36
Ceresa			68.63± 20.54	133
Chinon	0.00			30
Chinon			0.00	88
Chinon		71.58± 7.43		737
Mt Cheval Blanc	0.00			36
Mt Cheval Blanc			82.85± 7.76	221
Mt Sagra	0.00			30
Mt Sagra			52.86± 22.14	132
Mt Lure	0.00			54
Mt Lure			68.92± 17.59	118
Mt Merle	0.00			30
Mt Merle			0.00	49
Mt Raton	0.00			45
Mt Raton		1.24±1.30		1251
Mt Raton			62.03± 14.29	690
Mt Rié	0.00			30

Mt Rié		47.13± 10.84		290
Mt Chaudière	0.00			51
Mt Chaudière		0.35±0.40		300
Mt Chaudière			69.57± 11.31	219
Rocamadour	0.00			30
Rocamadour			10.76± 12.37	90
Total of means of GP (± s.d.)	**0.00**	**25.55±29.72**	**51.65±32.22**	**4860**

Table 3: Summary of models and ANOVA for the dependent variable (GP %) germination percentage and the linear regression's F-test, P values, R, R square and Std, the error of the estimate; a. predictors: (constant), period of seeds storage (month); b. predictors: (constant), period of seeds storage (month), altitude; c. predictors: (constant), period of seeds storage (month), altitude, latitude; d. predictors: (constant), altitude, latitude; e. predictors: (constant), altitude, latitude, longitude; f. predictors: (constant), condition of seeds storage, latitude, ploidy levels, longitude, altitude, period of seeds storage (month)

Models	**R**	**R square**	**Std. the error of the estimate**	**F**	**P values**
period of seeds storage	.350[a]	0.122	19.37623	13.811	.000[a]
period of seeds storage, altitude	.409[b]	0.168	18.9675	9.863	.000[b]
period of seeds storage, altitude, latitude	.558[c]	0.312	17.33442	14.651	.000[c]
altitude, latitude	.551[d]	0.304	17.34666	21.377	.000[d]
altitude, latitude, longitude	.616[e]	0.379	16.46044	19.772	.000[e]
condition and period of seeds storage, latitude, ploidy levels, longitude, altitude	.633[f]	0.4	16.43872	10.455	.000[f]

Fig. 1 (A and B): The seed germination percentages in the potting soil experiments for four populations of *Arenaria grandiflora* species complex in function of three factors: (A) type of seeds (normal and deformed); (B) geographical origin with mixed seeds; N = normal seeds; D = deformed seeds; NS= no significance; *** levels of Pearson's tests are represented by asterisk: p<0.000

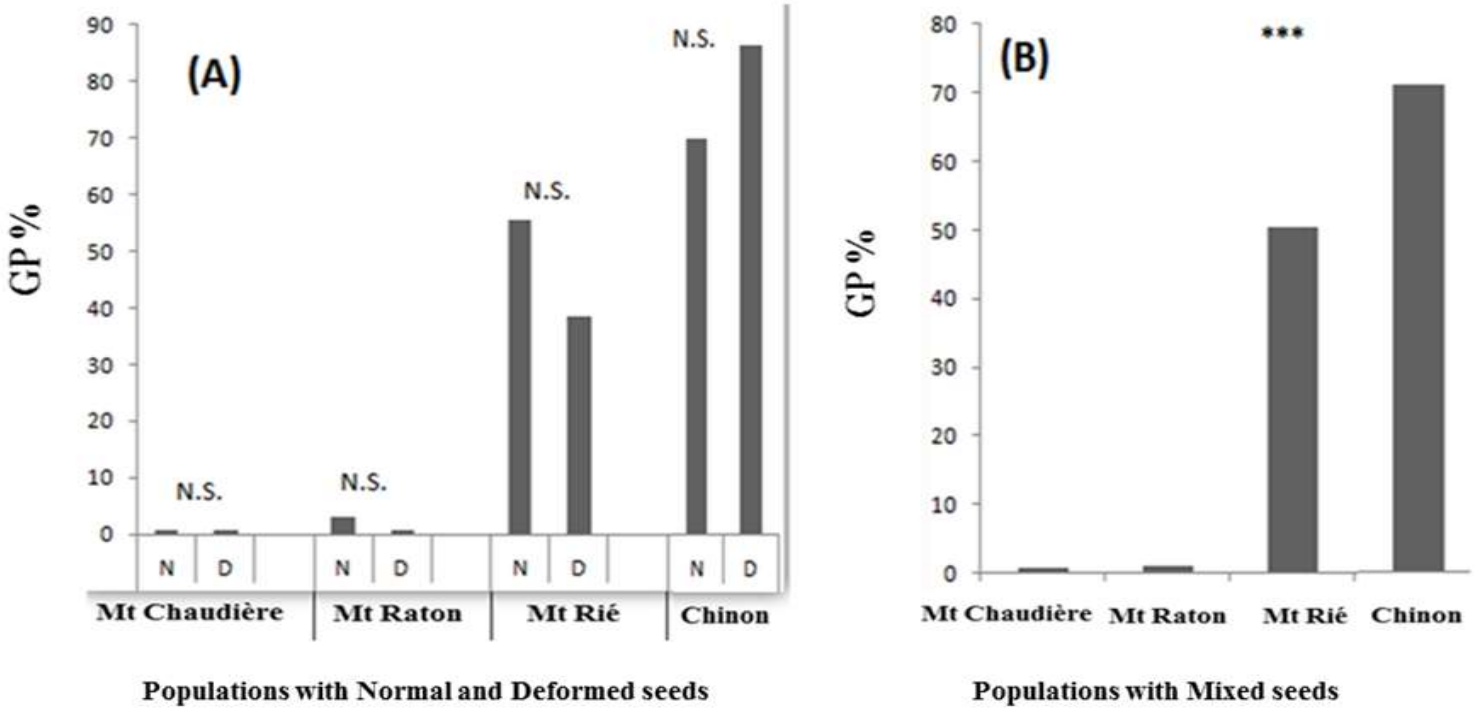


Fig. 2 Histogram shows the means of germination percentage (GP) for cultivated seed provenances *in vitro* (scarification), with error bars in function of the ploidy levels for each population. An ANOVA with welch test for both of seed provenances and their ploidy levels are represented by (F=44.002. p = 0.000; F=54.009. p = 0.000 respectively).

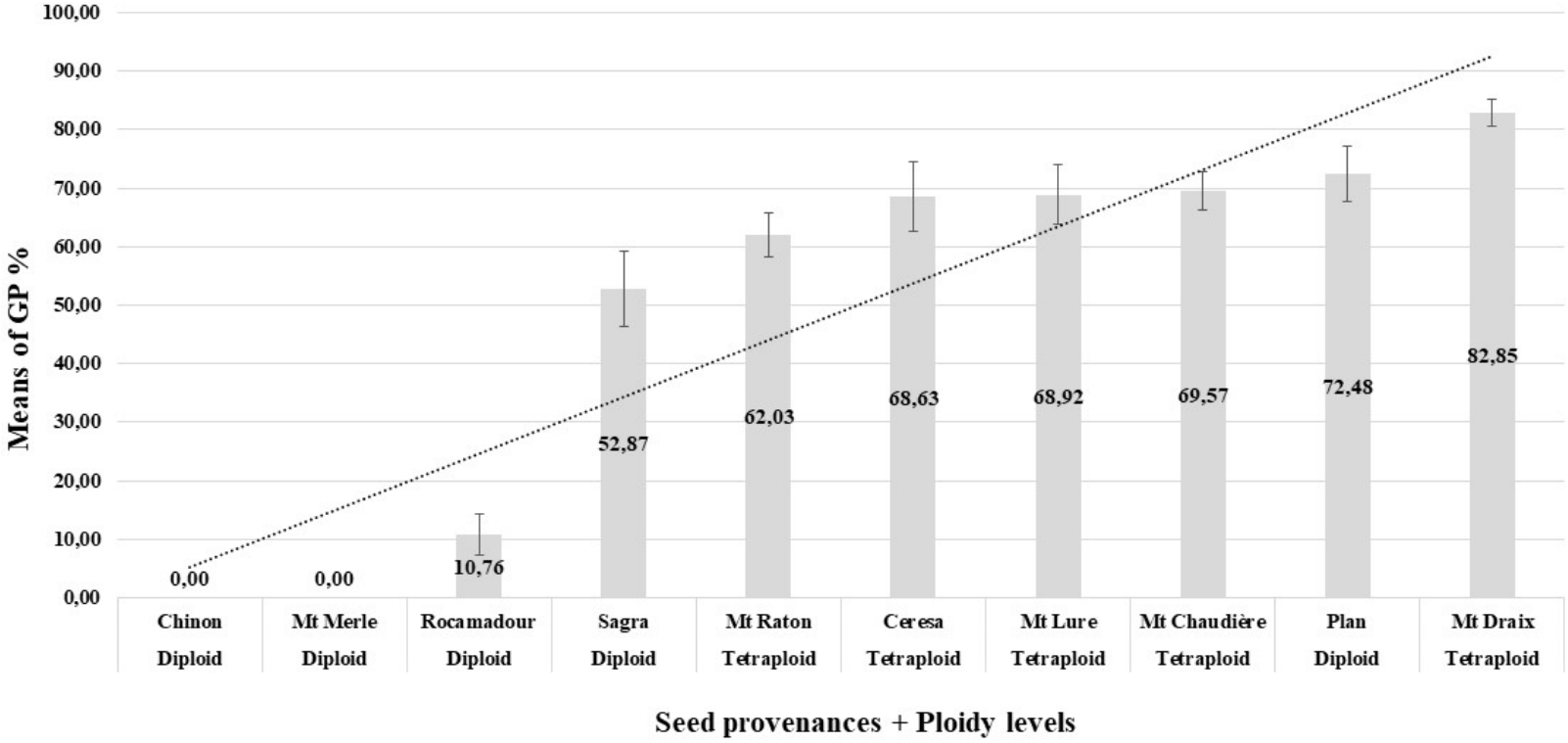
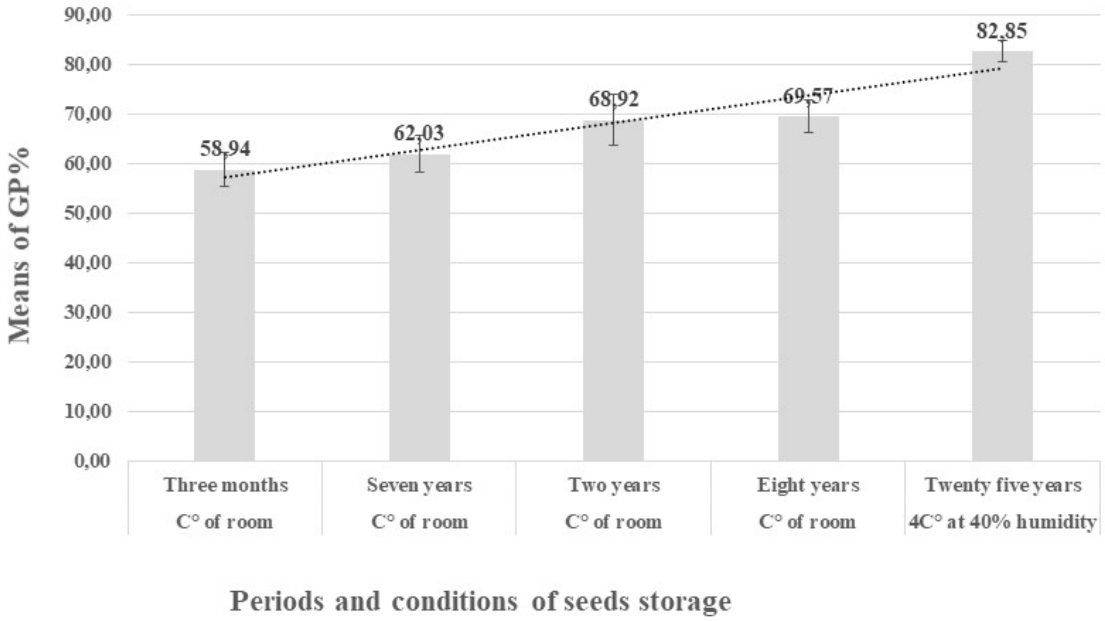


Fig. 3 Histogram shows the means of germination percentage (GP) for good germinated populations of *A. grandiflora* species complex in all trials (without GP< 10 %) with error bars in function of both of periods and conditions of seeds storage for each population. An ANOVA with welch tests are represented by (F=4.129. P=0.004; F=11.743. P=0.001 respectively)



Supplementary1: Multiple comparisons: Games-Howell (dependent variable: the germination percentage). The average of difference between each two populations cultivated *in vitro* (with scarification), standard error and the P values

(I) Population	(J) Population	Average of Difference (I-J)	Std Error	P values
Mt Sagra	Ceresa	-15.76271	8.71690	0.724
	Chinon	**52.86977***	**6.39067**	**0.000**
	Mt Chaudière	-16.70482	7.17575	0.422
	Mt Cheval Blanc	**-29.98060***	**6.77218**	**0.015**
	Mt Lure	-16.05429	8.16151	0.628
	Mt Merle	**52.86977***	**6.39067**	**0.000**
	Mt Raton	-9.15690	7.37915	0.955
	Plan	-19.61039	7.92548	0.337
	Rocamadour	**42.86646***	**7.24653**	**0.001**
Ceresa	Mt Sagra	15.76271	8.71690	0.724
	Chinon	**68.63248***	**5.92822**	**0.000**
	Mt Chaudière	-0.94211	6.76717	1.000
	Mt Cheval Blanc	-14.21789	6.33763	0.473
	Mt Lure	-0.29158	7.80470	1.000
	Mt Merle	**68.63248***	**5.92822**	**0.000**
	Mt Raton	6.60581	6.98248	0.992
	Plan	-3.84768	7.55754	1.000
	Rocamadour	**58.62917***	**6.84218**	**0.000**
Chinon	**Mt Sagra**	**-52.86977***	**6.39067**	**0.000**
	Ceresa	**-68.63248***	**5.92822**	**0.000**
	Mt Chaudière	**-69.57459***	**3.26355**	**0.000**
	Mt Cheval Blanc	**-82.85037***	**2.24094**	**0.000**

	Mt Lure	**-68.92406***	**5.07637**	**0.000**
	Mt Merle	0.00000	0.00000	
	Mt Raton	**-62.02667***	**3.68935**	**0.000**
	Plan	**-72.48016***	**4.68750**	**0.000**
	Rocamadour	-10.00331	3.41637	0.204
Mt Chaudière	Mt Sagra	16.70482	7.17575	0.422
	Ceresa	0.94211	6.76717	1.000
	Chinon	**69.57459***	**3.26355**	**0.000**
	Mt Cheval Blanc	-13.27578	3.95886	0.074
	Mt Lure	0.65053	6.03493	1.000
	Mt Merle	**69.57459***	**3.26355**	**0.000**
	Mt Raton	7.54792	4.92565	0.866
	Plan	-2.90557	5.71169	1.000
	Rocamadour	**59.57128***	**4.72465**	**0.000**
Mt Cheval Blanc	**Mt Sagra**	**29.98060***	**6.77218**	**0.015**
	Ceresa	14.21789	6.33763	0.473
	Chinon	**82.85037***	**2.24094**	**0.000**
	Mt Chaudière	13.27578	3.95886	0.074
	Mt Lure	13.92630	5.54900	0.335
	Mt Merle	**82.85037***	**2.24094**	**0.000**
	Mt Raton	**20.82370***	**4.31661**	**0.003**
	Plan	10.37021	5.19562	0.613
	Rocamadour	**72.84706***	**4.08576**	**0.000**
Mt Lure	Mt Sagra	16.05429	8.16151	0.628
	Ceresa	0.29158	7.80470	1.000
	Chinon	**68.92406***	**5.07637**	**0.000**
	Mt Chaudière	-0.65053	6.03493	1.000

	Mt Cheval Blanc	-13.92630	5.54900	0.335
	Mt Merle	**68.92406***	**5.07637**	**0.000**
	Mt Raton	6.89740	6.27542	0.979
	Plan	-3.55610	6.90958	1.000
	Rocamadour	**58.92076***	**6.11892**	**0.000**
Mt Merle	**Mt Sagra**	**-52.86977***	**6.39067**	**0.000**
	Ceresa	**-68.63248***	**5.92822**	**0.000**
	Chinon	0.00000	0.00000	
	Mt Chaudière	**-69.57459***	**3.26355**	**0.000**
	Mt Cheval Blanc	**-82.85037***	**2.24094**	**0.000**
	Mt Lure	**-68.92406***	**5.07637**	**0.000**
	Mt Raton	**-62.02667***	**3.68935**	**0.000**
	Plan	**-72.48016***	**4.68750**	**0.000**
	Rocamadour	-10.00331	3.41637	0.204
Mt Raton	Mt Sagra	9.15690	7.37915	0.955
	Ceresa	-6.60581	6.98248	0.992
	Chinon	**62.02667***	**3.68935**	**0.000**
	Mt Chaudière	-7.54792	4.92565	0.866
	Mt Cheval Blanc	**-20.82370***	**4.31661**	**0.003**
	Mt Lure	-6.89740	6.27542	0.979
	Mt Merle	**62.02667***	**3.68935**	**0.000**
	Plan	-10.45349	5.96523	0.756
	Rocamadour	**52.02336***	**5.02821**	**0.000**
Plan	Mt Sagra	19.61039	7.92548	0.337
	Ceresa	3.84768	7.55754	1.000
	Chinon	**72.48016***	**4.68750**	**0.000**
	Mt Chaudière	2.90557	5.71169	1.000

	Mt Cheval Blanc	-10.37021	5.19562	0.613
	Mt Lure	3.55610	6.90958	1.000
	Mt Merle	**72.48016***	**4.68750**	**0.000**
	Mt Raton	10.45349	5.96523	0.756
	Rocamadour	**62.47685***	**5.80037**	**0.000**
Rocamadour	**Mt Sagra**	**-42.86646***	**7.24653**	**0.001**
	Ceresa	**-58.62917***	**6.84218**	**0.000**
	Chinon	10.00331	3.41637	0.204
	Mt Chaudière	**-59.57128***	**4.72465**	**0.000**
	Mt Cheval Blanc	**-72.84706***	**4.08576**	**0.000**
	Mt Lure	**-58.92076***	**6.11892**	**0.000**
	Mt Merle	10.00331	3.41637	0.204
	Mt Raton	**-52.02336***	**5.02821**	**0.000**
	Plan	**-62.47685***	**5.80037**	**0.000**

*. Significantly difference is at the 0.05 level.

4. Chapter IV:

Morphometric variation within *Arenaria grandiflora* L. species complex

4.1 Introduction of Chapter IV

At the beginning of my PhD, I visited many European herbaria in order to collect samples of *Arenaria* sp. for a phylogeny study. This work was abandoned due to lack of results in molecular biology, but actually, I realized that we could observe an important morphological diversity within the specimens of *A. grandiflora* species complex, and particularly at the level of the leaves. Because the subject of this thesis turned to the study of variability in the *A. grandiflora* complex, we had the idea to examine also if the morphology variation could be attributed to environmental and/or genetic factors. This study is presented in the following paper carried out with the help of Florian Jabbour and Raphael Cornette from the Institute of Systematics, Evolution, and Biodiversity laboratory (ISYEB, MNHN).

4.2 Paper 3:

Leaf shape discriminates among populations with different ploidy levels and from different habitats in the *Arenaria grandiflora* L. (Caryophyllaceae) species complex

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Keywords: herbarium material. intraspecific variation. leaf elongation. log-shape ratio. principal component analysis

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Leaf shape discriminates among populations with different ploidy levels and from different habitats in the species complex of *Arenaria grandiflora* L. (Caryophyllaceae)

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Morphology

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Number of colored figures: 3

Leaf shape discriminates among populations with different ploidy levels and from different habitats in the species complex of *Arenaria grandiflora* L. (Caryophyllaceae)

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Abstract

Polyploidization in plants is sometimes associated with differences in leaf shape. Because of the environmental impacts on the genotype/phenotype relationships, we wondered how is leaf shape variation distributed in a species complex comprising plants from different ploidy levels and growing in different habitats. The leaf shape variation between diploids and tetraploids in the *Arenaria grandiflora* species complex has been observed by eye at the French National Herbarium. A sample of leaves from herbarium specimens has been examined using quantitative methods to test the validity of this distinction. The effect of ploidy levels, habitats, flowering time on leaf shape variation was assessed with morphometric methods consisting of the Log-Shape Ratio (LSR) of leaf measurements. The results show that the two cytotypes (diploid and tetraploid) have significantly distinct patterns of both leaf shape and elongation. The leaf elongation has revealed three groups, two of which corresponding to diploid populations differing in broad leaf shape (acicular or linear). Leaf shape varies a little with habitat and flowering time, but no explanatory pattern separating the specimens studied could be identified. Our results suggest that leaf shape can be used as a discriminant factor within this complex species.

Keywords: Herbarium material, Leaf elongation, Log-Shape Ratio (LSR), Principal Component Analysis (PCA)

Introduction

According to Mitteroecker et al. 2013, “the shape of an object is the geometric properties that are invariant to translation, rotation, and scaling”. Studies on plant species identification using morphometrics (Conesa et al. 2012; Cope et al. 2012; Viscosi and Cardini 2011; Viscosi et al. 2012) have shown that, among other traits, leaf shape is of great significance to help distinguish between different taxa. Both leaf shape and leaf dimensions differ within species as in *Atriplex canescens* (Pursh) Nutt (Dunford 1985) and in *Buddleja macrostachya* Wallich ex Benth (Chen et al. 2009). In addition, polyploidy could be one of the features that influenced the most vegetative morphological features (Chansler et al. 2016; Padoan et al. 2013). For example, the polyploidization is sometimes associated with differences in both leaf shape and elongation (Van Laere et al. 2011; Sun et al. 2011). As a further

matter, the leaf shape depends on the interaction between genetic features of the individuals and the environments in which they grow (Dkhar and Pareek 2014). Nevertheless, because the influence of environment could disturb the genotype/phenotype relationships, we wonder how is leaf shape variation distributed in a species complex comprising plants from different ploidy levels and growing in different habitats.

We performed a morphometric study on the *Arenaria grandiflora* species complex that is characterized by two cytotypes. Individuals can be diploid or tetraploid, with a basic number $x = 11$ (see more Daoud et al. submitted; Favarger 1959; Küpfer 1974) and grow at different altitudes in western Europe.

According to the Flora of France (Coste 1937), plants in the *A. grandiflora* species complex are organized into perennial caespitose tuft with woody rootstocks, but the stems are herbaceous, short, erected or ascendant, and usually about ten cm high. The leaves are coriaceous (stiff and tough, but somewhat flexible), opposite and sessile, glabrous or glabrescent, with an entire and thick margin. They are lanceolate-linear, oblong-lanceolate or ovate-orbicular. The leaf apex is often subulate (e.g. more than 3:1 L/w), usually prolonged, straight and stiff. The leaf is characterized by a very prominent middle rib.

The populations of plants belonging to the *A. grandiflora* species complex occur in disjunctive areas of Europe. In addition, the splitting of this species complex into discrete entities at the sub-specific level is controversial, because of the different numbers of subspecies that were mentioned in different databases. For example, three, five or seven accepted subspecies according to Flora Europaea, the Global Biodiversity Information Facility (GBIF; international open data infrastructure) and to the International Plant Names Index, respectively. As demonstrated by Daoud et al. (submitted) the patterns of genetic variation among populations of different ploidy levels discriminate among the studied populations as presented in Fig. 1. For instance, the populations from the plains are predominantly diploids, and the populations of high altitude are mostly tetraploids.

Morphometric methods in both plants and animals are powerful tools to reveal differences in organismal shapes. We used the collection of *A. grandiflora* species complex from the French National Herbarium (acronym P), consisting of 610 specimens, some being more than 200 years old. We

described the variation in leaf shape using a morphometric approach. The main objective was to identify both the leaf shape and elongation that could discriminate the sampled specimens belonging to different cytotypes and collected from different habitats.

Materials and Methods

Sampling and data collection

Forty-two herbarium specimens from the *A. grandiflora* species complex from Western Europe were used. Seventeen and 25 specimens were collected from populations that had been formerly described as diploid or tetraploid (see Daoud et al. submitted; Favarger 1959 and Küpfer 1974, for details). A map of the collection sites was drawn using ArcGIS (Fig. 1). For each specimen, we sampled five leaves from the central part of the individual. Measurements were hence made on a total of 210 leaves. We used simmering water to hydrate the dry leaves. We glued them on microscope slides (10 leaves [i.e. two specimens] on each slide) with white glue (Fig. 2). The microscope slides were numbered and the correspondence between these numbers and the specimen barcodes is given in Table 1. We put all the microscope slides on an inverted scanner (Herb*Scan*) and then took a digital 1200 DPI image of the set of leaves (Fig. 2a). Morphological linear measurements of the sampled leaves were made on the resulting picture using tpsDig version 2.26 (Rohlf 2015).

Leaf measurements

The following leaf measurements were performed in microns (Fig. 2b): the overall leaf length (L) and the leaf width at three levels dividing the leaf into three equally long segments (Fig. 2b). Thus, the raw linear measurements of the leaves composed the raw dataset.

Shape parameters: Log-Shape Ratio (LSR)

LSR (Mosimann 1970) is one of the methods which is used to compare shape parameters among-geographically distributed populations while removing size by partitioning the form into size and shape and to provide study differences in shape proportions. First, the isometric size was computed as a geometric mean of all logarithmic measurements by specimen for each leaf, then each measurement was divided by isometric size to obtain the LSR.

Leaf elongation

Based on the relation with leaf elongation (leaf length to maximal leaf width (L / W2)), three leaf types were defined: acicular: L / W2 > 12:1; linear: ratio between 12:1 and 6:1; and lanceolate: ratio between 6:1 and 3:1.

Sampling approach with a priori grouping

A sampling approach with a priori grouping was performed on the leaf shape dataset by using each one of the factors as a priori grouping. The factors were as follows: (1) The ploidy level (diploids and tetraploids) of our studied populations that were recovered from previous studies (see more Daoud et al. submitted; Favarger 1959; Küpfer 1974); (2) Their habitats which were classified according to the altitude (alt.> 900m: mountains; alt. < 250m: plains; 250 < alt. < 500m: hills); (3) The century of the collection (19^{th} century or 20^{th} century); (4) The flowering time that was extrapolated from the collection month of our selected specimens (May, June, July, August, and October).

Data analysis

The statistical analyses were done using the Rmorph statistical software packages (R Core Team, 2016). A Principal Component Analysis (PCA) was performed on the leaf shape dataset, to visualize the structure of leaf shape variation within the species complex of *A. grandiflora*. The results were then presented as scatter plots. A multivariate analysis of variance (MANOVA) was run on the principal component scores, to reveal significant statistical differences among populations and to test the significance of the following factors: the ploidy levels, the habitats, the century of collection and the flowering time.

Results

Leaf shape discriminates between two cytotypes

The ploidy level separates well the populations according to their leaf shape. The results showed that the polyploidy was discriminant in the species complex of *A. grandiflora* and we could distinguish evidently the data of leaf shape into the diploids and the tetraploids (Fig. 3a-b). A MANOVA of significance performed on the PCA of leaf shape data showed that there were significant differences among the specimens of different ploidy levels ($F=97.467$, $P = 0.000$).

Leaf elongation

The leaf shape variation within the species complex of *A. grandiflora* could be also explained in relation with leaf elongation, that included some of information about the leaf types and separated the leaves sampled into three groups (acicular leaves, linear leaves, and lanceolate leaves). The specimens (Fig.3b) were separated according to the variables of *PC*1, both the leaf length (L) and the leaf width (W2). Along the *PC*1, in the diploids, leaf length increases while leaf width is decreased, thus we found two of leaf types: acicular and linear leaves. In contrast, in the tetraploids, leaf length decreases while the leaf width is increased (one of leaf types: lanceolate leaves).

Leaf shape data analysis

The scatter plot of Principal Component Analysis (PCA) showed the structure of leaf shape variation in the species complex of *A. grandiflora* (Fig. 4). The first principal component (PC1) axis accounted for 66.04 % of the total leaf shape variation while the second principal component (PC2) accounted for 21.47 % and PC3 12.49 % (see Fig. 4). The first principal component is clearly congruent with the separation of the tested specimens; it opposes leaf length with its maximal width W2. The second principal component axis contrasts leaf width at the highest point (W1) to leaf width at the basal point (W3).

Leaf shape discriminates within the diploids

We found that leaf shape varied within the diploids of *A. grandiflora* species complex: acicular and linear leaves were defined with significant variation (F= 41.173; P = 0.000) (see Fig.3b). We could distinguish the leaf shape of the specimens that are belonging to the diploid population from Sainte Baume from the leaves of specimens which are belonged to other diploid ones (Fontainebleau, Chinon, Rocamadour, and Gèdre) (see Fig.5). In addition, the leaves of plants harvested from the diploid specimens of Mt Sagra population seem different from the other ones that were collected from the diploid populations of Fontainebleau, Chinon, Rocamadour, and Gèdre. Moreover, the leaves of the diploid populations of Fontainebleau, Chinon, Rocamadour, and Gèdre are not distinguishable.

Significant effects for both flowering time and habitat

The results of the PCA was performed on the leaf shape dataset using a sampling approach with three out of four priori groupings. Two out them (flowering time, habitats) had a significant variation ($P < 0.05$), but the century of collection, the fourth priori grouping, had no statistical significance effects. The MANOVA of significance performed on PCA of the leaf shape data showed significant differences among the studied populations for the flowering time, their habitats ($F = 2.974$, $P = 0.000$; $F = 13.707$, $P = 0.000$ respectively).

Discussion

Intraspecific leaf shape variation

Leaves of individuals from the species complex of *A. grandiflora* showed distinct patterns of shape and elongation variation, that were well related to the two cytotypes (diploids and tetraploids). Moreover, the leaf elongation in the *A. grandiflora* species complex ranged from acicular or linear to lanceolate, with an aristate apex, perfectly continuous margins, glabrous and entire. Leaves of tetraploids were typically lanceolate and the leaves of diploids were typically acicular or linear. These results agree partially with the general morphological description of the lanceolate or linear-lanceolate leaves of our model *A. grandiflora* in the French Flora (Coste 1937) and the Flora of the Jurassic Chains (Grenier 1865). Furthermore, the tetraploid specimens that were investigated in this study were restricted to the high-altitude areas in both the Swiss Jura mountains and the alpine mountains.

Why does leaf shape differ in the *A. grandiflora* species complex?

Many hypotheses have been proposed to explain the leaf shape variation. In fact, leaf shape reflects complex assemblages of shape-determining elements: genetic control, environmental factors (Dkhar and Pareek 2014); reviewed in a model plant, *Arabidopsis thaliana* by (Tsukaya 2005), and hormonal control. The gibberellin (GA) reduce marginal elaborations of tomato leaves (Hay et al. 2002; Jasinski et al. 2008). Moreover, many genes have been described that control leaf development and shape (reviewed by Byrne 2005; Li et al. 2009; Tsukaya 2005). Several studies reported important relationships between the DNA amount and many phenotypic characteristics such as leaf and flower characters (both discrete and continuous quantitative characters) in the polyploid group of *Ranunculus*

parnassifolius L.(Cires et al. 2010), and a negative relationship with leaf mass per unit area (LMA) in angiosperms by (Beaulieu et al. 2007). However, a considerable variation in genome size was detected among populations of diploid plants. For example, the two populations Mt Sagra and Ste Baume had a genome size of about 2.44 pg and 2.70 pg respectively (Daoud et al. submitted), when the others had about 2.11 pg. Thus, we could suppose that this high genome size for both the Mt Sagra and Ste Baume in the Mt Mediterranean, could explain their distinguishable leaves from the other diploid ones growing on the plains or in the Pyrenees chain.

Nevertheless, the morphological diversity in leaf shape could be a response to abiotic stress in their natural habitat (see Hovenden and Vander Schoor 2006; Xu et al. 2008; Royer et al. 2009). For instance, the simple leaves of *Quercus acutissima* become narrower in the xeric environments (Xu et al. 2009). Thus, it seems that leaf shape is sensitive to climate change. For example, Royer et al. (2009) highlighted the leaf-climate relationships in *Acer rubrum*. The leaf shape of native European hardwoods correlates strongly with temperature (Traiser et al. 2005), and it can also change according to salinity in *Vitis vinifera* L. (Sinclair and Hoffmann 2003). Our results showed that the habitats are not sufficient to separate clearly the leaf shape dataset into distinct groups, although they suggest a significant statistical variation. We observed that leaf shape of *A. grandiflora* species complex could be linked with the habitats in which they grow. Indeed, *A. grandiflora* species complex occurs in the dry mountains of North Africa, the Mediterranean islands and the Pyrenees in Spain and France where this species complex survives the harshest conditions. When growing in dry rocks or screes, in the alpine mountains, they produce small, thin and pointed leaves which may help them to bear extreme frost and dryness (such as lanceolate leaves of the tetraploids). Furthermore, as expected, the leaves of plants have grown in sunny environments were generally smaller than leaves of plants of shaded environments. This result might be due to the shade avoidance response as explained in Franklin (2008) or Tsukaya (2005), which is expressed by the populations exposed to the weak light of the Fontainebleau forest (compared to alpine ones) resulting in different leaf elongation (acicular and linear leaves).

Conclusion

Our results showed a limited effect of habitats on leaf shape variation in the species complex of *Arenaria grandiflora*. This result is probably due to genetic founding and drift effects. Because this species complex is so sparsely distributed, gene flow among populations is limited and thus leads to differentiated genomes. This phenomenon could explain a large part of the differences observed among the leaves. In this study, we assume that the respective influences of many factors, that likely drive the leaf shape variation in the *Arenaria grandiflora* species complex, are difficult to assess because some of them are confounding (ploidy levels, habitats, flowering time). This study would be interesting to replicate in the future on plants from current natural populations to estimate, for such a trait, the speed of the drift and the impact of climate change.

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Figure Captions

Fig.1 Distribution map of the herbarium specimens sampled from the collection of *Arenaria grandiflora* species complex, with their associated ploidy levels as mentioned in the literature (see Daoud et al. submitted, Favarger 1959, Küpfer 1974); 1: Fontainebleau; 2: Chinon; 3: Mt Chasseral 4: Mt Chasseron; 5: Mt Suchet; 6: Mt Ventoux; 7: Col de Tende; 8: St Baume; 9: Rocamadour; 10: Mt Gèdre; 11: Mt Sagra.

Fig.2 a: Set of microscope slides on which leaves sampled from herbarium specimens identified as belonging to the A*renaria grandiflora* species complex are glued. Each slide gathers 10 leaves, corresponding to two individuals sampled from two different specimens; **b:** Schematic representation of a leaf showing the four distances measured: W1, W2, W3 (for the width measured at the second third and first third of the total leaf length, and at the basis of the leaf), and L (for length). Distances are given in microns (*mu*).

Fig. 3 Plots of Principal Component Analysis (PCA) of leaf shape variation within the species complex of *Arenaria grandiflora*: a: plot of the diploids and the tetraploids; b: plot of two ploidy levels combining with leaf elongation: the leaves of diploids are acicular or linear, and the leaves of tetraploids are lanceolate. The differences among clusters are significantly different ($P = 0.000$).

Fig. 4 A scatter plot of Principal Component Analysis (PCA) reveals the structure leaf shape variation within *Arenaria grandiflora* species complex; L: leaf length; W2: maximal width; W1: leaf width at the highest point; W3: leaf width at the basal point.

Fig. 5 Plot of Principal Component Analysis (PCA) of the leaf shape variation among the diploid populations of the *Arenaria grandiflora* species complex. The difference among clusters is significant ($P = 0.000$). Chasse.: Mt Chasseron; Ftbleau : Fontainebleau; Rocamad : Rocamadour; Ventoux : Mt Ventoux.

Table 1 List of herbarium specimens sampled from the collection of *Arenaria grandiflora* species complex, corresponding microscope slides and collection year. High-resolution images of the herbarium specimens can be accessed using the following link: https://science.mnhn.fr/institution/mnhn/collection/p/item/search/form

N° studied sample	N° microscope slide	Specimen barcode	Collection year
1	2	P04933087	1891
2	3	P04936269	1877
3	4	P04936299	1840
4	5	P04936290	1889
5	7	P04936267	1869
6	8	P04933084	1853
7	9	P04936139	1854
8	10	P05162981	1891
9	17	P04933086	1891
10	21	P04936144	1874
11	22	P04936143	1859
12	26	P04933116	1916
13	27	P04933065	1865
14	28	P04933061	1844
15	29	P04933064	1844
16	30	P04932285	1895
17	38	P05160377	1978
18	44	P05162986	1873
19	45	P05109875	1881
20	50	P05161419	1881
21	52	P05167087	1845
22	53	P05109867	1898

23	58	P04936164	1898
24	60	P04936172	1867
25	61	P05106778	1881
26	64	P05109879	1898
27	67	P04936250	1878
28	68	P04936255	1867
29	79	P04936152	1861
30	80	P04936152	1861
31	81	P04936154	1881
32	84	P04936156	1877
33	87	P04936157	1881
34	89	P04936153	1847
35	97	P05163002	1881
36	99	P04936167	1844
37	103	P04936162	1844
38	104	P04936168	1898
39	105	P04936170	1972
40	125	P04978999	1845
41	143	P05109872	1847
42	146	P04936173	1898

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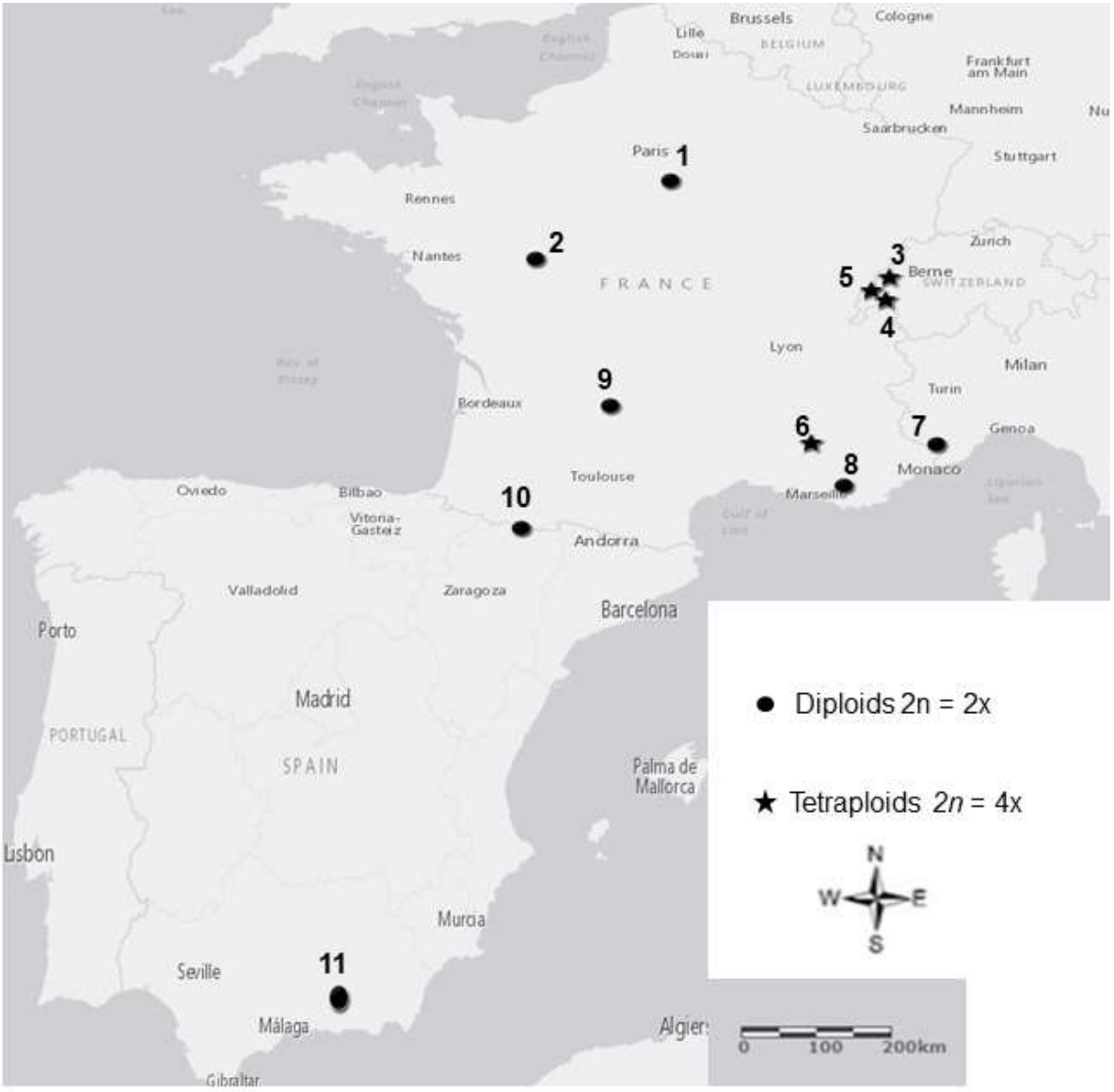

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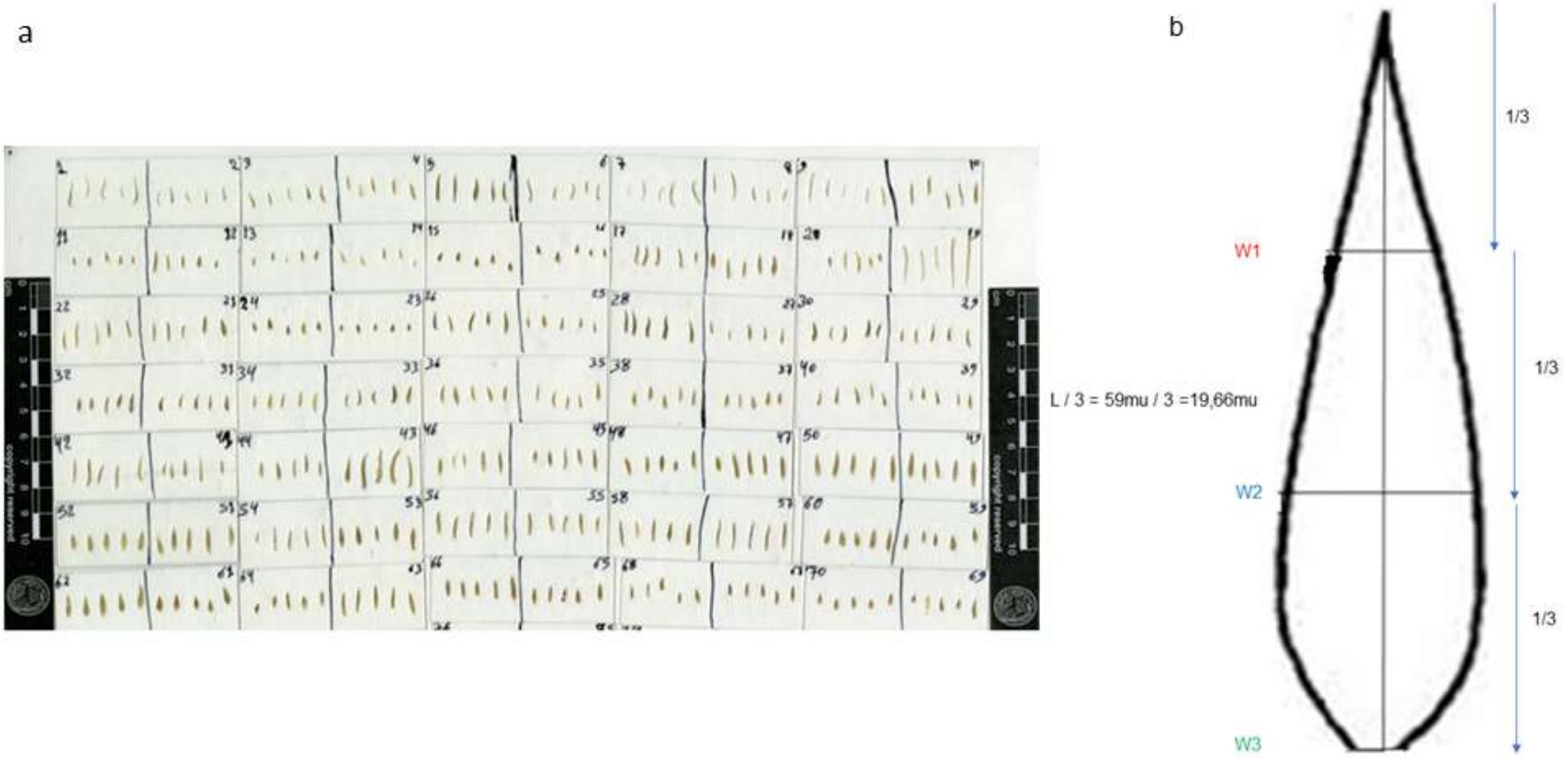

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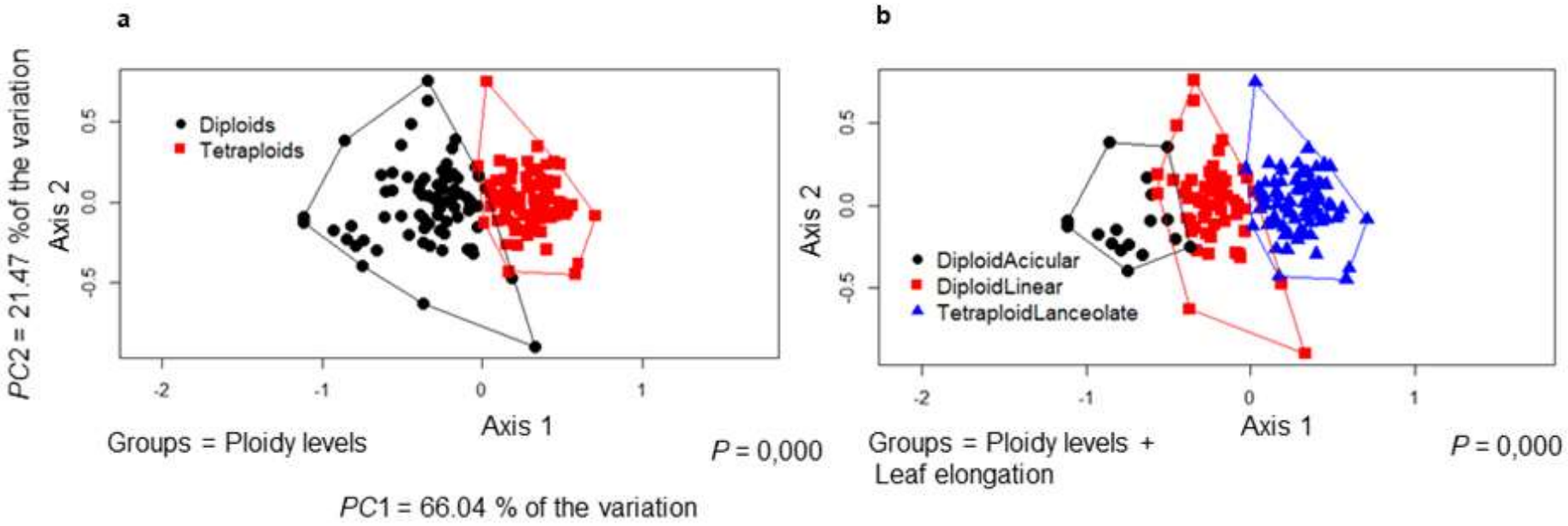

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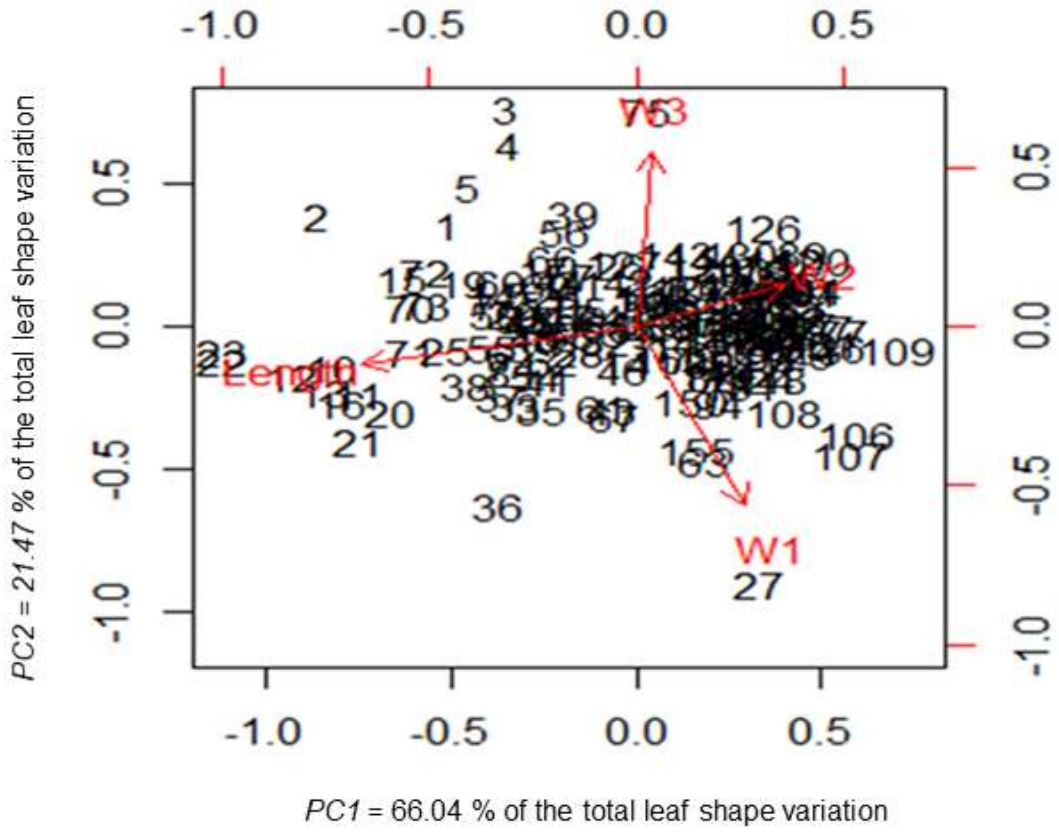
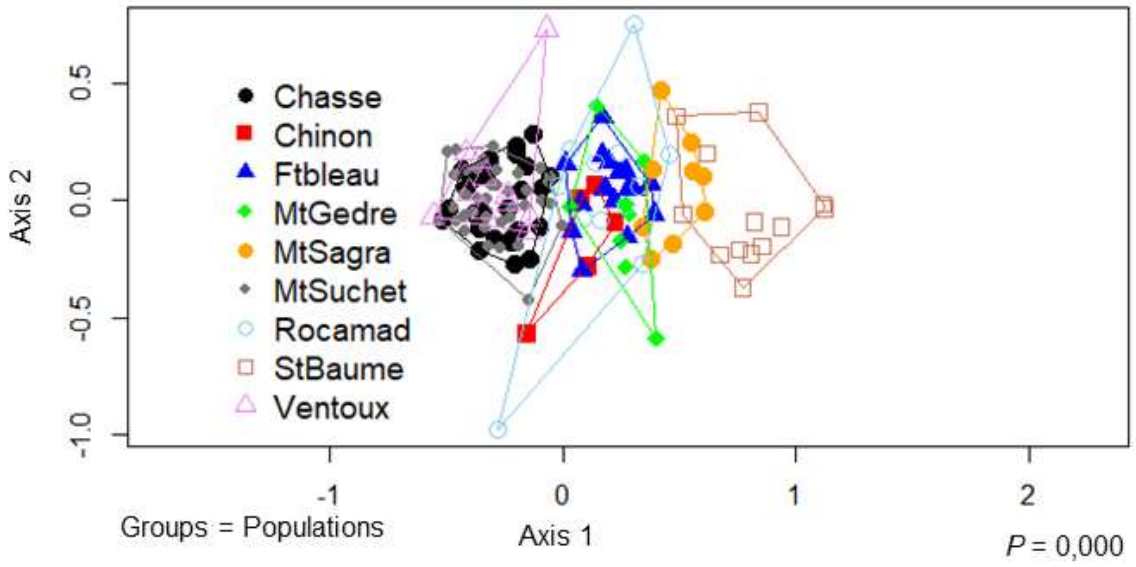


Fig. 5 Plot of Principal Component Analysis (PCA) of the leaf shape variation among the diploid populations of the *Arenaria grandiflora* species complex. The difference among clusters is significant (P = 0.000). Chasse.: Mt Chasseron; Ftbleau : Fontainebleau; Rocamad : Rocamadour; Ventoux : Mt Ventoux.



5. Chapter V:

General Discussion

The investigation within a widespread species of plants at large spatial scales in nature exhibits opportunities to characterize the importance of differentiation in many domains of biological research. The general objective of this doctoral thesis was to give a faithful portrait about the variation within the species complex of *Arenaria grandiflora*, with a view to providing complementary information useful for improving the management decisions for the future population-level conservation plans.

Differentiations among populations

Understanding the genetic or phenotypic patterns of differentiation among natural populations is one of the central concerns of many types of research such as population genetics, ecology, taxonomy, and conservation research. Moreover, the plant population is one of the most basic levels of plant studies, and because of naturally fragmented distributions of plant populations, an important genetic differentiation should be found among these naturally fragmented populations. For example, the isolated populations of a rare alpine species Campanula thyrsoides L. show a high genetic diversity and a considerable population differentiation (Aægisdóttir et al. 2009). In the case of the *Arenaria grandiflora* species complex, within its range of the widespread distribution, there were isolated, small populations (< 20 individuals; in Mt Chasseral and Ste Baume (obs. perso.)), and often threatened (or extinct; on the French side of Mt Jura and in the Fontainebleau forest (Prunier 2001) and perso. obs.), and genetically distinct population (Favarger 1959, 1962; Küpfer 1974). Thus, the present project provides a synthesis of results from studies on the natural populations of this species complex (in total 27 population) from Western Europe. This study has also proven interesting to ongoing research into patterns of genetic, cytogenetic and phenotypic variation within *A. grandiflora* species complex. The variation patterns of *A. grandiflora* populations seemed to be characterized by their ploidy levels i.e. chromosome numbers as well as their genome size, likewise, their seed germination percentage and leaf shape. As far as we know, this is the first integrative study of genetic and phenotypic diversities of *A. grandiflora* species complex on a large geographical scale across Western Europe, for which, we applied different approaches.

Firstly, the microsatellite analysis was one of the most important indications that revealed a differentiation among populations. Though microsatellite markers are routinely used to investigate the genetic structuring of natural populations, reliable estimates of differentiation among populations could

be frequently surveyed by microsatellite markers, for example; a genetic differentiation among local populations of Magnolia stellata (Siebold & Zucc.) Maxim., was estimated using the microsatellite markers (Setsuko et al. 2007) and (Walser et al. 2005) addressed a genetic differentiation among lichen populations using the microsatellite markers. In this study, we used the microsatellite markers in a different way a little bit, it has been tested whether such markers are used as a considerable indication to the ploidy levels, for the first time. Here, the microsatellites markers reveal a remarkable differentiation among populations at the ploidy levels within the species complex of *A. grandiflora,* using the allelic phenotypes at the ten microsatellite DNA loci that were amplified in some of the investigated populations. Some of the microsatellite loci in some of the populations were characterized by one or two alleles per locus per individual and made recognizable, in some of the cases, diploid heterozygosity. However, in populations of both Mt Jura and Mt Alps, the same loci displayed more than two alleles (up to four alleles) per individual, signal of tetraploid heterozygotes.

Furthermore, a classical karyological approach using conventional staining techniques was another proof demonstrating that explicit differentiation among populations exists within the *A. grandiflora* species complex. In this species complex, the occurrence of intraspecific chromosome number variation has been reported by two basic chromosome numbers of x=11,10 (Küpfer 1974; Luque & Lifante 1991, respictively). However, our results showed one basic chromosome numbers of x=11.Two chromosome numbers corresponding to two different ploidy levels were detected, diploid (2n=2x=22) and tetraploid (2n=4x=44), both with small chromosomes and characterized by the basic chromosome number of x = 11. Because of many obstacles like seed dormancy, crystals of oxalate of calcium that occurred in the roots and the presence of endophytic fungi in seeds, it has been very difficult to apply this classical karyological technique in A. grandiflora species complex. Thus, we used the technics of flow cytometry that can be a powerful tool for rapid and detailed analysis of populations of threatened species by using various materials such as leaf tissue (small amount), stored leaves in silica gel, cotyledons of germinated seedlings or dried seeds, which are adequate to estimate the variability among populations in genome size of the species complex of *A. grandiflora*. As well as a range of uses in plant researchers, flow cytometry offers a useful approach for monitoring the stability or the rapid evolution of genome size within an individual, likewise within a species, the genome size is usually constant within a species such

as some of species of the Mediterranean genus Cistus L., (Ellul et al. 2002), and (Lysak et al. 2000) demonstrated that Sesleria albicans Deyl., belongs to the plant taxa with a highly stable genome size, although there are notable exceptions, where a rapid evolution of genome size causes remarkable variation such as intraspecific differences in nuclear DNA content were found among populations of Koeleria macrantha (Ledeb.) Schult., (Pecinka et al. 2006), also an intraspecific genome size variation was observed in Camellia sinensis (L.) Kuntze (Huang et al. 2013). In addition, a significance of intraspecific genome size variation was found in quinoa (Chenopodium quinoa Willd.) using flow cytometry technic, with propidium iodide as the DNA stain (Kolano et al. 2012). The present study suggests that the species complex of *A. grandiflora* has no stable genome size, because of a considerable intraspecific variation in genome size among its natural and fragmented populations (21 populations) was identified.

Furthermore, (Severns & Liston 2008) uncovered large gaps in the current state of cytological knowledge for threatened and endangered plants and a lack of attention given to the topic of intraspecific chromosome number variation and plant conservation. Thus, our assessment of the distribution of this cytogenetic variability present within the species complex of *A. grandiflora* with its fragmented distribution in Western Europe was critically and important requirement for the success of any genetic conservation plan in the future.

Nevertheless, if the phenomenon of differentiation among populations in seed germination rates is well known (Pérez-García et al. 2003) (Ginwal et al. 2005) (Bahru et al. 2014) (Fredrick et al. 2016) (Xu et al. 2016), the seed germination response variability in the investigated populations of *A. grandiflora* species complex was described for the first time. The seed provenances were significantly differentiated by the seed germination percentages. In spite of the different designs in the experiments, such as potting soil and agar substrates, the findings were convergent. The germination percentages (GP) greatly varied from 0% to 80 % within the species complex of *A. grandiflora*. Thus, we suppose that this high intraspecific variation in germination rate of seeds could be interpreted by various factors like the maternal environmental effect of seed provenances (both altitude and longitude of seed provenances impacted the final germination percentage), the ploidy levels of individuals (the tetraploids germinated better than the diploid ones) and both period and condition of seeds storage (seed viability for the longer

period is retained at + 4 °C temperature and 40% humidity). Our results in seed germination trials provide valuable and baseline information of populations of *A. grandiflora* species complex to address conservation concerns for this endangered species.

Finally, the phenotypic patterns of differentiation among populations have been detected in herbarium' specimens belonging to some of populations of *A. grandiflora* species complex, that were stored in the French National Herbarium (in Paris). The distinct patterns of both shape and elongation variation have been exhibited in the leaves of *A. grandiflora* species complex by using the morphometric approaches. This variation in both of leaf shape and elongation has been distinguishable between the two cytotypes (diploids and tetraploids) of the species complex of *A. grandiflora*. In other words, we found that the leaves of diploids were typically acicular or linear and the leaves of tetraploids were typically lanceolate. An interesting leaf elongation variation within the diploids of *A. grandiflora* species complex has been found. Both the acicular and the linear leaves were shown, the leaves of specimens from Mt Sagra and Ste Baume populations are acicular, different from the linear leaves of specimens which are included in other populations as like as Fontainebleau, Chinon, Rocamadour and Gèdre.

As mentioned by Otto and Whitton (2000) and (Levin 2002), the process of polyploidization is often accompanied by changes in morphology, physiology and associated with some geographic locations, types of environment as well as the life history of populations. Here, we observe an impressive incidence of polyploidy among the natural and fragmented populations of *A. grandiflora* complex over its spatial scale in Western Europe. Moreover, this incidence of polyploidy seemed to influence the tested characters such as 1) molecular genotypes (variant number of alleles at some of the microsatellite loci), 2) seed germination responses (GP variation) and 3) both leaf shape and elongation variability. The results on ploidy levels within perennial herbaceous of *A. grandiflora* species complex agree with the current evidences which suggest that the frequency of polyploidization events differ among different life forms. For example, (Stebbins 1938) and Otto and Whitton (2000) reported that the high proportion of polyploids is generally among perennial herbaceous plants, compared to annuals species. Furthermore, the polyploids frequently differ in geographic ranges from diploid relatives, and often have larger geographic distribution and occupy more extreme environments such as high altitudes than diploids (Otto and Whitton 2000; Levin 2002). These differences in geographic ranges have been

explained through different aptitudes of polyploids such as best ecological tolerance (plasticity), due to higher heterozygosity and allelic diversity (Otto and Whitton 2000).

Protocol for seed germination and influential factors

To understand the effects of many factors on the germination percentage, we validated a linear model of regression with the 6 explanatory variables: altitude, longitude, latitude, ploidy levels and both period and condition of seed storage.

As well known, the reinforcement with new individuals is often recommended to preserve some populations for extinction (Bottin et al. 2007). Nevertheless, producing the individuals from seeds can be challenging because little is known about the germination requirements of many endangered species. Here, we established for the first time, a reliable protocol of seed germination that achieved a higher germination rate for most of the tetraploid populations of *A. grandiflora* species complex. As mentioned above, the seed coat dormancy is one of the obstacles that have been found in many populations of *A. grandiflora* species complex (especially tetraploid ones) and have been sideswiped by using in vitro seed germination with mechanical scarification. In vitro culture after mechanical scarification has been applied to seeds which have been collected from different altitudes and have been stored in two types of conditions for different periods. Four culture media with and without sugar have been prepared to germinate the seeds. Furthermore, the protocol of the surface sterilization has been used for pre-treatment the seeds to avoid the contamination. According to our novel protocol of seed germination, the propagation from seeds will be an efficient method for the in-situ restoration of these tetraploid populations.

Diploid cytotypes

Here, we present and discuss some of the diploid populations that we consider particularly remarkable for the future conservation plans for *A. grandiflora* species complex.

One of the most interesting populations is Mt Sagra population in the Mediterranean mountains that occurs in Granada (Andalusia, Spain) at high elevation (2279 m). According to Ryder (1986) who identified the populations as ESUs, the Mt Sagra population is considered by this study as distinct ESU, presenting a particular monoploid genome size which has been detected for the first time. Our findings exhibited that the Mt Sagra population had a maximum of two alleles per locus by using the

microsatellite markers and showed a mean 2C value in pg ±SD (2.44±0.96) detected by flow cytometry approach, Küpfer (1974) reported that one diploid population occurred in the Sierra Nevada (another massif in Granada-Andalusia, Spain). Thus, the population of Mt Sagra is worthy of follow-up, especially because it presents a good seed germination percentage (52,86± 22,14) in in vitro culture (with mechanical scarifying) and a significant difference from the GP for seeds of tetraploid population of the alpine mountains (Mt Cheval Blanc at 2280 m, in the same altitude).

So, the different seed germination responses we observed could be explained by the different types of climate i.e. the Mediterranean mountain and the alpine mountain ones, marked especially by seasonal changes that are affecting seed viability and storability. Moreover, the leaf shape of herbarium specimens of Mt Sagra population is another argument for which this population could be considered as an interesting distinct population among other diploids ones. Its leaves are acicular and seem different from the linear ones of the other diploid populations. The phenotypic findings on leaves could be explained by a large monoploid genome size that has been found and already counted as a distinct cytogenetic character in this population. Consequently, the Mt Sagra population revealed a distinguished pattern of genetic, cytogenetic and phenotypic variation and thus could be considered as a unit for population-level conservation to stop the biodiversity loss within a species complex such as *A. grandiflora* (Purvis & Hector 2000).

Another interesting diploid population that could be considered as a good ESUs, is the Ste Baume population. This diploid population of the species complex of *A. grandiflora* exhibits both in a restricted geographical distribution (1034 m) and a narrower and more marginal niche than other species of Arenaria, in dry rocky in the southern Provence of France (Youssef et al. 2011). In addition, it is a very small population (< 25 individual, obs. perso.2016). Here, the cytogenetic results show that Ste Baume population has the largest monoploid genome size with an interesting significant difference with other diploid ones. Furthermore, the leaves of herbarium specimens of Ste Baume population are acicular and look distinguishable from the linear leaves of the other diploid ones in the plains or in the Pyrenees chain. Thus, we have a distinct unit for population-level conservation in Mediterranean mountains that has presented a restricted geographical pattern of both cytogenetic and phenotypic variation. Because of all above, we suggest a serious and effective interest in the future for the Ste Baume population.

Tetraploid cytotypes

However, all the investigated populations (except Mt Chasseral population in Swiss Jura, already protected since 2005) with high genome size (from 4.30 to 5.27 pg.) have been considered, in this project, as distinct ESUs. The tetraploidy of all the following populations: the alpine and pre-alpine populations (Mt Lure, Mt Cheval Blanc, the Chaudière and Mt Raton), the Swiss Jura population (Mt Chasseral) and the Spanish Pyrenees populations (Ceresa and Gabas) has also been detected for the first time. The present results have showed that the seeds of all investigated tetraploids were physically dormant (seed coat dormancy). Though all those populations have been defined as ESUs, the Mt Cheval Blanc population, the highest population (i.e. situated at 2300 m) deserves to be interesting issue in the future studies. It differed significantly in genome size (4.56±0.03 pg.) from other both alpine (Mt Lure) and pre-alpine populations (Chaudière and Mt Raton). In addition, the seeds from Mt Cheval Blanc population could keep its viability and germinability (GP ± s.d. = 82.85± 7.76 %) for a longer period (until 300 months) with storage at the +4°C temperature and 40% humidity. Moreover, the germination percentage for the Mt Cheval Blanc population showed a significant difference from both pre-alpine populations (Mt Raton; GP ± s.d. = 62.03± 14.29 %; and Mt Chaudière; GP ± s.d. = 69.57± 11.31) that were stored during 84 and 96 months respectively.

As observed in the French National Herbarium (in Paris), the specimens from the tetraploid alpine and pre-alpine populations (Mt Lure, Mt Cheval Blanc, the Chaudière and Mt Raton) have not been harvested, except tetraploid specimens from the Swiss Jura populations (Mt Chasseron, Mt Suchet, and Mt Chasseral) and from the alpine population of Mont Ventoux. Moreover, the leaves of tetraploids (from the Swiss Jura populations and the alpine population of Mont Ventoux) were typically lanceolate and differ significantly from both the acicular and linear ones in the diploid populations. Thus, it will be very interesting to study the morphometric traits of leaves of the alpine and pre-alpine populations from its natural distributed range (Mt Lure, Mt Cheval Blanc, Chaudière and Mt Raton) in the future researches about this species complex of *A. grandiflora.*

Furthermore, one of the most interesting populations, that is already protected in Switzerland, is the Mt Chasseral population, that grows in rock debris and at a full sun exposure in very well-drained soil (Favarger 1959; (Hess et al. 1976). This population has only 8 individuals (obs. perso., 2013), so,

both leaf sampling and seed harvesting had been very difficult. The chromosome number of individuals from Mt Chasseral population were detected by Favarger (1959, 1962) and Küpfer (1974) (2n =4x = 44). Here, we reveal, for the first time for this population, both the number of alleles for microsatellite loci and the mean of 2C DNA content (4.39±1.54 pg.).

The presence of populations with different ploidy levels in the same mountain chain such as in the Spanish Pyrenees (Huesca), e.g. two tetraploid populations (Ceresa and Gabas) co-occur with two diploid ones (The Vilas del Turbon and Plan), arises new questions about the origins of polyploid populations.

General Conclusion

Taxonomic revision_ point of view

The Large Flowered Sandwort (*A. grandiflora*) is a widely distributed species complex with disjunctive populations in Western Europe but has an uncertain taxonomic status. Seven, sometimes five or three geographic subspecies, two varieties and two forms had sometimes been described. In this thesis, *A. grandiflor*a had been considered as species complex, because of the existence of numerous synonyms and intraspecific karyotypic variability, whereas, two cytotypes (diploid and tetraploid) have been detected among its fragmented populations. As demonstrated, the thesis has shown the patterns of genetic and phenotypic variation in *A. grandiflora* species complex. A correlation was found between polyploidy and phenotypic variation, there were differences between the two cytotypes (diploid and tetraploid). We found polymorphism for all the characters we studied, such as the genotyping of microsatellite loci, the chromosome number, the 2C-values of nuclear DNA content, the seed germination percentage and the leaf shape. The recent approach of flow cytometry has allowed more accurate and rapid estimates of the genome size and its variation within the species complex of *A. grandiflora*, including the correlations with the geographic range of its distribution and morphometric traits of its leaves. The mean 2C DNA value within the species complex *of A. grandiflora* ranges from 2.11±0.74 pg to 2.70±0.11 pg for the diploids and from 4.30±1.51 pg to 5.27±0.14 pg for the tetraploid ones. These results substantially support that both the genome size and the polyploidy are probably major causes of diversity within this species complex. However, as known, the plant genome sizes range from the smallest (1C = 0·0648 pg in Genlisea margaretae Hutch, Bennett and Leitch 2011) to the largest (1C = 152·23 pg in Paris japonica Franch. (Pellicer et al. 2010), there is no evidence supporting a link between overall genome size and diversification. Nevertheless, the rate of genome size evolution enhances the acquisition of the novel traits, the reproductive barriers and the movement into new niches, which have aided the diversification of angiosperms (Puttick et al. 2015). Moreover, when the changes in the overall genome size, the ploidy level and the chromosome number occur continuously in plants, it is not surprising that some of these changes should be fixed evolutionarily (Kellogg and Bennetzen 2004). Thus, a taxonomic revision of this species complex *A. grandiflora* is a critical step in the future, based on the observations of wild populations and the extensive herbarium material. Furthermore, the

ploidy levels, the genome size, the seed germination percentage and the leaf shape could be used with a degree of confidence to discriminate among the disjunctive populations of studied species complex.

Consequently, these patterns of genetic, cytogenetic and phenotypic variation could suggest a development of the intraspecific taxonomic keys problematic for this species complex and describe the taxonomic subgroups in ways that are useful to systematists, field botanists, and plant conservation biologists.

Evolutionarily significant units (ESUs) _ points of view

Over the past three decades, there has been an intense debate about the concept of the evolutionarily significant units (ESUs) (Ryder 1986; Moritz 1994; Crandall et al. 2000). Moritz (1994a) proposed a definition based on neutral genetic divergence at microsatellite loci. In contrast, Crandall et al. (2000) argued that the degree, to which populations are adapted to the same ecological niche and are thus exchangeable, must be considered. In this thesis, we found significant differences both genetically and morphologically among and within populations of *A. grandiflora* species complex by combining the genetic, phenotypic (seed germination rates and morphometric) technics. In our study, the combination of genetic and morphological differences indicated that diploid and tetraploid populations could likely be reproductively isolated. This strongly suggests that these populations should represent different ESUs (see fig. 7). Most of the habitats of diploids and tetraploids differ considerably. Thus, we argue that the identification of conservation units, could base on both species' genetics and ecology. It means that both Moritz (1994) and Crandall et al. (2000) concepts are compatible for this species complex because genetics and ecology are obviously closely linked.

Fig.7 A map presents some of proposed ESUs within the species complex of *Arenaria grandiflora* in Western Europe; the stars are the diploids and each colored star represents an evolutionarily significant unit (ESU) within the diploids; also, the circles are the tetraploids and each colored circle symbolizes an ESU within the tetraploids. The map was created using ArcGIS online.

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Résumé

La conservation au niveau population est extrêmement nécessaire pour limiter la perte de biodiversité au sein d'une espèce ou d'un complexe d'espèces. Ainsi, l'évaluation de la variabilité inter-populationnelle dans le complexe est reconnue comme première étape importante pour bien définir les plans de conservation des espèces menacées.

Arenaria grandiflora forme un complexe d'espèces herbacé pérenne à courte durée de vie (4 ans en moyenne) menacé dans certains sites de ses zones de distribution en Europe. A ce jour, sa taxonomie n'est pas bien résolue, ce qui entraîne des problèmes potentiels pour mettre en œuvre une conservation efficace de ce taxon. Une variation inter-populationnelle du complexe d'espèces *A. grandiflora* est présentée dans cette étude aux niveaux génétiques, cytogénétiques et morphométriques.

Quatre méthodes ont été utilisées : des marqueurs microsatellites nucléaires, une approche cytogénétique, la cytométrie en flux, et enfin la morphométrie sur les feuilles. De plus, les études phénotypiques de variation de taux de germination entre stocks de graines ont été développées.

Une différenciation significative entre les profils de variations moléculaires, cytogénétiques et phénotypiques a été détectée dans le complexe d'espèces. Deux cytotypes (diploïdes 2n = 2x = 22 et tétraploïdes 2n = 4x = 44) ont été mis en évidence en utilisant à la fois des méthodes classiques et des méthodes plus récentes (marqueurs microsatellites, nombres chromosomiques et cytométrie de flux). Le complexe d'espèces d'*A. grandiflora* présente une forte variation de la valeur de l'ADN 2C, la taille du génome varie de 2.11 ± 0.74 pg à 2.70 ± 0.11 pg pour les populations diploïdes et de 4.30 ± 1.51 pg à 5.27 ± 0.14 pg pour les populations de tétraploïdes. En outre, les graines de tétraploïdes germent significativement mieux que les graines des diploïdes. Les feuilles diffèrent considérablement entre les diploïdes (aciculaires et linéaires) et les tétraploïdes (lancéolées).

Cette étude peut être considérée comme préliminaire pour une révision taxonomique de ce complexe d'espèces. D'autre part, grâce à l'ensemble des résultats obtenus, il est également possible de revisiter le concept d'unités évolutives significatives (ESUs) dans le complexe d'espèces *A. grandiflora* et donc de définir les groupes de populations devant faire l'objet de mesures distinctes. Ainsi, il est possible d'évaluer la pertinence de plans déjà entrepris et de proposer de nouveaux plans de restauration efficaces pour l'avenir de ce complexe d'espèces.