Science Faculty
Department of Animal Biology, Vegetal Biology and Ecology
Botany Area

ESTUDOS EN BIOLOXÍA REPRODUTIVA NO XÉNERO *ANTHOXANTHUM* L. (POACEAE)

ESTUDIOS EN BIOLOGÍA REPRODUCTIVA EN EL GÉNERO *ANTHOXANTHUM* L. (POACEAE)

REPRODUCTIVE BIOLOGY STUDIES IN GENUS *ANTHOXANTHUM* L. (POACEAE)

Sara Gestal Jiménez

Final Degree Project

29/07/2015

Advisors: Dr. Manuel Pimentel Pereira and Dr. Elvira Sahuquillo Balbuena

TRABALLO DE FIN DE GRAO

Manuel Pimentel Pereira e Elvira Sahuquillo Balbuena autorizan a presentación do Traballo de Fin de Grao "Reproductive Biology studies in genus *Anthoxanthum* L. (Poaceae)" presentado por Sara Gestal Jiménez para a súa defensa ante o tribunal calificador.

En A Coruña, a 22 de Xullo de 2015

Asdo.: Dr. Manuel Pimentel

Asdo.: Dra. Elvira Sahuquillo

Resumen

En este proyecto se ha llevado a cabo un estudio sobre el efecto del nivel de ploidia en caracteres micromorfológicos y en un parámetro de la biología reproductiva de las especies, la viabilidad polínica. El trabajo se centra en dos especies del gén. *Anthoxanthum*: *Anthoxanthum odoratum* (x4) y *Anthoxanthum amarum* (x18). Para la realización del estudio se han analizado tres caracteres micro-morfológicos de la anatomía foliar de ambas especies relacionados con la ploidía. A continuación, se estudió la viabilidad del polen de ambas especies para comprobar si la especie poliploide, *A. amarum*, tenía una menor fertilidad polínica que el tetraploide, *A. odoratum*. El método de tinción del polen con FDA fue puesto a punto para obtener resultados óptimos.

Los datos morfológicos se correlacionan estadísticamente con la ploidía de las especies, por lo que se concluye que la especie poliploide tiene un mayor tamaño que la especie tetraploide para los caracteres analizados. Sin embargo, la viabilidad del polen no fue significativamente diferente entre ambas especies (14,31% en *A. odoratum*, 16,68% en *A. amarum*). Dado que el tamaño muestral para la viabilidad del pollen no fue significativo, para relacionar la fertilidad del polen con la ploidía de la especie son necesarios futuros ensayos con más individuos y poblaciones, además de estudios citogenéticos y moleculares.

Abstract

In this project, a study has been carried out on the impact of the ploidy level on leaf micromorphology and on a parameter of the reproductive biology of taxa, pollen viability. This work focuses on the polyploid complex composed by *Anthoxanthum odoratum*. (x4) and *Anthoxanthum amarum* (x18). To achieve our aims we have analysed three traits of the foliar epidermis that has been correlated with ploidy. Subsequently, we assessed pollen viability in both taxa to test if the polyploid species, *A. amarum*, had lower pollen viability than the tetraploid *A. odoratum*. The FDA technique was adapted for its use in *Anthoxanthum*.

Morphological data statistically differ between species (and ploidy levels). We conclude that the polyploid species has larger characters than the tetraploid one. However, pollen viability was not significantly different between both species (14.31% in *A. odoratum*, 16.68% in *A. amarum*). Given that sample size for pollen viability was rather small, in order to relate pollen fertility and species ploidy further studies with more individuals and populations are needed. Additional cytogenetic and molecular test will also be essential.

INDEX

1. **Introduction** 2

Aims 7

2. **Materials and methods** 8

Plant material 8

Morphology study 9

Pollen viability 11

Data analysis 11

3. **Results** 13

Morphological study 13

Pollen viability 18

4. **Discussion** 22

5. **Conclusions** 24

6. **References** 26

INTRODUCTION

The role of polyploidy

Polyploid organisms are those that have three or more complete sets of homologous chromosomes. While less common in the animal and fungi kingdom, polyploid species are highly prevalent in the plant kingdom (Soltis & Soltis, 2012). At present, polyploidy is recognized as a major force in plant evolution, affecting diversification and speciation (Weiss *et al*., 2013). The frequency and the level of ploidy vary among and within different taxonomic groups. Thus, it is suggested that polyploidization trends differ among lineages; polyploidy is more common in angiosperms and, within them, in monocots, especially grasses. The incidence of polyploidy correlates negatively with certain genomic features, such as genome size and chromosome numbers (Husband *et al*., 2013).

Even though the role of polyploidy is still controversial (Mayrose *et al*., 2011), recent authors emphasize the evolutionary potential and advantages of polyploids, such as: (i) an increased number of alleles, compensating the possible existence of deleterious mutations, (ii) an often stable heterosis, allowing transgressive performance and (iii) the potential of duplicated genes to acquire new or slightly varied functions (Weiss *et al*., 2013). Several lines of evidence suggest this positive role. The majority of polyploid taxa are of multiple and recurrent origin potentially increasing the polyploid's genomic diversity. Also, the fossil and genetic evidence indicate that polyploidy occurs in all angiosperm lineages, showing traces of at least one ancient whole genome duplication event (Soltis & Soltis, 2009; Weiss *et al*., 2013).

The observation that many polyploids display new phenotypic variation as compared to their progenitors indicates that they may have a fitness advantage, so polyploidy is considered an important factor shaping the geographical range of a species. This new phenotypic variation is supposed to enable polyploids to exploit different environmental niches better than their progenitors (Soltis & Soltis, 2012). However, the effects of polyploidy on physical and morphological traits that would determine the ecology of plant

are little known. The establishment of a polyploid, at least after its origin, triggers morphological changes, like larger size and seeds, and usually more and larger flowers per inflorescence (Weiss *et al*., 2013).

Genus Anthoxanthum. *Characteristics*

Polyploidy is especially recurrent among the grasses (F. Poaceae), and the genus *Anthoxanthum* (sweet vernal grasses) constitutes a well-known model of a polyploidy complex (Hedberg, 1986). *Anthoxanthum* L. *sensu stricto* (excluding genus *Hierochloë* R.Br.) is widely distributed, including temperate and alpine regions of Europe, Asia and Africa, comprising 18-22 taxa (Pimentel *et al.* 2013). The taxonomy of the sweet vernal grasses has been highly controversial due to their morphological similarity, ecological diversity and different ploidy levels (Pimentel *et al*., 2013). The species in this genus are characterized by flat and sheathing leaves and membranaceous, truncated or pointed ligules, never reduced to a line of hairs. Plants can be glabrous or pubescent, with condensate panicles bearing several laterally compressed spikelets. Each spikelet presents just one fertile floret and two awned lemmas of sterile florets of larger size than the fertile one. The glumes are unequal; the lower is shorter with one nerve and the upper is longer than the florets with three nerves. Lemmas and paleas have differences between the sterile and fertile florets. In sterile florets, they are pubescent while in fertile florets, they are smaller, membranous and glabrous. Fertile flowers lack lodicules. The cariopsis is glabrous, shiny and oval (Tutin, 1980; Fig. 1).

In Galicia, there are populations of three perennial and annual species of the genus with different ploidy levels, geographical and ecological distribution (Romero, 2008).

Within the perennial species, *Anthoxanthum odoratum* L. is a tetraploid (2n = 20) grass, the most commonly occurring species of the genus in Europe, western North Africa, Asia Minor, the Caucasus and Northern Asia (Fig. 2A). Occasionally, morphological traits of *A. odoratum* and annual species overlap, hampering their unambiguous identification, but the perennial might be distinguished by its rigid stem, long and narrow panicles and the shape and size of the sterile lemmas (Borril, 1963; Drapikowska *et al*., 2013). This tetraploid

shows a great variability and adaptability, colonizing a tremendous range of habitats, being even considered an invasive species (Borrill, 1961, 1963; Drapikowska *et al*., 2013).

Figure 1. Scheme of floral structure of *Anthixanthum amarum*. Autor. David Romero

A less well known perennial species of the genus is *Anthoxanthum amarum* Brot., an endemism of northwest Iberian Peninsula (Fig. 2B). This grass is a high polyploid (2n = 18x = 90) morphologically and genetically related to *A. odoratum.* It is characterized by the width of the leaves, greater than 1 cm; larger plant size and higher sterile floret length. It grows mainly in mesic, stable habitats with a temperate climate and relatively little seasonal fluctuation in temperature, such as forests or riverine areas, wastelands, ravines, or pine plantations (Pimentel *et al*., 2007a).

Figure 2. A) *Anthoxanthum odoratum* L., from science.halleyhosting.com **B)** *Anthoxanthum amarum* Brot. from flora-on.pt.

Anthoxanthum aristatum Boiss. is a diploid annual species (2n = 10) native to the western part of the Mediterranean region (Drapikowska *et al*., 2013). In *A. aristatum,* panicles are elongated, tapering at the base, usually not very dense and green at anthesis. The fertile lemma is equal to half the size of the sterile lemmas and the awn of the upper sterile lemma is prominently exserted (Pimentel *et al*., 2007a). It occupies a great range of habitats on acid and basic soils, and occurs over a wide range of altitudes, but towards the southern limit it is restricted to mountains (Borril, 1963).

Genus Anthoxanthum. *Phylogenetic context*

The current diversity in *Anthoxanthum* appears to have been shaped by recurring hybridization and polyploidization events (Pimentel *et al*., 2013; Fig. 3). Thus, a recent origin for *A. amarum* from *A. odoratum* ancestors has been suggested (Pimentel *et al*., 2007b), although recent phylogenies also indicate a clear proximity between the former and *A. aristatum* (Pimentel *et al*., 2013).

Figure 3. Phylogenetic tree of *Anthoxanthum* species, modified from Pimentel *et al*. 2013.

Relationships between polyploidy, morphology and fitness traits associated to fertility are still under discussion (Ortiz *et al*., 2011; Drapikowska *et al*., 2013; Hardion *et al.,* 2015). In genus *Anthoxanthum*, a strong relationship between ploidy and leave anatomy has been found for different lineages (Felber, 1987). It has been hypothesized that polyploidy could introduce new genomic entities possessing higher adaptability and phenotypic plasticity than their progenitors (Soltis & Soltis, 2012; Hardion *et al*., 2015). For these new genotypes, the maintenance of sexual reproduction could represent a risk due to intercrossing with the progenitors. Also, the frequent formation of multivalents could cause strong meiotic irregularities leading to non-viable gametes, which dramatically reduces reproductive success (Hardion *et al*., 2015). In this study, the relationship between morphology, pollen viability and ploidy in *Anthoxanthum* species is explored.

AIMS

The principal aim of this study is to analyse the possible relationship between taxonomy (and, therefore, ploidy level) and some morphological and fertility characters in the polyploid complex *A. odoratum*-*A. amarum* in Galicia. The following specific aims are included in the study:

- Study the distribution of three micro-morphological traits in the *A. odoratum*-*A. amarum* polyploid complex.
- Analyse micromorphology in each species of the complex and test its association with ploidy level.
- Analyse pollen viability in each species of the complex and test its association with ploidy level.

MATERIALS AND METHODS

Plant material

A total of 60 specimens of *A. amarum* and 40 of *A. odoratum* were sampled in four and three different locations in Galicia, Spain, respectively (Table 1; Fig. 4). All individuals were collected in anthesis for optimum pollen testing.

Table 1. Populations studied. NM: number of plants used to study morphological traits, NP: number of plants used to study pollen viability.

Species	Ploidy	Location	Date	NM	NP
Anthoxanthum amarum	18x	Chelo, Coirós, A Coruña	16/05/2015	15	5
Anthoxanthum amarum	18x	San Xoán, Guitiriz, Lugo	09/05/2015	15	5
Anthoxanthum amarum	18x	San Marcos, Fene, A Coruña	10/04/2015	15	5
Anthoxanthum amarum	18x	Valadares, Vigo, Pontevedra	04/05/2015	15	5
Anthoxanthum odoratum	4x	Arillo, Oleiros, A Coruña	23/03/2015	10	5
Anthoxanthum odoratum	4x	O Corgo, Lugo	02/05/2015	15	5
Anthoxanthum odoratum	4x	A Zapateira, A Coruña	22/05/2015	15	5

In each population, individual plants are difficult to define due to the prevalence of clonal reproduction. Each isolated *Anthoxanthum* stand in the area was taken as an individual plant. Plants were collected, stored separately in plastic bags and taken to the laboratory. The first population, *A. odoratum* of Arillo, Oleiros, was potted and kept in a culture chamber for testing different pollen viability stain methods. The other populations were kept in their bags inside a refrigerator with enough humidity until they were analyzed.

Morphology study

Three micro-characters of the leaf epidermal morphology were analyzed in this study (characters defined according to Devesa, 1992 ; table 2). Characters were chosen due to their usefulness in grass taxonomy (Devesa, 1992) and due to their common relationship with ploidy (Felber, 1987). Samples were processed following Devesa (1992), with minor modifications to adjust the method to the different species. First, 15 individual plants were selected from each population. In each plant, traits were measured on the second leaf from the base. The lower third of the leaf was used to perform the analysis. This part of the leaf was excised and then submerged in boiling water; 20 seconds for *A. odoratum* and 30 for *A. amarum*. Subsequently, the upper epidermis and the mesophyll were eliminated using a lancet moistened with lactic acid (50% solution). The lower epidermis was then put in a slide with lactic acid and observed at the optic microscope.

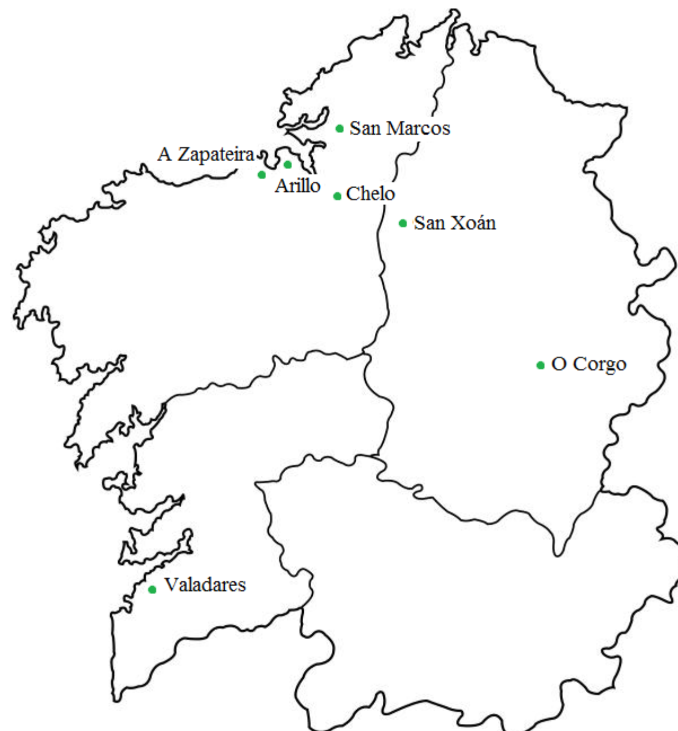

Figure 4. Location of populations.

The leaf anatomy in Poaceae is of great utility in taxonomical studies, especially micro-characters (Devesa, 1992; Pimentel *et al.* 2007a). In this study, three micro-characters of leaf anatomy were analyzed to differentiate morphologically each species (Table 2).

Table 2. Micro-characters measured in leaves.

Analyzed characters	Type	Units
Stomata length	Quantitative	µm
Stomata width	Quantitative	µm
Long cells length	Quantitative	µm

Three characters were measured: stomata length, stomata width and long cells length (Table 2). The stomata length is the distance between the points where the two guard stomatic cells are in touch. The stomata width is the largest distance between the walls of the guardian cells that are in contact with epidermal cells. The long cells are the most abundant cells in the epidermis, long and narrow cells that run parallel to the nerves of the leaf. In *Anthoxanthum amarum* a total of 600 stomata and long cells were measured and a total of 400 stomata and long cells in *Anthoxanthum odoratum* (Table 3).

Table 3. Number of measurements taken. NM: number of plants used to study morphological traits, NS: number of measured stomata, NLC: number of measured long cells.

Species	Location	NM	NS	NLC
Anthoxanthum odoratum	Arillo	10	100	100
	O Corgo	15	150	150
	A Zapateira	15	150	150
		40	400	400
Anthoxanthum amarum	Chelo	15	150	150
	San Xoán	15	150	150
	San Marcos	15	150	150
	Valadares	15	150	150
		60	600	600

Pollen viability

In an initial test to ascertain the pollen viability method, five different populations of four species were used: *A. aristatum* from Oleiros, *A. odoratum* from Oleiros, *A. odoratum* from Montenegro, *A. amarum* from A Zapateira and *Briza media* L. from A Zapateira. The aim of this preliminary test was to determine the best anther development stage to perform pollen viability tests. From this test, it was concluded that the anthers should be mature but not dehiscent, being the perfect moment when the anthers are yellow or purple and when they are protruding from the floret, but not out of it.

The method used to assess the pollen fertility was the Fluorescein Diacetate (FDA) Pollen Viability Assay, following Yarnes (2011). FDA is a nonpolar substrate incorporated into plant cells, where it is hydrolyzed in viable cells by the esterases to produce a polar product, fluorescein. Fluorescein is a fluorescent molecule that is retained intracellularly because it is weakly transported through the plasma membrane. After staining with FDA, fluorescein-positive viable cells can be visualized by fluorescence microscopy.

A powdered FDA (Sigma, Taufkirchen, Germany) was used to stain *Anthoxanthum* pollen cells. A stock solution of FDA was made with 200 µm of FDA and 100 mL of acetone. A working solution with 25 µL of stock solution of FDA (2 mg FDA/mL acetone) and 100 mL of 0.5M sucrose solution was used to stain pollen samples. The working solution was separated in vials of 1 mL and stored in a refrigerator. For staining the pollen, anthers from two or three florets were taken and smashed to liberate the pollen. This pollen was put inside a vial of FDA stain during one hour for the enzymatic reaction to take place. After the incubation, samples were taken to a centrifuge at 1200 rpm during 10 minutes. Two thirds of the supernatant was discarded and a sample of the remaining liquid saturated in pollen grains was collected and put on a microscope slide. At least 100 pollen grains per individual plant were counted with a Nikon Y-FL fluorescence microscope.

Data analysis

Several exploratory analyses were conducted on the data. More particularly, boxplot diagrams were built and different descriptive statistics were obtained. These statistics included mean values, maximum and minimum values, standard deviation (σ) and the

coefficient of variation (C_v). These values were estimated per population and ploidy level with the software SPSS Statistics 17.0 (IBM SPSS Inc, Armonk, USA).

Different tests were conducted in order to explore the effect of the variable "ploidy level" (i.e. species) in our morphological data. The analysis of variance (ANOVA) was used to detect the differences among variables across the different ploidy levels. The hierarchical cluster analysis (CA) was conducted in order to represent the statistical relationships among samples and their similarity. In this test we measured the distance among individuals using the Manhattan distance and the dendrogram was built using the *linkage between groups* method (UPGMA). Finally, a Principal Components Analysis (PCA) was carried out to summarize the variation in the data in a reduced component space. The varimax rotation was included to maximize the variation among the groups. Pollen viability between ploidy levels was assessed through a t- Student test. All analyses were conducted following Legendre & Legendre (1998) and Pimentel *et al* (2007a). All tests were performed using the software SPSS Statistics 17.0 (IBM SPSS Inc, Armonk, USA).

RESULTS

Morphological study

Overall, for the morphological study 600 stomata and long cells were measured for *Anthoxanthum amarum* in 60 samples, whereas 400 stomata and long cells were assessed for *Anthoxanthum odoratum* (Table 4) in 40 samples.

Table 4. Number of measurements taken. NM: number of plants used to study morphological traits, NS: number of measured stomata, NLC: number of measured long cells.

Species	Population	NM	NS	NLC
Anthoxanthum odoratum	Arillo	10	100	100
	O Corgo	15	150	150
	A Zapateira	15	150	150
		40	400	400
Anthoxanthum amarum	Chelo	15	150	150
	San Xoán	15	150	150
	San Marcos	15	150	150
	Valadares	15	150	150
		60	600	600

The descriptive statistics calculated are shown in Table 5. Three figures (Fig. 5-7) were constructed from these data to illustrate the variance in each population. Figures are referred to mean values.

Table 5. Descriptive statistics of morphological traits measured. SL: stomata length, SW: stomata width, LCL: long cells length. From these three variables, other statistics were calculated: max: maximum value, min: minimum value, σ: standard deviation, C_v: coefficient of variation.

	Anthoxanthum amarum					*Anthoxanthum odoratum*			
	Chelo	**San Xoán**	**San Marcos**	**Valadares**	**Total Sps**	**Arillo**	**O Corgo**	**Zapateira**	**Total Sps**
SL (µm)	74,28	70,90	73,91	71,13	72,56	52,25	47,53	53,37	50,90
SW (µm)	34,83	34,27	34,47	34,70	34,57	25,03	28,28	25,07	26,26
LCL (µm)	980,5	720,7	581,8	576,8	721,93	610,1	261,4	661,1	474,00
SL_{max} (µm)	86,21	83	88,5	84,25	88,5	60,75	52,5	62	62
SL_{min} (µm)	61	63,25	65,25	61,25	61	45,25	41,25	48	41,25
SW_{max} (µm)	43	40	47,25	43	47,25	28,75	32,75	27,25	32,75
SW_{min} (µm)	27,75	27	30,5	27	27	22,25	25,5	21,75	21,75
LCL_{max} (µm)	1247	970	747	795	1247	714	364,9	769	769
LCL_{min} (µm)	776	503	404	469	404	508	134,5	258,5	134,5
σ_{SL}	7,12	5,44	6,08	6,2	6,27	4,05	2,91	4,66	4,67
σ_{SW}	4,36	3,97	4,33	4,51	4,19	2,08	2,11	1,85	2,52
σ_{LCL}	150,4	133,4	115,2	103,6	193,923	66,1	67,7	161,1	188,3
$C_{V\,SL}$	0,096	0,077	0,082	0,087	0,086	0,078	0,061	0,186	0,092
$C_{V\,SW}$	0,125	0,116	0,126	0,130	0,121	0,083	0,075	0,003	0,096
$C_{V\,LCL}$	0,153	0,185	0,198	0,180	0,269	0,108	0,259	2,598	0,397

In Fig. 5 we can see that stomata length values were clearly higher in *A. amarum* populations than in *A. odoratum* populations. Data dispersion is low in all populations.

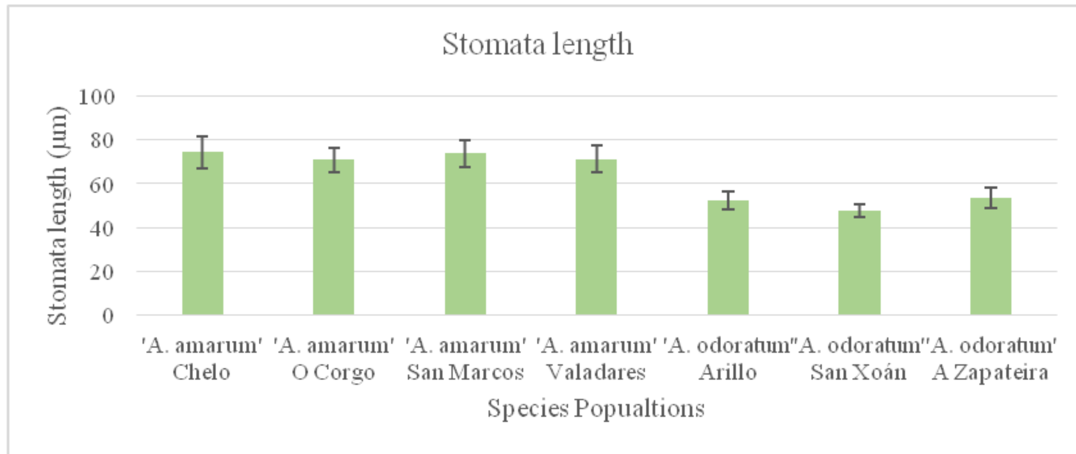

Figure 5. Stomata length values in *A. amarum* and *A. odoratum* populations. Green columns refer to mean values. Black lines refer to standard deviation.

Similar results were found with respect to stomata width (Fig. 6). Again, the octoploid *A. amarum* showed the highest values and low variation among populations. On the other hand, the tetraploid *A. odoratum* displayed a higher differentiation among populations with populations A Zapateira and Arillo showing similar values (Table 5).

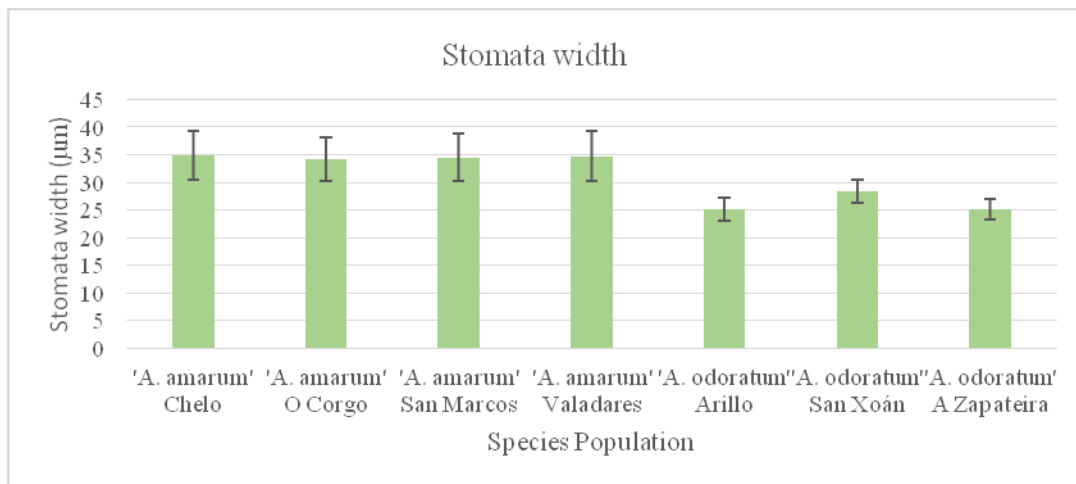

Figure 6. Stomata width values in *A. amarum* and *A. odoratum* populations. Green columns refer to mean values. Black lines refer to standard deviation.

The character that presents more variation in both species was long cells length (Figure 7, Table 5). In this case the differentiation between species was not clear, with A Zapateira population occupying an intermediate position between the two species (and ploidy levels).

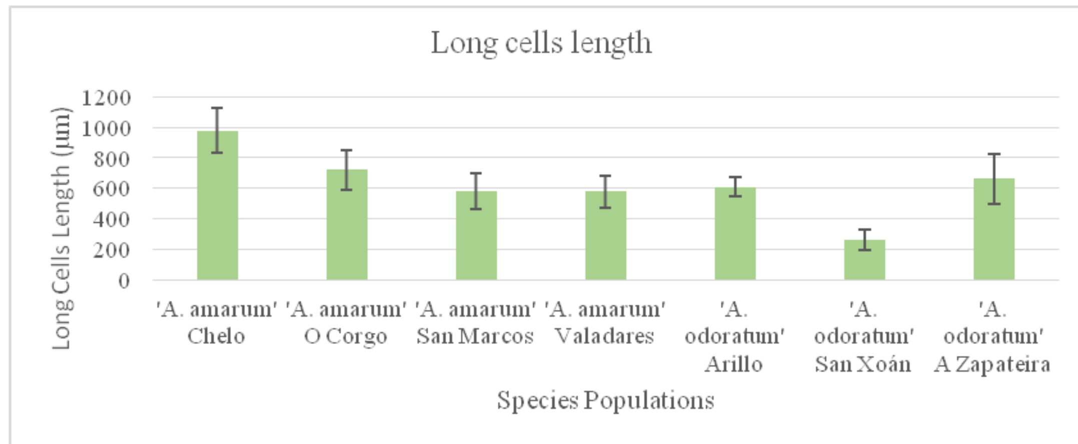

Figure 7. Long cells length values in *A. amarum* and *A. odoratum* populations. Green columns refer to mean values. Black lines refer to standard deviation.

Boxplots were also built in order to analyse the dispersion of data and the existence of outliers (Fig. 8). In these two species data distribution was symmetric and no outliers were detected.

The ANOVA test was conducted to trace the existence of significant differences between the ploidy levels. Prior to the calculation of the ANOVA, normality was checked through a Kolmogorov-Smirnov test. In this case, all three ANOVAs gave a p-value<0.05 (table 6). This p-value indicates that there are significant differences between species in each morphological variable. This fact is consistent with the boxplots built (fig. 7).

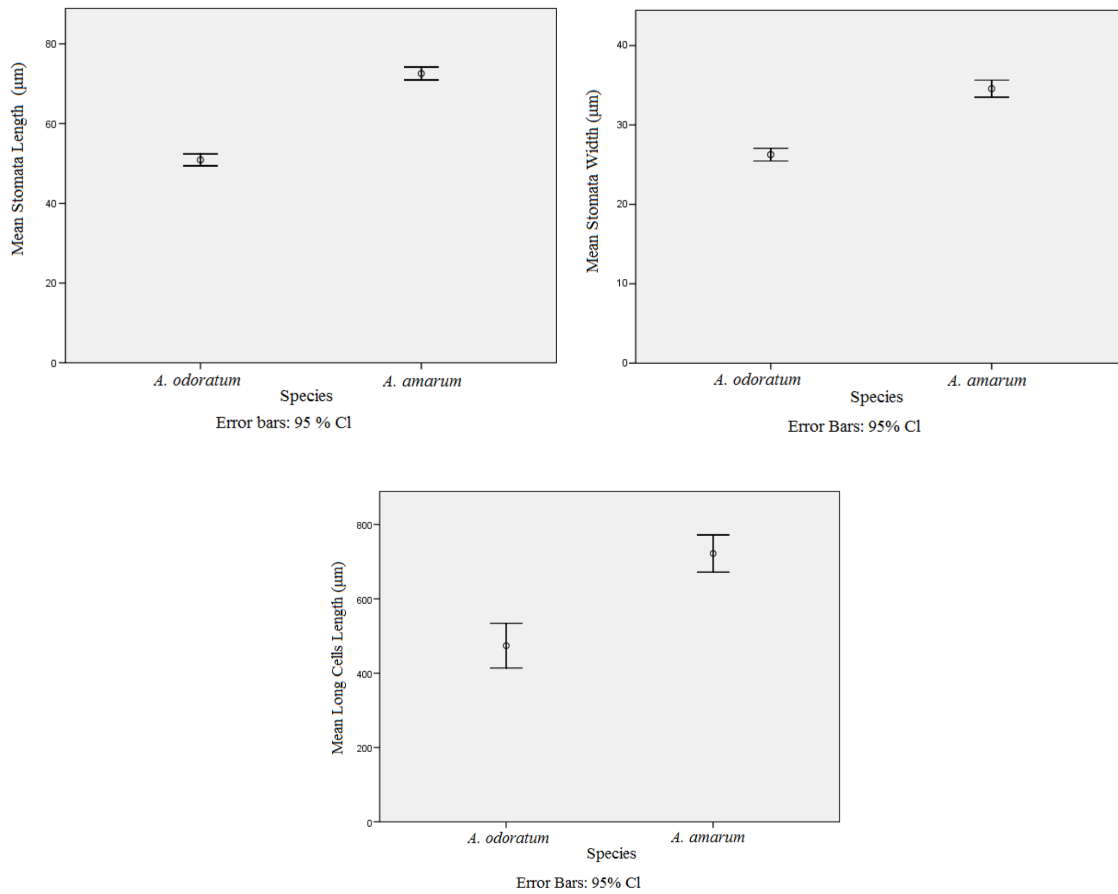

Figure 8. Boxplots of the morphological variables measured in *A. odoratum* and *A. amarum*.

Table 6. ANOVA summary table.

	Sum of Squares	df	Mean Square	F	p-value
Stomata length between groups	11257,569	1	11257,569	347,953	,000
Stomata width between groups	1655,020	1	1655,020	126,332	,000
Long cells length between groups	1474886,008	1	1474886,008	40,139	,000

The dendrogram derived from the hierarchical cluster analysis (Fig. 9) indicates that only two groups in the tree correspond to just one species (Fig. 9). The individuals in the first of those groups belong mostly to the Chelo population (*A. amarum*, 18x), whereas the second group includes mainly specimens from Arillo (*A. odoratum*, 4x) that particular group mostly belong to the Chelo population. The remaining populations are not distributed according to their geographic origin/ploidy level.

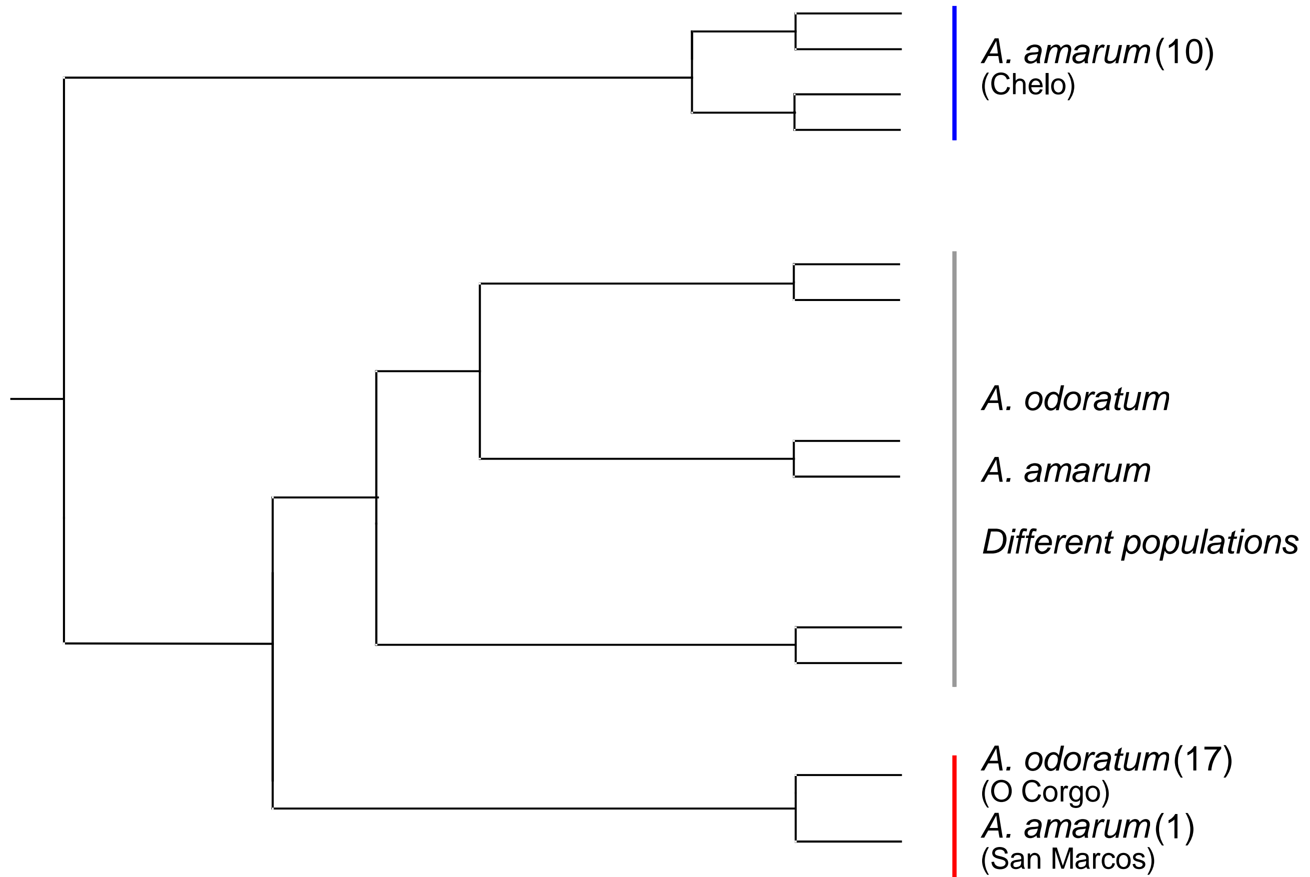

Figure 9. Dendrogram built from the hierarchical cluster analysis using the Manhattan distance and the UPGMA (linkage between groups) algorithm. The name of the populations and the number of specimens is only indicated in the clades where there is dominance of individuals from a particular population.

The PCA conducted extracted only one significant component, to which all measured characters belong to (Tables 7, 8). This component was responsible for 67.8% of variation

observed among samples, indicating a high correlation among the variables. The weight of each variable in the component is indicated in table 7.

Table 7. Weight of each variable in the PCA first component.

Variables	Component 1
Stomata length	0.895
Long cells length	0.805
Stomata width	0.765

In table 8, the components with total eigenvalues superior to one are indicated (only one significant component).

Table 8. Variance explained by the components generated with the PCA.

Total Variance Explained						
Component	Initial Eigenvalues			Extraction Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	2,034	67,795	67,795	2,034	67,795	67,795
2	,640	21,322	89,117			
3	,326	10,883	100,000			

Pollen viability

Table 9 shows the population distribution of the number of pollen grains counted. Overall, a total of 2224 *A. odoratum* pollen grains and 2568 *A. amarum* pollen grains were assessed for viability. Pollen grains were separated in two groups depending on their fluorescence; they were considered viable when there was green fluorescence and non-viable when there was no fluorescence (or a very faint one). The percentage values are displayed in Table 9.

The Arillo population was tested during a first test to ascertain the method. All pollen grains were non-viable due to their immature state. The Arillo population was excluded from all pollen viability statistical analysis.

The chosen method in a pollen viability study must essentially be reliable. A lot of pollen staining methods can't distinguish between viable pollen and death pollen and fail to assess pollen fertility (Rodriguez-Riano & Dafni, 2000; Wang *et al*., 2004). FDA staining method was then selected with proven results (Pinillos & Cuevas, 2008; Sutyemez, 2011).

The method was first tested to find out the most adequate anthers' maturation state. It was proved that the method worked, and pollen viable grains were easily distinguished from non-viable grains. Viable pollen had greater size and a bright green shine, while non-viable pollen was much smaller, deformed and dark (Fig. 10).

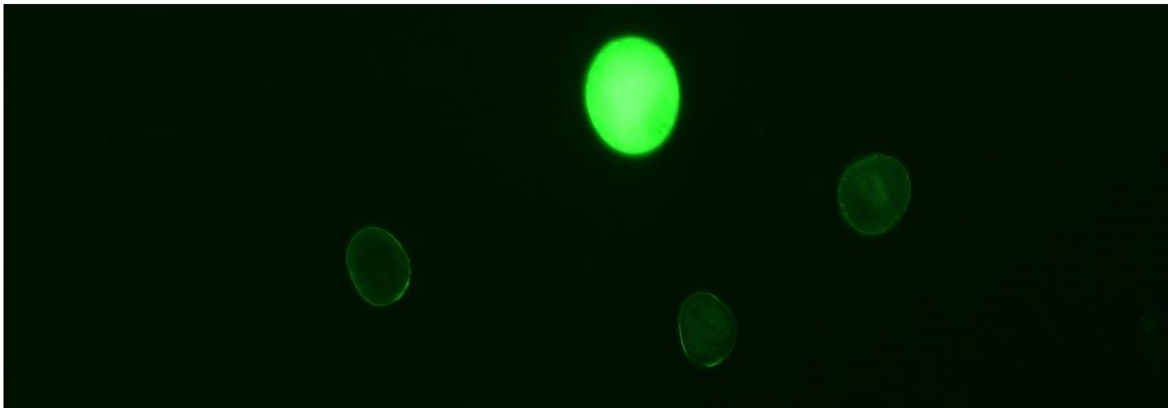

Figure 10. Stained pollen observed in a fluorescence microscope at 400X. In the middle, a bright viable grain surrounded by three non-viable smaller pollen grains.

Figure 11 shows pollen viability values for each species. The population from San Marcos showed the highest value of viability for *A. amarum.* In addition to this, *A. amarum* was more variable than *A. odoratum* as regards this trait. The population from Chelo (*A.amarum*) showed the lowest values of all sampled populations in the complex.

Table 9. Number of pollen grains counted. NP: number of plants used to study pollen viability, NPG: number of pollen grains counted, both viable and non-viable. *The pollen viability value of Arillo is not included in the statistical analysis.

Species	Population	NP	NPG	Pollen viability (%)
A. odoratum	Arillo	5	500	0*
	O Corgo	5	1116	13.89
	A Zapateira	5	608	14.72
		15	2224	14.31
A. amarum	Chelo	5	560	13.17
	San Xoán	5	625	17.15
	San Marcos	5	829	21.58
	Valadares	5	554	14.83
		20	2568	16.68

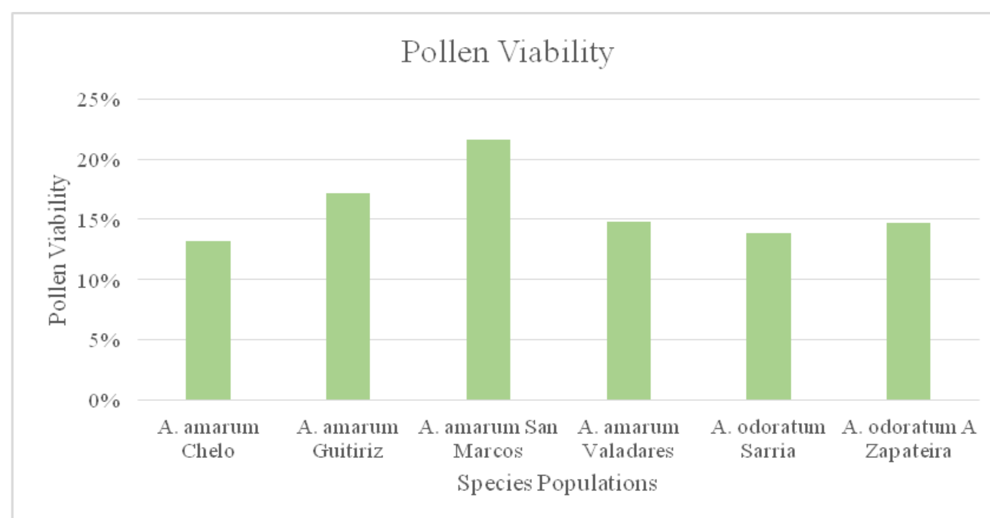

Figure 11. Pollen viability in the different populations studied.

The student-t test conducted (Table 10) in order to compare pollen viability between species (or ploidy levels) showed not significant differences between both species (p-value>0.05), which agrees with what is observed in figure 11.

Table 10. t-Student test values comparing pollen viability data of two species of *Anthoxanthum*.

Viability	Levene's Test for Equality of Variances		t-test for Equality of Means				
	F	p-value	t	df	p-value	Mean Differen ce	Std. Error Difference
Equal variances assumed	2,777	,107	-,763	28	,452	-2,3775	3,115702484

DISCUSSION

Morphological study

Following our results, *Anthoxanthum odoratum* adaxial epidermis is characterised by long cells with a length ranging between 300 and 700 µm. Besides, stomata are 46-56 µm long and 23-29 µm wide. *Anthoxanthum amarum* possesses larger long cells 500-900 µm long. Stomata are also rather longer, with length ranging between 66 and 79 µm and width oscillating in an interval of 30-39 µm (Table 5). Larger morphological traits are a common feature of high polyploids such as *A. amarum* (Stebbins, 1985).

These results agree with Pimentel & Sahuquillo (2003) as regards long cells length in *A. odoratum* populations, but not in *A. amarum*. Moreover, the description of *A. odoratum* given in Devesa (1992) is consistent with the results of Pimentel and Sahuquillo (2003). As regards stomata values, our results in both *A. odoratum* and *A. amarum* are similar to those in Pimentel & Sahuquillo (2003). Even if long cells measurements in *A. amarum* are higher than the ones published in Pimentel & Sahuquillo (2003), the analysed specimens and populations follow the same trend observed in that work. Populations near coastal areas (San Marcos and Valadares, Table 5) have lower values of long cell length than those from inland areas (Chelo and San Xoán, Table 5). The fact that Pimentel & Sahuquillo (2003) included populations from across the distribution range of the species makes it likely that we need to add new specimens to obtain similar results. Another possible explanation for the inconsistencies observed between the different studies is the high impact that environmental parameters have in leaf micro-anatomical traits (Aiken *et al.*, 1984), something that has been already observed in *A. amarum* (Pimentel & Sahuquillo, 2007).

The dendrogram conducted fails to reveal clear groupings in the data with two remarkable exceptions (Fig. 9). First, specimens from the Sarria population form an almost pure group in the tree. This population is located in an inland area of Galicia quite far from the sea (unlike all the other populations analysed for *A. odoratum*, Arillo and A Zapateira). Differences in environmental conditions, especially temperature and humidity, might explain the phenotypical variation in this species (Aiken *et al.*, 1984; Pimentel &

Sahuquillo, 2003). Another possible explanation for this differentiation lies in the multiple origins of the morphological species *A. odoratum* (Pimentel *et al*., 2013). Phylogenetic and cytological analyses have shown that different *A. odoratum* populations originated through different polyploidization events. It is possible that the Sarria population has a separate origin with respect to Arillo and A Zapateira, which may impact in its different morphology (and micromorphology). To solve this, cytogenetic and molecular techniques need to be applied. The second clear group observed corresponds to the Chelo population of *A. amarum*. These specimens were very large even for this rather big plant species. In addition to this, the population was very small and grew in an area of high humidity and permanent shade, which might affect the observed results.

The PCA analysis did not succeed in creating more than one significant component, which might highlight a high correlation between the analysed traits. However, the PCA results (Table 7) clearly indicate that the biometrics of stomata plays an important role in the variation between species, which was supported by previous studies (Devesa, 1992, Humbert-Droz & Felber, 1992).

Pollen viability

The chosen method was adequate and reliable for assessing pollen viability in *Anthoxanthum*, as it has been proved useful in many other plant taxa (Rodriguez-Riano & Dafni, 2000; Wang *et al*., 2004; Pinillos & Cuevas, 2008; Styemez, 2011). It is important to highlight the key role of the anther maturation stage in the comparability and reliability of the genus. The short period in which pollen viability can be measured makes it very important to find long-term preservation methods for pollen grains, especially if long-term studies are planned (Rodriguez-Riano & Dafni, 2000).

Pollen viability percentages were, in general, very low, with only 14.31% in *A. odoratum* and 16.68% in *A. amarum*. Our expectation, that pollen viability was higher in the tetraploid than in the high polyploid could not be justified with our data. The higher pollen viability for the diploids or low polyploids observed in other polyploid complexes (Ortiz *et al.*, 2011; Husband *et al.*, 2013; Weiss *et al*., 2013) is often explained due to the occurrence of irregular chromosomal segregation of univalent and multivalents during meiosis in

polyploids (Weiss *et al*., 2013). In addition to this, asexual reproduction, common in polyploids, makes pollen viability less essential for a new plant species to establish. Our failure to detect differences in our study system may be due to the fact that both species are already polyploids. Actually, studies including the annual diploid *A. aristatum* show higher pollen viability for this latter taxon (Salutregui, 2015). Clonal spread is very common in *A. amarum*, but no so much in *A. odoratum*. However, the prevalence of sexual reproduction involving the formation of a seed (apomixis) has not been tested in these species. Another explanation for our results might simply be the low number of specimens analysed, although the number of counted pollen grains per individual were considerable (Table 9). In further studies, an increase in the number of populations and specimens analysed should be considered. In addition to this, cytogenetic, reproductive biology and molecular studies are also necessary.

CONCLUSIONES

El estudio realizado permite extraer las siguientes conclusiones:

- Los caracteres micro-morfológicos de la anatomía foliar se correlacionaron estadísticamente con el nivel de ploidía en *Anthoxanthum odoratum* y *Anthoxanthum amarum*. Se puede concluir que para la especie poliploide *A. amarum*, los caracteres analizados presentan mayor tamaño que en la especie tetraploide *A. odoratum*. Las especies podrían diferenciarse taxonómicamente en base a estas variables morfológicas, aunque hay que tener en cuenta el reducido tamaño muestral. A pesar de esta diferenciación, nuestros resultados parecen señalar una fuerte influencia del medio ambiente en la micro-anatomía foliar.
- El método de tinción con FDA permite diferenciar entre granos de polen viables y no viables, por lo que es adecuado para evaluar este parámetro en especies de *Anthoxanthum*, una vez establecido el momento de madurez adecuado en las anteras.
- En el complejo poliploide *Anthoxanthum odoratum-Anthoxanthum amarum*, la viabilidad del polen es similar y no hay evidencias que correlacionen la fertilidad

del polen con su nivel de ploidía, por lo que son necesarios futuros estudios con un tamaño muestral mayor.

CONCLUSIONS

The present study allows extracting the following conclusions:

- The analyses conducted ascertain that the micro-morphological leaf traits correlate with the ploidy level of *Anthoxanthum odoratum* and *Anthoxanthum amarum*. It can be concluded that the polyploid species *A. amarum* is larger than the tetraploid *A. odoratum* for all the characters analysed. Therefore, these species can be taxonomically differentiated based on these morphological variables.
- The fluorescein di-acetate staining method is reliable and adequate to differentiate between viable and non-viable pollen grains in Anthoxanthum provided that the anthers are in the right maturation stage.
- In the polyploid complex *Anthoxanthum odoratum-Anthoxanthum amarum*, pollen viability is similar between both species and there are no evidences for a correlation between their pollen fertility with their ploidy level. Additional studies with an increased sample size are needed.

REFERENCES

Aiken, S.G., Darbyshire, S.J., Lefkovitch, L.P. 1984. The taxonomic value of using epidermal characteristics in Canadian rough fescue complex (Festuca altaica, F. campestris, F. halii, F.scabrella). *Canadian Journal of Botany* 62: 1864-1870.

Borril, M. 1961. The experimental taxonomy of *Anthoxantum* species. *Proceedings of the Linnean Society of London* 173: 106-109.

Borril, M. 1963. Experimental studies of evolution in *Anthoxanthum* (Gramineae). *Genetica* 34: 183-210.

Devesa, J.A. 1992. *Anatomia foliar y palinología de las gramíneas extremeñas*. Monografías botánicas. Universidad de Extremadura. Badajoz.

Drapikowska, M., Susek, K., Hasterok, R., Szkudlarz, P., Celka, Z., Jackowiak, B. 2013. Variability of stomata and 45S and 5S rDNAs loci characteristics in two species of Anthoxanthum genus: *A. aristatum* and *A. odoratum* (Poaceae). *Acta Biologica Hungarica* 64: 352–363.

Felber, F. 1987. *Contribution à l'étude phytogéographique, biosystématique et expérimentale du complexe polyploïde* Anthoxanthum odoratum *L. s. la*. Ph.D. thesis, University of Neuchâtel, Switzerland.

Hardion, L., Verlasque, R., Rosato, M., Roselló, J.A., Vila, B. 2015. Impact of polyploidy on fertility variation of Mediterranean *Arundo* L. (Poaceae). *Comptes Rendus Biologies* 338: 298-306.

Hedberg, I. 1986. The genesis of tetraploid *Anthoxanthum odoratum*. *Acta Univ. Ups., Symb. Bot. Ups.* 27: 147-154.

Humbert-Droz, C., Felber, F. 1992. *Etude biométrique des stomates et des grains de pollen comme indicateurs du degré de polyploïdie chez* Anthoxanthum alpinum *Löve et Löve*. Institut de Botanique, Université de Neuchâtel. Neuchâtel.

Husband, B.C., Baldwin, S.J., Suda, J. 2013. The incidence of polyploidy in natural plant populations: major patterns and evolutionary processes. In: Leitch IJ, Greilhuber J, Doležel J, Wendel JF, editors. *Plant Genome Diversity 2: Physical Structure, Behaviour and Evolution of Plant Genomes*. Springer. Wien.

Legendre, P. & Legendre, L. 1998. *Numerical Ecology 2nd edition*. Elsevier, Amsterdam.

Mayrose, I., Zhan, S.H., Rothfels, C.J., Magnuson-Ford, K., Barker, M.S., Rieseberg, L.H., Otto, S.P. 2011. Recently Formed Polyploid Plants Diversify at Lower Rates. Science 333: p. 1257.

Ortiz, A. M., Seijo, J. G., Fernández, A., Lavia, G.I. 2011. Meiotic behavior and pollen viability of tetraploid Arachis glabrata and A. nitida species (Section Rhizomatosae, Leguminosae): implications concerning their polyploid nature and seed set production. *Plant Systematics and Evolution* 292: 73-83.

Pimentel M, Sahuquillo E. 2003. Study of the leaf anatomy of the Iberian species of genus Anthoxanthum (Poaceae). *Bocconea* 16: 683-689.

Pimentel, M., Estévez, G., Sahuquillo, E. 2007a. European sweet vernal grasses (*Anthoxanthum*, Poaceae; Pooideae; Aveneae): a morphometric taxonomical approach. *Systematic Botany* 32: 43-59.

Pimentel, M., Sahuquillo, E., Catalán, P. 2007b. Genetic diversity and spatial correlation patterns unravel the biogeographic history of the European sweet vernal grasses (*Anthoxanthum* L., Poaceae). *Molecular Phylogenetics and evolution* 44: 667-684.

Pimentel, M., Sahuquillo, E., Torrecilla, Z., Popp, M., Catalán, P., Brochmann, C. 2013. Hybridization and long-distance colonization at different time scales: towards resolution of long-term controversies in the sweet vernal grasses (*Anthoxanthum*). *Annals of Botany* 112: 1015-1030.

Pinillos, V., Cuevas, J. 2008. Standardization of the fluorochromatic reaction test to assess pollen viability. *Biotechnic & Histochemistry* 83: 15-21.

Rodriguez-Riano, T., Dafni, A. 2000. A new procedure to assess pollen viability. *Sex Plant Reprod* 12: 241-244.

Romero, M.I. 2008. *Catálogo da flora de Galicia*. Monografías do Ibader 1. Universidade de Santiago de Compostela. Lugo.

Salutregui, S. 2015. Estudios en biología reproductiva en el género *Anthoxanthum* L. (Poaceae). Trabajo Fin de Grado. Universidad de A Coruña, A Coruña.

Soltis, P.S., Soltis, D.E. 2009. The role of hybridization in plant speciation. *Annual Review of Plant Biology* 60: 561-588.

Soltis, P.S., Soltis, D.E. 2012. *Polyploidy and Genome Evolution*. Springer. Alphen aan den Rijn (the Netherlands).

Stebbins, G.L. 1985. Poliploidy, hybridization and the invasion of new habitats. *Annals of the Missouri Botanical Garden* 72: 824-832.

Sutyemez, M. 2011. Pollen quality and pollen production in some almond cultivars under Kaharamanmaras (Turkey) ecological conditions. *African Journal of Agricultural Research* 6: 3078-3083.

Tutin, T.G. 1980. *Anthoxanthum* L. En: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA. eds. *Flora Europaea, Vol. 5*. Cambridge: Cambridge University Press, 229–230.

Wang, Z.Y., Yaxin, G., Scott, M., Spangenberg, G. 2004. Viability and longevity of pollen from transgenic and nontransgenic tall fescue (*Festuca arundinacea*) (Poaceae) plants. *American Journal of Botany* 91: 523-530.

Weiss-Schneeweiss, H., Emadzade, K., Jang, T.-S., Schneeweiss, G. M. 2013. Evolutionary consequences, constraints and potential of polyploidy in plants. *Cytogenet Genome Res* 140: 137-150.

Yarnes, S. 2011. *Pollen Conservation and FDA Protocol*. Available at: http://www.acffarms.org/papers/