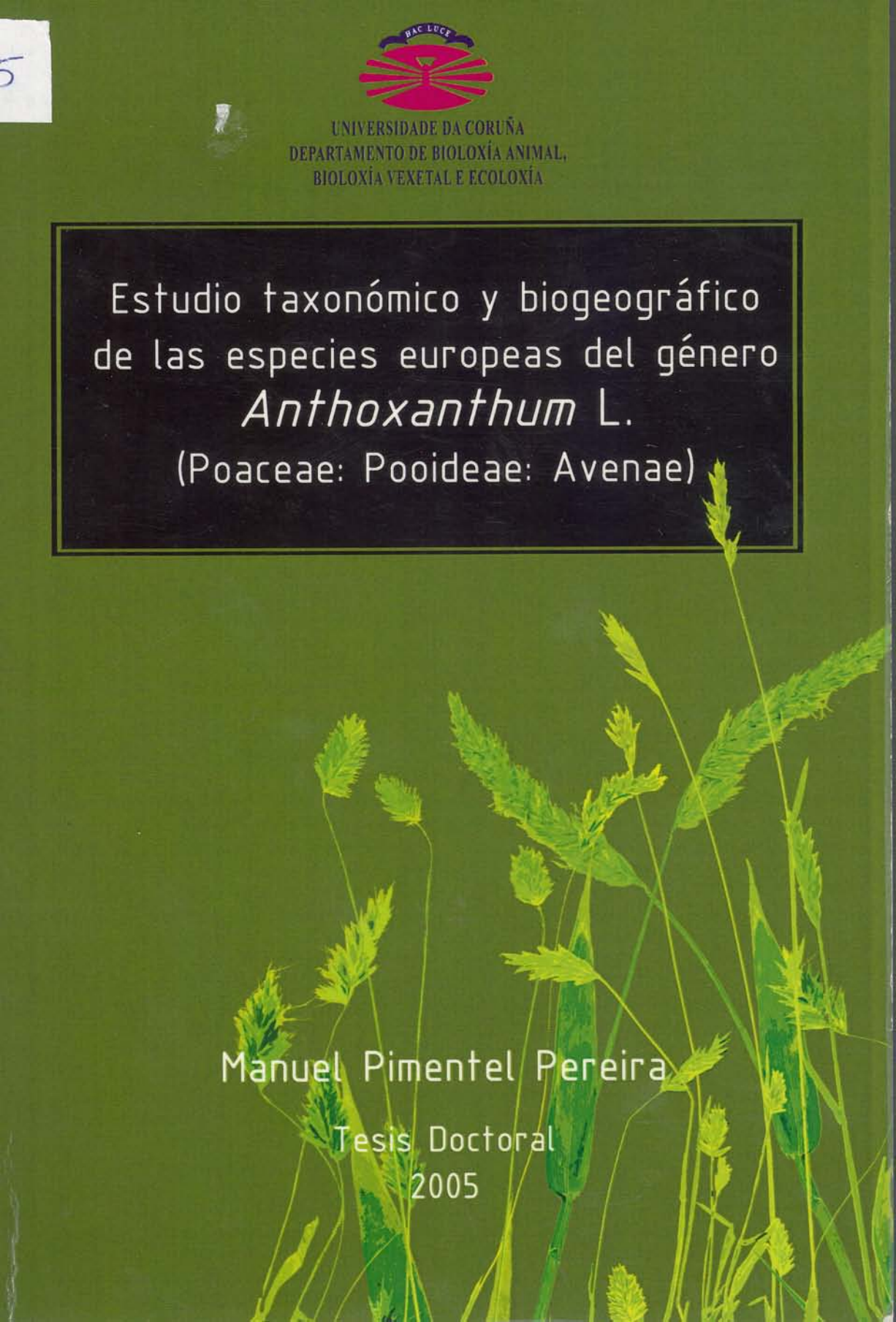
UNIVERSIDADE DA CORUÑA
DEPARTAMENTO DE BIOLOXÍA ANIMAL,
BIOLOXÍA VEXETAL E ECOLOXÍA

Estudio taxonómico y biogeográfico de las especies europeas del género *Anthoxanthum* L. (Poaceae: Pooideae: Avenae)



Manuel Pimentel Pereira

Tesis Doctoral
2005

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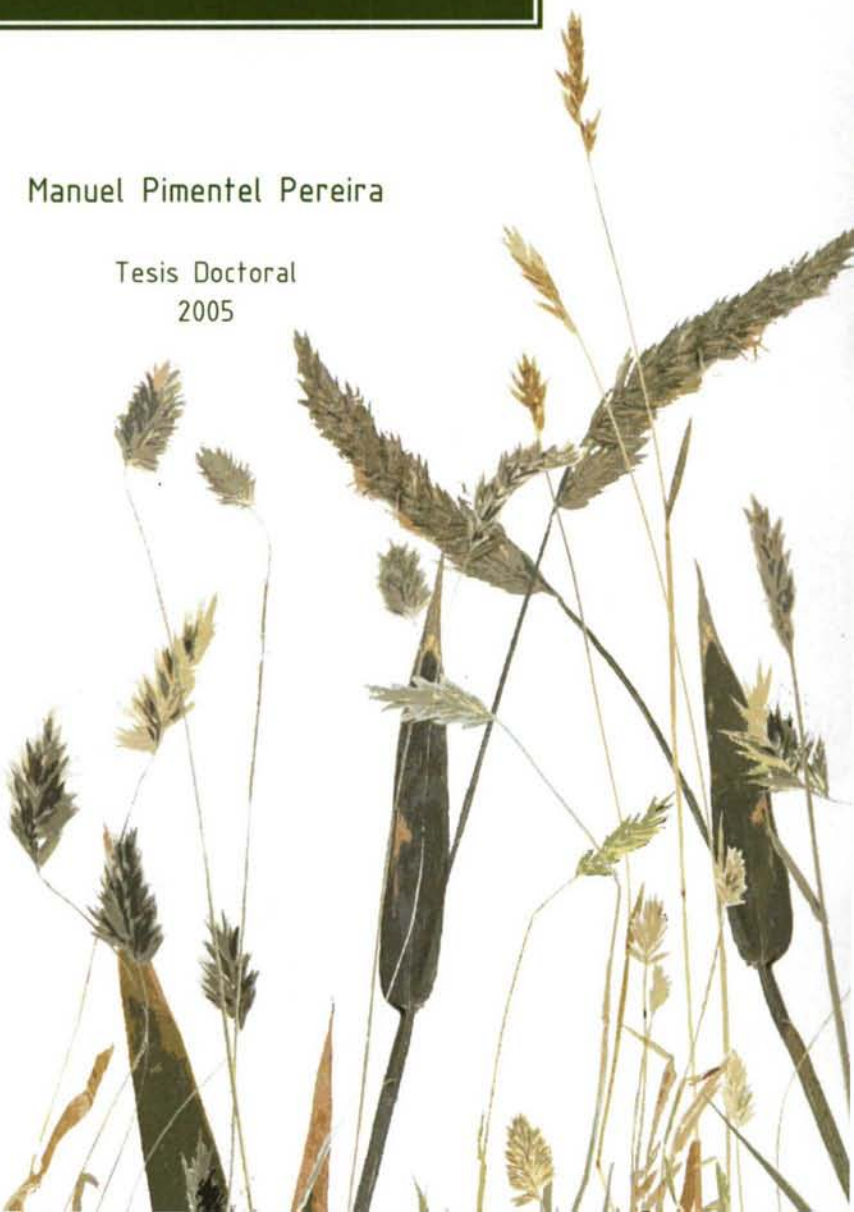

UNIVERSIDADE DA CORUÑA
DEPARTAMENTO DE BIOLOXÍA ANIMAL,
BIOLOXÍA VEXETAL E ECOLOXÍA

LABORATORIO DE PLANTAS VASCULARES
CIENCIAS

ELVIRA SAHUQUILLO BALBUENA, Profesora Titular del Departamento de Bioloxía Animal, Bioloxía Vexetal e Ecoloxía de la Facultade de Ciencias de la Universidade da Coruña,

CERTIFICA:

que la Memoria presentada por el licenciado Manuel Pimentel Pereira para optar al título de Doctor en Biología, titulada "Estudio taxonómico y biogeográfico de las especies europeas del género *Anthoxanthum* L. (Poaceae: Pooideae: Aveneae)", ha sido realizada en este departamento bajo su dirección. Considerando que se halla concluída, autoriza su presentación para que pueda ser juzgada por el tribunal correspondiente.

Para que conste, firma el presente certificado en A Coruña, a 1 de septiembre de 2005

Fdo.: *Elvira Sahuquillo Balbuena*

Índice

A grass so little has to do...

Emily Dickinson

Ós meus pais
Ós meus irmáns
A José Luis e Estrella

Capítulo 1

1 Introducción y Objetivos

Introducción

Anthoxanthum es un complicado e interesante género perteneciente a la familia Poaceae cuya caracterización, composición y situación taxonómica sigue siendo fuente de controversias. Fue descrito por Linneo (1753), quien utilizó para denominarlo los vocablos griegos "*anthos*" (flor) y "*xanthos*" (amarillo), haciendo referencia al color de los estambres y de la panícula tras la floración. Posteriormente ha recibido otras denominaciones como *Flavia* Fabric. (1759) y *Xanthoxanthus* St-Lager (1880), referidas ambas a su coloración amarillenta, o *Foenodorum* Krause (1911), por el característico y agradable olor a cumarina que desprendren, por lo que alguno de sus representantes ha sido cultivado como ornamental (Tava, 2001).

Es un género ampliamente distribuido por el Hemisferio Norte, y es especialmente interesante para contrastar diferentes hipótesis sobre el origen y diversificación de las especies como resultado del efecto de los procesos geológicos y climáticos. La composición de este género, que incluye un complejo poliploide asociado a la Europa boreal y templada, especies diploides de distribución variable en la cuenca medirerránea y la Macaronesia, además de varios poliploides de elevado número cromosómico y distribución restringida, indica la complejidad de su estructura.

Se caracteriza fundamentalmente por su inflorescencia, una panícula condensada a modo de espiga, que presenta numerosas espiguillas comprimidas lateralmente. Éstas contienen tres flores, la superior fértil y las inferiores estériles, reducidas a lemas (Figura 1.1). Las glumas son muy desiguales, membranosas, carenadas y mucronadas. La gluma inferior es más corta que las flores y uninervada, mientras que la superior es más larga y trinervada. Las lemas de las flores estériles son escariosas en la parte inferior y membranosas en la superior, dentadas o con 2 lóbulos oblongos u obtusos en el ápice,

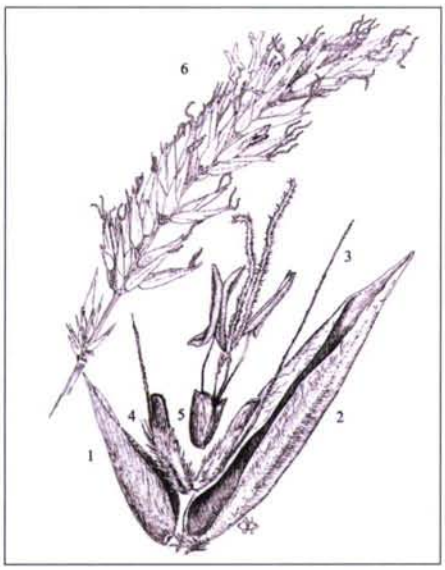

Figura 1.1. Inflorescencia y espiguilla de *Anthoxanthum amarum* Brot: (1), gluma inferior; (2), gluma superior; (3), arista; (4), lema estéril; (5), flor fértil; (6), inflorescencia.

presentan además una arista dorsal. Las glumillas (lema y pálea) de la flor fértil son más cortas que las lemas de las flores estériles. La lema presenta de 5 a 7 nervios y la pálea es uninervada. La flor fértil presenta dos estambres y no tiene lodículas.

El género *Anthoxanthum* está constituido por especies anuales y perennes, que se distribuyen de forma natural por las regiones templadas y subárticas de Europa y Asia, en las altas montañas tropicales de África y Asia, en Sudáfrica e Indonesia (Clayton, 1970; Valdés, 1973; Schouten & Veldkamp, 1985; Phillips, 1995; Bown, 1996; Soreng *et al.*, 2003). Algunos representantes han sido introducidos en extensas áreas de América y en las islas oceánicas (Cheeseman, 1906; Hitchcock, 1951; Hultén, 1962), donde *A. odoratum*, el taxon más ampliamente distribuido del género, se comporta como una especie invasora que presenta difícil control y erradicación (Nicora & Rúgolo de Agrassar, 1987; Gould & Shaw, 1992; Tovar, 1993).

EL GÉNERO *ANTHOXANTHUM* EN LA F. POACEAE

En la actualidad tanto la caracterización de este género como sus relaciones con otros representantes de la familia Poaceae siguen siendo controvertidas. Su proximidad a los géneros *Hierochloë* R.Br. y *Phalaris* L. ha sido tradicionalmente admitida (Bennett & Brown, 1838; Roshevits, 1942; Hitchcock, 1951; Jansen, 1951; Paunero, 1953; Prat 1960; Royen, 1968) y, recientemente evidenciada mediante estudios moleculares (GPWG,

2001). En estos tres géneros (*Hierochloë*, *Phalaris* y *Anthoxanthum*) las espiguillas están formadas por tres flores que presentan diferentes grados de reducción de sus piezas florales. Así, en *Anthoxanthum* las dos flores inferiores son estériles y están reducidas a lemas mientras que la flor superior es hermafrodita y presenta dos estambres. En *Hierochloë* las dos flores inferiores son masculinas con 3 estambres cada una y la terminal hermafrodita con 2 estambres. *Phalaris* posee dos flores inferiores estériles y una terminal hermafrodita con 2 estambres. Por otro lado, tanto *Hierochloë* como *Anthoxanthum* contienen cumarina, pero ambos géneros se diferencian en su número básico de cromosomas, siendo de 5 para las especies de *Anthoxanthum* y 7 para *Hierochloë* (Borril, 1962; Jones, 1964; Schouten & Veldkamp, 1985).

La existencia en Malasia, Nueva Zelanda y Nueva Guinea de formas intermedias entre ambos taxones, que eran incluidas de forma arbitraria en el género *Ataxia* R.Brown, en *Hierochloë* o en *Anthoxanthum*, llevaron a Schouten & Veldkamp (1985), en su estudio sobre la flora de Malasia y Tailandia, a plantear la necesidad de reunir las especies de los géneros *Hierochloë* y *Anthoxanthum* bajo la denominación de *Anthoxanthum s. lat.* Esta postura ha sido apoyada por algunos autores como Soreng *et al.* (2003) en el catálogo de las gramíneas del Nuevo Mundo; aunque otros como Clayton y Renvoize (1986) manifestaron su oposición a este cambio, por considerar que las diferencias existentes siguen siendo suficientes para mantener ambos géneros separados.

Las particularidades que presenta *Anthoxanthum* y su relación con *Hierochloe* y *Phalaris* llevó a separarlos en una tribu propia denominada *Phalaridae* Dumort. 1829 o *Anthoxantheae* Endl. 1830; aunque inicialmente habían sido incluidos en la tribu *Aveneae* Dumort. 1824 (Roshevits 1942). Esta diferenciación taxonómica fue apoyada por autores como Hitchcock (1950), Paunero (1953) y Prat (1960) y cuestionada por otros como Stebbins y Crampton (1961), Tateoka (1966, 1967) y Clayton (1970). El estudio morfométrico realizado por Hilu y Wright (1982), incluyendo un elevado número de caracteres morfológicos y anatómicos además de una amplia representación de las tribus de la familia Poaceae, relacionó estos tres géneros con *Holcus*, *Alopecurus* y *Phleum*, los cuales presentan también una sola flor fértil y tradicionalmente han sido considerados miembros de la tribu *Aveneae*. Posteriormente, la reorganización taxonómica de la familia realizada por Clayton y Renvoize (1986), incluyendo toda la información morfológica, anatómica, bioquímica o fisiológica conocida hasta el momento, confirman su pertenencia a la tribu *Aveneae*. Dentro de esta tribu forman un pequeño grupo caracterizado por la estructura de su espiguilla al que estos autores adjudican la categoría

de subtribu *Phalaridineae* Reichenb. 1828 (posteriormente denominada *Anthoxanthinea* por el mismo autor en 1846) (Figura 1.2).

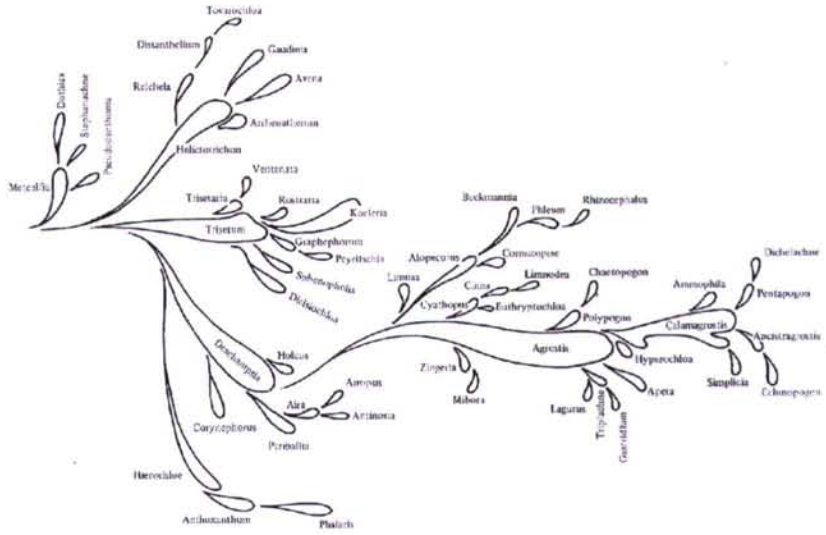

Figura 1.2. Relaciones filogenéticas entre los diferentes géneros de la tribu *Aveneae* según Clayton & Renvoize (1986).

Los estudios filogenéticos más recientes, que integran datos moleculares y morfológicos, han propuesto la unificación de las tribus *Aveneae* y *Poeae* (Figura 1.3), bajo la denominación de la tribu *Poeae.* Dentro de esta tendencia se reconoce un elevado número de subtribus entre las que se encuentra la subtribu *Phalaridinae* que incluye los tres géneros *Hierochloë*, *Phalaris* y *Anthoxanthum* (Soreng & Davis, 2000; GPWG, 2001).

Tradicionalmente la constitución de las espiguillas (nº flores, nº nervios, arista, etc.) es la base para definir las tribus en la familia Poaceae, mientras que las características anatómicas delimitan las subfamilias. Estudios fenéticos utilizando datos morfológicos, anatómicos, inmunológicos o moleculares (Esen & Hilu, 1989; Hilu & Wright, 1982; Hilu & Johnson, 1991; Watson & Dallwitz, 1992) apoyaron la división de la familia en 5-7 subfamilias comúnmente reconocidas, entre las que se encuentra la subfamilia *Festucoidea* que incluiría la tribu *Poeae.* Recientes trabajos cladísticos en los que se integran datos morfológicos y genéticos subdividen la familia Poaceae en 13 subfamilias. Según estos estudios la subfamilia *Pooideae*, que incluiría a la antigua subfamilia *Festucoidea*, sería un grupo monofilético y basal (Kellogg & Campbell, 1987; Davis &

Soreng, 1993; Hsiao *et al.*, 1999; Kellogg, 1998; Soreng & Davis, 1998; Clark *et al.*, 2000, GPWG, 2001).

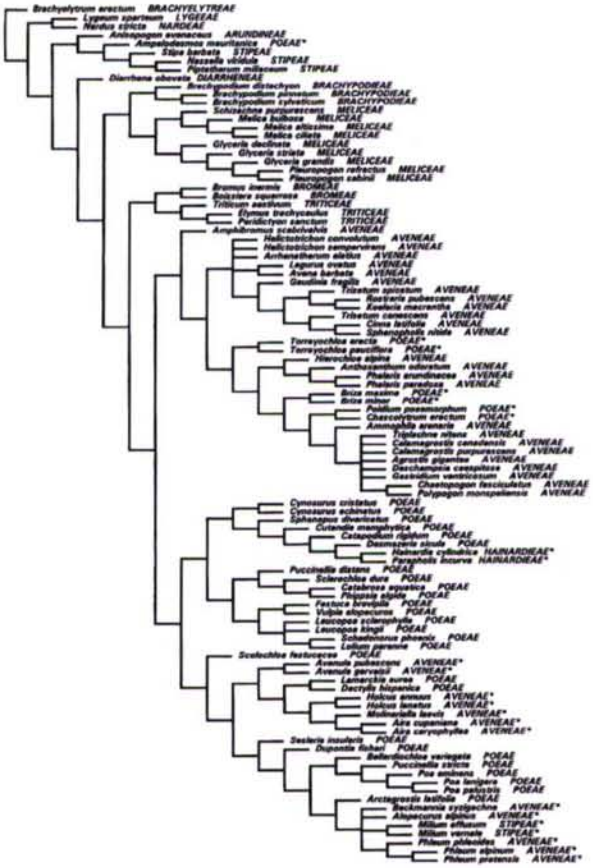

Figura 1.3. Relaciones filogenéticas entre las diferentes tribus de la subfamilia *Pooideae* según Soreng & Davis (2000).

La particular inflorescencia llevó a algunos autores a diferenciar una subfamilia que agrupase estos géneros bajo el nombre de *Anthoxanthoideae* Link 1824 (Soreng *et al.*, 2003). Sin embargo, la inclusión de la subtribu *Phalaridinae* dentro de la subf. *Pooideae* ha sido tradicionalmente admitida (Roshevits, 1946; Tateoka, 1957; Clayton & Renvoize, 1986; Dahlgren *et al.*, 1985; Gould & Shaw, 1992; Takhtajan, 1997; GPWG, 2001). Actualmente, la presencia de células subsidiarias de lados paralelos, la ausencia de pelos pluricelulares y lodículas, la presencia del epiblasto y la ausencia de una fisura escutelar en el embrión apoyan esta inclusión (Kellogg & Campbell, 1987; Catalán *et al.*, 1997; Soreng & Davis, 1998).

ORGANIZACIÓN TAXONÓMICA DEL GÉNERO *ANTHOXANTHUM*

El número de especies que componen el género ha sido y sigue siendo fuente de controversias. La elevada variabilidad morfológica y la plasticidad ecológica que presentan algunos de sus representantes, unido a la existencia de diferentes niveles de ploidía, han llevado a la descripción de nuevos taxones a los que se adjudicaron distintas categorías taxonómicas, según el grado de diferenciación morfológica o ecológica observada. Este número oscila entre las 4 especies indicadas por Gould y Shaw (1992) y las 20 propuestas por Dahlgren, Clifford & Yeo (1985), aunque las aproximaciones más aceptadas incluyen entre unas 15 especies (Nicora & Rugolo, 1987) y 18 (Clayton & Renvoize, 1986; Phillips 1995).

En Europa se considera que este género está representado por 3 especies anuales (*A. ovatum*, *A. aristatum* y *A. gracile*) y 5 perennes (*A. odoratum*, *A. alpinum*, *A. amarum*, *A. maderense* y *A. puciflorum*). Sin embargo, sus relaciones taxonómicas todavía son muy discutidas, resultando especialmente problemáticos los grupos formados por: *A. odoratum-A. alpinum*, *A. ovatum-A. aristatum-A. odoratum* y *A. puciflorum*. Además, se ha indicado la existencia de subespecies y variedades dentro de las especies anuales con distinto grado de aceptación (Paunero, 1953; Valdés, 1973; Tutin 1980; Pignatti, 1982; López González, 1994).

Dos especies asiáticas parecen estar relacionadas con las europeas: *A. nipponicum* Honda, (Honda, 1926, 1930) perenne diploide que presenta una fuerte afinidad ecológica, citológica y morfológica con *A. alpinum* por lo que algunos autores han señalado la posibilidad de que se trate de la misma especie (Löve & Löve, 1948; Tateoka, 1966; Hedberg, 1967; Phillips, 1995) y *A. japonicum* (Maxim.) Hack., poliploide perenne de difícil adscripción taxonómica, que fue descrito inicialmente como perteneciente a *Hierochloë*, posteriormente situado en el género *Ataxia* por Bentham y Hooker (1883) y finalmente en una sección de *Anthoxanthum* por Honda (1930). Su elevado número cromosómico (2n=70) que puede presentar como básico 5 ó 7, no ha permitido clarificar su situación (Tateoka, 1967; Schouten & Veldkamp, 1985).

En África también se han descrito varias especies cuyas afinidades con los taxones europeos han sido señaladas: *A. nivale* K. Schum. (1895), hemicriptófito cespitoso muy parecido a *A. odoratum* y *A. amarum*, que se caracteriza por sus hojas anchas pubescentes, por presentar vainas blancas "esponjoso-papiráceas" que cubren la base del tallo y por su cilíndrica, erguida y densa panícula. Es propio de pastizales y brezales de zonas húmedas en alta montaña, a menudo cerca de cursos de agua. Ha sido citado en las altas montañas

de Kenia, Tanzania y Uganda entre los 2400 y 4800 m. Se han indicado varios números cromosómicos, 2n=20,60 (Hedberg, 1967; Clayton, 1970; Phillips, 1995; Teppner, 2002). Sinonimias: *A. scaposum* Peter 1930 [*A. amarum sensu* Peter *non* Brot.].

A. ecklonii (Nees) Stapf. Especie considerada una variante del taxon precedente, originaria de Sudáfrica y Malawi. Caracterizada por sus tallos delgados y ascendentes, por sus panículas delgadas, con aristas exertas y por presentar un corto internudo bajo la flor fértil (Clayton, 1970; Teppner, 2002).

A. aethiopicum Hedberg (1976). Especie perenne rizomatosa de tallos delgados y erectos (20-50 cm). Hojas de 2-4 mm ancho, glabras, con vainas basales no libres y esponjosas. Panícula delgada, 3,5-7 cm, abierta. Espiguillas estrechas, oblongas, 7-8,4 mm de largo, con aristas poco aparentes y escasamente sobresalientes. Propia de zonas húmedas y umbrías de montaña (2700-4500 m). Está considerada como endemismo de Etiopía, el cual es reemplazado en las montañas del Este africano por *A. nivale* (Phillips, 1995).

Otras especies han sido descritas en este género con distribución asiática o sudafricana, algunas consideradas como pertenecientes a los género *Hierochloe* o *Ataxia*, pero la información existente es todavía escasa: *A. borii* Jain & Pal. de la India, *A. brevifolium* Stapf. de la Región del Cabo, *A. formosanum* Honda, *A. horsfieldii* (Kunth ex Bennett) Mez ex Reeder, *A. madagascariense* Stapf. de Madagascar; *A. pallidum* (Hand.-Mazz.) Keng y *A. sikkimense* (Maxim.) Ohwi de Japón (Schouten & Veldkamp, 1985).

Los trabajos realizados sobre el género se han centrado, fundamentalmente, en el estudio del complejo poliploide formado por *A. odoratum-A. alpinum*, con la finalidad de analizar el origen de los poliploides y su papel en la evolución de la diversidad vegetal. Entre estos trabajos destacan los realizados por Böcher (1961), Rozmus (1958, 1960), Borril (1962, 1963), Jones (1964), Felber (1986, 1988) y Hedberg (1961, 1986, 1990).

Los trabajos que presentan un enfoque más taxonómico son escasos y entre ellos destacan las revisiones de Paunero (1953) para la Península Ibérica, la de Pinto da Silva y Teles (1971) para las especies anuales portuguesas, Valdés (1973) para las anuales ibéricas y Lambion y Deschâtres (1991) y Felber (1993) para las anuales de la isla de Córcega. En la caracterización de las especies perennes *A. odoratum* y *A. alpinum* desde un punto de vista morfológico y ecológico destacan los trabajos de Rozmus (1960), Hedberg (1964) y Felgrová y Krahulec (1999). Otros estudios se han centrado en la ecología de *A. odoratum*, una especie muy agresiva y con un fuerte carácter alelopático.

Por otra parte, este taxon presenta una elevada resistencia a la presencia de metales pesados (Wu & Subodh, 1980; Felber, 1987; Yamamoto, 1995; Sammul *et al.*, 2000).

EL GÉNERO *ANTHOXANTHUM* EN EUROPA

Como se ha mencionado anteriormente, en Europa se han descrito 8 especies de las cuales 5 son perennes: *Anthoxanthum odoratum, A. alpinum, A. amarum, A. puciflorum* y *A. maderense*, y 3 anuales: *A. aristatum*, *A. ovatum* y *A. gracile* (Tutin *et al.*, 1980; Teppner, 2002). Además, se definieron dos subespecies y dos variedades en *A. aristatum* y dos variedades en *A. ovatum* cuya validéz taxonómica ha sido discutida (López González, 1994; Pignatti, 1982).

Problemática del complejo poliploide *A. odoratum-A. alpinum*

***A. odoratum* L., Sp. Pl. (1753)**

Es la especie que presenta una distribución más amplia dentro del género, encontrándose en los cinco continentes, si bien en América y Oceanía ha sido introducida (Figura 1.4). Desde su descripción y debido a su gran variabilidad morfológica, ha sido disgregada en un elevado número de formas o variedades, algunas de las cuales han sido elevadas a rangos específicos o subespecíficos. Las variaciones observables en cuanto a su tamaño y pubescencia han sido señaladas en otras denominaciones para este taxon como:

Anthoxanthum villosum Dumort (1823, 1824)

Anthoxanthum pilosum Döll (1843)

Anthoxanthum glabrescens Celak. (1867)

Xanthoxanthos odoratum (L.) St.-Lag. (1880)

Anthoxanthum odoratum var.*altissimum* Eaton (1917)

Se diferencia del resto de las especies por ser una planta cespitosa perenne que no presenta raíces tuberosas en la base, por la anchura de sus hojas (2-8 mm), por el tamaño de sus espiguillas (7-9 mm) y por el grado de pubescencia en las lemas de las flores estériles que presentan, además, una arista geniculada.

Fue considerada inicialmente como tetraploide (Katterman, 1931; Parthasarathy,

1939; Hunter, 1934) hasta que Oestergren (1942) encontró un citotipo diploide en muestras procedentes de los Alpes y Escandinavia. Este citotipo sería posteriormente descrito como la especie *A. alpinum* por Löve & Löve (1948). Desde el descubrimiento de la existencia de estos dos citotipos (tetraploide y diploide) en *A. odoratum s. lat.*, los intentos de caracterizarlos aplicando diferentes aproximaciones (cariológicas, morfológicas, ecológicas, isoenzimáticas, fenológicas, etc.) han dado resultados contradictorios. Son destacables los trabajos de Rozmus (1958, 1960) quien, analizando muestras polacas de ambos citotipos, estableció una serie de diferencias morfológicas, anatómicas, ecológicas, fenológicas y citológicas (cariotipo) que justificarían la separación de ambas especies. Este autor propone que *A. alpinum* debe ser considerada "la fuente ancestral" del origen de *A. odoratum*.

Por el contrario, Böcher (1961) con material de ambos citotipos transplantados a un jardín botánico, concluye que no se pueden diferenciar los individuos diploides de los tetraploides, pero detecta la existencia de otros diploides de altitudes medias de Italia (600-1100 m), que sí se diferencian por la fuerte pilosidad de las glumas, las largas panículas y el elevado número de espiguillas. Este autor sugiere que este diploide podría haber estado involucrado en el origen alopoliploide de *A. odoratum* (2n=4x=20) junto con *A. alpinum*, al que le otorga la categoría de subespecie.

Borril (1963) intenta establecer las relaciones genéticas entre estos dos taxones realizando cruzamientos entre los diploides (*A. alpinum, A. ovatum* y *A. aristatum*) y el tetraploide (*A. odoratum*). Sus trabajos indicaron que *A. ovatum* podría ser la otra especie implicada en el origen del citotipo tetraploide. Los estudios cariológicos realizados por Jones (1964) con el material analizado por Borril (l.c.), apoyan el origen alopoliploide de *A. odoratum* por hibridación con posterior duplicado de la dotación cromosómica entre varios taxones diploides: *A. alpinum* y *A. ovatum*, *A. alpinum* y un citotipo diploide detectado en Creta y finalmente, entre *A. ovatum* y el diploide cretense. Ambos autores sugieren el estatus específico para *A. alpinum*.

Los trabajos de Hedberg (1967, 1970), Felber (1987) y Teppner (1970, 1998) profundizan en la relación entre ambos taxones pero desde enfoques diferentes. Así los resultados citogenéticos de Hedberg (1967, 1970) con poblaciones suecas, suizas y austriacas de ambos citotipos, parecen indicar la existencia de autopoliploidía en el origen de *A. odoratum s. str.* Este resultado, unido a su débil diferenciación morfológica, ya que sólo las poblaciones escandinavas diploides parecen estar caracterizadas morfológicamente por la ausencia de pubescencia en las glumas (carácter que no se

mantiene en las poblaciones centroeuropeas), lleva a esta autora a concluir que *A. alpinum* debe considerarse una subespecie de *A. odoratum*. Posteriormente, Hedberg (1970) describe un citotipo tetraploide resultado del duplicado cromosómico de *A. alpinum* en la Región des Morgins (Suiza) y Felber (1987) en el Macizo Central francés, suponiéndole una distribución más amplia por el Jura meridional y en la franja NW de los Alpes Suizos. Teppner (1970), analizando las poblaciones centroeuropeas, encuentra un nuevo citotipo diploide en *A. odoratum*, perenne, que ocupa zonas bajas y medias de las montañas italianas y los Balcanes. Este autor plantea un origen alopoliploide de *A. odoratum* tetraploide, siendo el resultado de la hibridación entre este citotipo diploide perenne y un ancestro de *A. alpinum*, que no presentaría constricciones secundarias. Por todo esto, Teppner defiende la separación de ambas especies.

Además, otros estudios habían indicado la existencia de *A. odoratum* diploides en Córcega (Litardière, 1937; Contandriopoulos, 1957, 1962; Grosstête, 1982) considerados como *A. odoratum* var. *corsicum*. En Creta, Jones (1964) describe un citotipo diferente de *A. alpinum* que considera más próximo a *A odoratum* como *A. odoratum* "cretense" y en Bulgaria también encuentran otro citotipo Böcher (1961) y Pundeva (1974).

Es importante señalar que todos los autores de estos trabajos destacan la dificultad que supone trabajar cariológicamente con los representantes de este género, debido a que se observa una elevada variabilidad en cuanto a sus cariotipos incluso a nivel poblacional.

Para caracterizar *A. odoratum* frente *A. alpinum* se han utilizado datos morfológicos y anatómicos, aunque nuevamente los resultados son contradictorios. Rozmus (1960) y Polatschek (1966) detectan una cierta diferenciación en cuanto al tamaño del polen entre ambas especies, utilizando el citotipo más representativo de cada especie. En cambio, Hedberg (1967) y Grossetête (1982), que analizan además el tamaño estomático no consideran este carácter totalmente fiable. Los trabajos de Humbert-Droz y Felber (1992), incluyendo ambos citotipos de *A. alpinum* y comparándolos con los publicados para *A. odoratum* tetraploide, tampoco son concluyentes, puesto que aunque encuentran ciertas diferencias significativas al comparar los valores medios, los rangos de variación se solapan entre ambas especies y citotipos. Estos autores consideran que el análisis conjunto del diámetro del polen y longitud de los estomas pueden servir para deducir los citotipos cuando los medios materiales (material herbario, estudios poblacionales) no permiten realizar otro tipo de análisis, aunque reconocen que existe una marcada variación dentro de las poblaciones (dependiendo de en qué época son analizadas) y del taxon.

***A. alpinum* Á. Löve & D. Löve,**
Icel. Univ. Inst. appl. Sci. Dept. Agric. Rep.Ser. B 3: 1-131 (1948)

Inicialmente descrita como un citotipo diploide de *A. odoratum* que se caracterizaba por su menor tamaño (< 25 cm), por la anchura de sus hojas (> 5 mm), que son glabras al igual que las glumas, por el tamaño de la panícula (< 2 cm) y por las lemas escábridas (Tutin, 1950; Knaben, 1950; Borril, 1962; Hedberg, 1961; Lauber & Wagner, 2001). Trabajos posteriores han puesto de manifiesto que las diferencias morfológicas no eran constantes en toda su área de distribución (Hedberg 1964; Felber 1987). Así, el material Escandinavo estudiado sí presentaba esas características, al igual que el originario de los Balcanes y los Cárpatos (Rozmus, 1958; Böcher, 1961). Por contra, en los Alpes, las hojas y glumas eran pubescentes y las lemas lisas (Borril, 1962; Jones, 1963: Hylander, 1953). Hedberg (1964) destaca que la variación en la pubescencia no es sólo geográfica, sino que puede encontrarse dentro de la misma población e incluso en el mismo individuo. La utilización de este carácter llevó a Jones (1963) a citar la presencia de *A. alpinum* en las Islas Británicas.

Está considerada como una especie ártico-alpina (Figura 1.4) cuya área de distribución se extiende por el N de Europa, desde Scandinavia a través de Siberia, probablemente, hasta Japón (Hedberg, 1986). Está presente también en las montañas centroeuropeas, excepto en Pirineos (Hedberg, 1990) y puntualmente ha sido citada a baja altitud en un afloramiento de serpentinas en Austria (Melzer, 1986). *A. odoratum*, por el contrario, se encuentra más al sur o se asocia con zonas de menor altitud en el norte (Rozmus, 1958; Felber, 1988; Bogenrieder, Bühler & Härringer., 1993; Felgrová & Krahulec, 1999). Esta diferenciación ecológica es discutida por algunos autores y Hedberg (1986) señaló que, si bien en la actualidad los límites son más difusos, esto se debe a la actividad humana. Los trabajos de Felgrová y Krahulec (1999) parecen indicar la existencia de cierta diferenciación en cuanto a la preferencia de hábitats (Figura 1.5). En un gradiente altitudinal a lo largo del cual se desarrollan ambas especies, los transplantes invertidos realizados se tradujeron en un pobre éxito reproductivo en ambas especies. En *A. alpinum* los autores asocian este resultado a la competitividad con otras especies y en *A. odoratum* a la reducción en su capacidad de producir semillas debido a factores ambientales.

Otros estudios que apoyan la diferenciación específica se basan en la resistencia a la infección de *Puccinia sardonensis* propia de *A. alpinum* y en la existencia de diferencias fenológicas, puesto que *A. alpinum* es más precoz que *A. odoratum* (Felber, 1988).

También Bogenrieder, Bühler & Härringer. (1993) destacan diferencias en cuanto a sus características bioquímicas (clorofilas, proteínas, etc.) y a su biología.

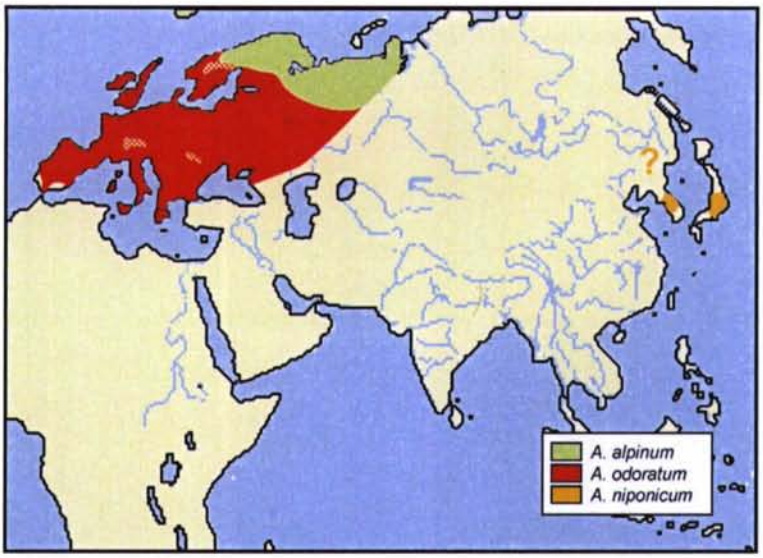

Figura 1.4. Distribución de las especies perennes *Anthoxanthum alpinum*, *A. odoratum* y *A. niponicum* según Hedberg (1990).

Figura 1.5. (A), Población de *Anthoxanthum odoratum* en Marei, Lugo, España; (B), Población de *A. alpinum* en Piccolo San Bernardo, Italia.

Como resultado de los estudios realizados siguen existiendo varias hipótesis para explicar las relaciones entre *A. odoratum-A. alpinum*:

-Origen autopoliploide:

-Por duplicación cromosómica de *A. alpinum* diploide.

-Por duplicación cromosómica del citotipo diploide *A. odoratum*.

-Por duplicación de un citotipo diploide mediterráneo.

-Origen alopoliploide:

-Por hibridación del *A. alpinum* diploide con otro citotipo diploide de origen mediterráneo (*A. ovatum* o *A. odoratum* diploide). Esto justificaría la elevada variabilidad observada en meiosis en *A. odoratum* tetraploide y la ausencia de constricciones secundarias en cuatro pares cromosómicos.

-Por hibridación de *A. odoratum* diploide con un citotipo mediterráneo (*A. ovatum*, *A. aristatum*, citotipo cretense).

En la actualidad, se considera que el origen de *A. odoratum* debe ser alopoliploide, formando parte de su dotación *A. alpinum* y probablemente un diploide de origen mediterráneo (Jones 1964; Hedberg, 1990). Los estudios de hibridación entre especies realizados por Borril (1963) le llevaron a considerar *A. ovatum* como posible ancestro.

Los resultados de estos estudios, en muchos casos contradictorios, han llevado a que la consideración taxonómica de estas especies resulte conflictiva y no exista un criterio definido a la hora de describir la riqueza florística de una zona o realizar estudios filogenéticos o biogeográficos. Así queda de manifiesto en la revisión de algunos trabajos florísticos de amplia repercusión como son: "Flora Helvética" (Lauber & Wagner 2001) donde se consideran ambas especies; "Flora Europaea", en la que se incluye dentro de *A. odoratum* aunque cuestionada (Tutin, 1980) y "Flora Italiana" (Pignatti, 1982) que la califica como especie "hermana o microespecie" dentro de *A. odoratum* gr.

A. amarum Brot., Phyt. Lusit. Fasc.I, nº 3: 4 (1800)

Especie endémica del NW de la Península Ibérica, incluyendo NW de España y N y C. de Portugal (Figura 1.6). Ha sido citada en otras zonas de la Península Ibérica, como Euskadi (Aizpurúa *et al.*, 2000), Salamanca y Valencia (Moreno-Saiz & Sainz-Ollero, 1992). Es una especie propia de zonas húmedas y umbrías, bordes de ríos o sotobosque de roble o pino (Figura 1.7)

Es una planta perenne que se caracteriza por su mayor porte (130 cm) y por la presencia, en ocasiones, de raíces tuberosas o rizomas. Son también características la anchura de sus hojas, (> 10 mm) y el tamaño de las espiguillas (< 10 mm). Los trabajos

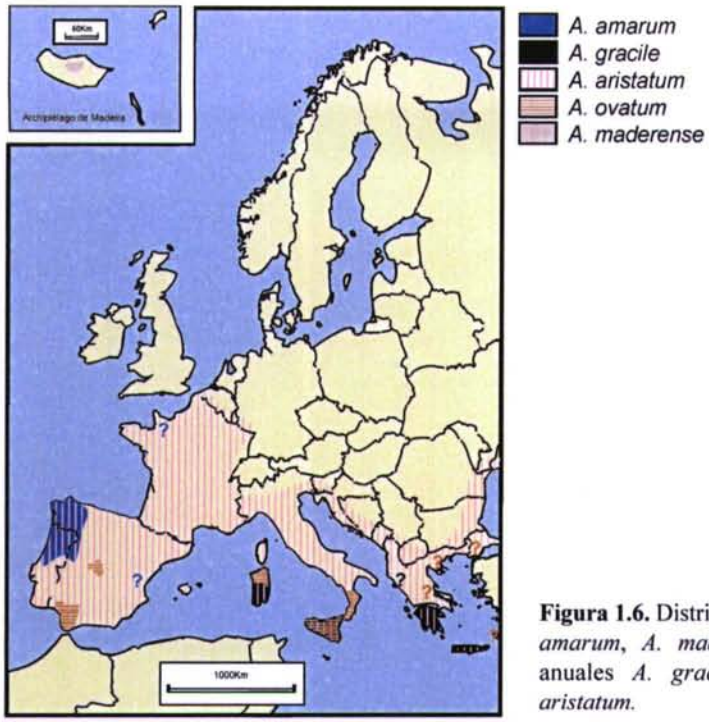

Figura 1.6. Distribución de *Anthoxanthum amarum*, *A. maderense* y las especies anuales *A. gracile*, *A. ovatum* y *A. aristatum*.

Figura 1.7. Poblaciones de *Anthoxanthum amarum* en: (A), San Andrés de Teixido, A Coruña, España; (B) Amorín, Pontevedra, España.

de Ostergren (1942), Fernandes y Queiros (1969) y Teppner (2002) han indicado que se trata de un poliploide de elevado número cromosómico, 2n = 80, 86, 88, 90.

Sinónimo: *A. odoratum* var. *majus* Haeckel.

A. maderense Teppner, Phyton (Horn) 38(2): 309 (1998)

Originaria de la isla de Madeira (Figura 1.6) y propia de zonas altas y expuestas. Se trata de una especie perenne, de bases lignificadas y tallos rígidos. Su cariotipo diploide y su ecología han sido la base para diferenciar esta especie (Figura 1.8).

Figura 1.8. Población de *Anthoxanthum maderense* en el Pico Areeiro, Madeira, Portugal.

A. pauciflorum Adamovic, Denkschr. Akad. Wiss. Math.-Nat. Kl. (Wien) 74:116 (1904)

Especie poco conocida que, según Tutin (1980), podría tratarse de una forma extrema de *A. odoratum* puesto que sus caracteres diferenciales coinciden con los valores extremos inferiores de este taxon. Considerada endemismo del Sur de Yugoslavia, su área de distribución es desconocida. Se caracteriza por su pequeño porte (< 10 cm) y por el tamaño de sus inflorescencias (0.8-1.2 cm) y espiguillas (5.5-6 mm). Asimismo, la anchura de las hojas (3-3.5 mm) y su distribución (alcanzan la base de la inflorescencia) se han utilizado en la diferenciación de este taxon.

Problemática del complejo *A. aristatum-A. ovatum*

Aunque en "Flora Europaea" Tutin (1980), reconoce la existencia de estas dos especies anuales y sus subespecies, la plasticidad morfológica observada en los caracteres

utilizados para diferenciarlas (forma y densidad de la panícula, tamaño de la arista o forma de las lemas estériles) han provocado discrepancias y por ello la circunscripción de estos taxones sigue siendo controvertida.

Por otro lado, además de la problemática relación entre estos taxones, su diferenciación de *A. odoratum*, basada fundamentalmente en la duración de su ciclo biológico, ha sido señalada como de validez relativa, puesto que no es infrecuente encontrar individuos de *A. aristatum* o *A. ovatum* bienales (Paunero, 1953; Borril, 1963; Valdés, 1973) o de *A. odoratum* que en determinadas situaciones puede comportarse como anual (Paunero, 1953; Böcher, 1961; Borril, 1962; Valdés, 1973). Otras características como talla, pilosidad del tallo y hojas, tamaño y pilosidad de la panícula y glumas, resultan muy variables dentro de las poblaciones y en las especies. Según Paunero (1953) y Valdés (1973) son las características de la florúncula (forma y tamaño relativo de las lemas fértiles y estériles; Figura 1.9) las que proporcionan caracteres más constantes y de validez taxonómica. Los intentos por caracterizarlas con mayor precisión realizados por Paunero (1953) o Valdés (1973), analizando un mayor número de individuos y añadiendo a los caracteres de las florúnculas el número de cromosomas, la estructura de la epidermis foliar o las características del polen, han aportado nuevos datos pero no resultan concluyentes. Debido a lo críptico de estos caracteres y la variabilidad que presentan a lo largo del desarrollo de la panícula hasta la fructificación, han sido la causa de la confusión entre las diferentes especies y subespecies, siendo por ello cuestionados (López González, 1994).

A. aristatum Boiss., Voy. Bot. Midi Esp. 2:638 (1842)

Especie propia de la región mediterránea (Figura 1.6), presente también en la mayor parte de la Europa occidental, llegando de forma natural hasta Bélgica, si bien se ha extendido más al norte hasta Polonia y Suecia (Warcholinska & Sicinski, 1976, Valdés, 1973). Suele aparecer en cultivos de cereales y viñedos y ha sido introducida en la India (Jain & Pal, 1975).

Se trata de una especie diploide, anual o bienal, glabra o poco pilosa, cuyo tamaño oscila entre 5 y 60 cm, con hojas de hasta 5 mm de anchura. Su panícula es oval-oblonga, con espiguillas de hasta 5 mm longitud, con una arista que supera claramente la espiguilla en la lema superior estéril. La flor fértil es de un tamaño mucho menor que las estériles.

Dentro de este taxon han sido descritas dos subespecies (subsp. *aristatum* y subsp.

macranthum) y tres variedades, diferenciadas basicamente por la longitud de la arista y la forma de la lema esteril inferior (Valdés, 1973; Tutin *et al.*, 1980; Valdés *et al.*, 1987; López-González, 1994). La subespecie *aristatum* aparece en todo el rango de distribución de la especie, mientras que la subsp. *macranthum* se localiza únicamente en Andalucía occidental, N de Marruecos e Italia, (Valdés, 1973; Tutin *et al.*, 1980; Valdés *et al.*, 1987).

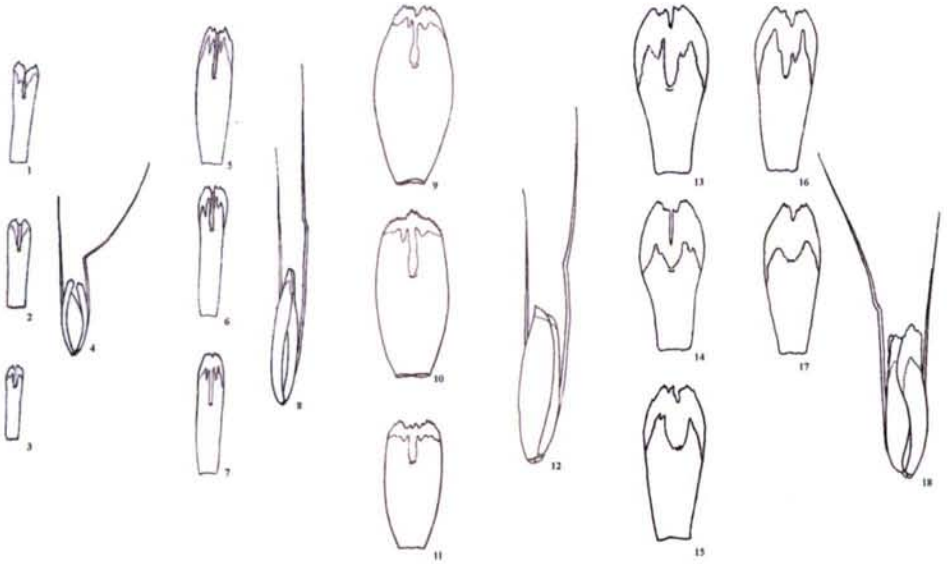

Figura 1.9. Morfología de las piezas florales en el complejo *A. aristatum-A. ovatum* según Valdés (1973). (1-4), *Anthoxanthum ovatum* var. *ovatum*: 1-3, lemas estériles inferiores; 4, flor fértil. (5-8), *A. ovatum* var. *exertum*: 5-7, lemas estériles inferiores; 8, flor fértil. (9-12), *A. aristatum* subsp. *macranthum*: 9-11, lemas estériles inferiores; 12, flor fértil. (13-18), *A aristatum* subsp. *aristatum* var. *aristatum*: 13-17, lemas estériles inferiores; 18, flor fértil.

A. aristatum subsp. *aristatum*. Se caracteriza básicamente porque la arista de la lema estéril superior es bastante más larga que la gluma superior. Las lemas estériles son más anchas en la parte superior que en la parte inferior, presentando una escotadura muy marcada (Figura 1.9).

Dentro de esta subespecie se han descrito dos variedades (*A. aristatum* subsp. *aristatum* var. *aristatum* y *A. aristatum* subsp. *aristatum* var. *welwitschii* Ricci 1881) reflejo del polimorfismo que puede presentar este taxon. Ambas variedades presentan una distribución coincidente, aunque Valdés (1973) señala cierta diferenciación ambiental, indicando que la var. *aristatum* es preferentemente atlántica y la var. *welwitschii*, más continental. Se diferencian por el distinto grado de cobertura que presentan las lemas

estériles sobre la flor fértil.

A. aristatum subsp. *macranthum* Valdés 1973. Se caracteriza por presentar la arista de la lema estéril más corta o casi igual que la gluma superior, siendo las lemas estériles más anchas en la parte media o basal que en la parte superior. Es considerada por Valdés (1973) como un caso extremo de la subsp. *aristatum* y en particular de la variedad *aristatum*, por una reducción de la arista y de la zona membranosa de la lema estéril (Figura 1.9). Se distribuye por el SW de la Península Ibérica, N Marruecos y C Italia. El tipo procede del Coto de Doñana (Huelva), en El Martinazo (Figura 1.10, B).

A. ovatum Lag., Gen. Sp. Nov.: 2 (1816)

En esta especie se incluyen plantas dioicas, anuales o bienales, caracterizadas por su panícula ovada, densa y truncada en la base. Presenta lemas estériles de lados paralelos o ligeramente más anchos en la parte superior, pero que dejan ver la flor fértil. En esta especie se han diferenciado dos variedades (*ovatum* y *exertum* Lindberg, 1932) según la longitud de la arista (var. *exertum*) y la relación entre la lema fértil y la estéril (LE = LF, var. *ovatum*; LE > LF, var. *exertum*). Valdés (1973), en su revisión de las especies anuales, les otorga categoría de variedad debido a que suelen encontrarse en la misma población e hibridan con relativa facilidad (Figura 1.9).

Esta especie se considera repartida por la cuenca mediterránea, fundamentalmente en la mitad occidental (Figura 1.6 y 1.10). Autores como Dogan (1985) la excluyen de Turquía, Strid (1991) de Grecia y Gamisans (1985) de Córcega, aunque este último autor menciona la existencia de un *A. aristatum* muy variable y perenne que en 1910 fue descrito como *A. odoratum* var. *corsicum* (Briq.) Rouy. La variedad *exertum*, también muy criticada, sólo estaría presente en el Mediterráneo Occidental [España, Italia (Sicilia), Marruecos y Argelia].

A. gracile Bivona, Stirp. Rar. Sic. Descr. 1:13. (1813)

Especie relativamente bien caracterizada con respecto al resto de las anuales diploides. Su menor tamaño, la inflorescencia laxa y pauciflora, el mayor tamaño de las espiguillas (10-12 mm), de las lemas estériles (< 6-9 mm) y de las fértiles (> 2,5 mm), además de la longitud de su arista (dos veces más larga que la gluma superior) permiten su rápida y fácil identificación.

Su distribución se considera asociada a las islas mediterráneas (Malta, Cerdeña y Sicilia) y al N de África (Túnez), aunque también fue citada en el Peloponeso y Albania

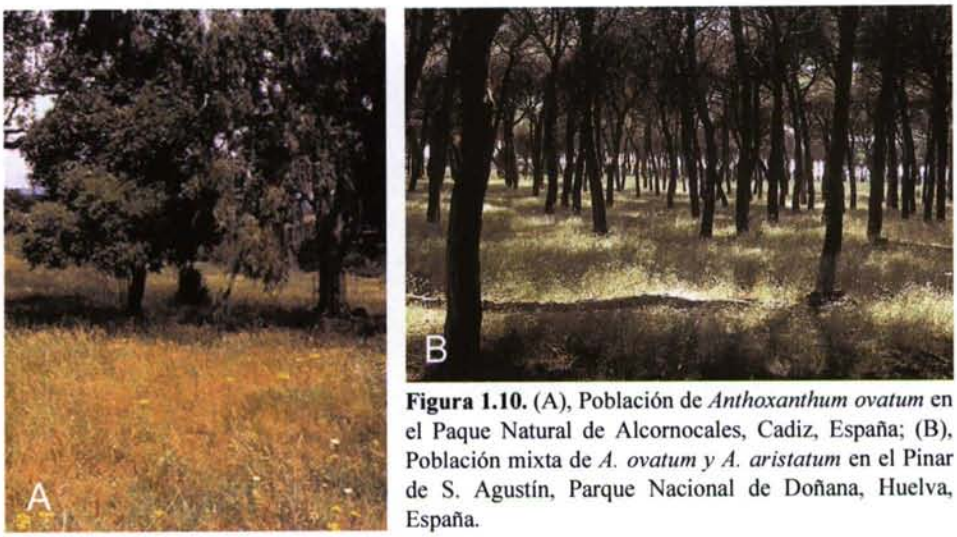

Figura 1.10. (A), Población de *Anthoxanthum ovatum* en el Paque Natural de Alcornocales, Cadiz, España; (B), Población mixta de *A. ovatum y A. aristatum* en el Pinar de S. Agustín, Parque Nacional de Doñana, Huelva, España.

(Tutin *et al.*, 1980) y según Pignatti (1982) no está presente en Sicilia (Figura 1.6).

CONSIDERACIONES METODOLÓGICAS

Durante el desarrollo de la presente Tesis Doctoral y a medida que se profundizaba en la organización taxonómica del género *Anthoxanthum*, han surgido nuevas incógnitas que se han abordado mediante el empleo de diversas técnicas. En primer lugar, se analizaron las relaciones morfológicas entre los taxones de este género presentes en Europa mediante técnicas de Taxonomía Numérica. Los métodos estadísticos permitieron seleccionar los caracteres macro y micromorfológicos que podrían ser más útiles a la hora de identificar estos taxones. Al mismo tiempo, se detectaron las especies o grupos de especies que requerían un análisis más profundo para clarificar su situación taxonómica (**capítulo 2**). A continuación, se englobaron datos morfológicos y moleculares para abordar la problemática de los complejos *A. odoratum*/*A. alpinum* (**capítulo 3**), *A. aristatum*/*A. ovatum* (**capítulo 4**) y para analizar la variabilidad morfológica y ecológica observada en el endemismo del NW Ibérico *A. amarum* (**capítulo 5**).

Si bien los principales esfuerzos realizados se han centrado en la clarificación de la taxonomía del género, los datos moleculares obtenidos se han empleado también en establecer su filogeografía en Europa, esto es, analizando las relaciones genéticas entre las

especies e interpretando su distribución geográfica actual a la luz de los cambios geológicos que se han producido en el continente durante el Cuaternario (**capítulo 6**).

Tanto los métodos fenéticos o de Taxonomía Numérica como los métodos moleculares han sido muy utilizados en la resolución de problemas taxonómicos y sistemáticos en diversos grupos de plantas (*e.g.*, Gengler-Nowak, 2002; Reales *et al.*, 2004; Mehrnia, Zarre & Sokhan-Sanj, 2005). Sin embargo, estos estudios han sido considerados como atomísticos en exceso, ya que reducen los organismos a los caracteres que los describen, siendo los estudios moleculares un ejemplo extremo de esta tendencia (Weber, 2003). De acuerdo con Stevens (2000) y Endress (2003), la morfometría, considerada especialmente útil en los estudios taxonómicos a nivel de especie o población, tiene la desventaja de presentar una gran carga de homoplasia y un gran riesgo de caer en analogías. Por otro lado, Thorne (2000) y Stuessy (2003) consideran que, pese a los grandes avances que han aportado las técnicas moleculares a la Taxonomía y Sistemática de las plantas, la morfología es esencial para comprender la naturaleza de las mismas, su clasificación y su interacción con el medio ambiente.

De acuerdo con Thorne (2000) la diferenciación entre los estudios morfológicos y moleculares es artificial, ya que, si bien cada tipo de datos ofrece una información diferente, todos se centran en estructuras del organismo. Por su parte, Stuessy (2003) considera que en un estudio morfológico todos los niveles estructurales del organismo deben considerarse, desde el nanomorfológico (datos moleculares) hasta el macromorfológico. Siguiendo ese enfoque, hemos intentado realizar un estudio morfológico en su sentido más profundo, con el objetivo de aproximarnos de la forma más completa posible a las especies europeas del género *Anthoxanthum*, su taxonomía y su biogeografía. Se han incluido en el presente trabajo datos macromorfológicos, micromorfológicos (anatomía foliar) y nanomorfológicos (marcadores moleculares RAPDs y AFLPs). La integración de diversos tipos de datos ha sido muy utilizada en los estudios sistemáticos de plantas durante los últimos años (*e.g.*, Hansen, Elven & Brochmann, 2000; Scheen, Elven & Brochmann, 2002; James *et al.*, 2004).

ESTUDIO MORFOMÉTRICO

La morfometría es el conjunto de métodos numéricos empleados en la descripción de los patrones de variación morfológicos. Se ha considerado de gran utilidad en los niveles más bajos de la jerarquía taxonómica, donde la variación en las formas es difícilmente

captable de forma intuitiva (Sneath & Sokal, 1973; Gengler-Nowak, 2002). Dos tipos de datos se han utilizado en el estudio morfométrico, datos macromorfológicos y micromorfológicos, tanto de la epidermis foliar superficial como de la sección transversal del limbo.

Caracteres macromorfológicos. Los caracteres macromorfológicos se seleccionaron en base a dos criterios. (i) Aquellas características que han sido tradicionalmente empleados en la diferenciación de las especies del género *Anthoxanthum* y (ii) caracteres que hemos observado una cierta variabilidad inter o intrapoblacional. El listado de variables empleadas es ligeramente diferente en los diversos capítulos, ya que los rasgos a tener en cuenta variaban según el problema planteado o los taxones estudiados. En términos generales, los caracteres cuantitativos y cualitativos se han analizado por separado para poder comprobar la utilidad diferencial de ambos tipos de datos (Greimler, Hermanowski & Jang, 2004), así como para evitar el proceso de transformación de los datos cuantitativos a cualitativos, que presenta un alto grado de subjetividad (Crowe, 1994; Stevens, 2000). En ningún caso, salvo en los capítulos 3 y 4, se han incluido ratios en el conjunto de las variables. De acuerdo con Goldman, Berg y Griffith (2004) el incluir estos caracteres derivados reduce la efectividad de los métodos estadísticos. En los capítulos 3 y 4, sin embargo, se han utilizado, ya que los cocientes entre distintas variables de la espiguilla y entre la longitud y anchura de la hoja son importantes en la diferenciación de los complejos *A. odoratum*/*A. alpinum* (Hedberg, 1990) y *A. aristatum*/*A. ovatum* (Paunero, 1953; Valdés, 1973) respectivamente.

Se tomaron diversas medidas para estandarizar la toma de datos y evitar las analogías. Todos los individuos recogidos estaban florecidos y completamente desarrollados. En el caso de individuos con más de un eje floral, se tuvo en cuenta el mayor de todos ellos. Las medidas foliares y del carácter de la lígula se tomaron en la segunda hoja empezando por la base de la planta, descartándose aquellos especímenes en los que la lámina foliar estuviese dañada. La anchura de la hoja se tomó en el punto más ancho de la lámina. En el caso de las medidas florales, se tomaron en la espiguilla basal de la cuarta rama empezando por la base de la inflorescencia, descartándose las plantas en las que tal espiguilla estuviese ausente. La pilosidad de la vaina se comprobó en el área comprendida entre la inserción de la segunda y la tercera hoja.

Algunas de las variables consideradas lo fueron a pesar de su naturaleza "pseudocualitativa", dada la importancia que se les viene otorgando en diversas monografías, como en el caso de la medida de intensidad de la pilosidad de las gluma y

la lema (Lauber & Wagner, 2001). Lo mismo ocurre en ciertos caracteres en los que establecer la homología es difícil, tales como las medidas de anchura de los distintos órganos (Stevens, 1984).

En todos los casos, los datos macromorfológicos cuantitativos fueron estandarizados mediante su transformación en logaritmos (Castro *et al.*, 2005). Sin embargo, las variables transformadas sólo se utilizaron cuando los resultados diferían de los obtenidos con los datos puros (Dufrêne, Gathoye & Tyteca, 1991). En el capítulo 4 se eliminaron aquellos caracteres que estaban altamente correlacionados. Esta operación no se realizó en el resto de capítulos, puesto que los diferentes métodos multivariantes empleados ya eliminan la información redundante que pudiera haber en los datos (Legendre & Legendre, 1998).

Caracteres micromorfológicos. Los rasgos de la anatomía foliar han permitido establecer las relaciones filogenéticas y la posición taxonómica de numerosas especies dentro de la familia Poaceae (*e.g.*, Davila & Clark, 1990; revisado por Giraldo-Cañas, 2003). Estos caracteres han sido utilizados también en estudios de interacción de las plantas con su medio, detectándose una gran influencia de diversos parámetros ambientales en varios aspectos de la micromorfología foliar (Connor, 1960; Böcher, 1979; Hauenstein, Arriagada & Latsague, 1990). Asimismo, la anatomía foliar se ha utilizado para estimar patrones fisiológicos en las especies (Cutler, 1978).

Los caracteres micromorfológicos analizados en el presente estudio se han registrado en la epidermis abaxial de la hoja y en el corte transversal del limbo foliar. Ambos aspectos han sido utilizados por diversos autores en la taxonomía de *Anthoxanthum* (*e.g.*, Valdés, 1973; Devesa, 1992). Para la preparación de las muestras se ha seguido el procedimiento de Devesa (1992) con ciertas modificaciones en el caso de las especies anuales, que presentan hojas muy frágiles (Pimentel & Sahuquillo, 2003b). Para una mejor comprensión de las estructuras, los cortes histológicos se tiñeron con safranina y azul-alcián según el procedimiento de Martoja y Martoja-Pierson (1970).

Para todas las especies, las preparaciones histológicas se llevaron a cabo en el tercio inferior de la segunda hoja empezando por la base. Para los caracteres cuantitativos, los datos utilizados estadísticamente para cada espécimen son la media de 12 medidas. La terminología empleada en las descripciones fue la de Ellis (1976; 1979), Devesa (1992), Nicora y Ruogolo (1987), Snow (1996) y Aliscioni y Arriaga (1998). Las variables medidas fueron las mismas para todos los capítulos en los que se tuvo en cuenta la anatomía foliar (Capítulos 3 y 4), y se seleccionaron a partir de los trabajos de Cutler

(1978), García-González (1983) y Devesa (1992).

Dado el bajo número de caracteres micromorfológicos cuantitativos analizados (9 de 32), éstos fueron transformados en binarios a pesar de la ambigüedad inherente al proceso. Para llevar a cabo la transformación se utilizó el método de "gap coding", que a pesar de ser arbitrario en exceso para estudios cladísticos o filogenéticos, se considera válido en Taxonomía Numérica (Rae, 1998).

Análisis estadísticos. Los métodos estadísticos realizados para describir y representar los patrones de variación morfológica entre las especies europeas de *Anthoxanthum* se pueden clasificar en multivariantes y univariantes. Los primeros se caracterizan por manejar datos múltiples, en los que cada organismo estudiado está caracterizado por más de una variable, mientras que en los univariantes cada variable es analizada por separado (Krzanowski, 1988).

Todos los análisis multivariantes empleados permitieron resumir, en unas pocas dimensiones, la mayor parte de la variabilidad presente en la matriz de dispersión formada por todos los casos estudiados (Legendre & Legendre, 1998). Por otro lado, los distintos análisis permitieron una representación gráfica del conjunto de los datos por medio del nuevo sistema de coordenadas generado. Los tests multivariantes llevados a cabo se dividen en: canónicos, aquellos que expresan la variabilidad de la matriz de datos en función de una variable externa o explicativa (*explanatory variable*) y no canónicos, los que simplemente representan la varianza observada en el nuevo marco de referencia (*ordination space*) (Legendre & Legendre, 1998).

Se emplearon los siguientes análisis multivariantes no canónicos: (i) El *Análisis de Componentes Principales* (PCA) (**capítulos 1-4**). Este test se usó como parte del análisis exploratorio por su capacidad para representar gráficamente la diferenciación entre los grupos en función de las nuevas variables generadas, llamadas componentes. Por otro lado, mediante este test se pudo conocer también la importancia relativa de cada carácter en la variabilidad total observada (*component loadings*) (Visauta, 1998). Previamente a la realización de este análisis, la adecuación de nuestros datos al test se estimó mediante una prueba de Kaiser-Mayer-Olkin (KMO) (Almeida-Pinheiro de Carvalho *et al.*, 2004). Por otro lado, en algunos de los análisis llevados a cabo se realizó la rotación de los ejes (rotación Varimax), con el objetivo de maximizar la varianza de cada una de las componentes (Visauta, 1998). Todos los PCA realizados en el presente trabajo se basaron en la matriz de correlaciones. (ii) El *Análisis del Escalamiento Multidimensional no Métrico* (NMDS) (**capítulo 4**) (Kruskal, 1964) se llevó a cabo en casos en los que el

método anterior fallaba en detectar variabilidad entre los grupos. En el PCA, las distancias entre los individuos en el nuevo marco de referencia generado por las componentes son las mismas que en la matriz de datos original. Por el contrario, el NMDS maximiza las distancias entre los más alejados y las minimiza entre los más próximos (Legendre & Legendre, 1998). (iii) El *Análisis de Coordenadas Principales* (PCoA) (**capítulos 1-5**) fue aplicado en el caso de las variables macro y micromorfológicas cualitativas ya que los métodos anteriores sólo son válidos para los caracteres cuantitativos. En este test, las nuevas variables generadas como combinación lineal de las originales se llaman coordenadas principales. La representación de los casos en función de estas nuevas componentes permite visualizar la diferenciación entre los individuos (Torrecilla *et al.*, 2003). (iv) Los *Análisis de Asociación* (Clustering analyses) (**capítulos 1-5**), de especial interés ya que tienen como objetivo la organización del conjunto de individuos en unidades más pequeñas en base a su proximidad. Este procedimiento permitió, asimismo, que la variabilidad de los datos se resumiese en una estructura jerárquica llamada dendrograma (Legendre & Legendre, 1998). Los Análisis de Asociación se han llevado a cabo con los datos macro y micromorfológicos, tanto cualitativos como cuantitativos, radicando la diferencia en la unidad de distancia elegida y en el método de asociación. Para los datos cualitativos se ha utilizado la distancia Phi, que valora tanto las presencias como las ausencias. En el caso de las variables cuantitativas, la distancia empleada fue la Euclídea, de uso tradicional en Taxonomía (Rohlf, 1993). Con respecto a los métodos de agrupación, fueron utilizados UPGMA (*Unweighted pair-group method, arithmetic average*) y *Neighbor-joining*.

Los análisis estadísticos canónicos se emplearon como métodos de contraste de las hipótesis taxonómicas o ecológicas planteadas en las diferentes fases de este estudio. Los tests canónicos utilizados para los caracteres cuantitativos fueron: (i) El *Análisis Canónico de Varianzas* (CVA) (**capítulos 2, 4**) (Legendre & Legendre, 1998). En este análisis, los agrupamientos de individuos, se proponen *a priori* y son reflejo de las hipótesis que se desean verificar, ya sean ecológicas o taxonómicas. El test busca diferencias significativas entre los grupos predefinidos en base a las supuestas variables explicativas que se establecen inicialmente. Asimismo, este análisis permite conocer la importancia relativa de cada uno de los caracteres analizados en las funciones discriminantes que diferencian a los grupos propuestos (Legendre & Legendre, 1998). (ii) El *Análisis Discriminante de Clasificación* (CDA) (**capítulos 2, 3**). Está muy relacionado con el CVA. En este análisis también se define el número de grupos *a prioi* pero indicando el grupo al que cada caso pertenecerá con mayor probabilidad (Legendre & Legendre,

1998; Repka, 2003). Las funciones discriminantes se calculan tantas veces como individuos haya en la muestra y, en cada cálculo, uno de los casos es excluido de la prueba. A continuación, cada uno de los individuos excluidos es clasificado teniendo en cuenta las funciones generadas por el resto de la muestra, registrándose si pertenece o no al grupo esperado (Legendre & Legendre, 1998). Para los caracteres cualitativos, el test empleado fue: (iii), El *Análisis Canónico de Correspondencias* (CCA) (**capítulos 2, 5**) (Ter Braak & Smilauer, 1998). Investiga la relación entre los caracteres anatómicos y diversos parámetros ambientales. Este test estadístico permite correlacionar la matriz de datos con una matriz respuesta (*response matrix*), constituida por el conjunto de las condiciones ambientales estudiadas.

En los distintos apartados de esta tesis se han utilizado, además, diversos procedimientos estadísticos univariantes. En los casos en los que se comprobó la normalidad de los datos (**capítulo 1**), la comparación entre las medias de los distintos grupos se realizó mediante el test *t* de Student. Por otro lado, para la detección de híbridos entre *A. ovatum* y *A. aristatum* (**capítulo 4**) se utilizó el procedimiento del "*recuento de caracteres*" (*character count procedure*, Wilson, 1992). En este método, el número de caracteres que, en el presunto híbrido, presentan un fenotipo intermedio entre los padres, es comparado con el número de rasgos no intermedios mediante un test *de los signos*. Con respecto a los caracteres cualitativos, la comparación entre los grupos se estableció por medio de las *Tablas de Contingencia de una vía*, prueba análoga al AMOVA para las variables cuantitativas (Legendre & Legendre, 1998) (**capítulos 2-4**). Como test asociado a la Tabla de Contingencia se calcularon los valores Lambda, que son medidas de asociación entre variables. Estos valores indican en qué porcentaje se reduce el error cometido al predecir el valor de la variable dependiente si la independiente es tenida en cuenta (Visauta, 1998).

Para realizar los tests estadísticos; CVA, PCA, CDA, Tablas de Contingencia y test *t* de Student, se utilizó el programa SPSS 11.5 para Windows (SPSS Inc., Chicago). Por otro lado, los PCoA, NMDS y el Análisis de Asociación se realizaron con el programa NTSYS pc Versión 2.1 (Rohlf, 2002). Para la representación gráfica de los dendrogramas se utilizó el programa MEGA2 Versión 2.1 (Kumar *et al.*, 2001). Finalmente, el CCA se calculó mediante el programa CANOCO Versión 4.0 (Ter Braak & Smilauer, 1998).

ESTUDIO MOLECULAR

El desarrollo de las técnicas moleculares basadas en la PCR han permitido un gran avance en el estudio de la distribución de la variabilidad genética a distintos niveles, desde el poblacional hasta el supraespecífico (Karp, Seberg & Buiatti, 1996; Segarra-Moragues, 2004). A su vez, el conocimiento de la proximidad genética entre los taxones y las poblaciones ha propiciado un gran desarrollo de los estudios biogeográficos y taxonómicos en plantas. En la actualidad existe una gran variabilidad de marcadores moleculares disponibles, y la elección de uno o de otro dependerá de los objetivos que se desee conseguir. De acuerdo con Segarra-Moragues (2004), es necesario el uso conjunto de varias técnicas moleculares para abordar el estudio de especies cercanas, como es el caso de los taxones europeos del género *Anthoxanthum*.

En el presente trabajo se han empleado dos tipos de marcadores moleculares diferentes, los RAPDs (Random Amplified Polymorphic DNA; Williams *et al.*, 1990) y los AFLPs (Amplified Fragment Length Polymorphism; Vos *et al.*, 1995). A continuación se describirán los aspectos más importantes de ambas técnicas.

***Random Amplified Polymorphic DNAs*, (capítulos 2, 4).** Esta técnica se basa en los principios de la PCR. Para la obtención del perfil de bandas que caracteriza a cada muestra o individuo, al DNA de la muestra se le añade un único primer (generalmente decámero) de secuencia aleatoria, de forma que se unirá a gran cantidad de sitios complementarios en la cadena molde, tanto en el sentido directo como en el reverso. Cuando algunos de estos sitios de unión estén orientados de manera opuesta, y lo suficientemente cerca para permitir la amplificación, se generará un producto en la PCR (Cerdán *et al.*, 1997; Segarra-Moragues, 2004). Para la visualización de los resultados, alícuotas de la reacción de PCR son sometidas a electroforesis de agarosa o acrilamida, siendo registrada la presencia o ausencia de bandas como una variable binaria (0/1). En la interpretación de los resultados, los fragmentos comigrantes son considerados homólogos y representantes del mismo alelo. Esta asunción es generalmente aceptada a nivel infraspecífico o entre especies muy relacionadas.

Las principales ventajas de los RAPDs como marcadores moleculares son: (i) son marcadores hipervariables, esto es, detectan una gran cantidad de polimorfismo a escala individual (Williams *et al.*, 1990; Segarra-Moragues, 2004). (ii) Los puntos de unión del decámero de secuencia aleatoria están distribuidos a lo largo de todo el genoma, por lo que mediante este método se registra toda su variabilidad. (iii) De entre los diversos métodos de análisis genético, son de los más rápidos y que requieren un menor coste. Por

otra parte, la concentración del ADN de partida es menor que en otros marcadores dominantes como los AFLPs. Entre las desventajas, su herencia es dominante, lo que no permite diferenciar homozigosis de heterozigosis. Por otro lado, estos marcadores presentan una baja reproducibilidad, y hay una alta probabilidad de homoplasia cuando se analizan grupos no muy próximos entre si (Thormann *et al.*, 1994; Catalán *et al.*, 1995).

Dadas sus propiedades, los RAPDs han sido muy utilizados en estudios taxonómicos en grupos complejos, en los que los caracteres morfológicos no presentaban suficiente resolución (Díaz-Lifante & Aguinagalde, 1996; Zeid *et al.*, 1997; Lázaro & Aguinagalde, 1998; Klaas, 1998; Adams, 2000; Jorgensen *et al.*, 2003; Román *et al.*, 2003; revisado en Segarra-Moragues, 2004). Asimismo, se ha demostrado la eficacia de estos marcadores moleculares en estudios genético-poblacionales y filogeográficos (Gabrielsen *et al.*, 1997; Scheen, Elven & Brochmann, 2002; Segarra-Moragues & Catalán, 2003).

***Amplified Fragment Length Polymorphisms*, (capítulos 3, 5).** En esta técnica, la obtención de los perfiles de bandas para cada muestra mayor complejidad que en los RAPDs (Vos *et al.*, 1995; Segarra-Moragues, 2004). Los AFLPs fueron diseñados originalmente para la construcción de mapas genómicos, y están basados en la detección de fragmentos de restricción mediante amplificación por PCR. El protocolo de los AFLPs se articula en las siguientes fases. (i) Restricción del ADN. Se utilizan dos enzimas diferentes (en nuestro caso *EcoR* I y *Mse* I), una de corte frecuente y otra que posee pocas secuencias diana en el genoma ("de corte raro"). La enzima de corte frecuente produce gran cantidad de fragmentos del tamaño adecuado para su amplificación. Sin embargo, la enzima de corte raro actuará como limitante del número de marcadores generados, ya que se amplificarán preferentemente aquellos fragmentos con extremos diferentes (Vos *et al.*, 1995). De este modo, será el número de dianas de restricción de la enzima de corte raro la que determine el número de amplicones. (ii) En una segunda fase, la de ligamiento, se introducen fragmentos "adaptadores" de doble cadena, específicos de los extremos cohesivos de los fragmentos de restricción, a los que se unen mediante una ligasa. (iii) En la fase de amplificación, se añaden primers complementarios de las secuencias formadas por las zonas de restricción y los adaptadores. Estos primers tienen una serie de bases extra, llamadas bases selectivas, que reducen el número de amplicones generados por PCR, lo que permite la visualización de los resultados. La amplificación se puede llevar a cabo en una o varias fases y mediante el uso de distintas bases selectivas se puede modular el número de amplicones producidos (Vos & Kuiper, 1998). Sin embargo, el

tamaño del genoma debe ser tenido en cuenta a la hora de tomar una decisión sobre el número de bases selectivas que se ha de utilizar (Fay, Cowan & Leitch, 2005).

Para la visualización de los resultados, alícuotas del resultado de la PCR de amplificación son sometidas a electroforesis en gel de acrilamida y posterior tinción con Nitrato de Plata (Bassam, Caetano-Anolles & Gresshofff 1991). Como en la técnica anterior, los fragmentos comigrantes se consideran homólogos, y de nuevo la presencia o ausencia de las bandas se registra como una variable binaria (1/0).

Las principales ventajas de la técnica de los AFLPs coinciden en parte con las de los RAPDs. Son también marcadores hipervariables que analizan la variabilidad de todo el genoma. Sin embargo, los AFLPs ofrecen una mayor reproducibilidad. Entre las desventajas, algunas también son compartidas, como el hecho de que presenten herencia dominante (y por lo tanto, el homocigoto es indistinguible), o que exista un alto riesgo de homología cuando se comparan grupos alejados. Por otro lado, la técnica de los AFLPs requiere una mayor cantidad de ADN de partida y es notablemente más laboriosa. Distintos estudios en los que el uso de ambos marcadores moleculares ha sido comparado, muestran una alta coherencia entre los resultados (Palacios & González-Candelas, 1997; Palacios, Kresovich & González-Caldelas, 1999).

Los AFLPs han sido muy utilizados para resolver problemas taxonómicos en grupos complejos, en los que la morfología o el ADN cloroplástico no mostraron variabilidad (Schaeffer *et al.*, 2002; Zhang & Kadereit, 2002; Richardson *et al.*, 2003). Asimismo, se han empleado en estudios genético-poblacionales y biogeográficos (*e.g.*, Sasanuma, Endo & Ban, 2002).

Análisis genéticos y estadísticos. Tanto en los estudios de AFLPs como en los de RAPDs, el perfil de bandas de cada individuo se registra en una matriz binaria (0/1) reflejo de la ausencia o presencia de las bandas observadas. Para calcular la diversidad genética de las poblaciones se utilizan distintos estimadores basados en la frecuencia de las bandas. Estos estimadores son independientes del nivel de ploidía y no requieren una codificación del genotipo, que será imposible en los marcadores dominantes (Segarra-Moragues, 2004). En el presente trabajo se han utilizado los índices de Nei (Nei, 1987) y el de Shannon-Weaver (según Legendre & Legendre, 1998) (**capítulos 2, 4, 5**). Otros parámetros calculados habitualmente para describir el polimorfismo genético de las poblaciones son el índice de alelos raros (N_{RI}) y el de alelos privados (N_p) (Després *et al.*, 2002; Schönswetter *et al.*, 2004).

Para el cálculo de las distancias genéticas entre individuos se han empleado distintas medidas, concretamente los índices de similaridad de Dice y Jaccard y la distancia Euclídea. El índice de Dice es considerado especialmente válido por no tener en cuenta las dobles ausencias, que pueden estar producidas por fallos en la PCR (Rohlf, 1993). En nuestro caso se estimaron las distancias con los tres algoritmos, y su equivalencia se comprobó mediante un test de correlación de Mantel (Mantel, 1967).

Hemos empleado diversos análisis estadísticos multivariantes para resumir la variabilidad genética observada y representarla gráficamente. Entre estos métodos, el Análisis de Coordenadas Principales (PCoA) y los Análisis de Asociación (UPGMA y *Neighbor-joining*) ya han sido mencionados en el apartado de estudio morfométrico. Además, se aplicó el *Minimum Spanning Tree* (Gower & Ross, 1969) (**capítulos 2-5**) que resulta muy útil para detectar distorsiones en los datos y outliers multivariantes mediante su superposición con el PCoA (Rohlf, 1993).

La diferenciación genética entre las poblaciones se calculó por medio del estimador no sesgado F_{ST} de Weir y Cockerham (1984) (**capítulos 2, 4, 5**). Para este análisis se utilizó el programa TFGPA (Miller, 1997) que permite calcular el F_{ST} a distintos niveles jerárquicos. Por último, se establecieron niveles de confianza para los estimadores mediante el procedimiento bootstrap.

Para calcular la distribución de la varianza genética entre los distintos niveles establecidos, ya sean taxonómicos o geográficos, se utilizó el *Análisis de Varianza Molecular* (AMOVA) (**capítulos 2, 4, 5**) (Excoffier, Smouse & Quattro, 1992), que implica la definición, *a priori*, de una estructura jerárquica en las poblaciones (Vila, 2004). En esta prueba, la suma de los cuadrados de las distancias genéticas calculada es distribuida entre los distintos niveles jerárquicos. Por otro lado, los intervalos de confianza para la varianza se establecieron mediante un test de permutación. El AMOVA se llevó a cabo con el programa ARLEQUIN 2000 (Schneider *et al.*, 2000).

El test de correlación de Mantel (Mantel, 1967; Legendre & Legendre, 1998) se calculó para cuantificar la distancia genética entre pares de individuos en relación a su distancia geográfica (*e.g.*, Stehlik, Schneller & Bachmann, 2001; Després *et al.*, 2002). Para todos los tests de Mantel, se calculó el estadístico normalizado R_M, cuya significación se estableció mediante un test de permutación.

El test de Mantel (**capítulos 2, 4, 5**) se utilizó de dos maneras diferentes. (i) Mediante la comparación directa entre la matriz de distancias genéticas y la matriz de distancias

geográficas medidas en un mapa y, (ii) mediante la construcción de correlogramas, en los que las diferencias genéticas entre las poblaciones se representan como función de las distancias geográficas que las separan. Este segundo procedimiento, que puede realizarse a distintos niveles (regional, poblacional, etc.), se basa en la transformación propuesta por Oden y Sokal (1986), por la que la matriz de distancias geográficas se transforma en una matriz modelo que representa la hipótesis que se desea verificar (Legendre & Legendre, 1998; Stehlik, Schneller & Bachmann, 2001).

Finalmente, para identificar la repartición de las poblaciones más acorde con los datos, se aplicó el método Bayesiano de asignación propuesto por Pritchard, Stephens y Donnelly (2000) (**capítulo 4**). Este método es implementado por el algoritmo Markov Chain Monte Carlo (MCMC) y no requiere ningún conocimiento previo de la estructura de las poblaciones (Caizergues *et al.*, 2003). Estos análisis se llevaron a cabo con el programa STRUCTURE 2.1 (Pritchard, Stephens & Donnelly, 2000), que asigna a los individuos a un número predefinido de subgrupos (*K*) y devuelve un valor de probabilidad para cada partición de los datos. Para marcadores dominantes, como los RAPDs o los AFLPs, se asume el modelo de *non admixture*, según el cual cada individuo pertenece en exclusiva a una población (Carlsson, Söderberg & Tegelström, 2003; Pritchard & Wen, 2003). Para seleccionar el número más apropiado de subpoblaciones se siguió el método de Evanno, Regnaut y Goudet (2005).

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Objetivos

La revisión bibliográfica realizada sobre el género *Anthoxanthum* ha puesto de manifiesto que las relaciones taxonómicas y biogeográficas entre los taxones que conforman este género todavía no han sido resueltas. En la actualidad, tanto la caracterización del género como de los taxones que lo componen sigue siendo fuente de controversias debido a que se basa en caracteres no fácilmente observables que presentan cierta variación en el espacio y a lo largo del tiempo. Los trabajos realizados hasta el momento no permiten obtener conclusiones generales dado que el estudio de las especies europeas del género nunca se ha abordado globalmente. Asimismo, el número de individuos utilizado suele ser bajo y proceder de muestras de herbario o de jardín botánico. Esto implica que el grado de representatividad de las poblaciones analizadas con respecto al área de distribución de los taxones suele ser limitado y, la caracterización de los taxones, subjetiva.

Por todo ello, se ha planteado la realización de este estudio intentando abarcar la mayor representación posible del género en el ámbito europeo, analizar los taxones desde una perspectiva poblacional y complementar los datos morfológicos, anatómicos, ecológicos obtenidos con datos moleculares que permitan establecer las relaciones biogeográficas a nivel poblacional y taxonómico.

Los objetivos planteados son los siguientes:

-Estudiar a nivel poblacional y específico el valor discriminatorio de los caracteres morfológicos y anatómicos utilizados tradicionalmente en la diferenciación de los taxones incluidos en el género *Antoxanthum* en el ámbito europeo.

-Profundizar en las relaciones biogeográficas y taxonómicas del complejo formado por *A.odoratum* y *A.alpinum* analizando sus poblaciones desde un punto de vista morfológico, anatómico y genético.

-Utilizar métodos morfométricos y moleculares para clarificar las relaciones taxonómicas en las especies anuales *A. aristatum* y *A. ovatum* y sus taxones infraespecíficos.

-Analizar la influencia de los factores ambientales en la caracterización de *A. amarum*, su relación con *A. odoratum* y su organización biogeográfica.

Capítulo 2

The European sweet vernal grasses (*Anthoxanthum* L., Poaceae, Pooideae, Aveneae): a morphological and anatomical analysis

The European sweet vernal grasses (Anthoxanthum L, Poaceae, Pooideae, Aveneae): a morphological and anatomical analysis

ABSTRACT

Genus *Anthoxanthum* includes around 20 species from Europe, Asia and Africa that have not been well characterized morphologically. In the present study, multivariate analyses were carried out in order to assess the taxonomic relationships among the European species of the genus. An overall of 1787 *Anthoxanthum* specimens from all over Europe were analysed. Thirty macromorphological (13 quantitative and 17 qualitative) and 26 microanatomical characters were considered in the study. A principal components analysis was applied to quantitative macromorphological data in order to find out the most discriminant traits. Next, a canonical discriminant analysis was conducted to assess the accuracy of the present taxonomic structure of the genus. Regarding qualitative and micromorphological data, principal coordinates analyses and contingency tables were applied summarize the relationships among samples and to look for the most discriminant traits. Finally, dendrograms were constructed with both sorts of traits to dislay the pairwise distances between specimens. According to our results, only two taxa, *Anthoxanthum gracile* Biv. and *Anthoxanthum amarum* Brot. were clearly differentiated morphologically. In addition to this, two groups of species were observed: an annual group, comprising the diploids *Anthoxanthum ovatum* Lag. and *Anthoxanthum aristatum* Boiss. and a perennial one, including *Anthoxanthum maderense* Teppner, *Anthoxanthum alpinum* Á & D Löve and *Anthoxanthum odoratum* L. Moreover, most of the traits that have been traditionally used in the differentiation of these taxa were not able to characterize them in this study.

ADDITIONAL KEYWORDS: *Anthoxanthum* L. - canonical discriminant analysis - Europe - leaf anatomy - morphometrics - Numerical Taxonomy - multivariate statistics.

INTRODUCTION

Anthoxanthum L. *s. str.* (not including *Hierochloë* R. Br.) has traditionally comprised between 15 and 20 annual and perennial species (Clayton, 1970; Dahlgren, Clifford & Yeo, 1985; Nicora & Rúgolo de Agrassar, 1987). *Anthoxanthum* is naturally distributed mainly in temperate and arctic-alpine regions of Europe, Asia and Africa (Polunin, 1959; Clayton & Renvoize, 1986; Watson & Dallwitz, 1992). Besides, it has been introduced in wide areas of America and Australia (*e.g.*, Cheeseman, 1906; Tovar, 1993). More recently, different studies based on the evolution of the spikelet have pointed out that the separation between *Anthoxanthum* and the broad genus *Hierochloë* might be artificial (Schouten & Veldkamp, 1985; Soreng, 2003). However, in the present study, European species belonging to former genus *Hierochloë* have not been considered for the analysis. On the one hand, European species of *Hierochloë* can be easily distinguished morphologically. On the other hand, the transitional forms that justify the merging of both genera do not exist in the European Flora.

Eight *Anthoxanthum* species have been described in Europe and the Macaronesia (Tutin, 1980; Teppner, 1998), and different taxonomic arrangements have been proposed to encompass the variability observed in these regions (*e.g.*, Willkomm & Lange, 1870; Paunero, 1953; Valdés, 1973; Tutin, 1980). However, the systematics of this group of species is still puzzling because of the extreme morphological diversity and ambiguous boundaries among the taxa, most of which have not been well characterized morphologically. Among the European sweet vernal grasses, five are perennials (*Anthoxanthum odoratum* Boiss., *Anthoxanthum alpinum* Löve & Löve, *Anthoxanthum maderense* Teppner, *Anthoxanthum amarum* Brot. and *Anthoxanthum puciflorum* Adamovic) and three annuals (*Anthoxanthum gracile* Biv., *Anthoxanthum aristatum* Boiss. and *Anthoxanthum ovatum* Lag.). However, this distinction is rather unclear, since *A. odoratum* can behave as annual, and bisannual plants of *A. aristatum* and *A. ovatum* have been found (Borrill, 1963; Hedberg, 1964). The morphological differentiation of the these taxa has been traditionally based on plant height and spikelet length, sterile florets shape, spike colour, plant pubescence and ligule type. Nevertheless, none of this traits is able to ditinguish unambiguously among them (*e.g.*, Tutin, 1980; López-González, 1994; Pimentel & Sahuquillo, 2003a).

Among the European sweet vernal grasses, the ecological, karyological, anatomical and biogeographical relationships between the widespread perennial tetraploid *A. odoratum* and the arctic-boreal diploid *A. alpinum* have been widely studied (*e.g.*, Tutin,

1950; Rozmus, 1958, 1960; Borrill, 1962; Böcher, 1961; Jones, 1964; Hedberg, 1970, 1990; Teppner, 1970; Pundeva, 1974; Felber, 1988; Felgrová & Krahulec, 1999). Moreover, the systematic position of the Mediterranean annuals *A. aristatum* and *A. ovatum* and their infraspecific taxa has been also addressed by different authors (*e.g.*, Valdés, 1973; López-González, 1994). Conversely, few attention has been paid to the other European *Anthoxanthum* species, and a thorough taxonomical study of the whole genus based on statistical methods is lacking. The present work deals with the different European species of *Anthoxanthum,* and aims to statistically analyze morphological and anatomical variation in relation to boundaries among taxa. Moreover, the usefulness of the traits that have been traditionally used to delimit species within the genus is evaluated. The use of statistical methods has rendered very useful results in plant systematics (*e.g.*, Sneath & Sokal, 1973; Baum, 1986). In addition to this, the importance of micromorphological traits in the taxonomy of the Poaceae has been highlighted by different authors (reviewed in Giraldo-Cañas, 2003).

In the present study, the rather unknown taxon *A. pauciflorum* has not been considered since, according to Tutin (1980) and to our observations of herbarium material, this taxon is an extreme form of the widespread *A. odoratum*.

MATERIALS AND METHODS

PLANT MATERIAL

A total of 1787 plants belonging to the European species of *Anthoxanthum s. str.* but *A. pauciflorum* were sampled on 60 European populations, several of which were sympatrid. Collection sites were subjectively chosen in order to cover a high morphological, ecological and biogeographical variation. To identify the most interesting areas, we examined specimens deposited in several European Herbaria (SANT, UNEX, BC, MA, LOU, SEV, VAL, UPS, G, BEO, WU, CLF, FI, and PAL. For Herbarium codes see Holmgren, Holmgren & Barnett, 1990). The selection of localities was also based on different publications on the genus (*e.g.*, Paunero, 1953; Hedberg, 1967; Valdés, 1973; Felber, 1988). In addition to this, six individuals belonging to *Anthoxanthum aethiopicum* Hedberg and *Anthoxanthum nivale* K. Schum. from East Africa were included as outgroups. These specimens were borrowed from the Kew Gardens Herbarium (K, for voucher details see Appendix 2.1). These African species were chosen as outgroups for

they are closely related morphologically to European *Anthoxanthum* taxa, but their populations are totally isolated with respect to the European species.

In overall, 1793 adult and flowering plants were considered on the analysis. Between 15 and 140 plants per locality were dried and employed in the morphometric (from 15 to 120 specimens) and anatomical (up to 20 plants) studies. The number of collected individuals depended on the morphological and taxonomical variability observed in the population. All the specimens gathered were tentatively identified following Paunero, (1953) and Valdés, (1973). The origin of plant material is listed on Appendix 2.1, and voucher specimens were deposited in the SANT Herbarium. Outgroups were excluded from anatomical analyses.

MORPHOMETRIC AND ANATOMICAL DATA

The morphometric analysis was performed by measuring 30 macromorphological characters (Table 2.1) that were selected according to: (i), their common use in *Anthoxanthum* taxonomy; (ii), the variability observed between or within populations and (iii), other traits of interest in the grasses taxonomy were also included. Among the considered characters, 13 were quantitative and 17 qualitative. All the qualitative traits were scored as binary variables. Each measured specimen was treated as an independent operational taxonomic unit (OTU) for all the statistical tests but the cluster analysis performed with quantitative data. Given the high volume of data, for this latter analysis the measurements for all specimens of a population were averaged into one OTU score for each of the characters (following Abdel-Khalik *et al.*, 2002). We constructed different datasets for qualitative and quantitative traits in order to calibrate the different usefulness of each type of data (Legendre & Legendre; Greimler, Hermanowski & Jang, 2004).

For the anatomical analysis, we measured 29 anatomical traits (14 from the epidermal surface and 15 from the leaf-blade as viewed in transverse section, Table 2.2). Samples were processed following Devesa (1992) and Pimentel & Sahuquillo (2003b), with minor modifications for the annual species, that present specially fragile leaves. The terminology used was that by Ellis (1976, 1979). All the quantitative anatomical data obtained per specimen are the mean value of twelve different measures. Finally, figures were transformed into binary variables following the gap coding method (Rae, 1998). Data from the abaxial surface and the leaf transverse section were analysed separately.

QUANTITATIVE CHARACTERS	QUALITATIVE CHARACTERS
Plant height (cm) (PS)	Life cycle (LC) (0 annual, 1 perennial)
Leaf length[1] (cm) (LL)	Presence of stolons (0 non-stoloniferous, 1 stoloniferous) (S)
Leaf width[1] (cm) (LW)	Presence of bulbils (0 bulbils present, 1 bulbils absent) (B)
Inflorescence length (cm) (IL)	Sheath pubescence (0 sheath glabrous, 1 sheath hairy) (SP)
Spike lower branch length (cm) (SLBL)	Convolute leaves (0 planar, 1 convolute) (CL)
Spike lower internode length (cm) (SLIL)	Ligule shape (0 round, 1 pointed) (LS)
Spikelet length[2] (cm) (SpL)	Ligule fimbriate (0 ligule entire, 1 ligule fimbriate) (LF)
Upper glume width (cm) (UGW)	Reddish spikelets (0 green spikelets, 1 spikelets reddish) (R)
Lower glume length (cm) (LGL)	Presence of pruine (Pr) (I)
Upper sterile floret length (cm) (STL)	Lower glume pubescence (LgP) (II)
Upper sterile floret width (cm) (STW)	Upper glume pubescence (UGP) (III)
Awn length (cm) (AL)	Mucronate glumes (MG) (IV)
Fertile floret length (cm) (FfL)	Upper sterile lemmas pubescence (SFP) (V)
	Sterile lemma hairs longer than sterile lemma (SFL) (VI)
	Presence of a forked apex on upper sterile lemma (N) (VII)
	Amount of overlap of fertile floret by sterile lemmas (C) (VIII)
	Fertile floret scabridity (FP) (IX)

Table 2.1. List of characters measured for morphometric analyses. Character codes are listed between parenthesis. [1] Characters measured in the second leaf from the base of the plant; [2] Characters measured in the fourth spikelet form the base of the inflorescence. (I) Leaves not pruinose (0) or pruinose (1); (II) Lower glume glabrous (0) or hairy (1); (III) Upper glume glabrous (0) or hairy (1); (IV) glumes not mucronate (0) or mucronate (1); (V) Upper sterile lemma glabrous (0) or hairy (1); (V) Sterile lemma hairs shorter (0) or longer than the sterile lemma (1); (VI) Sterile lemma hairs shorter (0) or longer than the sterile lemma; (VII) Upper sterile lemma without (0) or with a forked apex; (VIII) Fertile floret not covered by the sterile lemmas (0) or covered (1); (IX) fertile floret smooth (0) or hairy (1).

STATISTICAL ANALYSES

For all morphometric quantitative traits, exploratory analyses including boxplots, descriptive statistics, the coefficient of variation (*cv*) and Kolmogorov-Smirnov normality tests were performed on each population to eliminate outliers. Subsequently, to limit the influence of allometry on the results, quantitative data were $\log_{10}$ transformed (Dufrêne, Gathoye & Tyteca, 1991; Almeida-Pinheiro de Carvalho *et al.*, 2004). However, comparison with the results obtained from the original characters indicated only minor differences with logarithmically transformed variables. Therefore, we decided to use the original data set.

	EPIDERMAL TRAITS	LEAF BLADE TRANSVERSE SECTION
intercostal zones	Abaxial epidermis type (^{A})	Bundle sheath outline (0, round; 1, elliptical)
	Long cell type (^{B})	Bundle sheath cell shape (0, round; 1, angular)
	Long cells length* (^{E}) (LCI)	Bundle sheath cells equal in size (0) or not (1)
	Presence of short cells (0, absent; 1, present) (SI)	Median vb. sheath type (0, single; 1, complete)
	Short cells length* (^{G}) (LSI)	Median vb. sheath cell diameter (0, < 15 μm; 1, > 15 μm) (M)
	Exod. elements (0, presence; 1, abscence)	N° of cells in vb sheath (0, < 17 cells; 1, > 17 cells)
	Stomate type (0, parallel-sided; 1, dome-shaped)	N° of bulliform cells per group (0, < 6 cells; 1, > 6 cells)
costal zones	Long cell type (^{B})	N° of vb. per leaf (0, <15; 1, > 15) (VB)
	Long cell length* (^{F}) (LCN)	Median vb. type (^{D}) (VBT)
	Presence of short cells (0, absent; 1, present) (SN)	Median vb. diameter (^{C}) (0, <75 μm; 1, > 75 μm) (VBD)
	Short cell length (0, < 50 μm; 1, > 50 μm)	Presence of a radial chlorenchyma (0, absent; 1, present)
		Colorless parenchyma (0, absent; 1, present)
	Exod. elements (0, presence; 1, abscence)	Ribs and furrows (0, absent; 1, present)
	Presence of bulliform cells (0, absent; 1, present)	Developed keel (0, absent; 1, present)
	Leaves pruinose (0, not pruinose; 1, pruinose)	Bulliform cell diameter (0, < 30 μm; 1, > 30 μm)

Table 2.2. List of anatomical traits considered in the analyses. (A), abaxial epidermis homogeneous (0) or siliceous-homogeneous (1). (B), long cells l2 (0) or l3 (1) Devesa (1992). (C), maximum diameter of the vascular bundle measured horizontally. (D), median vascular bundle free or joined to the epidermis by schlerenchyma girders. (*) Multistate characters. Each of the states is interpreted as an independent binary variable. (E), LCI1, 101-200 μm; LCI2, 301-400 μm; LCI3, 401-500 μm; LCI4, 501-600μm; LCI5, 601-700μm. (F), LCN1, (101-200μm; LCN2, 201-300μm; LCN3, 301-400μm; LCN4, 401-500μm. (G), LSI1, 11-20μm; LSI2, 21-40μm; LSI3, 41-50μm). Exod. elements, exodermic elements (Devesa, 1992). vb., vascular bundle.

A correlation-based principal component analysis (PCA) was used as an objective method to summarize variation patterns produced by all characters. This procedure allowed the variance within all the phenotypic traits to be considered simultaneously (Sargent *et al.*, 2004). Prior to the PCA, a Kaiser-Meyer-Olkin (KMO) test was performed to assess the suitability of our data for multivariate analyses (Almeida-Pinheiro de Carvalho *et al.*, 2004). The varimax rotation was used to maximize the variance of each factor (Visauta, 1998). Subsequently, a classification discriminant test (CDA, cross-validation) was carried out to determine the group to which each specimen belonged with the highest probability (Legendre & Legendre, 1998; Saint-Laurent *et al.*, 2000; Repka, 2003). Two different arrangements of specimens were tested in this analysis: (i) specific classification following Tutin (1980) and (ii) PCA-based clustering of species.

The differentiation among taxa was also assessed by means of Student's *t*-tests that were performed for each quantitative character accross every species pairs. Finally, the

Euclidean distance among each pair of individuals was estimated, and the relationships among specimens and populations was represented in a dendrogram using the UPGMA clustering method.

Regarding qualitative and anatomical traits, the statistical treatment was the same for both sorts of data. First, a principal coordinate analyses (PCoA) was performed to represent the relationships among specimens and populations. Secondly, univariate contingency tables were constructed and the Lambda coefficients were estimated for each qualitative character and for both groupings of populations (Legendre & Legendre, 1998; Saint-Laurent *et al.*, 2000). Finally, the relationships among samples were established by means of an UPGMA cluster analysis based on the Phi coefficient.

All the statistical tests but the PCoA and the cluster analysis were performed with the program SPSS 11.5 for Windows (SPSS Inc., Chicago, USA). The program NTSYS pc Version 2.1 (Rohlf, 2002) was used to construct the PCoA and the dendrograms.

RESULTS

MACROMORPHOLOGICAL ANALYSES

Descriptive statistics and boxplots showed that only two taxa, *A. amarum* (a high polyploid endemism from NW Iberian Peninsula) and *A. gracile* (an annual diploid with a rather unknown Mediterranean distribution) were clearly differentiated. Conversely, the remaining species overlapped in all the studied quantitative characters. Vegetative traits such as plant height and leaf width were specially important differentiating *A. amarum*, whilst floral characters such as awn and sterile floret length characterized *A. gracile* (Figure 2.1). Regarding the coefficient of variation (Table 2.3), *A. aristatum* and *A.*

TRAITS	*A. amarum*	*A. aristatum*	*A. odoratum*	*A. ovatum*	*A. maderense*	*A. gracile*	*A. alpinum*	MEAN
PS	0.29	0.46	0.34	0.49	0.21	0.18	0.33	0.33
LL	0.28	0.55	0.46	0.49	0.27	0.26	0.42	0.39
LW	0.32	0.37	0.41	0.39	0.19	0.16	0.26	0.30
IL	0.20	0.29	0.26	0.25	0.17	0.18	0.20	0.22
SLBL	0.19	0.22	0.25	0.20	0.18	0.14	0.21	0.20
SLIL	0.32	0.36	0.34	0.40	0.27	0.36	0.32	0.34
SpL	0.10	0.13	0.09	0.08	0.05	0.05	0.10	0.09
UGW	0.08	0.13	0.16	0.11	0.11	0.04	0.16	0.12
LGL	0.12	0.13	0.12	0.12	0.06	0.07	0.11	0.11
STL	0.12	0.21	0.11	0.22	0.05	0.04	0.11	0.12
STW	0.22	0.28	0.46	0.42	0.09	0.07	0.24	0.26
AL	0.10	0.22	0.1	0.21	0.04	0.03	0.10	0.12
FfL	0.13	0.17	0.09	0.12	0.04	0.04	0.08	0.1
MEAN	0.19	0.28	0.25	0.27	0.13	0.12	0.21	
MEAN CV IN VEGETATIVE CHARACTERS: 0.31					MEAN CV IN FLORAL CHARACTERS: 0.13			

Table 2.3. Coefficient of variation (CV) observed for each trait accross the different taxa. For character codes see Table 2.1.

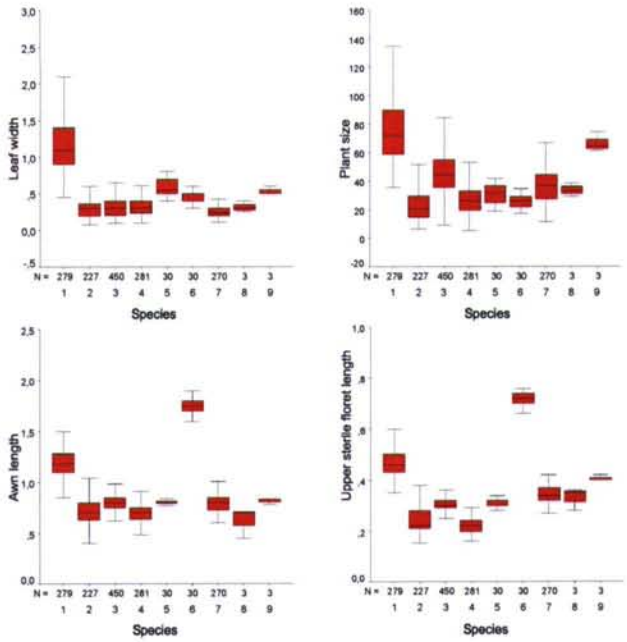

Figure 2.1. Boxplots for plant height, leaf width, uppersterile floret length and awn length. 1, *A. amarum*; 2, *A. aristatum*; 3, *A. odoratum*; 4, *A. ovatum*; 5, *A. maderense*; 6, *A. gracile*; 7, *A. alpinum*; 8, *A. aethiopicum*; 9, *A. nivale*.

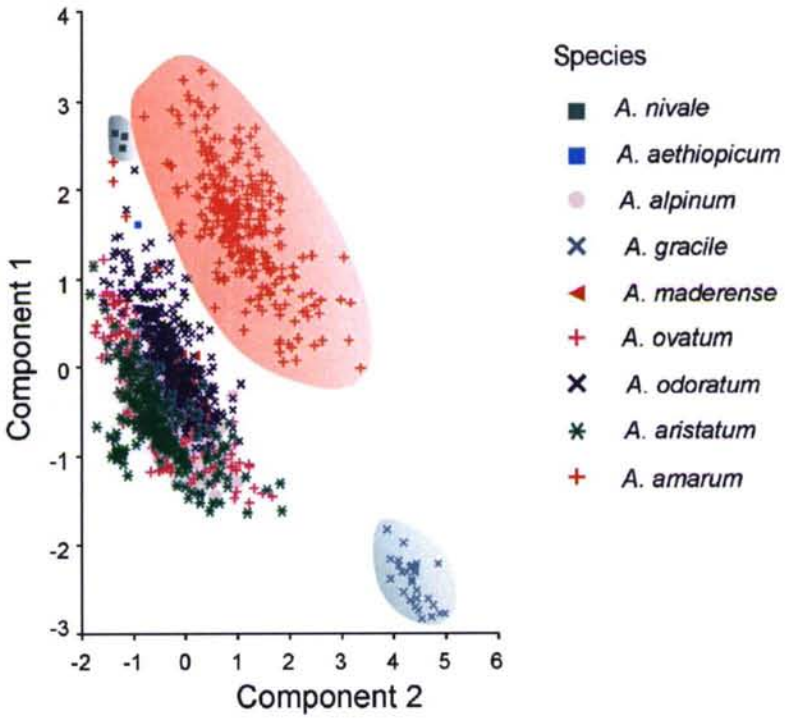

Figure 2.2. Scatterplot of the PCA performed with the complete dataset.

ovatum showed the highest variability for all the considered traits except leaf width, lower branch of the inflorescence length and upper glume width, whereas *A. gracile* and *A. maderense* showed the lowest for most of the studied characters. On the other hand, vegetative traits were much more variable than floral ones accross all the species and populations. The most variable traits were leaf length and width, plant height and lower internode of the inflorescence length. As showed by the Kolmogorov-Smirnoff test, all characters presented a normal distribution in the analysed populations

The KMO analysis performed rendered a value of 0.78 which indicates that our sample is adequate for multivariate analyses such as the PCA. In this analysis (Figure 2.2), our accessions clustered in three main groups: (i), *A. amarum* and *A. nivale*; (ii), *A. odoratum*, *A. aristatum*, *A. ovatum*, *A. maderense* and *A. aethiopicum* and (iii), *A. gracile*. The two main components explained 78% of the variance. Floral characters were specially important in the second component, responsible for the differentiation of *A. gracile*, whereas vegetative traits presented high loadings in the first axis that differentiated the *A. amarum*/*A. nivale* group (Table 2.4).

	Component loadings	
Traits	Component 1	Component 2
IL	**0.86**	0.35
LL	**0.83**	0.32
PS	**0.8**	0.2
SLIL	**0.8**	0.3
SLBL	**0.76**	0.4
LW	**0.75**	0.43
UGW	**0.64**	0.53
AL	0.22	**0.9**
STL	0.26	**0.89**
STW	0.41	**0.75**
FfL	0.52	**0.72**
SpL	0.62	**0.67**
LGL	0.55	**0.64**

Table 2.4. Matrix of rotated components (Varimax rotation) of the PCA performed with the complete dataset. In bold, morphological traits showing highest factor loadings on the first two principal components. For character codes, see Table 2.1.

A new principal component analysis was performed excluding *A. nivale*, *A. amarum* and *A. gracile* (Figure 2.3, Table 2.5). In this test, the first two components accounted for 82% of the variance. As it can be seen in the scatterplot, annuals and perennials are slightly separated in the first axis (vegetative characters), although separation is not clear. Regarding perennials, floral traits were more important than vegetative ones in the distinction of species, although variation was continuous. Moreover, *A. maderense* occupies an intermediate position between *A. alpinum* and *A. odoratum*.

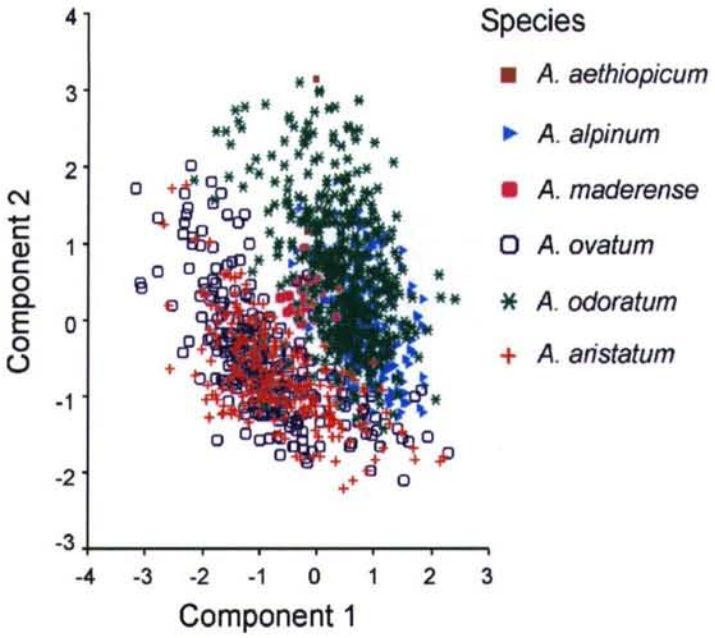

Figure 2.3. Scatterplot of the PCA performed excluding *A. amarum*, *A. nivale* and *A. gracile*.

	Component loadings		
Traits	Component 1	Component 2	Component 3
IL	**0.83**	0.12	0.31
PS	**0.77**	0.16	0.013
SLIL	**0.77**	0.43	-0.03
LL	**0.66**	-0.28	0.4
SLBL	**0.64**	0.29	0.21
LW	**0.44**	-0.37	0.4
STL	0.2	**0.84**	0.008
STW	0.09	**0.69**	0.02
AL	-0.03	**0.66**	0.35
FfL	0.33	**0.65**	0.2
SpL	0.19	0.23	**0.83**
LGL	0.15	0.12	**0.75**
UGW	0.1	0.06	**0.69**

Table 2.5. Matrix of rotated components (Varimax rotation) of the PCA performed excluding *A. amarum*, *A. nivale* and *A. gracile*. In bold, morphological showing highest factor loadings on the first three principal components. For character codes, see Table 2.1.

Using the discriminant functions produced in the CDA based on the specific classification (Table 2.6, A), the percentage of well-classified individuals ranges from 100% in *A. gracile* and the outgroups to 49.1% in *A. aristatum*. Three species presented more than 30% mismatched individuals: *A. aristatum* (50.1%), *A. ovatum* (31%) and *A. maderense* (34%). Conversely, when the PCA groups are used as explanatory variable (Table 2.6, B), the mean percentage of well-classified specimens raised from 84% to

98%. It is noteworthy that most of the misclassified *A. aristatum* plants corresponded to *A. aristatum* subsp. *macranthum* Valdés, whereas misclassified specimens of *A. ovatum* belonged mainly to *A. ovatum* var. *exertum* Lind.

A		PREDICTED GROUP								
		1	2	3	4	5	6	7	8	9
ACTUAL GROUP	1	97%	0%	0.7%	0%	0%	0%	0%	0%	2.3%
	2	0%	49.1%	2.2%	44.7%	0.5%	0%	3.5%	0%	0%
	3	0%	0.6%	92%	2.9%	0%	0%	4.1%	0%	0.4%
	4	0%	24.9%	5.1%	69%	0.7%	0%	0.3%	0%	0%
	5	0%	3.7%	26.6%	3.7%	66%	0%	0%	0%	0%
	6	0%	0%	0%	0%	0%	100%	0%	0%	0%
	7	0%	0.2%	9.6%	0.2%	0%	0%	90%	0%	0%
	8	0%	0%	0%	0%	0%	0%	0%	100%	0%
	9	0%	0%	0%	0%	0%	0%	0%	0%	100%

B		PREDICTED GROUP					
		1	2	3	4	5	6
ACTUAL GROUP	1	97%	0%	2.4%	0%	0%	0.6%
	2	0%	97.5%	0%	0%	0%	2.5%
	3	6.9%	0%	93.1%	0%	0%	0%
	4	0%	0%	0%	100%	0%	0%
	5	0%	0%	0%	0%	100%	0%
	6	0%	0%	0%	0%	0%	100%

Table 2.6. Results of the CDA (cross-validation) performed on quantitative data. A: 1, *A. amarum*; 2, *A. aristatum*; 3, *A. odoratum*; 4, *A. ovatum*; 5, *A. maderense*; 6, *A. gracile*; 7, *A. alpinum*; 8, *A. aethiopicum* (outgroup); 9, *A. nivale* (outgroup). B: Group 1, *A. odoratum*, *A. alpinum* and *A. maderense*. Group 2, *A. amarum*. Group 3, *A. ovatum* and *A. aristatum*. Group 4, *A. gracile*. Group 5, *A. aethiopicum* (outgroup). Group 6, *A. nivale* (outgroup).

The Student's *t*-tests performed revealed that all quantitative characters were significantly different between *A. amarum* or *A. gracile* and all the other taxa. In addition to this, all characters except leaf width were significantly distinct between the perennial and the annual groups. Conversely, a high number of traits (mainly floral) were not statistically different in the pairs *A. odoratum*/*A. maderense*, *A. odoratum*/*A. alpinum* and *A. aristatum*/*A. ovatum* (Table 2.7). Finally, the cluster analysis performed with quantitative data failed to reveal any consistent arrangement of specimens and populations (not shown).

Regarding qualitative traits, the PCoA performed showed no clear grouping of specimens (not shown). Moreover, the Lambda values associated to the different variables were low regardless the taxa or groups of taxa (Table 2.8). The highest values corresponded to ligule shape (useful distinguishing annuals and perennials), presence of mucronate glumes, presence of stolons (in some *A. amarum* populations and in *A. maderense*), life cycle and the distribution of pubescence in the sterile floret.

	A. odoratum		*A. amarum*		
CHARACTER	N	MEAN ± SD	N	MEAN ± SD	SIGNIFICANCE
PS	450	46 ± 15.7	279	76.2 ± 22.2	***
LL	450	6 ± 2.8	279	14.8 ± 4.15	***
LW	450	0.31 ± 0.13	279	1.19 ± 0.4	***
IL	450	4.4 ± 1.17	279	8.3 ± 1.7	***
SLBL	450	1.29 ± 0.33	279	2.3 ± 0.45	***
SLIL	450	0.76 ± 0.09	279	1.52 ± 0.5	***
SpL	450	0.77 ± 0.07	279	1.1 ± 0.12	***
UGW	450	0.35 ± 0.04	279	0.48 ± 0.04	***
LGL	450	0.41 ± 0.05	279	0.57 ± 0.07	***
STL	450	0.3 ± 0.04	279	0.47 ± 0.05	***
STW	450	0.1 ± 0.018	279	0.18 ± 0.04	***
AL	450	0.8 ± 0.09	279	1.15 ± 0.14	***
FIL	450	0.2 ± 0.03	279	0.29 ± 0.04	***

	A. odoratum		*A. ovatum*		
CHARACTER	N	MEAN ± SD	N	MEAN ± SD	SIGNIFICANCE
LL	450	6 ± 2.8	276	4.95 ± 2.4	***
LW	450	0.31 ± 0.13	276	0.33 ± 0.16	NS
PS	450	46 ± 15.7	276	28.2 ± 14	***
IL	450	4.4 ± 1.17	276	2.9 ± 0.7	***
SpL	450	0.77 ± 0.07	276	0.71 ± 0.06	***
LGL	450	0.41 ± 0.05	276	0.4 ± 0.04	***
AL	450	0.8 ± 0.09	276	0.72 ± 0.15	***
STW	450	0.1 ± 0.018	276	0.07 ± 0.03	***
STL	450	0.3 ± 0.04	276	0.23 ± 0.05	***
FIL	450	0.2 ± 0.03	276	0.17 ± 0.02	***
SLIL	450	0.76 ± 0.09	276	0.27 ± 0.11	***
SLBL	450	1.29 ± 0.33	276	0.97 ± 0.2	***
UGW	450	0.35 ± 0.04	276	0.33 ± 0.04	***

	A. odoratum		*A. maderense*		
CHARACTER	N	MEAN ± SD	N	MEAN ± SD	SIGNIFICANCE
LL	450	6 ± 2.8	30	5.85 ± 1.6	***
LW	450	0.31 ± 0.13	30	0.57 ± 0.11	***
PS	450	46 ± 15.7	30	30.6 ± 6.5	***
IL	450	4.4 ± 1.17	30	3.62 ± 0.64	***
SpL	450	0.77 ± 0.07	30	0.75 ± 0.04	NS
LGL	450	0.41 ± 0.05	30	0.5 ± 0.03	***
AL	450	0.8 ± 0.09	30	0.8 ± 0.05	NS
STW	450	0.1 ± 0.018	30	0.09 ± 0.009	NS
STL	450	0.3 ± 0.04	30	0.3 ± 0.014	NS
FIL	450	0.2 ± 0.03	30	0.2 ± 0.01	NS
SLIL	450	0.76 ± 0.09	30	0.51 ± 0.14	***
SLBL	450	1.29 ± 0.33	30	1.26 ± 0.23	NS
UGW	450	0.35 ± 0.04	30	0.35 ± 0.04	NS

	A. odoratum		*A. aristatum*		
CHARACTER	N	MEAN ± SD	N	MEAN ± SD	SIGNIFICANCE
LL	450	6 ± 2.8	228	4.4 ± 2.4	***
LW	450	0.31 ± 0.13	228	0.29 ± 0.013	NS
PS	450	46 ± 15.7	228	22.9 ± 10.6	***
IL	450	4.4 ± 1.17	228	2.75 ± 0.8	***
SpL	450	0.77 ± 0.07	228	0.7 ± 0.09	***
LGL	450	0.41 ± 0.05	228	0.37 ± 0.05	***
AL	450	0.8 ± 0.09	228	0.74 ± 0.16	***
STW	450	0.1 ± 0.018	228	0.07 ± 0.02	***
STL	450	0.3 ± 0.04	228	0.24 ± 0.04	***
FIL	450	0.2 ± 0.03	228	0.17 ± 0.03	***
SLIL	450	0.76 ± 0.09	228	0.25 ± 0.09	***
SLBL	450	1.29 ± 0.33	228	0.95 ± 0.21	***
UGW	450	0.35 ± 0.04	228	0.31 ± 0.04	***

	A. odoratum		*A. alpinum*		
CHARACTER	N	MEAN ± SD	N	MEAN ± SD	SIGNIFICANCE
PS	450	46 ± 15.7	270	36.5 ± 12.2	***
LL	450	6 ± 2.8	270	3 ± 1.2	***
LW	450	0.31 ± 0.13	270	0.26 ± 0.07	***
IL	450	4.4 ± 1.17	270	3 ± 0.6	***
SLBL	450	1.29 ± 0.33	270	1.14 ± 0.25	***
SLIL	450	0.76 ± 0.09	270	0.62 ± 0.2	***
SpL	450	0.77 ± 0.07	270	0.65 ± 0.07	***
UGW	450	0.35 ± 0.04	270	0.3 ± 0.002	***
LGL	450	0.41 ± 0.05	270	0.35 ± 0.04	***
STL	450	0.3 ± 0.04	270	0.34 ± 0.03	NS
STW	450	0.1 ± 0.018	270	0.1 ± 0.002	NS
AL	450	0.8 ± 0.09	270	0.79 ± 0.09	NS
FIL	450	0.2 ± 0.03	270	0.2 ± 0.017	***

	A. ovatum		*A. aristatum*		
CHARACTER	N	MEAN ± SD	N	MEAN ± SD	SIGNIFICANCE
LL	276	4.95 ± 2.4	228	4.4 ± 2.4	***
LW	276	0.33 ± 0.16	228	0.29 ± 0.013	***
PS	276	28.2 ± 14	228	22.9 ± 10.6	***
IL	276	2.9 ± 0.7	228	2.75 ± 0.8	NS
SpL	276	0.71 ± 0.06	228	0.7 ± 0.09	***
LGL	276	0.4 ± 0.04	228	0.37 ± 0.05	***
AL	276	0.72 ± 0.15	228	0.74 ± 0.16	NS
STW	276	0.07 ± 0.03	228	0.07 ± 0.02	NS
STL	276	0.23 ± 0.05	228	0.24 ± 0.04	***
FIL	276	0.17 ± 0.02	228	0.17 ± 0.03	NS
SLIL	276	0.27 ± 0.11	228	0.25 ± 0.09	NS
SLBL	276	0.97 ± 0.2	228	0.95 ± 0.21	NS
UGW	276	0.33 ± 0.04	228	0.31 ± 0.04	***

Table 2.7. Mean value and standard deviation for each quantitative character for different *Anthoxanthum* taxa. Mean values were compared by means of a Student's t-test. All characters were indicated in cm. Significance levels: (NS), not significant; (***), significant ($P \leq 0.05$). For character codes see Table 2.1

QUALITATIVE TRAITS	LAMBDA VALUES	
	SPECIES	PCA GROUPING
LC	**0,25**	**0,6**
S	**0,11**	**0,14**
B	0,057	0,08
SP	0	0
CL	0,085	0
LS	**0,16**	**0,428**
LF	0,004	0
R	0,059	0
Pr	0	0
LGP	0,098	0
UGP	0,062	0
MG	**0,142**	**0,2**
SFP	0,012	0,016
SFL	**0,185**	0,04
N	0,04	0
C	0,074	0
FP	0	0

Table 2.8. Lambda values obtained for each of the qualitative traits considered. For character codes see Table 2.1. Group 1, *A. odoratum*, *A. alpinum* and *A. maderense*. Group 2, *A. amarum*. Group 3, *A. ovatum* and *A. aristatum*. Group 4, *A. gracile*. Group 5, *A. aethiopicum* (outgroup). Group 6, *A. nivale* (outgroup).

The dendrogram constructed with qualitative data (Figure 2.4) showed a clear differentiation of the *A. amarum*/*A. nivale* cluster, whereas the rest of the taxa but *A. maderense* appeared rather intermingled. It is noteworthy that all annual taxa including *A. gracile* and *A. aethiopicum* cluster together, and that there is a clear geographic differentiation in *A. odoratum*.

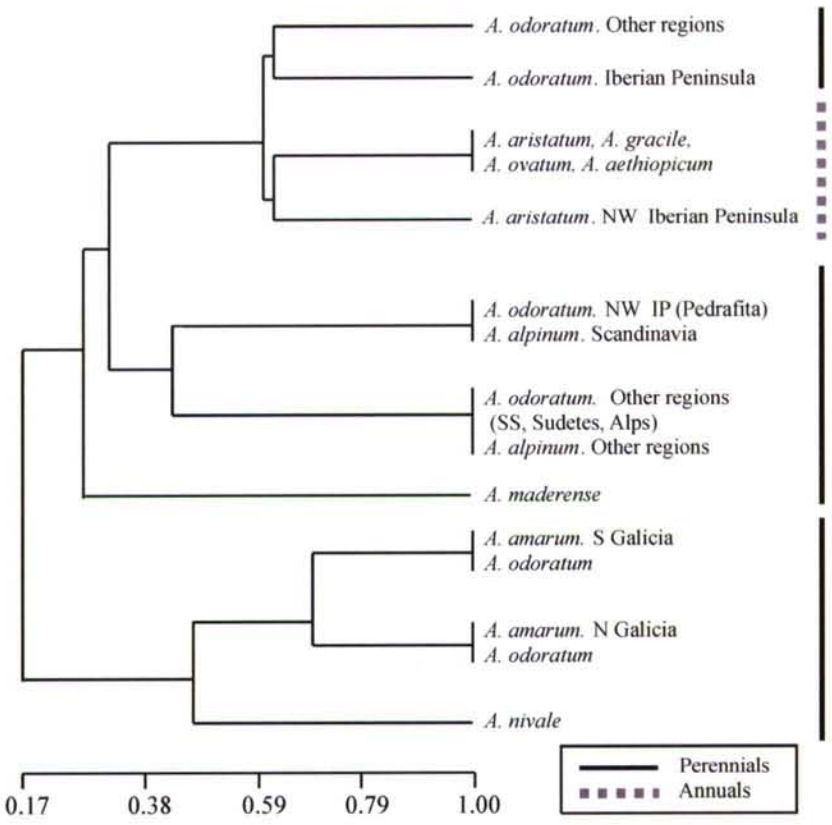

Figure 2.4. Dendrogram performed with qualitative morphological data. SS, Southern Scandinavia; IP, Iberian Peninsula.

MICROMORPHOLOGICAL ANALYSES

Both the cluster analysis and the contingency tables performed with micromorphological data from the epidermal surface and the leaf transverse section (not shown) were useless to reveal any clear grouping of populations or any consistent pattern of variation. Only the epidermal surface data-based PCoA (Figure 2.5) showed a slight differentiation between *A. amarum*, *A. odoratum* and the rest of the taxa, that appeared totally intermingled. The first three coordinates of the analysis explained only 38% of the total variance. The most important traits in the differentiation of groups were the presence of the short cells in costal and intercostal areas and the typology of long cells in intercostal areas.

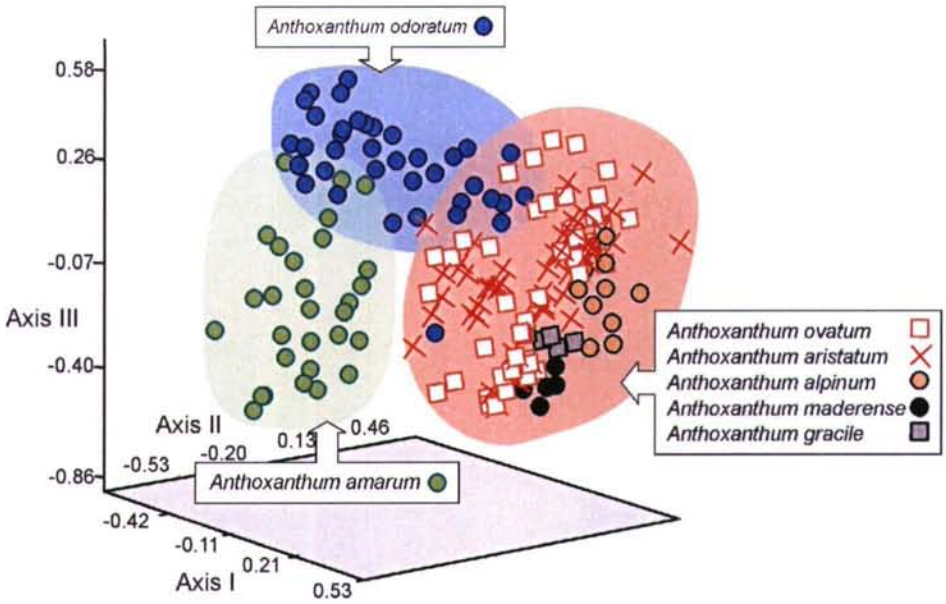

Figure 2.5. Scatterplot of the PCoA performed with the micromorphologicall data from the epidermal abaxial surface.

DISCUSSION

According to our results, the variability in floral characters was much lower than in vegetative traits in all *Anthoxanthum* species and populations. This fact has been already observed in previous studies on this genus (Pimentel & Sahuquillo, 2003a). According to Cresswell (2000) and Herrera (2005), infraspecific differences concerning the floral structure might easily turn into an obstacle for reproduction in zoophilous plants, so morphologically constant flowers would be expected. It is noteworthy that the same pattern was observed in anemophilous plants like *Anthoxanthum*.

Both vegetative and floral structures have been traditionally used in the characterization of *Anthoxanthum* species. Among the most commonly employed traits, floral ones such as spikelet length and fertile floret length presented low variability levels. Conversely, vegetative traits such as leaf width and length and plant height, important in the characterization of perennial species (Paunero, 1953; Tutin, 1980), are rather variable. This fact, as well as the overlapping observed in the these traits indicated that their taxonomic value is arguable.

The different multivariate analyses performed with the macro- (qualitative and

quantitative) and micromorphological data showed that the differentiation among the European *Anthoxanthum* species is rather difficult. Only the Mediterranean diploid *A. gracile* (Figure 2.2) and the Iberian high polyploid *A. amarum* (Figures 2.2 and 2.4) can be easily distinguished based on morphological characters. On the one hand, sterile lemma length and width, awn length and fertile floret length are specially important in the characterization of *A. gracile*. These traits have been extensively used to differentiate this taxon in monographs and Regional Floras (Fiori, 1969; Tutin, 1980; Pignatti, 1982). On the other hand, *A. amarum* is characterized mainly by its plant height, leaf and inflorescence length and the presence of stolons or bulbils. According to Merino (1909), this taxon should be considered a variety inside the widespread tetraploid *A. odoratum* (*A. odoratum* var. *majus* Hackel). However, our results indicate that both taxa are clearly differentiated macro- and micromorphologically. Moreover, the different ploidy level constitues a reproductive barrier between these species (Fernandes & Queiros, 1969).This fact supported the the assignment of a specific rank for this taxon. The dendrogram constructed with qualitative characters (Figure 2.4) revealed a morphological distinction between *A. amarum* populations from Northern and Southern Galicia (NW Iberian Peninsula). To find out the basis of such differentiation, further research involving molecular markers would be needed. It is also noteworthy that the African taxon *A. nivale* is closely related morphologically to the Iberian *A. amarum*. Both species are high polyploids (*A. nivale*; 2n=60; *A. amarum* 2n=86, 88, 90) that share their habitat with tetraploid relatives (Hedberg O, 1957; Hedberg I, 1976; Phillips, 1995; Teppner, 2002). However, the number of *A. nivale* specimens considered has been too low to draw any definite conclusion.

The PCA performed with quantitative data excluding *A. amarum*, *A. nivale* and *A. gracile* (Figure 2.3, Table 2.5) revealed two different groups based mainly on vegetative characters such as plant height and inflorescence length. (i) An "annual group" composed by the Mediterranean diploids *A. aristatum* and *A. ovatum* and (ii), a "perennial group" including *A. odoratum*, *A. maderense*, *A. alpinum* and *A. aethiopicum*. In the annual cluster, all samples from both taxa were intermingled. However, it is noteworthy that sympatric populations and infraspecific categories occupied intermediate positions in the ordination space, whereas allopatric populations were placed on outermost areas. This pattern could be explained by hybridization between these taxa, as it has been suggested by (Valdés 1973). In addition to this, and according to the CDA (Table 2.6, A), the percentage of misclassified individuals was rather high in *A. aristatum* and *A. ovatum* when the specific classification is taken into account (50% and 30% respectively). Most

of the misclassified individuals belonged to *A. aristatum* subsp. *macranthum* Valdés and *A. aristatum* var *exertum* Lindb. Conversely, when the PCA-derived groups are used in the CDA (Table 2.6, B), the percentage of well-classified individuals in the annuals raised until 93.1%.

Considering our results, we coincide with those that conclude that no infraspecific category should be described in the *A. aristatum*/*A. ovatum* complex (*e.g.*, Pérez-Lara, 1866; Paunero, 1953; López-González, 1994) and that the specific differentiation between these taxa is impossible (Battandier & Trabutt, 1885; López-González, 1994).

The PCA performed excluding *A. amarum*, *A. nivale* and *A. gracile* (Figure 2.3), revealed that a slight morphological differentiation between the *A. aristatum*/*A. ovatum* complex and the perennial group, specially the tetraploid *A. odoratum*, is possible. However, the separation is not complete, since 7% of the *A. maderense* specimens clustered with the annuals in the CDA. These results support the point of view of those who consider that *A. odoratum* and the *A. aristatum*/*A. ovatum* complex should be considered different species (*e.g.*, Paunero, 1953; Tutin *et al.*, 1980). On the other hand, the different ploidy level would reinforce the isolation of these taxa, although different hybridization experiments conducted have rendered viable offspring (Borrill, 1962; Grosstête, 1982). Nevertheless, to clarify this situation further research involving molecular analyses would be needed.

Despite the morphological closeness between the annuals and *A. alpinum* or *A. maderense*, these taxa are isolated ecologically and biogeographically. Moreover, hybridization between *A. alpinum* and the annuals is rather restricted (Borrill, 1962). This fact might indicate homoplasy in the characters.

Regarding the perennial group, composed by *A. odoratum*, *A. alpinum, A. maderense* and *A. aethiopicum*, we agree with those who consider that morphological differentiation between *A. odoratum* and *A. alpinum* is difficult (Figure 2.3) (reviewed in Hedberg, 1990). Several qualitative and quantitative traits such as awn length, glume hairiness, spikelets colour and the distribution of the pubescence in the sterile floret (e.g. Knaben, 1950; Tutin, 1950; Rozmus, 1958; Tateoka, 1966), have been used to characterize one or the other cytotype. However, according to our results, there are no significant differences in awn length between both cytotypes (Table 2.7). In addition to this, lambda values were low for all the qualitative traits (Table 2.8).

Despite the lack of morphological differentiation revealed by the PCA, the percentage

of misclassified individuals is negligible for both taxa in the CDA based on the specific classification (Table 2.6, A). On the other hand, *A. odoratum* and *A. alpinum* are quite distinct karyologically (Jones, 1964) and ecologically (Felgrová & Krahulec, 1999). Besides, the PCoA performed with micromorphological data from the epidermal surface (Figure 2.5) showed a clear separation between both taxa that had been already claimed by Rozmus (1960). Further molecular analyses would be needed to clarify the relationships between both cytotypes.

Pubescence of leaves and glumes have been also used to characterize several infraspecific categories within *A. odoratum* such as f. *glabrescens* Celak, f. *glabrum* Norman or f. *pubescens* Norman (Knaben, 1950). Pubescence has been pointed out as a spurious character by Hedberg (1964) in common garden experiments, and we have observed that it is extremely variable even in the same population. The taxonomic use of this trait is, therefore, untenable.

None of the morphological quantitative or micromorphological characters used has been able to characterize *A. maderense*. However, this species, that occupies inland cliffs of Madeira Island, is clearly ecologically and biogeographically distinct from *A. odoratum*. We consider that the lack of differentiation observed may be due to homoplasy in the selected traits. Moreover, aspects such as the presence of woody stems, the number of nodes or the shrubby habit, that are important in the diagnosis of other macaronesian Poaceae such as *Dactylis smithii* Link. subsp. *hylodes* Parker were not taken into account. This latter taxon occupies similar habitats than *A. maderense* in Madeira and the Canary Islands (Parker, 1972; Sahuquillo & Lumaret, 1995).

CONCLUSIONS

1. *Anthoxanthum gracile* can be easily distinguished morphologically within the genus *Anthoxanthum* using floral quantitative traits. Characters such as awn length, sterile floret length and width and fertile floret length are useful to characterize this taxon.

2. *Anthoxanthum amarum* can be characterized in the basis of quantitative and qualitative vegetative traits such as plant height, leaf and inflorescence length and the presence of stolons or bulbils. In addition to this, micromorphological traits such as the length of long cells in costal and intercostal areas are also useful to differentiate this taxon.

3. Two clusters of species could be distinguished using quantitative traits, An annual

group including *A. aristatum* and *A. ovatum* and a perennial one comprising *A. maderense*, *A. odoratum* and *A. alpinum* can be distinguished using quantitative traits. None of the considered characters was able to clearly differentiate groups within the clusters.

4. In our opinion, the specific distinction between *A. aristatum* and *A. ovatum*, as well as the definition of infraspecific categories within these taxa is untenable. However, molecular analysis are also needed to clarify this question.

5. Further research involving molecular markers would be needed to definitely assess the taxonomic relationships among *A. odoratum*, *A. alpinum* and *A. maderense*.

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	COLLECTION DATA	MORPH.	ANAT.	VOUCHER NUMBER
A. amarum Brot.	SPAIN: A Coruña, A Capela, Fraga do Belelle. 18.iii.2000. M. Perille & M. Pimentel	32	5	SANT-53449
	SPAIN: A Coruña, Cedeira, San Andrés de Teixido. 12.vi.1999. M. Perille, M. Pimentel & D. Romero	32	5	SANT-52216
	SPAIN: A Coruña, Coirós, Couto de Chelo. 27.vi.2000. M. Pimentel & E. Sahuquillo	31	5	SANT-53451
	SPAIN: Pontevedra, Vigo, Valadares. 3.iv.2000. C. Cortizo & E. Sahuquillo	30	5	SANT-53448
	SPAIN: Pontevedra, Tomiño, Amorín. 4.v.1999. C. Cortizo & E. Sahuquillo	30	5	SANT-52217
	SPAIN: Ourense, Montederramo, Gabin. 2.vi.2002. M. Perille, M. Pimentel & E. Sahuquillo	34	5	SANT-52222
	SPAIN: Lugo, Xove, Praia de Esteiro. 20.v.2004. M. Perille & M. Pimentel	30	-	SANT-52215
	SPAIN: Pontevedra, As Neves. 9.iv.2004. M. Pimentel	30	-	SANT-53455
	SPAIN: A Coruña, Ferrol, Esmelle, Valón. 12.v.2004. M. Perille & M. Pimentel	30	-	SANT-53453
A. aristatum Boiss.	**SPAIN: Huelva, Parque Nacional de Doñana, Caño del Tío Antoñito. 24.iv.2002. M. Pimentel & E. Sahuquillo**	30	5	SANT-53375
	SPAIN: Huelva, Parque Nacional de Doñana, Navazo del Toro. 24.iv.2002. M. Pimentel & E. Sahuquillo	25	5	SANT-53368
	SPAIN: Huelva, Parque Nacional de Doñana, El Martinazo. 24.iv.2002. M. Pimentel & E. Sahuquillo	7	5	SANT-53365
	SPAIN: Huelva, Parque Nacional de Doñana, Laguna de Santa Olalla. 24.iv.2002. M. Pimentel & E. Sahuquillo	30	5	SANT-52187
	SPAIN: Cáceres, Parque Nacional de Monfragüe, Fuente de Tres Cantos. 4.iv.2001. C. Cortizo & E. Sahuquillo	30	5	SANT-52197
	SPAIN: A Coruña, Ferrol, San Cristovo. 7.v.2001. M. Perille, M. Pimentel, D. Romero & E. Sahuquillo	15	5	SANT-52196
	SPAIN: Ourense, Larouco. 5.iv.1998. C. Cortizo, M. Perille, M. Pimentel & E. Sahuquillo	-	5	SANT-53405
	SPAIN: Pontevedra, Baiona, Cabo Silleiro. 1.vi.2002. C. Cortizo & E. Sahuquillo	15	5	SANT-52193
	SPAIN: Lugo, Quiroga, Sequeiros. 2.v.2004. M. Perille, M. Pimentel, D. Romero & E. Sahuquillo	-	5	SANT-52195
	SPAIN: Ourense, O Barco de Valdeorras, Entoma. 12.iii.1999. M. Perille, M. Pimentel, & E. Sahuquillo	-	5	SANT-53426
	SPAIN: Ourense, Pena Trevinca, Campa do Hotel. 20.vi.1999. M. Perille, M. Pimentel & E. Sahuquillo	-	5	SANT-53410
	SPAIN: Madrid, Montejo de la Sierra. 18.v.2002. C. Cortizo & E. Sahuquillo	15	-	SANT-53404
	***SPAIN: Huelva, Parque Nacional de Doñana, Caño del Tío Antoñito. 24.iv.2002. M. Pimentel & E. Sahuquillo**	30	5	SANT-52185
	***SPAIN, Huelva, Parque Nac'ional de Doñana, Pinar de San Agustín. 24.iv.2002. M. Pimentel & E. Sahuquillo**	30	5	SANT-52181
A. ovatum Lag.	**SPAIN, Huelva, Parque Nacional de Doñana, Pinar de San Agustín. 24.iv.2002. M. Pimentel & E. Sahuquillo**	30	5	SANT-52183
	SPAIN: Huelva, Parque Nacional de Doñana, Caño del Tío Antoñito. 24.iv.2002. M. Pimentel & E. Sahuquillo	30	5	SANT-53357
	SPAIN: Huelva, Parque Nacional de Doñana, Pinar de El Acebuche. 5.vi.2001. R. Pimentel & M. Pimentel	57	5	SANT-53378
	SPAIN: Huelva, Parque Nacional de Doñana, Navazo del Toro. 24.iv.2002. M. Pimentel & E. Sahuquillo	30	5	SANT-52180
	SPAIN: Huelva, Parque Nacional de Doñana, El Martinazo. 24.iv.2002. M. Pimentel & E. Sahuquillo	22	5	SANT-53379
	SPAIN: Cádiz, Los Barrios, Montera del Torero. 6.vi.2001. M. Pimentel & R. Pimentel	29	5	SANT-52186
	SPAIN, Cádiz, Los Barrios, Arroyo del Tiradero. 5.vi.2001. M. Pimentel & R. Pimentel	-	5	SANT-53400
	SPAIN: Madrid. El Paular. 4.vi.2002. C. Cortizo & E. Sahuquillo	15	-	SANT-52221
	°SPAIN: Huelva, Parque Nacional de Doñana, Caño del Tío Antoñito. 24.iv.2002. M. Pimentel & E. Sahuquillo	30	5	SANT-53384
	°SPAIN: Huelva, Parque Nacional de Doñana, El Martinazo. 24.iv.2002. M. Pimentel & E. Sahuquillo	8	5	SANT-53344
	°SPAIN: Huelva, Parque Nacional de Doñana, Laguna de Santa Olalla. 24.iv.2002. M. Pimentel & E. Sahuquillo	30	5	SANT-53383
A. odoratum L.	SPAIN: Lugo, O Courel, Alto de Pedrafita. 8.vi.2001. C. Cortizo, M. Perille, M. Pimentel & E. Sahuquillo	30	5	SANT-52190
	SPAIN: A Coruña, Oleiros, Arillo. 19.iii.2001. M. Pimentel & E. Sahuquillo	30	5	SANT-53361
	SPAIN: Lugo, O Corgo, Marei. 5.v.2005. C. Cortizo, M. Perille, M. Pimentel, D. Romero & E. Sahuquillo	30	5	SANT-53424
	SPAIN: Ourense, Vilardevós, Trasigrexa. 17.vi.2000. C. Cortizo, M. Perille, M. Pimentel & E. Sahuquillo	30	5	SANT-53412
	SPAIN: A Coruña, Ferrol, Doniños. 24.iii.2002. M. Perille, M. Pimentel & E. Sahuquillo	-	5	SANT-53423
	SPAIN: Ourense, Rubiá, Covas. 22.v.1999. C. Cortizo, M. Perille, M. Pimentel & E. Sahuquillo	-	5	SANT-53422
	SPAIN: Castellón, El Boixar. 21.v.2004. D. Giraldo, M. Pimentel, D. Romero & E. Sahuquillo	15	-	SANT-52213
	SPAIN: Soria, Embalse de la Cuerda del Pozo. 22.v.2004. D. Giraldo, M. Pimentel, D. Romero & E. Sahuquillo	15	-	SANT-52211
	SPAIN: Huesca, Benasque, La Renclusa. 6.vii.2004. M. Perille & M. Pimentel	15	5	SANT-52210
	SPAIN: Salamanca, La Alberca. 14.iv.2003. M. Pimentel & E. Sahuquillo	15	-	SANT-53391
	CZECH REPUBLIC: Krkonoše Mts., Rokytnice, Jehlanka. 9.vi.2003. L. Filipová & M. Pimentel	30	-	SANT-52208
	CZECH REPUBLIC: Krkonoše Mts, Modrý Dul Valley. 11.vi.2003. L. Filipová & M. Pimentel	15	-	SANT-52206
	CZECH REPUBLIC: Krkonoše Mts., Rokytnice, Svetlanka. 10.vi.2003. L. Filipová & M. Pimentel	30	-	SANT-52203
	SWITZERLAND: Vaud, Col des Mosses. 5.viii.2004. M. Perille, M. Pimentel & D. Romero	15	5	SANT-52192
	SWITZERLAND: Valais, Pas de Morgins. 5.viii.2004. M. Perille, M. Pimentel & D. Romero	15	-	SANT-52214
	FRANCE: Rhône-Alps, Savoie, La Rosière. 5.viii.2004. M. Perille, M. Pimentel & D. Romero	15	5	SANT-52218
	SWEDEN: Uppland, Alunda. 9.vi. 2002. M. Pimentel	30	5	SANT-53396
	SWEDEN: Vastmanland, Ångsö-Västerås. 11.vi.2002. M. Pimentel	30	-	SANT-53389
	SWEDEN: Vastmanland, Lövudden. 12.vi.2002. M. Pimentel	30	-	SANT-52199
	SWEDEN: Vastmanland, Skinnskatteberg. 12.vi.2002. M. Pimentel	30	-	SANT-52200
	SWEDEN: Vastmanland, Stalldalen. 12.vi.2002. M. Pimentel	30	-	SANT-52201
A. alpinum Löve & Löve	SWITZERLAND: Valais, Grimselpass. 6.viii.2004. M. Perille, M. Pimentel & D. Romero	15	5	SANT-52220
	FRANCE: Rhône-Alps, Haute-Savoie, Brevent Mt. 4.viii.2004. M. Perille, M. Pimentel & D. Romero	15	5	SANT-52191
	ITALY, Valle d'Aosta, Piccolo San Bernardo. 5.viii.2004. M. Perille, M. Pimentel & D. Romero	15	-	SANT-52209
	CZECH REPUBLIC: Hrubý Jesenik Mts., Vysoká Hole Mt. 11.vi.2003. L. Filipová & M. Pimentel	30	-	SANT-52207
	CZECH REPUBLIC: Krkonoše Mts., Dolní Misecky. 10.vi.2003. L. Filipová & M. Pimentel	30	-	SANT-52204
	BULGARY: Rodopi Mts. Vii.2004. A. Quintanar	15	-	SANT-53388
	SWEDEN: Dalama, Nipfjället Mt. 14.vi.2002. M. Pimentel	30	-	SANT-52182
	SWEDEN: Lappland, Björkliden. 9.vii.2002. M. Pimentel	30	-	SANT-52202
	SWEDEN: Lappland, 10 Km N Björkliden. 9.vii. 2002. M. Pimentel	30	-	SANT-53395
	SWEDEN: Lappland, Vittangi. 7.vii.2002. M. Pimentel	30	-	SANT-53393
	SWEDEN: Lappland, Nikkaluokta. M. Pimentel	30	5	SANT-52198
Am	PORTUGAL: Madeira, Poça da Neve, Estrada cara ó Arieiro. 2.viii.2004. M. Sequeira & P. Catalán	30	5	SANT-52179
Ag	MALTA: Wied Zembaq. 25.iii.2004. E. Lanfranco	30	5	SANT-52223
Aae	ETHIOPIA: Bale Mts. National Park. 2.ix.1986. S. Phillips	1	-	H2005/00137-15
	ETHIOPIA: Romacaru. 24.iii.1958. H. Smeds	1	-	H2005/00137-14
	ETHIOPIA: 50 Km N Dolo Menna. 29.x.1984. I. Friis, M.G. Gilbert & K. Vollesen	1	-	H2005/00137-13
Aniv	KENYA: Mt. Kenya. 20.x.1943. J. Belly	1	-	H2005/00137-8
	UGANDA: Elgon. 8.x.1961. F. Rose	1	-	H2005/00137-7
	UGANDA: Kiggi. ix.1946. J.W. Purseglow	1	-	H2005/00137-5
	TOTALS	**1573**	**220**	**1793**

APPENDIX 2.1. Material studied. (*), *A. aristatum* Boiss. subsp. *macranthum* Valdés; (°), *A. ovatum* Lag. var. *exertum* Lindb. **MORPH.**, number of specimens considered in morphometric analyses; **ANAT.**, Number of individuals studied in anatomical analyses. *Am*, *A. maderense* Teppner; *Ag*, *A. gracile* Biv., *Aae*, *A. aethiopicum* I. Hedberg; *Aniv*, *A. nivale* K. Schum. In bold, areas were more than one *Anthoxanthum* taxa were found.

Capítulo 3

Relationships among close congeners assessed through molecular and morphological methods. The case of the *Anthoxanthum odoratum s. lat.* complex

Relationships among close congeners assessed through molecular and morphological methods. The case of the *Anthoxanthum odoratum s. lat.* complex

3

ABSTRACT

The genus *Anthoxanthum s. lat.* (including *Hierochloë* R. Br.) (Poaceae, Pooideae, Aveneae) comprises between 35-50 species and has a cosmopolitan distribution. *Anthoxanthum alpinum* Löve & Löve was described as a diploid perennial that is distributed in northern Eurasia and in the high mountains of Central and East Europe. Difficulties in finding reliable morphological differences between this taxon and the widespread tetraploid *Anthoxanthum odoratum* L. have resulted in taxonomists treating them as conspecific, despite the cytological differentiation. The purpose of this study was to provide information which may help clarify the relationships between these taxa. Macromorphological, micromorphological and molecular data were gathered and analyzed for 14 populations representing both taxa from Scandinavia and the Iberian Peninsula. Different cluster analyses were performed to study the relatedness among individuals and populations. Subsequently, we computed a principal component analysis based on macromorphological quantitative traits, whereas principal coordinates analyses were used to analyse qualitative, micromorphological and RAPD data. An analysis of molecular variance was computed on molecular data, and the genetic differentiation among samples was measured by means of the F_{ST} estimator. Our results showed that the geographical origin was more important than the ploidy level when it comes to explain the relatedness among specimens and populations. Moreover, we found a strong correlation between the micromorphological traits and the environmental parameters. The results of the analyses do not support the assignment of the specific rank to *A. alpinum*.

ADDITIONAL KEY WORDS: *Anthoxanthum alpinum* - *Anthoxanthum odoratum s. str.* - infraspecific classification - leaf anatomy - morphometrics - RAPDs - Poaceae - poliploid complex.

INTRODUCTION

The use of a combined molecular and morphological approach has been recommended to test different hypotheses on taxonomic delimitations in complex taxa (*e.g.*, Black-Samuelsson *et al.*, 1997; Scheen, Elven & Brochmann, 2002). On the one hand, detailed morphological analyses are necessary to unravel the distribution of the variation in intrincate species complexes. However, the influence of the environment and the inclusion of characters that vary continuously across taxa may conceal their taxonomical structure. On the other hand, the use of molecular techniques that generate many genetic markers at low taxonomic levels provides the possibility to address the correlation between morphological and genotypic variation (*e.g.*, Hansen, Elven & Brochmann, 2000). In the present study, molecular, macro- and micromorphological data have been used to address different questions on the relationships between *A. odoratum s. str.* and *A. alpinum*.

Traditionally, the genus *Anthoxanthum* L. *s. str.* (Poaceae; Pooideae; Aveneae) comprises around 18 species, distributed in temperate and arctic-alpine regions of Europe, Asia and Africa (Watson & Dallwitz, 1992). More recently, different studies based on the evolution of the spikelets have pointed out that the separation between *Anthoxanthum* and *Hierochloë* R. Br., may be artificial (Schouten & Veldkamp, 1985; Soreng *et al.*, 2003; but see Clayton & Renvoize, 1986). As a result, 35 to 50 species would be included in *Anthoxanthum s. lat.*, growing mainly in temperate and arctic regions of both hemispheres, and also on tropical mountains (Clayton, 1970; Hedberg, 1976; Royen, 1979; Tutin, 1980; Phillips, 1995). Seven of these species are present in Europe: *A. odoratum s. lat.*, *Anthoxanthum aristatum* Boiss., *Anthoxanthum ovatum* Lag., *Anthoxanthum amarum* Brot., *Anthoxanthum gracile* Biv, *Anthoxanthum pauciflorum* Adamovic. and *Anthoxanthum nitens* (Web.) Schouten & Veldk., although this number is uncertain, since the status of some of these taxa is arguable and new species and subspecies have been proposed.

The most widely distributed species of the genus is the perennial *A. odoratum*, a morphologically extremely variable taxon that constitutes a diploid-tetraploid complex. It grows in a broad range of habitats in Europe, North Africa, Asia Minor, the Caucasus and northern Asia. It has been also introduced in North and South America and in Oceania, where it may constitute an ecological problem (Cheeseman, 1906; Hitchcock, 1951; Tovar, 1993).

Diploids of *A. odoratum* were first reported and described by Östergren (1942).

Subsequently, Löve and Löve (1948) named these diploids *A. alpinum*, despite the difficult morphological distinction from the tetraploids. Later, different names such as *A. odoratum* subsp. *alpinum* (Á. & D. Löve) Hultén and var. *montanum* Asch. et Graebn. were proposed for this taxon (Böcher, 1961; Pundeva, 1974).

Anthoxanthum alpinum is confined to the northernmost parts of the distribution range of *A. odoratum*, and also to the high mountains of Central and East Europe (Hedberg, 1969; Felber, 1993; Felgrová & Krahulec, 1999). It usually grows in arctic-alpine meadows, whereas the tetraploid *A. odoratum* occurs in a wide range of habitats.

Different karyotypes have been reported for both cytotypes. Jones (1964), studying Greek plants, was the first to point out the existence of a diploid karyotype very much like *odoratum* in morphology and distinct from *A. alpinum* whose taxonomic position has still to be determined. Later, it was assigned by Hedberg (1986) to *A. odoratum*. Also, Hedberg (1970) was the first to report the existence of two karyotypes in tetraploid *A. odoratum*, later confirmed by Teppner (1970).

Contradictory studies regarding morphological and karyological differences between the cytotypes and karyotypes (reviewed by Hedberg, 1990) have led to disagreements on the taxonomic rank that should be applied to the ploidy levels. Tutin (1950), Rozmus (1958, 1960), Borrill (1962, 1963), Jones and Melderis (1964), Mayová (1982), Felber (1986, 1988, 1993) and Lauber and Wagner (2001) considered the diploids and tetraploids to represent well-defined species that can be distinguished by certain morphological characters (Table 3.1), whereas Böcher (1961), Tutin (1980) and Hedberg (*e.g.*, 1970, 1986, 1990) concluded that the specific rank is not justified for *A. alpinum* since rather high morphological variability can be observed in natural populations.

Direct observation of morphological and anatomical features is still an essential element in taxonomic studies, and approaches combining morphometric (qualitative and quantitative characters) and micromorphological data have proven to be specially useful in Poaceae taxonomy (*e.g.*, Metcalfe, 1960; Dávila & Clark, 1990; Acedo & Llamas, 1999; Giraldo-Cañas, 2001). In addition to this, the random amplified polymorphic DNA technique (RAPD; Williams *et al.*, 1991) has been extensively used to address different questions in plant biology referred to taxonomy and biogeography (*e.g.*, Díaz-Lifante & Aguinagalde, 1996; Segarra-Moragues & Catalán, 2003).

The aims of this work are: (i) to study the macromorphological, micromorphological and molecular relationships among several Scandinavian and Iberian populations

belonging to both cytotypes, (ii) to evaluate macro- , micromorphological and molecular variation in relation to the *A. odoratum*/*A. alpinum* delimitation, (iii) to calibrate the usefulness of the different characters that have been traditionally used in the differentiation of both cytotypes and (iv) to determine if correlation exists between micromorphological characters and environmental variables. We provide new data that could help clarify the taxonomy of *A. alpinum* and *A. odoratum*.

Anthoxanthum odoratum	*Anthoxanthum alpinum*
Fertile floret smooth	Fertile floret scabridous
Unicellular hairs in the lower glume	Lower glume smooth
Glume hairiness variable	Glumes without long soft hairs
Leaf blade planar	Leaf blade convolute
Spikelets greenish	Spikelets reddish
Leaves concolorous	Leaves discolorous
Stomata 44-52 µm long	Stomata 40-46 µm long
Leaf length/leaf width ratio > than *A. alpinum*	Leaf length/leaf width ratio < than *A. odoratum*

Table 3.1. Traditional characteristics used in the cytotypes morphological differentiation. References in the text.

MATERIALS AND METHODS

PLANT MATERIAL

We sampled 14 populations of *A. odoratum* and *A. alpinum* from the Iberian Peninsula and Scandinavia during summer 2002 (Figure 3.1, Table 3.2). The areas were established to embrace a broad range of geographical and environmental variation in natural populations of *A. odoratum*. In addition to this, to ensure that both cytotypes were included in the analysis, the Scandinavian populations were selected following Hedberg (1967), wherein ploidy levels were reported. No populations of *A. alpinum* have been recorded in the Iberian Peninsula (*e.g.*, Hedberg, 1990). The sampled populations were also tentatively assigned to taxon on the basis of the morphological characters used by Lauber & Wagner (2001).

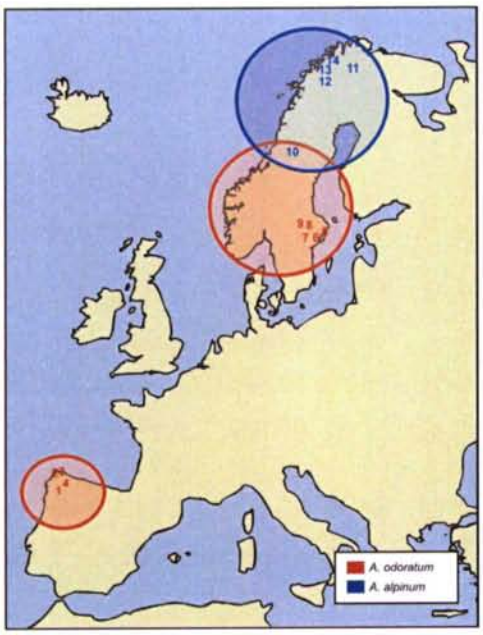

Figure 3.1. Map showing sample sites for the 18 *A. odoratum s. lat.* populations analysed. In red, tetraploids; in blue, diploids. Numbers of populations are listed on Table 3.2.

TAXON	POPULATION AND POPULATION CODES	HABITAT	VOUCHER NUMBERS
A. odoratum	Trasigrexa, Vilardevós, Ourense. I. (Vl)	Wet meadow	*Pimentel 53412 (SANT)*
	Arillo, Oleiros, A Coruña. I. (Ol)	Wasteland by a road	*Pimentel 53361 (SANT)*
	A Zapateira, A Coruña. I. (AZ)	*Eucalyptus* plantation	*Pimentel 52188 (SANT)*
	Taro Blanco, O Courel, Lugo. I. (TB)	Mountain meadow	*Pimentel 53364 (SANT)*
	Alunda, Uppland. SS. (Al)	Birch forest	*Pimentel 53396 (SANT)*
	Angsö-Västerås, Västmanland. SS. (AV)	Mixed forest	*Pimentel 53389 (SANT)*
	Lövudden, Västmanland. SS. (Lö)	Birch forest	*Pimentel 52199 (SANT)*
	Skinnskatteberg, Västmanland. SS. (Sk)	Birch forest	*Pimentel 52200 (SANT)*
	Ställdalen, Västmanland. SS. (St)	Spruce forest	*Pimentel 52201 (SANT)*
A. alpinum	Nipfjället Mt., Dalarna. NS. (Nip)	Mountain meadow	*Pimentel 52182 (SANT)*
	Vittangi, Lappland. NS. (Vit)	Wasteland by a road	*Pimentel 53393 (SANT)*
	Nikkaluokta, Lappland. NS. (Nik)	Birch forest	*Pimentel 52198 (SANT)*
	Björkliden, Lappland. NS. (Bjö)	Mountain meadow	*Pimentel 52202 (SANT)*
	5 km N Björkliden, Lappland. NS. (NB)	Mountain meadow	*Pimentel 53395 (SANT)*

Table 3.2. Populations sampled and corresponding habitats of *Anthoxanthum odoratum* and *A. alpinum*. I, Iberian Peninsula; SS, southern Scandinavia; NS, northern Scandinavia. Precise localities are provided on the voucher labels. Population codes are in parentheses.

A total of 40 individuals were collected per population. Since *A. odoratum s .lat.* is capable of limited vegetative reproduction, sampled plants were at least 3 m apart. Thirty and five individuals were employed in the morphometric and micromorphological

analyses, respectively. Lastly, fresh leaves from the remaining five plants per population were taken and stored in silica gel for molecular studies (Chase & Hills, 1991). A grand total of 560 plants were studied. Voucher specimens were deposited in Santiago de Compostela University Herbarium (SANT; Holmgren, Holmgren & Barnett, 1990). The voucher numbers are indicated in Table 3.2.

RAPD ANALYSIS

Total genomic DNA was extracted from 25 mg of dried leaves using the CTAB protocol (Doyle & Doyle, 1990). The quality of the DNA was checked on 1% TAE-agarose gels, and its concentration was quantified by spectrometry with the Cary 3E Spectrophotometer (Varian, Palo Alto, USA). A total of 30 decamer primers from kits OPA (Operon Technologies, Alameda, USA) and ABA (Advanced Biotechnologies, Epsom, United Kingdom) were used for preanalyzing RAPD variation among five plants. Nine arbitrary decamer primers (Table 3.3) that produced the most distinct, reproducible and polymorphic bands were selected for full RAPD analysis of all 70 plants. The PCR amplification program started with a melting step, thirty seconds at 94 ºC, followed by 40 cycles of thirty seconds at 92 ºC, 1 minute at 35 ºC and 2 minutes at 72 ºC and by a final extension cycle of 7 minutes at 72ºC. Reactions were performed in a 25 µl volume with 1x reaction buffer, 125 µM dNTPs (Roche Pharmaceuticals, Basel, Switzerland), 0.22 µM primer, 5mM $MgCl_2$, 20 ng genomic DNA and 2 U. Amplitaq (Applied Biosystems, Foster City, USA). Amplification products were electrophoresed in 1.5% agarose gel, stained with ethidium bromide and visualized under UV light.

Primer identification	Nucleotide seq. (5´to 3´)	Nº of amplification products					
		Overall	4x	2x (N Sweden)	S. Sweden	Iberia	Sweden (overall)
OPA-2	TGCCGAGCTG	7	6	6	6	5(1)	7
OPA-3	AGTCAGCCAC	13	12	8(1)	12	4	13
OPA-4	AATCGGGCTG	11	11	9	9(1)	11	9
OPA-5	AGGGGTCTTG	11	10	9(1)	9(1)	9	11(1)
OPA-9	GGGTAACGCC	9	8	5(1)	6	6	6
OPA-10	GTGATCGCAG	11(1)	11	7(2)	10	9(1)	11
OPA-11	CAATCGCCGT	8	8(1)	8(1)	5(1)	8(1)	8(1)
OPA-13	CAGCACCCAC	10	10	9	10	7	10
OPA-18	AGGTGACCGT	8	8	6	6	7	7
Total		88(1)	84(1)	67(6)	73(3)	66(3)	82(2)

Table 3.3. List of decamer oligonucleotides used as random primers, their sequences and number of products of amplification. Between brackets, number of monomorphic fragments. M, monomorphic primers. Seq., sequence.

Amplified bands were scored as presence (1) or absence (0) in a manually constructed matrix. A DNA molecular weight marker (weight marker XIV, Roche Pharmaceuticals) was included as a reference. Only clear bands with homogeneous intensity were recorded. Reproducibility of the observed patterns was assured by performing the whole RAPD protocol twice in 10 randomly chosen individuals.

The Euclidean and Jaccard's distances, as well as the Dice's similarity coefficient, were calculated for each pair of individuals using NTSYS-pc Version 2.1 (Rohlf, 2002). To assess the equivalence among the different distance measures, similarity matrices were compared by means of a Mantel correlation test (following Legendre & Legendre, 1998). Finally, the level of significance was estimated by a permutation test (1000 replicates).

We measured the mean genetic diversity within populations using the Shannon diversity index and the percentage of monomorphic and private fragments per population. Overall values of these parameters were obtained for each cytotype and region (Després, Loriot & Gaudeul, 2002). Genetic diferentiation between populations and regions was calculated using the unbiased F_{ST} estimator of Weir & Cockerham (1984) as implemented in TFGPA Version 1.3 (Miller, 1997). The 95% confidence interval for the estimator was obtained by bootstrapping 5000 replicates over loci.

To summarize the relationships among RAPD phenotypes, a neighbor-joining (NJ) tree was constructed with MEGA Version 2.1 (Kumar *et al.*, 2001) and a principal coordinates analysis (PCoA) with a superimposed minimum spanning tree (MST, Gower & Ross, 1969) was conducted using NTSYS-pc Version 2.1.

The Analysis of Molecular Variance (AMOVA, Excoffier, Smouse & Quattro, 1992) has been widely used to study the distribution patterns of RAPD phenotypes (Segarra-Moragues & Catalán, 2003 and references therein). To analyse the genetic structure of the studied populations, an AMOVA was performed at two different hierarchical levels: (i) among regions (Iberian Peninsula, S Scandinavia and N Scandinavia) and (ii) between cytotypes (*A. odoratum* and *A. alpinum*). Significance levels for the variance components were obtained by non-parametric permutation (1000 replicates). AMOVA analyses were performed using ARLEQUIN 2.000 (Schneider *et al.*, 2000).

MORPHOMETRIC DATA

For the macro-morphological study, 26 macroscopic characters (12 qualitative, 13 quantitative and one ratio) were chosen and scored (Table 3.4). All the characters listed

in Table 3.1 were included in the survey except the color of leaves, since previous papers on *Anthoxanthum* and other grasses reported that this character is highly influenced by the environment (Kjellqvist, 1961; Hedberg, 1967; Zeven, 1986; Sahuquillo, Fraga & Martínez, 1991). To standardize the gathering of the data, characters from the leaf blade and the ligule were taken in the second leaf. In addition to this, floral measures were taken in the basal spikelet of the fourth branch of the inflorescence. To carry out the statistical analyses the qualitative characters were transformed into binary variables. The mean and its 95% confidence interval, the standard deviation, as well as the maximum and minimum values per population are listed in Appendix 3.1.

The most informative taxonomic characters were identified by calculating the coefficient of variation (*cv*) for each character and performing a principal components analysis (PCA) for quantitative data. Moreover, contingency tables and a PCoA-MST were applied to qualitative characters. The similarity among samples was established using the Manhattan distance and the Phi coefficient for quantitative and qualitative data, respectively. The relationships among populations were summarized in phenograms obtained using UPGMA clustering methods. Statistical analyses were carried out with SPSS Version 11.0 for Windows (SPSS Inc., Chicago).

QUANTITATIVE CHARACTERISTICS AND RATIOS	QUALITATIVE CHARACTERISTICS
Plant height (cm) [Ph]	Leaf blade outline (0 planar; 1 convolute)
Leaf blade length (cm) [Ll]	Ligule shape (0 round; 1 pointed)
Leaf blade width at the middle (cm) [Lw]	Sheath hairiness (0 glabrous; 1 hairy)
Inflorescence length (cm) [Il]	Upper glume hairiness (0 glabrous; 1 hairy)
First internode of the inflorescence length (cm) [Intl]	Presence of a mucro (0 not mucronate; 1 mucronate)
First inflorescence branch length (cm) [Bl]	Lower glume hairiness (0 glabrous; 1 hairy)
Spikelet length (cm) [Sl]	Presence of a forked apex on upper sterile lemma (0 not forked; 1 forked)
Upper glume width (cm) [Ugw]	Ratio of sterile lemma hairs length to the sterile lemma length (0/1)(*)
Lower glume length (cm) [Lgl]	Sterile lemma hairiness (0 glabrous; 1 hairy)
Upper sterile lemma length (cm) [Sfw]	Amount of overlap of fertile floret by sterile lemmas (0/1)(**)
Upper sterile lemma width (cm) [Sfl]	Fertile floret scabridity (0 smooth; 1 scabrid)
Awn length (cm) [Al]	Colour of the spikelets (0 green; 1 reddish)
Fertile floret length (cm) [Ffl]	
Leaf blade length/leaf blade width [L/l]	

Table 3.4. Quantitative and qualitative macroscopic morphological characters used in the study. (*) Sterile lemma hairs shorter than the sterile lemma (0); sterile lemma hairs longer than the sterile lemma (1). (**) Fertile floret not covered by the sterile ones (0); fertile floret covered by the sterile ones (1). Character codes are between brackets.

MICROMORPHOLOGICAL DATA

A total of 29 leaf blade characters (14 from the abaxial epidermis and 15 from the transverse section) were chosen and scored (Table 3.5). The method used to obtain samples for microscopic observation was that of Devesa (1992) and Pimentel & Sahuquillo (2003b), and the terminology followed Ellis (1976, 1979), Nicora & Rúgolo de Agrassar (1987), Devesa (1992) and Aliscioni (2000). Regarding the exodermic elements as defined by Devesa (1992), we made no difference between prickles and hooks, since according to Metcalfe (1960), Snow (1996), and Aliscioni & Arriaga (1998) both elements are homologous. The quantitative data obtained per specimen were the mean values of 12 different measures. The final quantities were transformed into binary variables.

	EPIDERMAL TRAITS	LEAF BLADE TRANSVERSE SECTION
intercostal zones	Abaxial epidermis type (^{A})	Bundle sheath outline (0, round; 1, elliptical)
	Long cell type (^{B})	Bundle sheath cell shape (0, round; 1, angular)
	Long cells length* (^{E}) (LCI)	Bundle sheath cells equal in size (0) or not (1)
	Presence of short cells (0, absent; 1, present) (SI)	Median vb. sheath type (0, single; 1, complete)
	Short cells length* (^{G}) (LSI)	Median vb. sheath cell diameter (0, < 15 µm; 1, > 15 µm) (M)
	Exod. elements (0, presence; 1, abscence)	N° of cells in vb sheath (0, < 17 cells; 1, > 17 cells)
	Stomate type (0, parallel-sided; 1, dome-shaped)	N° of bulliform cells per group (0, < 6 cells; 1, > 6 cells)
costal zones	Long cell type (^{B})	N° of vb. per leaf (0, <15; 1, > 15) (VB)
	Long cell length* (^{F}) (LCN)	Median vb. type (^{D}) (VBT)
	Presence of short cells (0, absent; 1, present) (SN)	Median vb. diameter (^{C}) (0, <75 µm; 1, > 75 µm) (VBD)
	Short cell length (0, < 50 µm; 1, > 50 µm)	Presence of a radial chlorenchyma (0, absent; 1, present)
	Exod. elements (0, presence; 1, abscence)	Colorless parenchyma (0, absent; 1, present)
		Ribs and furrows (0, absent; 1, present)
	Presence of bulliform cells (0, absent; 1, present)	Developed keel (0, absent; 1, present)
	Leaves pruinose (0, not pruinose; 1, pruinose)	Bulliform cell diameter (0, < 30 µm; 1, > 30 µm)

Table 3.5. Anatomical characters used in the study. (A), abaxial epidermis homogeneous (0) or siliceous-homogeneous (1). (B), long cells l2 (0) or l3 (1) Devesa (1992). (C), maximum diameter of the vascular bundle measured horizontally. (D), median vascular bundle free or joined to the epidermis by schlerenchyma girders. (*) Multistate characters. Each of the states is interpreted as an independent binary variable. (E), LCI1, 101-200 µm; LCI2, 301-400 µm; LCI3, 401-500 µm; LCI4, 501-600µm; LCI5, 601-700µm. (F), LCN1, (101-200µm; LCN2, 201-300µm; LCN3, 301-400µm; LCN4, 401-500µm. (G), LSI1, 11-20µm; LSI2, 21-40µm; LSI3, 41-50µm). Exod. elements, exodermic elements (Devesa, 1992). vb., vascular bundle.

The statistical analyses applied to the micromorphological data were the same as those used for the qualitative macroscopic traits. To determine if correlations existed between the micromorphological characteristics that resulted more discriminant in the dendrogram and the environment, a canonical correspondence analysis (CCA) was performed using CANOCO Version 4.0. (Ter Braak, 1987; Ter Braak & Smilauer, 1998; Lepš & Smilauer, 2003). The environmental parameters considered were soil pH (following Guitián & Carballos, 1976), mean, minimum and maximum annual temperature (ºC), drought period and mean annual rainfall (mm). Climatic data from the Iberian Peninsula were gathered from Carballeira *et al.* (1983) and from Scandinavia at the website of the Swedish Meteorological and Hydrological Institute (www.smhi.se). The meteorological stations nearest to the collection localities were selected.

RESULTS

RAPD ANALYSIS

The 9 primers used in this study yielded 88 polymorphic bands for the 70 surveyed individuals (Table 3.3). A different RAPD profile was registered for each of the studied specimens. Eighty-four of the markers (95.5%) were present in tetraploids, and 67 (77.3%) in diploids. Regarding regions, 66 bands (75%) were present in the Iberian Peninsula, 73 (82.3%) in S Scandinavia and 67 (77.3%) in N Scandinavia. The percentage of monomorphic fragments was low in all the studied groups (cytotypes and regions), although it was quite variable accross the different populations (Table 3.6).

GROUP	Nº OF BANDS	MONOMORPHIC FRAGMENTS	PRIVATE FRAGMENTS	MONOMORPHIC AND PRIVATE FRAGMENTS	SHANNON INDEX
Oleiros	38	14 (36.8%)	0	0	7.26
Vilardevós	44	12 (27.3%)	0	0	9.96
Taro Blanco	36	14 (38.8%)	0	0	6.27
A Zapateira	38	16 (42.1%)	0	0	6.31
Iberian Peninsula	66	3 (4.5%)	6 (9%)	0	17.48
Alunda	41	16 (39%)	0	0	7.71
Ångsö	37	17 (46%)	1 (2.7%)	0	5.69
Nora	49	16 (32.6%)	3 (6.1%)	1	9.71
Skinnskatteberg	41	21 (51.2%)	0	0	4.94
Stalldalen	37	17 (46%)	2 (5.4%)	1	5.57
S. Sweden	73	3 (4%)	8 (10.5%)	0	21.12
Tetraploids	84	1 (1.2%)	21 (24.4%)	0	23.74
Nipfjället	34	24 (70.6%)	0	0	3.11
Nikkaluokta	45	19 (42.2%)	2 (5.4%)	1	8.22
Vittangi	42	22 (52.3%)	0	0	5.58
Björkliden	38	20 (52.6%)	0	0	5.89
N Björkliden	29	19 (65.5%)	0	0	3.06
Diploids/N Sweden	68	6 (8.8%)	3 (4.5%)	0	17.29
TOTAL	88	1 (1.1%)	-	-	23.88

Table 3.6. Number of bands, degree of polymorphism and Shannon index values registered per group.

The percentage of polymorphic loci and the Shannon index for each population were strongly correlated (Spearman's correlation coefficient; Polymorphism vs. H_{Sh}: $r^s = 0,92$). Mean genetic diversity was higher in S Scandinavia than in N Scandinavia or the Iberian Peninsula, and in tetraploids than in diploids (Table 3.6). However, these differences were not significant (*e.g.*, Mann-Whitney U-test; S Scandinavia vs. Iberia $P = 0.413$).

There was a high genetic differentiation among the populations in both diploids and tetraploids (global F_{ST}; 0.6, 95% confidence interval (CI) 0.48-0.72 and 0.65, CI 0.50-0.80, respectively). Within tetraploids, among-population differentiation was as high in the Iberian Peninsula as in S Scandinavia. Genetic differentiation among regions and cytotypes was much lower (0.14, CI 0.06-0.2 and 0.097, CI 0.05-0.14, respectively).

As shown by all the hierarchical AMOVAs performed, most of the genetic variation was found among populations within groups and within populations. Only between 10% and 18% of the variation was found among groups (cytotypes, regions), regardless of the considered grouping. The maximum value of among groups variation was obtained when the two main geographical regions (Iberian Peninsula and Scandinavia) were considered, whilst the lowest value was recovered when the arrangement of populations was based on their ploidy levels.

In a PCoA analysis of the total RAPD data set (Figure 3.2) the three first axis extracted only 32% of the total variation. The first axis (18%) clearly separated Iberian and Scandinavian populations, whereas the second one (9%) partially discriminated between Swedish diploids and tetraploids. Thus, *A. odoratum s. str.* population from Lovüdden grouped with diploid *A. alpinum* populations. The minimum spanning tree that was superimposed on this PCoA analysis connected the *A. alpinum* populations with the Iberian tetraploids.The Scandinavian and Iberian populations of *Anthoxanthum* were clearly separated in the NJ analysis. Interestingly, there was a clear differentiation between Scandinavian diploids and tetraploids (Figure 3.3). We observed some irregularities in the dendrogram. On the one hand, Lovüdden population (S Scandinavia) grouped with diploid *A. alpinum*. On the other hand, one of the samples from Vilardevós (Iberian Peninsula) grouped with diploids as well.

MORPHOMETRIC ANALYSES

The statistical exploratory analysis of morphometric data showed that vegetative characters were more variable than reproductive characters for all populations.

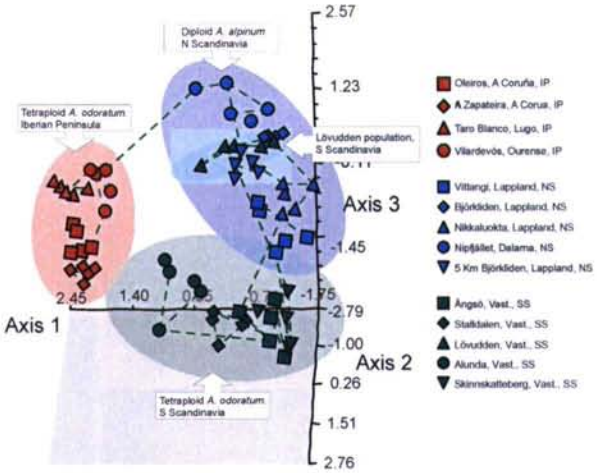

Figure 3.2. PCoA of RAPD data (70 plants) of *A. odoratum s. lat.* In blue, diploids; in red, Iberian tetraploids; in green, Scandinavian tetraploids. SS, S Scandinavia; NS, N Scandinavia; IP, Iberian Peninsula. Vast., Vastmanland.

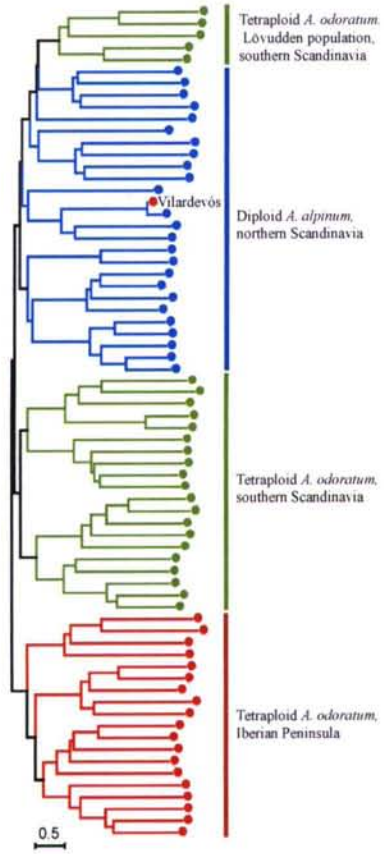

Figure 3.3. Dendrogram based on RAPD data. In blue, diploids; in red, Iberian tetraploids; in green, Scandinavian tetraploids.

Conversely, reproductive characters such as spikelet and lower glume length presented lower *cv* values (Table 3.7) and non overlapping variation ranges between both cytotypes, at least for values inside the 95% confidence interval (Appendix 3.1, Figure 3.4). The *cv* observed throughout the tetraploid populations was quite similar to that for diploid *A. alpinum* (*cv* = 0.17 and 0.16, respectively).

		Quantitative characters													
		Ll	Lw	Ph	L/l	ll	Bl	Intl	Sl	Lgl	Al	Ugw	Sfw	Sfl	Ffl	**MV**
Populations	Vl	0.24	0.23	0.32	0.37	0.23	0.16	0.17	0.09	0.14	0.15	0.11	0.2	0.12	0.11	**0.17**
	Ol	0.29	0.24	0.26	0.31	0.2	0.2	0.37	0.08	0.16	0.12	0.11	0.24	0.13	0.13	**0.19**
	AZ	0.28	0.22	0.18	0.3	0.17	0.29	0.35	0.08	0.1	0.12	0.07	0.11	0.1	0.09	**0.19**
	TB	0.28	0.3	0.24	0.27	0.34	0.31	0.26	0.1	0.09	0.15	0.08	0.24	0.17	0.1	**0.2**
	Al	0.3	0.24	0.13	0.32	0.17	0.18	0.27	0.05	0.08	0.1	0.09	0.11	0.07	0.05	**0.14**
	AV	0.35	0.22	0.2	0.34	0.15	0.18	0.27	0.1	0.11	0.13	0.12	0.12	0.1	0.08	**0.16**
	Lö	0.46	0.32	0.13	0.4	0.15	0.2	0.22	0.08	0.1	0,1	0.09	0.14	0.08	0.08	**0.16**
	Sk	0.44	0.21	0.23	0.45	0.19	0.2	0.31	0.08	0.08	0.09	0.09	0.15	0.09	0.07	**0.17**
	St	0.29	0.23	0.16	0.34	0.23	0.2	0.31	0.07	0.12	0.07	0.06	0.11	0.09	0.09	**0.15**
	Nip	0.36	0.25	0.15	0.35	0.23	0.25	0.24	0.05	0.1	0.08	0.14	0.08	0.08	0.11	**0.16**
	Vit	0.46	0.27	0.2	0.45	0.26	0.25	0.28	0.08	0.12	0.11	0.11	0.13	0.13	0.07	**0.19**
	Nik	0.45	0.3	0.19	0.4	0.24	0.15	0.23	0.08	0.13	0.1	0.12	0.18	0.07	0.08	**0.18**
	Bjö	0.53	0.23	0.17	0.45	0.19	0.18	0.26	0.07	0.07	0.08	0.1	0.09	0.09	0.05	**0.16**
	NB	0.33	0.15	0.15	0.37	0.1	0.18	0.18	0.07	0.05	0.05	0.1	0.05	0.06	0.07	**0.11**
Ov.		**0.58**	**0.41**	**0.35**	**0.47**	**0.32**	**0.25**	**0.35**	**0.14**	**0.17**	**0.12**	**0.2**	**0.23**	**0.13**	**0.1**	

Table 3.7. Coefficients of variation of each character for each population. For population and character codes see Table 3.2 and 4 respectively. Ov, overall values. MV, mean values per population.

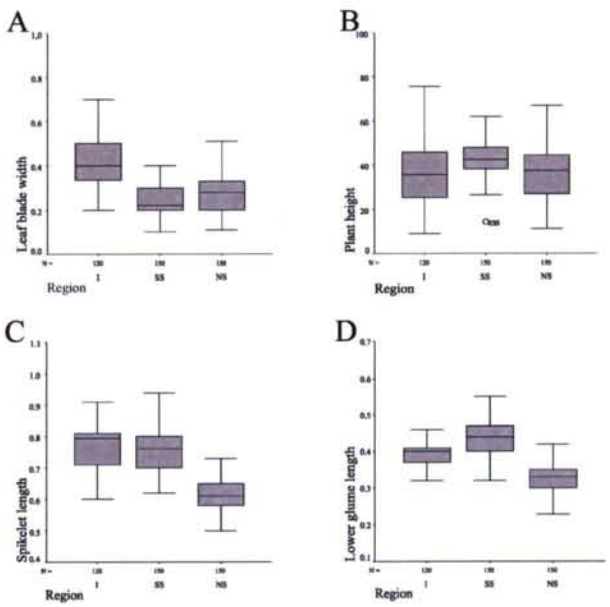

Figure 3.4. Boxplots for some of the quantitative characters studied. A, leaf blade width; B, plant height; C, spikelet length; D, lower glume length. I, Iberian Peninsula; SS, S Scandinavia; NS, N Scandinavia.

As a result of the principal components analysis, the first three components explained 61% of the variation (Table 3.8). The PCA plot of the first three components showed a continuum between *A. alpinum* and *A. odoratum*, although loosely cohesive groups for each region and cytotype were observed (Figure 3.5). This scarce separation among the groups was mainly due to the first component, that accounted for 31.6% of the variance. The characters with highest loadings in the first component were leaf width and length, the ratio leaf length/leaf width and inflorescence and spikelet length. In our results, only the spikelet length (Mann-Whitney U-test; tetraploids *vs* diploids, P = 0.003) and the ratio (tetraploids *vs* diploids, P = 0.006) were able to achieve a slight differentiation of the cytotypes. Interestingly, characters usually viewed as taxonomically useful, such as fertile floret length or upper sterile lemma width (Paunero, 1953; Valdés, 1973; Tutin, 1980), were weakly correlated with all three components, indicating their low discriminant value. Moreover, the same distribution of populations was observed in the cluster analysis performed with quantitative data (not shown).

CHARACTER CODE	COMPONENT 1	COMPONENT 2	COMPONENT 3
Ll	**0.87**	-0.28	-0.066
Lw	**0.53**	-0.36	-0.29
Ph	**0.44**	0.29	-0.37
L/l	**0.59**	0.03	0.2
Il	**0.88**	-0.07	0.14
Bl	**0.57**	0.03	-0.35
Intl	**0.61**	0.04	0.43
Sl	**0.74**	0.29	0.4
Lgl	**0.58**	0.46	0.36
Al	0.11	**0.71**	-0.17
Ugw	0.54	-0.27	**0.65**
Sfw	-0.016	-0.08	**0.63**
Sfl	-0.35	**0.58**	-0.19
Ffl	0.17	**0.65**	0.36
PV	**31.6%**	**15.1%**	**14.9%**

Table 3.8. Morphological characters showing highest factor loadings on the first three components of the PCA. PV, percentage of variance explained by each component. For characters codes see Table 3.4.

Regarding qualitative characters, significant differences among populations were common. However, the variation was not partitioned according to cytotypes (*A. alpinum* and *A. odoratum*). For instance, the character "lower glume smooth", considered diagnostic for *A. alpinum* (Knaben, 1950; Tutin, 1950; Jones & Melderis, 1964; Lauber and Wagner, 2001), was variable in some of the diploid populations analyzed such as Nipfjället Mt and Björkliden. This fact has been already observed by Borrill (1963) and Hedberg (1964, 1967) in diploid and tetraploid populations from the British Isles, Scandinavia and the Alps.

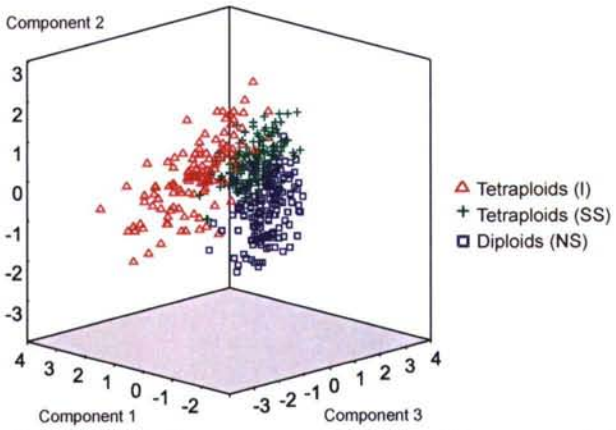

Figure 3.5. Plot from a principal components analysis of 13 macromorphological characters representing 30 plants from each of nine populations of *Anthoxanthum odoratum* (five scandinavian and four iberian) and five scandinavian populations of *A. alpinum*. I, Iberian Peninsula; SS, southern Scandinavia; NS, northern Scandinavia.

A cluster analysis of qualitative characters (Figure 3.6) showed some differentiation of the cytotypes, however, both taxa are somewhat intermingled. It is worth noting that while 12% of the tetraploids clustered with diploids, only 2% of the *A. alpinum* clustered with *A. odoratum* populations. Within *A. odoratum*, populations with different geographic origins were separated at low levels with one exception, the Taro Blanco population. This population is situated in a mountain area in northwestern Iberian Peninsula, characterized by extreme climatic conditions, in terms of minimum temperatures, compared to the remaining tetraploid Iberian populations. Noteworthy character states for which a population or cluster was fixed included reddish spikelets, glabrous glume and sheaths and the upper sterile lemma having a forked apex.

The PCoA performed with qualitative data failed to reveal differentiation among cytotypes. Conversely, populations from the same region clustered together (not shown).

To test the usefulness of leaf anatomy in the taxonomy of the *A. odoratum-A. alpinum* complex, a cluster analysis was performed using micromorphological data from the abaxial epidermis and transverse section of the leaf blade. In the resulting dendrogram, no clear grouping of populations or cytotypes could be distinguished (Figure 3.7). However, a certain separation between the Scandinavian and Iberian populations was observed. The differentiating characters were the presence of short cells in the intercostal and costal zones, the diameter of the median vascular bundle and the diameter of the vascular bundle sheath cells. Also, it is noteworthy that plants from the Taro Blanco population clustered

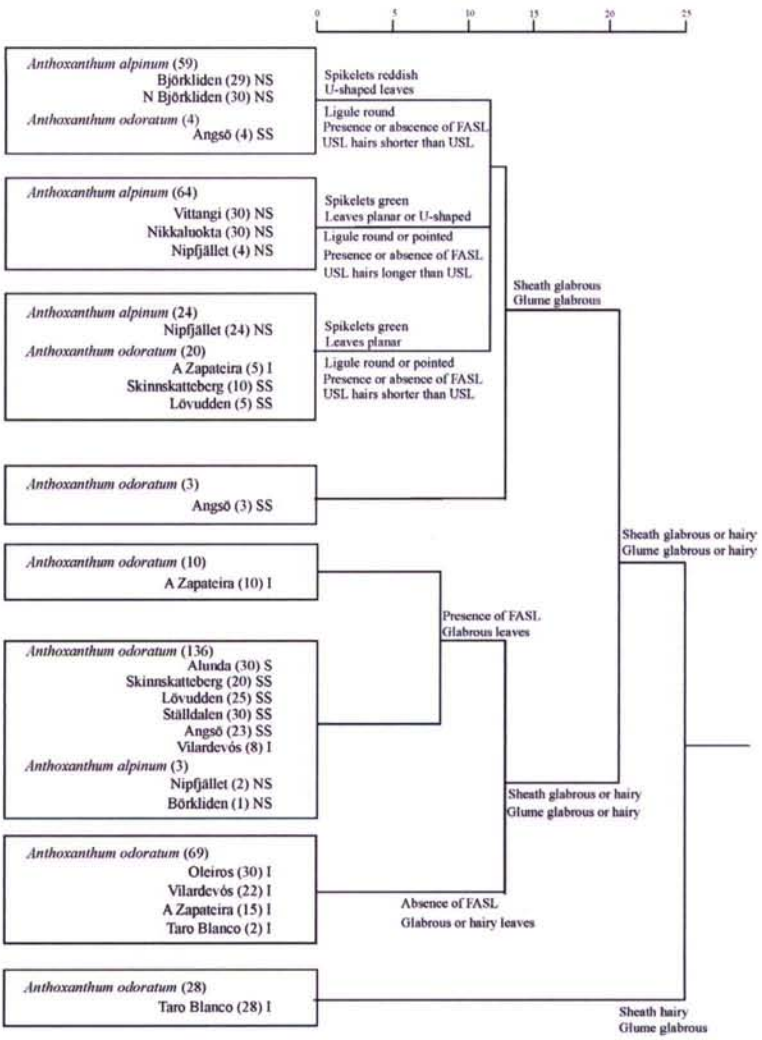

Figure 3.6. UPGMA phenogram based on 12 qualitative characters representing 30 plants from each of nine populations of *Anthoxanthum odoratum* (five scandinavian and four iberian) and five scandinavian populations of *A. alpinum*. FASL = Presence of a forked apex on upper sterile lemma; USL = Upper sterile lemma. The number of specimens per population included in each cluster is indicated between brackets. I, Iberian Peninsula; SS, southern Scandinavia; NS, northern Scandinavia.

with the Scandinavian ones.

The PCoA performed with micromorphological data revealed a clear separation between Iberian and Scandinavian populations (Figure 3.8). Interestingly, the MST constructed connected tetraploids from the Iberian Peninsula and S Scandinavia.

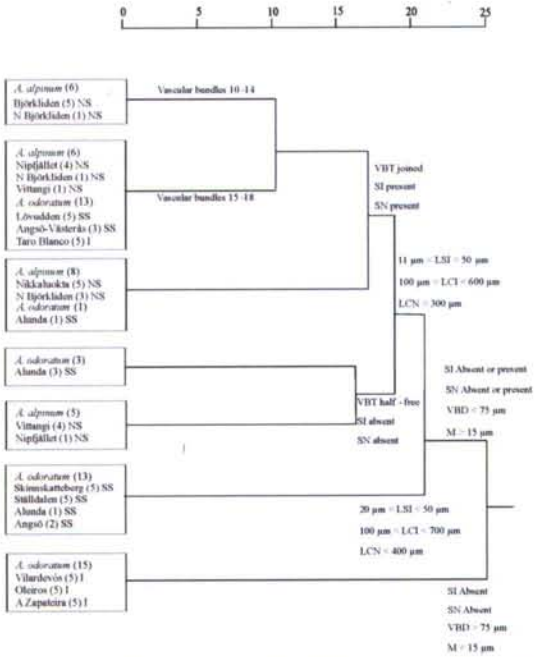

Figure 3.7. UPGMA phenogram based on 29 micromorphological characters representing 5 plants from each of nine populations of *Anthoxanthum odoratum* (five scandinavian and four iberian) and five scandinavian populations of *A. alpinum*. SCL = intercostal zone short cells length; SCI = presence of short cells in intercostal zones; SCN = presence of short cells in costal zones; NLCL = costal zone long cells length; LCL = Intercostal zone long cells length; VBD = median vascular bundle diameter; MVBS = Median vascular bundle sheath cell diameter. The number of specimens per population included in each cluster is indicated between parentheses. I, Iberian Peninsula; SS, southern Scandinavia; NS, northern Scandinavia.

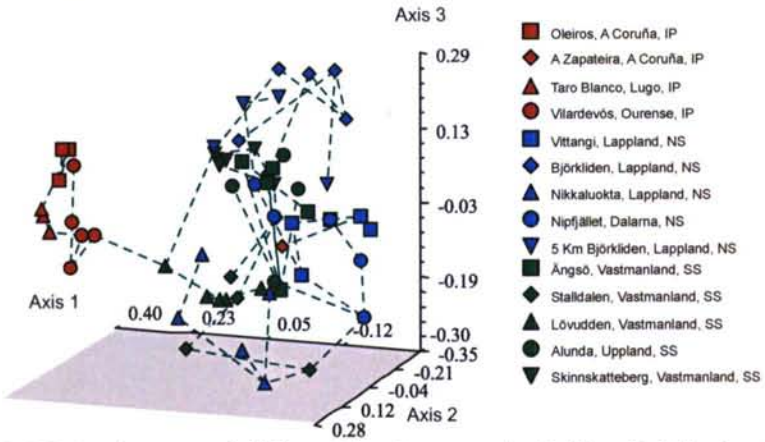

Figure 3.8. PCoA using anatomical data of *A. odoratum s. lat.* In blue, diploids; in red, Iberian tetraploids; in green, Scandinavian tetraploids. SS, S Scandinavia; NS, N Scandinavia; IP, Iberian Peninsula.

As revealed by the CCA, a high correlation between micromorphological characters and the environmental parameters was found (0.91 and 0.81, first and second axes, respectively; Figure 3.9). The lower length of long cells in costal and intercostal areas was related to high temperatures and the availability of water. Also, the number of vascular bundles per leaf and the diameter of the median vascular bundle increased with temperatures. Conversely, the presence and length of short cells were related to low temperatures. Regarding the populations, diploids appeared to be quite related to low temperatures, whereas all the Iberian tetraploids except Taro Blanco were associated to high temperatures.

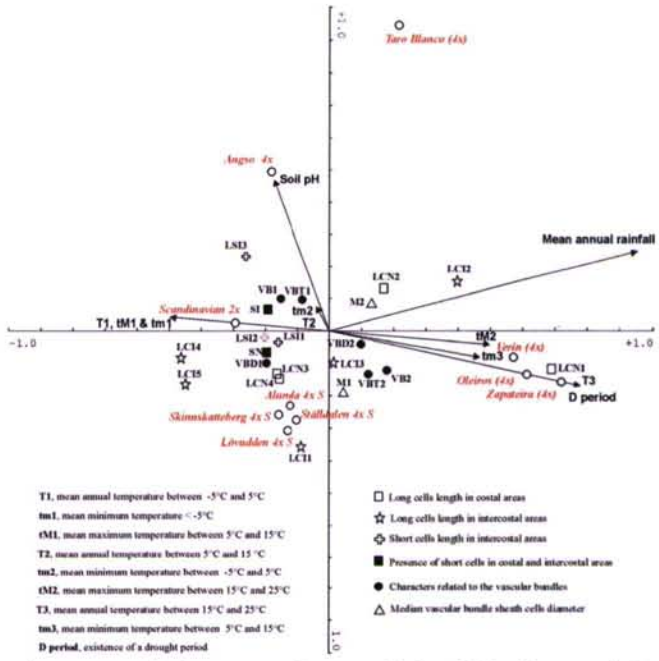

Figure 3.9. Plot from a canonical correspondence analysis of 29 micromorphological characters representing 5 plants from each of 14 populations of *Anthoxanthum odoratum* and *A. alpinum*. Empty circles represent the studied populations. All scandinavian diploid populations were plotted together. SI, presence of short cells in the intercostal zones; SN, presence of short cells in the costal zones; VB, number of vascular bundles per leaf (VB1 < 15; VB2 = 15); VBT, median vascular bundle type (VBT1, free or half free; VBT2, joined to the epidermis by schlerenchyma girders); VBD, median vascular bundle diameter (VBD1 < 75 μm; VBD2, = 75μm); M, median vascular bundle sheath cells diameter (M1, < 15μm; M2 = 15μm); LSI, short cells length in intercostal zones (LSI1, 11-20μm; LSI2, 21-40μm; LSI3, 41-50μm); LCI, long cells length in intercostal zones (LCI1, 101-200 μm; LCI2, 301-400 μm; LCI3, 401-500 μm; LCI4, 501-600 μm; LCI5, 601-700 μm.); LCN, long cells length in costal zones (LCN1, 101-200 μm; LCN2, 201-300 μm; LCN3, 301-400 μm; LCN4, 401-500 μm).

DISCUSSION

Both reproductive and vegetative traits such as fertile lemma hairiness, spikelet colour, and sheaths and glumes pubescence have been traditionally used in the diagnosis of *A. alpinum* (Felber, 1988; Lauber & Wagner 2001). According to our results, variability in the reproductive characters was lower than in the vegetative characters in all the populations. This fact had been already observed in other *Anthoxanthum* species such as *A. aristatum* or *A. ovatum* (Pimentel & Sahuquillo, 2003a), and reinforces the use of reproductive characteristics in the differentiation of *Anthoxanthum* taxa. It is worth noting that some vegetative characters used to circumscribe *A. alpinum*, such as plant height and leaf width showed high *cv* and variation ranges greatly overlapped (Appendix 3.1, Table 3.7, Figure 3.4). Higher variability could be expected in the tetraploid populations, since they were collected in a wider range of habitats over a greater geographical area, whereas the diploid populations were gathered in climatically homogeneous areas of northern Scandinavia (Table 3.2). However, only minor quantitative differences were observed between both cytotypes.

As showed by the PCA, the most important characters in the differentiation of groups were leaf width and length (and the ratio leaf length/leaf width), inflorescence size and spikelet length. However, only the ratio and the spikelet length were sigificantly different between both cytotypes. According to Östergren (1942), Knaben (1950) and Tutin (1950), a weak differentiation between *A. alpinum* and *A. odoratum* based on these traits may be achieved. However, the variation ranges they observed in populations from Scandinavia and the British Isles reduced their taxonomic usefulness. In addition to this, Hedberg (1967) showed in garden experiments that leaf width and length and inflorescence length were easily modified by the environment. Particularly, the abundance of snow during winter has been claimed the most important factor to determine the distribution of *A. alpinum* (Hedberg, 1967).

Traditionally, qualitative characters have been used more often than quantitative characters for the diagnosis of *A. alpinum* (Table 3.1). However, the separation between the cytotypes was rather uncomplete in the dendrogram performed with qualitative data (Figure 3.6). The pubescence of glumes and sheaths were the most important characters in the discrimination of both cytotypes in the cluster analysis. Different studies in grasses have suggested that pubescence is highly dependent on the conditions under which the plants grow and, therefore, can hardly be considered a distinctive character (Hedberg,

1964, 1967, 1990; Zeven, 1986; Sahuquillo, Fraga & Martínez, 1991; Ramesar-Fortner *et al.*, 1995; Chiapella, 2000). The fact that Taro Blanco population (Iberian Peninsula), that presented hairy sheaths and glabrous glumes clustered with the Scandinavian diploids seems to reveal this influence.

No differentiation between cytotypes was revealed by the cluster analysis conducted with micromorphological data. In addition to this, a strong influence of the environment on this characters was revealed by the CCA. Such an influence has been also reported by different authors in other grasses (*e.g.*, Aiken, Darbyshire & Lefkovitch, 1985; Ramesar-Fortner, Aiken & Dengler, 1995). As a consequence, these characters should be used with caution for taxonomic purposes in this group.

The different analyses performed with macro- and micromorphological data suggest that the geographical origin and the environmental conditions are as important as the ploidy level in the morphological differentiation of groups. To us, the specific status for *A. alpinum* would be, therefore, untenable and different infraspecific ranks should be considered. However, a limited morphological separation has led several authors to rather different taxonomic consequences. Thus, Chiapella (2000) and Gengler-Nowak (2002), studying *Deschampsia* P. Beauv. (Poaceae) and *Malesherbia* Ruiz and Pav. (Malesherbiaceae) respectively, consider that continuous morphological variation among groups prevents the assignment of a specific taxonomic rank. Conversely, Stace & Cotton (1991) consider that minor morphological and/or ecological differences, when associated with variations in ploidy level, are enough to differentiate species in the genus *Vulpia* Gmelin.

Regarding molecular data, the different analyses performed revealed a lack of genetic differentiation between the cytotypes. Moreover, the dendrogram (Figure 3.3) and the PCoA (Figure 3.2) performed with molecular data showed a geographical, rather than taxonomic, arrangement of populations.

To us, the scarce phenotypic and genotypic differentiation observed between both cytotypes did not justify the distinction of *A. odoratum* and *A. alpinum* as different species. Both an autoploid (Hedberg, 1986) and an alloploid (Jones, 1964) origin have been proposed for tetraploid *A. odoratum*, and it has been stated that polyploidization in this taxon has caused an ecological differentiation between both cytotypes (Felber-Girard, Felber & Buttler, 1996; Felgrová & Krahulec, 1999). According to our results, this process has not led to the morphological or genetic divergence between both forms, and a varietal rank seems to be more suitable for *A. alpinum*.

CONCLUSIONS

1. Most of the quantitative traits that have been traditionally used in the differentiation of *A. alpinum* and *A. odoratum* appear to be too variable or influenced by the environment.

2. Among the quantitative characters, only the spikelet and lower glume length and the ratio leaf length/leaf width were able to slightly separate the cytotypes.

3. Micromorphological and qualitative macroscopic characteristics are not discriminant in the *A. odoratum*/*A. alpinum* complex, and are more related to the geographical location of the populations than to ploidy level. Moreover, a high correlation between the anatomical traits and the environment was detected.

4. According to our results, there was no genetic differentiation between both cytotypes. RAPD genetic diversity is apparently more related to geography than to ploidy level. Nevertheless, it is mainly found within populations and among populations within regions.

5. The observed overlap between both cytotypes regarding macro- and micromorphology, anatomy and genetics suggested incomplete isolation and an overall continuum in phenotype and genotype. Our results indicated that a varietal taxonomic status would be more suitable for *A. alpinum*.

6. To build on this study, all *A. odoratum s. lat.* karyotypes should be included in the survey. Moreover, sampling new populations in other areas of interest, such as the Alps or the eastern Mediterranean mountains, would be also convenient.

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Pop.	Data	Ll	Lw	L/l	Ph	Il	Bl	Inl
Vl (I)	Mean (M-m)	7.78 (10.9-3.2)	0.36 (0.5-0.2)	22.8(8.2-54.5)	62,9(89,1-9,8)	5.37 (8.6-3.2)	1,3(1,7-1)	1,01(1,4-0,8)
	SD (CI)	**1.9 (7.7-8.5)**	**0.085 (0.32-0.39)**	**8.6(19.6-26)**	**20,5(55.2-70.5)**	**1.26(4.9-5.8)**	**0,21(1.2-1.4)**	**0,17(0.95-1.1)**
Ol (I)	Mean (M-m)	9.3 (13.9-3.6)	0.55 (0.8-0.3)	17.3(6.4-34.5)	41,6(70,4-21,6)	5.9 (8.1-3.8)	0,36(0,43-0,26)	1,56(2,2-1)
	SD (CI)	**2.72 (8.3-10.3)**	**0.13(0.5-0.6)**	**5.4(15.2-19.3)**	**10,7(37.6-45.6)**	**1.2 (5.4-6.3)**	**0,042(0.35-0.38)**	**0,32(1.4-1.68)**
AZ (I)	Mean (M-m)	7.23 (12.1-4.1)	0.46(0.7-0.3)	16.2(8.8-25.5)	34.6(45.4-19.2)	5.2(7.4-3.4)	0.39(0.44-0.32)	1.4(2.4-0.7)
	SD (CI)	**1.9(6.5-7.9)**	**0.103(0.42-0.5)**	**4.9(14.3-18)**	**6.16(32.3-36.9)**	**0.9(4.8-5.5)**	**0.027(0.38-0.4)**	**0.4(1.2-1.5)**
Tll (I)	Mean (M-m)	4.3(7.25-2.5)	0.36(0.62-0.2)	12.4(6.6-18.1)	19.8(26.7-10.3)	3.5(6.7-1.4)	1.2(2.1-0.7)	0.64(1-0.4)
	SD (CI)	**1.19(3.9-4.8)**	**0.11(0.32-0.4)**	**3.4(11.1-13.7)**	**4.73(18.1-21.6)**	**1.2(3.1-4)**	**0.38(1.07-1.36)**	**0.17(0.57-0.7)**
Al 4x	Mean (M-m)	4.2(6.7-0.8)	0.24(0.4-0.15)	17.7(4-31.4)	43.1(56.2-21.8)	4.4(6.2-3)	1.3(1.8-0.8)	0.78(1.3-0.4)
(SS)	**SD (CI)**	**1.32(3.75-4.7)**	**0.06(0.2-0.26)**	**5.7(15.6-19.9)**	**5.4(41.1-45.2)**	**0.7(4.2-4.8)**	**0.24(1.2-1.4)**	**0.2(0.7-0.86)**
AV 4x	Mean (M-m)	3.8(8.6-1.7)	0.23(0.36-0.16)	16.9(9.3-33.7)	48.1(64.7-26.6)	3.6(4.9-2.5)	1.2(1.9-1.8)	0.78(1.3-0.4)
(SS)	**SD (CI)**	**1.01(3.37-4.4)**	**0.05(0.21-0.25)**	**5.8(14.7-19.1)**	**9.45(44.5-51.6)**	**0.54(3.4-3.8)**	**0.2(1.13-1.2)**	**0.21(0.7-0.86)**
	Mean (M-m)	3.9(9.3-2.1)	0.23(0.4-0.1)	18.1(6.5-32.2)	41.2(53.9-28.9)	3.6(4.9-2.3)	1.15(1.9-0.6)	0.61(0.9-0.4)
(SS)	**SD (CI)**	**1.1(3.3-4.65)**	**0.07(0.2-0.26)**	**7.3(15.3-18.5)**	**5.4(39.9-43.9)**	**0.57(3.4-3.9)**	**0.23(1.07-1.25)**	**0.13(0.4-0.9)**
Sk 4x	Mean (M-m)	3.2(7-1.4)	0.21(0.31-0.13	15.8(6.4-35)	41.6(60.7-14.3)	3.3(5-2.2	1.06(1.7-0.8)	0.51(1-0.3)
(SS)	**SD (CI)**	**1.4(2.7-3.7)**	**0.04(0.19-0.22)**	**7.2(13-18.5)**	**9.7(37.9-45.2)**	**0.63(3.1-3.6)**	**0.2(0.9-1.14)**	**0.15(0.45-0.56)**
St 4x	Mean (M-m)	4.8(9.1-3)	0.29(0.5-0.2)	17.1(9-29.4)	41.1(60-29.5)	4.1(6-2.2)	1.17(1.9-0.8)	0.72(1.3-0.4)
(SS)	**SD (CI)**	**1.4(4.3-5.4)**	**0.07(0.27-0.32)**	**5.9(14.8-19.3)**	**6.53(38.7-43.5)**	**0.95(3.7-4.45)**	**0.23(1.08-1.25)**	**0.2(0.63-0.79)**
Nip 2x	Mean (M-m)	2.8(5.5-1.4)	0.36(0.65-0.2)	7.7(3.8-13.7)	33.1(40.6-22)	2.9(3.9-2)	1.17(1.9-0.6)	0.58(0.8-0.3)
(NS)	**SD (CI)**	**1.01(2.4-3.1)**	**0.09(0.33-0.4)**	**2.7(6.7-8.7)**	**5.02(31.3-35)**	**0.49(2.7-3.12)**	**0.29(1.06-1.27)**	**0.14(0.52-0.62)**
Vit 2x	Mean (M-m)	3(6.9-1)	0.21(0.4-0.13	14.6(5-36.3)	36.85(1.7-18.8	35.2-1.7	1.05(1.6-0.4)	0.69(1.2-0.3)
(NS)	**SD (CI)**	**1.4(2.5-3.5)**	**0.05(0.19-0.23)**	**6.7(12.1-17.1)**	**7.53(33.9-39.5)**	**0.78(2.7-3.3)**	**0.26(0.95-1.14)**	**0.19(0.61-0.75)**
Nik 2x	Mean (M-m)	3.4(6.8-1.3)	0.23(0.4-0.11	14.8(6.2-31.5)	40.65(5.4-21.6	3.24(8-2.1	11.4-0.7	0.7(1-0.3)
(NS)	**SD (CI)**	**1.52(2.8-3.9)**	**0.11(0.21-0.26)**	**5.9(12.5-17)**	**7.8(37.7-43.5)**	**0.75(2.9-3.5)**	**0.15(0.34-1.05)**	**0.15(0.6-0.71)**
	Mean (M-m)	1.7(5-0.9)	0.25(0.4-0.17)	6.7(2.5-17.9)	19.3(25.6-11.1)	2.6(3.9-1.8)	1.06(1.6-0.8	0.44(0.7-0.2)
(NS)	**SD (CI)**	**0.9(1.3-2.03)**	**0.06(0.23-0.27)**	**3.1(5.5-7.8)**	**3.3(18.1-20.5)**	**0.5(2.4-2.8)**	**0.2(0.99-1.14)**	**0.11(0.4-0.5)**
NB 2x	Mean (M-m)	2.9(4.8-1.1	0.33(0.41-0.21	9.1(3.9-17.1)	53.6(66.9-39.2	3.6(4.5-2.9	1.4(1.9-1)	0.9(1.2-0.7
(NS)	**SD (CI)**	**0.95(2.5-3.3)**	**0.05(0.31-0.35)**	**3.4(7.8-10.4)**	**8.1(50.6-56.6)**	**0.38(3.4-3.7)**	**0.26(1.3-1.51)**	**0.16(0.8-0.97)**

APPENDIX 3.1. Morphological variation in the studied populations of *A. odoratum* and *A. alpinum*. Measurements are given in cm. For population and character codes see Table 3.2 and 3 respectively. Pop., population. 4x, tetraploid; 2x, diploid. I, Iberian Peninsula; SS, southern Scandinavia; NS, northern Scandinavia. SD, standard deviation; M, maximum value; m, minimum value; CI, 95% confidence interval on the mean.

Pop.	Data	Sl	Lgl	Al	Ugw	Sfw	Stl	Ffl
Vl(I)	Mean (M-m)	0.79 (0.9-0.7)	0,39(0,5-0,3)	0,79(1,1-0,55)	0,35(0,42-0,29)	0.11(0.17-0.1)	0,31(0,41-0,29)	0.21 (0.25-0.15)
	SD (CI)	0.074(0.76-0.8)	0,055(0.3-0.5)	0,12(0.75-0.83)	0,04(0.34-0.37)	0.024(0.11-0.13)	0,036(0.29-0.32)	0.024(0.19-0.22)
Ol(I)	Mean (M-m)	0.77(0.87-0.63)	0,42(0.58-0,3)	0,77(0.9-0,44)	0,2(0,25-0,12)	0.08 (0.1-0.05)	0.29(0,4-0,26)	0.26 (0.32-0.2)
	SD (CI)	0.063(0.74-0.79)	0,066(0.39-0.44)	0,096(0.73-0.8)	0,025(0.19-0.21)	0.02(0.076-0.09)	0,04(0.27-0.33)	0.035(0.25-0.28)
A/(I)	Mean (M-m)	0.76(0.9-0.55)	0.4(0.5-0.3)	0.76(1-0.6)	0.2(0.27-0.14)	0.088(0.1-0.06)	0.3(0.36-0.24)	0.28(0.34-0.19)
	SD (CI)	0.08(0.73-0.8)	0.04(0.38-0.42)	0.09(0.72-0.8)	0.019(0.19-0.21)	0.01(0.08-0.09)	0.042(0.28-0.32)	0.028(0.28-0.3)
TB(I)	Mean (M-m)	0.77(0.91-0.62)	0.37(0.42-0.29)	0.82(1.02-0.62)	0.4(0.46-0.33)	0.12(0.18-0.07)	0.29(0.4-0.21)	0.19(0.23-0.15)
	SD (CI)	0.078(0.74-0.8)	0.035(0.36-0,39)	0.12(0.77-0.87)	0.03(0.38-0.4)	0.03(0.1-0.13)	0.05(0.27-0.31)	0.019(0.18-0.2)
Al 4x(SS)	Mean (M-m)	0.79(0.84-0.69)	0.5(0.55-0.41)	0.85(1.06-0.7)	0.3(0.39-0.27)	0.09(0.12-0.07)	0.31(0.36-0.27)	0.23(0.26-0.21)
	SD (CI)	0.04(0.76-0.79)	0.04(0.46-0.49)	0.09(0.81-0.88)	0.07(0.3-0.33)	0.01(0.09-0.1)	0.02(0.3-0.32)	0.012(0.22-0.24)
AV 4x(SS)	Mean (M-m)	0.77(1.01-0.65)	0.42(0.52-0.33)	0.81(1.08-0.52)	0.32(0.42-0.25)	0.105(0.13-0.08)	0.3(0.41-0.25)	0.21(0.27-0.19)
	SD (CI)	0.082(0.74-0.8)	0.046(0.4-0.43)	0.1(0.77-0.84)	0.038(0.31-0.34)	0.013(0.1-0.11)	0.031(0.29-0.32)	0.016(0.2-0.22)
	Mean (M-m)	0.75(0.84-0.62)	0.43(0.51-0.34)	0.86(1.02-0.72)	0.31(0.36-0.25)	0.1(0.13-0.08)	0.31(0.36-0.26)	0.22(0.25-0.18)
	SD (CI)	0.06(0.72-0.77)	0.04(0.42-0.45)	0.08(0.83-0.88)	0.03(0.29-0.32)	0.014(0.09-0.13)	0.025(0.3-0.32)	0.018(0.21-0.23)
Sk 4x(SS)	Mean (M-m)	0.74(0.86-0.74	0.43(0.49-0.35)	0.78(0.89-0.6)	0.32(0.41-0.26)	0.19(0.12-0.07	0.31(0.37-0.27)	0.21(0.23-0.18
	SD (CI)	0.06(0.72-0.77)	0.035(0.42-0.45)	0.07(0.76-0.81)	0.03(0.31-0.33)	0.015(0.09-0.11)	0.027(0.3-0.32)	0.014(0.21-0.22)
St 4x(SS)	Mean (M-m)	0.73(0.75-0.71)	0.42(0.51-0.32)	0.8(0.91-0.6)	0.31(0.35-0.28)	0.09(0.11-0.08)	0.3(0.33-0.23)	0.22(0.26-0.18)
	SD (CI)	0.05(0.71-0.75)	0.05(0.4-0.44)	0.06(0.78-0.83)	0.018(0.3-0.32)	0.01(0.09-0.1)	0.025(0.27-0.29)	0.02(0.21-0.23)
Nip 2x(NS)	Mean (M-m)	0.67(0.73-0.6)	0.37(0.45-0.31)	0.78(0.93-0.64)	0.29(0.35-0.22)	0.1(0.12-0.09)	0.36(0.4-0.31)	0.21(0.25-0.16)
	SD (CI)	0.036(0.66-0.69)	0.037(0.35-0.38)	0.06(0.76-0.81)	0.04(0.27-0.3)	0.008(0.1-0.12)	0.028(0.35-0.37)	0.02(0.2-0,22)
Vit 2x(NS)	Mean (M-m)	0.580.68-0.5	0.330.43-0.27	0.750.91-0.61	0.270.32-0.18	0.120.15-0.07	0.330.41-0.21	0.210.24-0.18
	SD (CI)	0.04(0.56-0.6)	0.038(0.31-0.34)	0.08(0.73-0.79)	0.03(0.26-0.28)	0.015(0.11-0.12)	0.04(0.31-0.34)	0.013(0.2-0.21)
Nik 2x(NS)	Mean (M-m)	0.58(0.67-0.44)	0.28(0.37-0.22)	0.73(0.89-0.6	0.26(0.34-0.2	0.11(0.15-0.07)	0.31(0.35-0.27)	0.18(0.21-0.14)
	SD (CI)	0.05(0.56-0.6)	0.037(0.27-0.3)	0.07(0.7-0.76)	0.03(0.25-0.28)	0.02(0.1-0.15)	0.02(0.3-0.32)	0.014(0.18-0.2)
	Mean (M-m)	0.6(0.66-0.47	0.31(0.37-0.28	0.78(0.95-0.65	0.27(0.35-0.23	0.1(0.12-0.09	0.34(0.41-0.29	0.21(0.24-0.2)
	SD (CI)	0.04(0.57-0.6)	0.02(0.3-0.32)	0.06(0.75-0.8)	0.027(0.26-0.28)	0.01(0.1-0.11)	0.03(0.33-0.35)	0.011(0.21-0.23)
NB 2x(NS)	Mean (M-m)	0.62(0.71-0.55	0.35(0.4-0.31)	0.96(1.04-0.85	0.17(0.21-0.15	0.05(0.06-0.05	0.36(0.41-0.34	0.19(0.23-0.18)
	SD (CI)	0.04(0.61-0.64)	0.017(0.34-0.35)	0.05(0.11-0.13)	0.016(0.16-0.18)	0.002(0.04-0.06)	0.02(0.35-0.38)	0.014(0.19-0.23)

APPENDIX 3.1. (Continued). Morphological variation in the studied populations of *A. odoratum* and *A. alpinum*. Measurements are given in cm. For population and character codes see Table 3.2 and 3 respectively. Pop., population. 4x, tetraploid; 2x, diploid. I, Iberian Peninsula; SS, southern Scandinavia; NS, northern Scandinavia. SD, standard deviation; M, maximum value; m, minimum value; CI, 95% confidence interval on the mean.

Capítulo 4

Morphological and molecular evidence of introgression between the grasses *Anthoxanthum ovatum* and *A. aristatum* in sympatry. Taxonomic consequences

Morphological and molecular evidence of introgression between the grasses *Anthoxanthum ovatum* and *A. aristatum* in sympatry. Taxonomic consequences

4

ABSTRACT

The sweet vernal grasses *Anthoxanthum aristatum* Boiss. and *A. ovatum* Lag. are very morphologically diverse when in sympatry. Occasional hybridization between these two rather polymorphic but quite differentiated species has been suggested to explain this fact. In this study, we aimed at determining whether the specific status holds or not in sympatric scenarios. For this, we collected and analysed macro-, micromorphological, and genetic (AFLP) data from six sympatric and two allopatric populations of *A. aristatum* and *A. ovatum*. We calculated principal coordinates analyses and contingency tables for both micromorphological and macromorphological qualitative data to summarize the morphological variance encountered and to look for possible distinctive characters. Subsequently, and regarding quantitative macromorphological traits, a principal components analysis and a canonical variate analysis were conducted to identify the most important characters in the differentiation of populations and taxa. In addition to this, the character count procedure was applied to try to identify putative hybrids within the sympatric populations. Next, the molecular relationships among samples were summarized by means of a principal coordinates analysis, and different dendrograms were constructed to represent the morphological and molecular distance among specimens, populations and taxa. Our results suggest extensive introgression between *A. aristatum* and *A. ovatum* in sympatry. According to our data, no infraspecific taxonomical distinction is justified within the *A. aristatum*/*A. ovatum* complex.

ADDITIONAL KEYWORDS: *Anthoxanthum aristatum* Boiss. - *Anthoxanthum ovatum* Lag. - sympatry - hybridization - multivariate morphometrics - leaf anatomy - AFLP - Doñana National Park - Taxonomy.

INTRODUCTION

Hybrids usually display a mosaic of parental and intermediate morphological characters, although new and extreme traits may also be common in the hybrid phenotype (Marhold *et al.*, 2002). Hybridization can be recognized by relatively small genetic differences between sympatric populations, where inbreeding may be common (Grant, Grant & Petren, 2005). Both interspecific hybridization and introgression between not well-differentiated taxa have been used to explain the existence of transitional forms between the annual diploids *Anthoxanthum ovatum* Lag. and *Anthoxanthum aristatum* Boiss. should they occur in sympatry (Valdés, 1973; López-González, 1994). In the present study, we used morphological and molecular tools to determine the causes of the morphological diversity observed in sympatric populations of these species.

A. aristatum and *A. ovatum* are distributed naturally in the Mediterranean basin and in Atlantic areas of southern Europe and northern Africa. The former taxon is native northwards up to northern France and eastwards up to the Balkans (Tutin *et al.*, 1980). The distribution of the latter is much more restricted. According to Valdés (1973) and Tutin (1980), *A. ovatum* grows mainly in dry open habitats of southern Iberia, Morocco and Algeria, but it is also present eastwards up to Greece and Turkey. However, following Dogan (1985) and Strid (1991) its presence in many of these eastern areas is doubtful (reviewed in López-González, 1994).

Different taxonomical arrangements have been proposed for the *A. aristatum*/*A. ovatum* complex during the last two centuries. On the one hand, the lack of distinctive characters between both taxa supported their taxonomical equivalence (Pérez-Lara, 1866; Soreng, 2003). On the other hand, their morphological differentiation was considered enough to maintain them as separate species (Willkomm & Lange, 1870; Paunero, 1953; Valdés, 1973; Tutin 1980; Pignatti, 1982).

Karyologically, these species are quite similar. They basically differ on the frequency and location of secondary constrictions (Jones, 1964). Experimental studies demonstrated that both taxa were able to hybridize, although this process involved some loss of fertility (Borrill 1963). It is worth noting that the identification of some of the individuals used in this latter study is uncertain, and the possibility of previous hibridization between some of the *A. ovatum* and *A. aristatum* samples used could not be discarded by the author (Borrill, 1963).

The relationships between these annual species and the perennial A. odoratum have

been also widely discussed. Thus, *Anthoxanthum aristatum* and *A. ovatum* have been classified as two races of the widespread tetraploid *Anthoxanthum odoratum* L. (*e.g.*, Battandier & Trabut, 1895; Maire, 1931; Fiori, 1969), that can behave as annual or biannual depending on environmental conditions (Borrill, 1962; Hedberg, 1964). According to López-González (1994), *A. odoratum* is morphologically undistinguishable from the *A. ovatum*/*A. aristatum* complex..

The high morphological variability found in the *A. aristatum*/*A. ovatum* complex led to define different infraspecific taxa, some of which have been widely accepted (Valdés, 1973; Soreng, 2003; but see López-González, 1994). Within *A. aristatum* two different subspecies have been distinguished: *Anthoxanthum aristatum* subsp *aristatum* and *Anthoxanthum aristatum* subsp. *macranthum* Valdés. Moreover, two varieties can be found in *A. ovatum*: *Anthoxanthum ovatum* var. *ovatum* and *Anthoxanthum ovatum* var. *exertum* Lindb. Reproductive traits have been usually employed to differentiate between *A. aristatum* and *A. ovatum* and among the different infraspecific taxa. The most commonly used characters are listed on Figure 4.1.

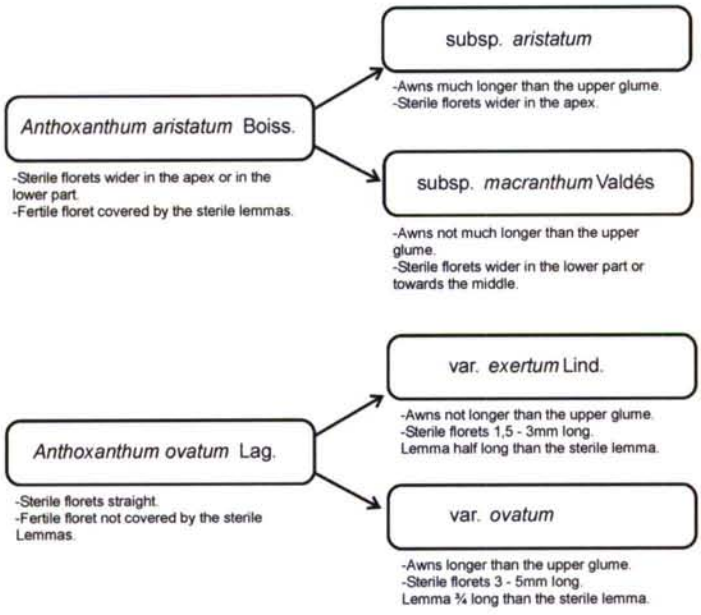

Figure 4.1. Infraspecific taxa described for *A. aristatum* and *A. ovatum* in the Iberian Peninsula.

The SW of the Iberian Peninsula is a suitable area to study the patterns and causes of morphological variability on these species and to assess their taxonomical status. On the

one hand, both *A. aristatum* and *A. ovatum* grow in this region, where all the infraspecific taxa can be found and both species are highly variable in morphology (López-González, 1994). On the other hand, according to Valdés (1987) *A. odoratum* is not present in this area, so that hybridization between this taxon and *A. ovatum* or *A. aristatum* can be excluded. The Doñana National Park (SW Iberian Peninsula) is a well-known highly preserved area where up to four annual *Anthoxanthum* taxa (*A. aristatum* subsp. *macranthum*, *A. aristatum* subsp. *aristatum*, *A. ovatum* var. *ovatum* and *A. ovatum* var. *exertum*) can be found growing sympatrically (Valdés, 1973; Rivas-Martínez *et al.*, 1980). In this mixed populations, transitional forms between the different taxa are common (López-González, 1994). To explain this pattern, hybridization between *A. aristatum* and *A. ovatum* (Valdés, 1973) and introgression between not well-differentiated taxa (López-González, 1994) have been proposed.

The main aims of the present study were: (i), to find out whether the sympatric scenario has resulted in a local morphological and genetic continuum of variation because of extensive inbreeding or whether the different taxa are maintained as well-separated entities and (ii), to clarify the taxonomical status of the iberian annual taxa of genus *Anthoxanthum*. To achieve these goals, different molecular and morphological methods were applied.

The study of hybridization among plants is largely based on morphological characters (Vázquez-Garcidueñas *et al.*, 2003). Multivariate analyses conducted with macro- and micromorphological data have been extensively used to distinguish between hybridization and common ancestry (Neuffer *et al.*, 1999; Hansen, Elven & Brochmann, 2000; Nielsen & Olrik, 2001; Hagen *et al.*, 2002; Marhold *et al.*, 2002; Tovar-Sánchez & Oyama, 2004). However, the accuracy of these statistical analyses is controversial, and other methods have been proposed. When interspecific hybridization occurs, it is expected that quantitatively inherited traits of hybrid individuals, mainly in the first generation, will be intermediate between their putative parents (Vázquez-Garcidueñas *et al.*, 2003). According to Wilson (1992), this intermediate state may not be revealed by multivariate statistics, and he proposed the "character count" procedure. In the light of this, we used different approaches, *i.e.*, multivariate statistics and the "character count" procedure to analyse the possible existence of hybridization in sympatric populations.

The analysis of morphology alone has the disadvantage of its limited usefulness in the description of the genetic relationships between closely related taxa. This weakness stresses the importance of using other sources of characteristics, mainly molecular, to

detect hybridization. (Rieseberg & Ellstrand, 1993). The amplified fragment length polymorphism (AFLP) technique (Vos *et al.*, 1995) proved to be useful to assess genetic differences between closely related species (Mueller & Wolfenbarger, 1999; Gaudeul *et al.*, 2004). Also, this procedure has been widely used in hybridization studies (*e.g.*, Han *et al.*, 2000; Marhold *et al.*, 2002), as well as in the resolution of taxonomic problems up to the species level (Sheen, Elven & Brochmann, 2002; Stuessy *et al.*, 2003).

The achieve our goals, we have applied a combined approach of morphological and molecular methods. This procedure has been considered useful in hybridization studies (*e.g.*, Neuffer *et al.*, 1999) and to address different taxonomic questions in closely related species (*e.g.*, Black-Samuelsson *et al.*, 1997; Hansen, Elven & Brochmann, 2000).

MATERIALS AND METHODS

PLANT MATERIAL

A total of 494 plants belonging to *A. ovatum* and *A. aristatum* were sampled on 6 mixed populations from the Doñana National Park (Andalusia, SW Spain) (Figure 4.2).

Collection sites were chosen in order to cover most of the morphological and taxonomic variation observed for the *A. aristatum*/*A. ovatum* group in the area. For this, herbarium specimens from this region were previously examined (BC, MA, SE.V;. Holmgren, Holmgren & Barnett, 1990). Also, 79 individuals belonging to two allopatric [*A. ovatum* var. *ovatum* (39 individuals) and *A. aristatum* subsp. *aristatum* (40 individuals)] populations were added to the survey (Figure 4.2). Lastly, one *Anthoxanthum alpinum* Löve & Löve population (35 individuals) was included in the analyses as an outgroup (Table 4.1). *A. alpinum*, a diploid perennial species with an alpine-boreal distribution, was included in the survey rather than the tetraploid *A. odoratum* since different studies suggested that *A. ovatum* is involved in the genesis of the latter (Borrill, 1963; Jones, 1964). Moreover, the *A. aristatum* and *A. ovatum* populations included as outgroups came from areas where only one annual *Anthoxanthum* taxa was present (Valdés, 1973, 1987; Devesa, 1991) and, therefore, a hybrid origin could be ruled out for them. All the collected specimens were tentatively identified following Valdés (1973) and Lauber & Wagner (2001).

Between 30 and 140 plants per locality were dried and employed in the morphometric (from 29 to 120 specimens) and micromorphological (up to 20 plants) studies. *A. alpinum* was excluded from micromorphological analyses. Also, fresh leaves from up to 10

individuals per locality were taken and stored in silica gel for genetic analyses (Chase & Hills, 1991). The origin of the plant material is listed in Table 4.1. Voucher specimens were deposited in the Santiago de Compostela University Herbarium (SANT; Holmgren, Holmgren & Barnett, 1990). Voucher numbers are listed on Table 4.1.

AFLP FINGERPRINTING

Figure 4.2. Map of distribution of sample sites of the analysed *Anthoxanthum* species. **1**, Pinar de El Acebuche; **2**, Navazo del Toro; **3**, Laguna de Santa Olalla; **4**, El Martinazo; **5**, Caño del Tío Antoñito; **6**, Pinar de San Agustín; **7**, Fuente Tres Cantos; **8**, Montera del Torero. The different taxa collected per locality are listed on Table 4.1.

Total genomic DNA was extracted from 25 mg of dried leaves using the Dneasy Plant Mini Kit (Quiagen, Hilden, Germany) according to the manufacturer's protocol. DNA quality and concentration were determined photometrically using the Cary 3E Spectrophotometer (Varian, Palo Alto, USA) and by electrophoresis on a 1% TAE-agarose gel.

The AFLP method was performed with the AFLP Analysis System I for plant genomic DNA (Invitrogen, Carlsbad, USA) following the manufacturer's protocol. Two PCR primer pairs with three selective bases, E-AAG/M-CTC and E-AGC/M-CAG, were selected over twelve pairs tested for the quality of produced bands (even distribution of

bands with relatively homogeneous intensity). To the best of our knowledge, the genome size of *A. aristatum* or *A. ovatum* has not been determined. Even so, the use of three selective bases has been recommended for *A. odoratum*, that is closely related to the *A. aristatum*/*A. ovatum* complex (Grime & Mowforth, 1982; Fay, Cowan & Leitch, 2005).

TAXA, ORIGIN, COLLECTION DATA AND VOUCHER NUMBER	Nº INDIVIDUALS	
	M./A.	AFLP
Anthoxanthum ovatum Lag. var. *ovatum*		
Spain, Doñana National Park, Navazo del Toro, 25.iv.2002, SANT-52180	30/5	5
Spain, Doñana National Park, Pinar del Acebuche, 25.iv.2002, SANT-53378	57/10	-
Spain, Doñana National Park, El Martinazo, 25.iv.2002, SANT-53379	22/5	-
Spain, Doñana National Park, Caño del Tio Antoñito, 25.iv.2002, SANT-53357	30/5	-
Spain, Doñana National Park, Pinar de San Agustin, 24.iv.2002, SANT-52183	30/5	5
Spain, Cádiz, Los Barrios, Los Alcornocales, Montera del Torero, 06.vi.2001, SANT-52186	29/5	5
Totals	198	15
Anthoxanthum ovatum Lag. var. *exertum* Lindb.		
Spain, Doñana National Park, Laguna de Santa Olalla, 25.iv.2002, SANT-52184	30/5	5
Spain, Doñana National Park, Caño del Tio Antoñito, 25.iv.2002, SANT-53384	30/5	-
Spain, Doñana National Park, El Martinazo, 25.iv.2002, SANT-53344	8/5	
Totals	68	5
Anthoxanthum aristatum Boiss. subsp. *aristatum*		
Spain, Doñana National Park, Navazo del Toro, 25.iv.2002, SANT-53368	25/5	-
Spain, Doñana National Park, El Martinazo, 25.iv.2002, SANT-53365	7/5	-
Spain, Doñana National Park, Caño del Tio Antoñito, 25.iv.2002, SANT-53375	30/5	5
Spain, Doñana National Park, Laguna de Santa Olalla, 25.iv.2002, SANT-52187	30/5	-
Spain, Monfragüe National Park, Fuente Tres Cantos, 04.iv.2001, SANT-52197	30/5	5
Totals	122	10
Anthoxanthum aristatum Boiss. subsp. *macranthum* Valdés		
Spain, Doñana National Park, Pinar de San Agustin, 24.iv.2002, SANT-52181	30/5	5
Spain, Doñana National Park, Caño del Tio Antoñito, 25.iv.2002, SANT-52185	30/5	5
Totals	60	10
Anthoxanthum alpinum Löve & Löve		
Sweden, Dalarna, Nipfjället Mt., 14.iv.2002, SANT-52182	30	5
TOTALS	478/85	45

Table 4.1. List of localities sampled per taxa, collection data, voucher number and number of individuals studied. M./A., individuals studied macro- and micromorphologically; AFLP, specimens used in the molecular analyses.

Products were electrophoresed on a 6% denaturing polyacrylamide gel and visualised through silver staining (Bassam, Caetano-Anolles & Gresshoff, 1991).

Amplified products were scored as presence (1) or absence (0) in a matrix that was manually constructed. Only clear bands with homogeneous intensity were taken into account. A DNA molecular weight (weight marker VI, Roche Pharmaceuticals, Basel, Switzerland) was included as a reference. We assessed the reproducibility of the observed patterns by reapiting the whole AFLP protocol twice for ten randomly chosen individuals.

The AFLP phenotype of each specimen was expressed as a vector of zeros and ones. Subsequently, we calculated the Euclidean and Jaccard's distances as well as the Dice's

similarity coefficient for each pair of individuals. The equivalence between the different distance measures was assessed by means of a Mantel correlation analysis (following Legendre & Legendre, 1998), whose level of significance was estimated by a permutation test (1000 replicates).

We used two different analytical approaches. Firstly, a principal coordinates analysis (PCoA) with minimum spanning tree (MST, Gower & Ross, 1969) was conducted to examine the structure of the data (Torrecilla *et al.*, 2003). It also shed some light on whether the obteined groups were consistent with the taxonomic arrangement proposed for the *A. aristatum*/*A. ovatum* complex or not. Secondly, we calculated a Neighbor-joining (NJ) dendrogram based on the Euclidean distance in order to display the relationships among samples. The pairwise distances, PCoA-MST analysis and the Mantel tests were calculated with the program NTSYS-pc Version 2.11a (Rohlf, 2002). We used the program MEGA Version 2.1 (Kumar *et al.*, 2001) in order to build the NJ topology.

MACRO- AND MICROMORPHOLOGICAL ANALYSIS

We studied 37 macromorphological vegetative and floral traits for each specimen. Twenty of them were quantitative traits (five consisting on ratios used to assess shape), whereas the remaining 17 were qualitative characters (Table 4.2). We selected these 38 traits following Paunero (1953), Valdés (1973), López-González (1994) and Pimentel & Sahuquillo (2003a). All traits commonly used to differentiate among the taxa within the *A. aristatum*/*A. ovatum* complex were considered in the present study. Also, other characters of interest in the Poaceae taxonomy have been included in the survey.

Leaf micromorphological data proved to be useful to solve different taxonomic and ecological questions in the Poaceae, as well as an indicator of ploidy level in different grass genera including *Anthoxanthum* (*e.g.*, Cutler, 1978; Humbert-Droz & Felber, 1992; Giraldo-Cañas, 2001). In the present study, we selected a total of 29 micromorphological traits (14 from the abaxial epidermal surface and 15 from the leaf-blade transverse section) (Table 4.3). Samples were processed following Devesa (1992), with minor modifications to adapt the method to the fragile leaves of these species. For the description of the samples we followed Ellis (1976, 1979), Nicora & Rúgolo de Agrassar (1987), Devesa (1992), Aliscioni & Arriaga (1998) and Pimentel & Sahuquillo (2003b). All the quantitative data obtained *per* specimen are the mean value of twelve different

measures. Finally, we transformed figures into binary variables following the gap coding method (Rae, 1998).

Quantitative Characters and Ratios	Qualitative Characters
Plant height (cm)	Life cycle (0 annual, 1 perennial)
Leaf length[1] (cm)	Culm structure (0 single, 1 ramose)
Leaf width[1] (cm)	Sheath pubescence (0 glabrous, 1 hairy)
Inflorescence length (cm)	Leaves pubescence (0 glabrous, 1 hairy)
Spike lower branch length (cm)	Convolute leaves (0 planar, 1 convolute)
Spike lower internode length (cm)	Ligule shape (0 round, 1 pointed)
Spikelet length[2] (cm)	Presence of auricles (0 present, 1 absent)
Upper glume width (cm)	Colour of auricles (0 green, 1 reddish)
Lower glume length (cm)	Presence of a bract in the base of the spike([I])
Upper sterile floret length (cm)	Rachis pubescence (0 glabrous, 1 hairy)
Lower sterile floret length (cm)	Upper glume pubescence (0 glabrous, 1 hairy)
Awn length (cm)	Mucronate glumes (0 not-mucronate, 1 mucronate)
Fertile floret length (cm)	Upper glume scabridity([II])
N° of long paracladia	Upper sterile lemma erose or entire([III])
Number of nodes	Sterile lemmas pubescence ([IV])
	Presence of a forked apex in the upper sterile floret([V])
	Amounto of overlap of fertile floret by sterile lemmas
Leave length/leave width	
Awn length/spikelet length	
Awn length/spikelet floret length	
Sterile floret length/sterile floret width	
Fertile floret length/sterile floret length	

Table 4.2. List of characters measured for morphometric analyses. [1] Characters measured in the second leaf from the base of the plant; [2] characters measured in the fourth spikelet form the base of the inflorescence. ([I]), bract in the base of the spike absent (0), or present (1); ([II]), upper glume smooth (0), or scabrid (1); ([III]), upper sterile lemma entire (0), or erose (1); ([IV]), sterile lemmas glabrous (0), or hairy (1); ([V]) upper sterile lemma lacks a forked apex (0), upper sterile lemmas present a forked apex (1).

In order to calibrate the different usefulness of each sort of data, we constructed different datasets for qualitative, quantitative (macromorphological) and micromorphological characters (following Dufrêne, Gathoye & Tyteca, 1991; Greimler, Hermanowski & Jang, 2004). In addition to this, quantitative variables and ratios were analysed separately in order to avoid decreasing the efectiveness of the multivariate analyses performed on quantitative characters (Goldman, van den Berg & Griffith, 2004). All the statistical analyses but the PCoA were performed using the program SPSS 11.5 for Windows (SPSS Inc., Chicago, USA). The principal coordinates analyses were computed using the NTSYS-pc Version 2.1 (Rohlf, 2002).

For all quantitative characters, boxplots, descriptive statistics and the coefficient of variation (*cv*) were computed on each population to eliminate outliers. To avoid misinterpretations of the results, and given that the variables were both continuous and discrete, data were $\log_{10}$-transformed (Castro *et al.*, 2005). Next, Spearman's and

Pearson's correlation coefficients were calculated between all morphological quantitative traits in order to avoid redundant information. All the characters that were strongly correlated were excluded from subsequent analyses (Lihová, Tribsch & Stuessy, 2004).

	EPIDERMAL TRAITS	LEAF BLADE TRANSVERSE SECTION
intercostal zones	Abaxial epidermis type (^{A})	Bundle sheath outline (0, round; 1, elliptical)
	Long cell type (^{B})	Bundle sheath cell shape (0, round; 1, angular)
	Long cells length* (^{E}) (LCI)	Bundle sheath cells equal in size (0) or not (1)
	Presence of short cells (0, absent; 1, present) (SI)	Median vb. sheath type (0, single; 1, complete)
	Short cells length* (^{G}) (LSI)	Median vb. sheath cell diameter (0, < 15 µm; 1, = 15 µm) (M)
	Exod. elements (0, presence; 1, abscence)	N° of cells in vb sheath (0, < 17 cells; 1, = 17 cells)
	Stomate type (0, parallel-sided; 1, dome-shaped)	N° of bulliform cells per group (0, < 6 cells; 1, = 6 cells)
costal zones	Long cell type (^{B})	N° of vb. per leaf (0, <15; 1, = 15) (VB)
	Long cell length* (^{F}) (LCN)	Median vb. type (^{D}) (VBT)
	Presence of short cells (0, absent; 1, present) (SN)	Median vb. diameter (^{C}) (0, <75 µm; 1, = 75 µm) (VBD)
	Short cell length (0, < 50 µm; 1, = 50 µm)	Presence of a radial chlorenchyma (0, absent; 1, present)
	Exod. elements (0, presence; 1, abscence)	Colorless parenchyma (0, absent; 1, present)
		Ribs and furrows (0, absent; 1, present)
	Presence of bulliform cells (0, absent; 1, present)	Developed keel (0, absent; 1, present)
	Leaves pruinose (0, not pruinose; 1, pruinose)	Bulliform cell diameter (0, < 30 µm; 1, = 30 µm)

Table 4.3. List of anatomical traits considered in the analyses. (A), abaxial epidermis homogeneous (0) or siliceous-homogeneous (1). (B), long cells l2 (0) or l3 (1) Devesa (1992). (C), maximum diameter of the vascular bundle measured horizontally. (D), median vascular bundle free or joined to the epidermis by schlerenchyma girders. (*) Multistate characters. Each of the states is interpreted as an independent binary variable. (E), LCI1, 101-200 µm; LCI2, 301-400 µm; LCI3, 401-500 µm; LCI4, 501-600µm; LCI5, 601-700µm. (F), LCN1, (101-200µm; LCN2, 201-300µm; LCN3, 301-400µm; LCN4, 401-500µm. (G), LSI1, 11-20µm; LSI2, 21-40µm; LSI3, 41-50µm). Exod. elements, exodermic elements (Devesa, 1992). vb., vascular bundle.

A correlation-based principal component analysis (PCA) was calculated to select the most taxonomically interesting traits. Next, a canonical variate analysis (CVA) was computed in order to assess the distinction among the groups as well as the discriminatory power of variables (Hörandl, 2002). Three different arrangements of populations were tested in the CVA: (i) infraspecific taxa; (ii) *A. aristatum vs A. ovatum vs A. alpinum*; (iii) Doñana populations *vs* allopatric populations.

A classification discriminant test (CDA, cross-validation) was carried out in order to determine the group into which the classified object belongs with the highest probability (Legendre & Legendre, 1998; Repka, 2003; Marhold *et al.*, 2005). Again, three

arrangements of population were tested: (i), infraspecific taxa; (ii), *A. aristatum vs A. ovatum vs A. alpinum* and (iii), Doñana *vs* outgroups. Finally, the pairwise distance between samples was calculated by means of the Manhattan distance and represented in a phenogram constructed using the UPGMA clustering method.

The "character count procedure" for the detection of hybrids (Wilson, 1992) was also applied to quantitative morphological data (excluding ratios). This method aims at determine whether there is a statistically significant proportion of intermediate character states in putative hybrids. As outlined by Wilson (1992), this can be achieved by means of a one-sided sign test of intermediate *vs* non-intermediate characters. The test will be significant at the 0.05 level when 7 out of 8 characters are intermediate. The criterion to consider a putative hybrid character as intermediate is that its mean value lies between the mean values of the putative parents for this character. To interpret the results of this analysis, it must be considered that the low morphological differentiation between parents reduce the accuracy of this method (Wilson, 1992; Henderson & Martins, 2002; Vázquez-Garcidueñas, 2003).

As putative parents we selected two different pairs of populations. (i) The allopatric populations of *A. aristatum* and *A. ovatum*. (ii) *Anthoxanthum ovatum* var *ovatum* and *A. aristatum* subsp. *macranthum* from Doñana National Park. Hybridization between the latter pair has been suggested by Valdés (1973).

Regarding qualitative and transformed micromorphological traits, the relationships among samples were established by means of a cluster analysis based on the Phi coefficient. Next, a PCoA was performed to detect clusters of populations. Finally, contingency tables were constructed and the Lambda (λ) coefficient was estimated to calibrate the differentiation among groups and to assess the power of independent variables (characters) to infer the values of the dependent ones (group or taxa).

RESULTS

AFLP ANALYSIS

The total number of bands per taxa, as well as the number of private markers found on each group are listed on Table 4.4. Overall, 121 reproducible AFLP markers were scored, allowing the differentiation of 80% of the samples on the basis of their AFLP profile. The number of private fragments was low accross all the studied taxa but in *A. alpinum*, where 23.9% of the markers were exclusive. Within the *A. aristatum*/*A. ovatum*

complex, the percentage of private bands ranged between 0% and 1.56% (Table 4.4). The number of shared bands between pairs of taxa was also estimated. Nearly all the markers were shared among more than two taxa. Conversely, only four markers were exclusive of a pair of taxa (Table 4.5).

TAXON (N)	Nº OF MARKERS	PRIVATE MARKERS
A. ovatum exertum (Doñana, 5)	69	0
A. ovatum ovatum (Doñana, 10)	72	0
A. aristatum macranthum (Doñana, 10)	89	1 (1.12%)
A. aristatum aristatum (Doñana, 5)	64	1 (1.56%)
A. aristatum (Doñana, overall, 15)	97	2 (1.02%)
A. ovatum (Doñana, overall, 15)	85	0
A. aristatum (Fuente de Tres Cantos, 5)	67	1(1.5%)
A. ovatum (Montera del Torero, 5)	68	1 (1.5%)
A. alpinum (outgroup, 5)	71	17(23.9%)
TOTAL	**121**	

Table 4.4. Overall number of AFLP bands and number of private markers scored for the analysed *Anthoxanthum* taxa. (n) - number of analysed individuals.

PAIR OF TAXA	ov ar	ov ox	ov oo	ov aa	ov am	ar ox	ar oo	ar aa	ar am	ox oo	ox aa	ox am	oo aa	oo am	aa am
SHARED CHAR.	42	45	56	44	60	49	51	45	60	56	52	60	56	62	54
P. S. C.	0	0	0	0	1	1	0	0	0	2	0	0	0	0	0

Table 4.5. Number of fragments shared by each pair of taxa (second row) and number of markers shared by the pair of taxa but not with any other taxon (third row); ov - *A. ovatum* from Montera del Torero (allopatric), ar - *A. aristatum* from Monfragüe (allopatric), ox - *A. ovatum* var. *exertum* from Doñana, oo - *A. ovatum* var. *ovatum* from Doñana, aa - *A. aristatum* subsp. *aristatum* from Doñana, am - *A. aristatum* subsp. *macranthum* from Doñana. Char., characters; p.s.c., private shared characters.

Mantel correlation coefficients between the different distance or similarity indices were high and significant (*e.g.*, Euclidean distance *vs* Dice's coefficient; R_M = -0.967, P = 0.001). The Euclidean distance was employed for the subsequent analyses.

The PCoA-MST performed with AFLP data (Figure 4.3) showed a clear differentiation between the annuals and the *A. alpinum*. Also, the allopatric populations from Montera del Torero (*A. ovatum* var. *ovatum*) and Fuente de Tres Cantos (*A. aristatum* subsp. *aristatum*) appared in opposite positions in the scatterplot. Regarding Doñana specimens, they were subdivided into two groups linked by the MST. The first group was composed by *A. aristatum* subsp. *aristatum*, *A. ovatum* var. *ovatum* and *A. ovatum* var.

exertum specimens, whereas the second included the *A. aristatum* subsp. *macranthum* populations. The NJ dendrogram constructed (Figure 4.4) showed a quite similar distribution of specimens. Again, the allopatric populations occupied opposite positions in the plot and the *A. aristatum* subsp. *macranthum* specimens clustered together and independently from the remaining Doñana specimens.

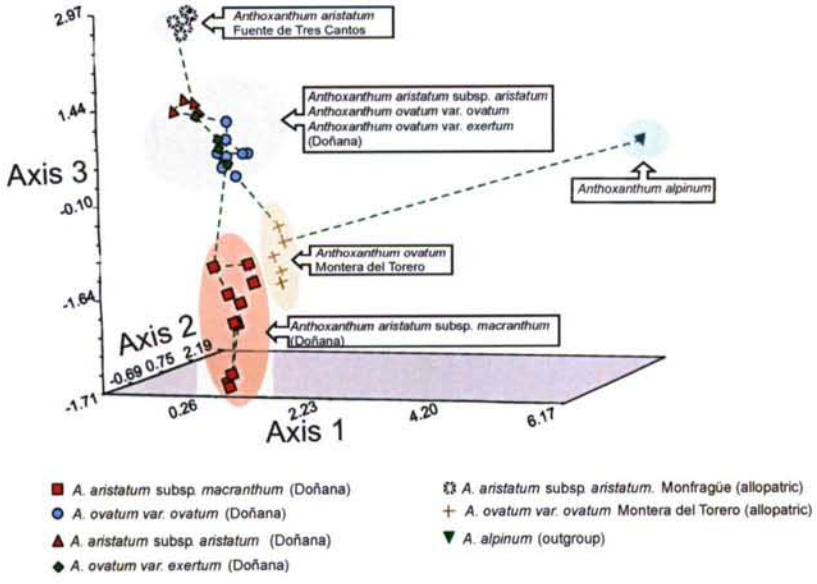

Figure 4.3. Principal coordinate analysis performed with AFLP data.

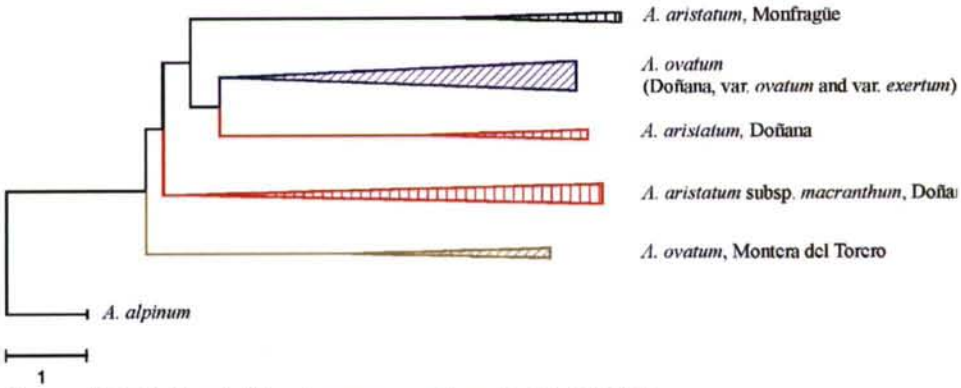

Figure 4.4. Neighbor-joining phenogram performed with AFLP data.

MORPHOMETRIC AND ANATOMICAL ANALYSES

Descriptive statistics and boxplots showed a lack of morphological differentiation among taxa and populations. Only *A. alpinum* was clearly separated by a few variables, particularly upper sterile lemma length, fertile floret length, number of long paracladia and spike lower internode length. Conversely, quantitative characters were not able to differentiate among annual taxa. Only plant height was significantly higher on the Montera del Torero population (*A. ovatum* var. *ovatum*, allopatric). Regarding the *cv*, vegetative characters were much more variable than floral ones through all taxa and populations (coefficient of variation, *cv* = 0.31 and 0.104 respectively). The same result has been observed in other *Anthoxanthum* species (Pimentel & Sahuquillo, 2003a). It is noteworthy that this behaviour, that has been already observed in many other plant species, is more common in zoophilous plants than in anemophilous species like *Anthoxanthum* (Herrera, 2005 and references therein).

Pearson's and Spearman's indices showed a high degree of correlation between several characters. Eight uncorrelated quantitative traits were used in the subsequent analyses (plant height, spike lower branch length, spike lower internode length, spikelet length, upper glume width, upper sterile lemma length, fertile floret length and number of long paracladia).

The PCA scatterplot showed no clear grouping of populations (not shown). Conversely, a clear pattern was observed in the CVAs performed using the following explanatory variables: (i), the specific classification (*A. aristatum vs A. ovatum vs A. alpinum*, Figure 4.5) and (ii), Doñana *vs* outgroups (Figure 4.6). In both cases, the *A. alpinum* population appeared clearly differentiated. Also, the populations belonging to the *A. aristatum*/*A. ovatum* complex formed a continuum with a clear morphological transition between *A. aristatum* and *A. ovatum*. It is noteworthy that allopatric populations ocupied extreme and opposite positions in the plot (Figure 4.5). In both analyses, the first three canonical functions accounted for more than 70% of the total variation.

None of the different multivariate analyses conducted with morphological quantitative data showed the same pattern obtained in the AFLP-based PCoA-MST (Figure 4.3). Canonical variate analyses were also performed with ratios; however, no clear pattern could be observed in the diagram (not shown).

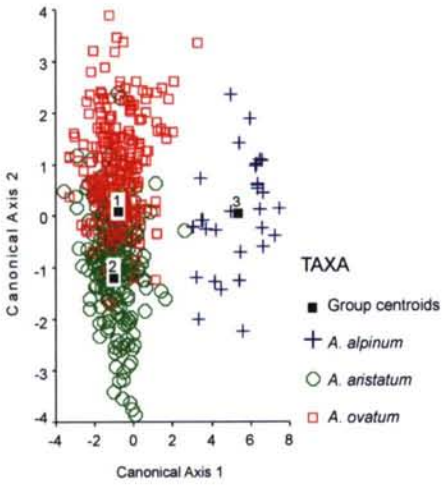

Figure 4.5. Canonical axis one and two of the canonical variate analysis (CVA) of all the studied populations. The specific classification was used as explanatory variable.

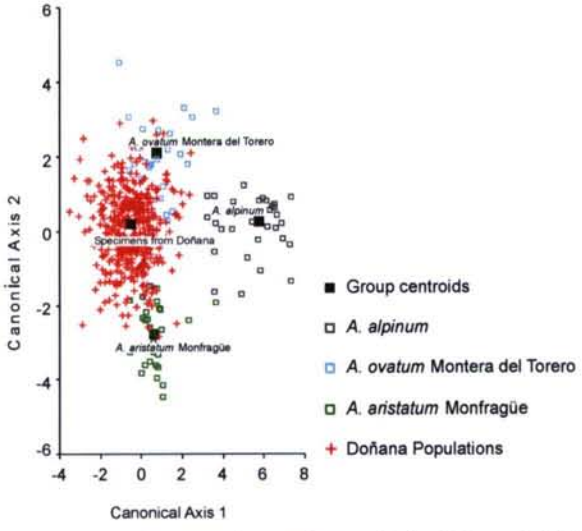

Figure 4.6. Canonical axis one and two of the canonical variate analysis (CVA) of all the studied populations. The differentiation between Doñana and the outgroups was used as explanatory variable.

The CDA carried out with quantitative data (Table 4.6) and ratios showed very similar results. When the infraspecific classification was used as explanatory variable (Table 4.6, A), 196 out of 478 individuals were misclassified (41%). Very similar results were obtained when the specific classification was used as explanatory variable (33.9% of

misclassified individuals, Table 4.6, B). Conversely, when the groups of populations were established according to their geographical origin (Doñana *vs* allopatric populations *vs A. alpinum*), only 25 out of 478 specimens were misclassified (5.2%, Table 4.6, C). The percentage of specimens uncorrectly placed varied a lot among taxa or group of populations. Only 1.6% of individuals identified as *A. aristatum* subsp. *macranthum* were correctly clustered, whereas 66.6% of them clustered with *A. ovatum* var. *ovatum* samples. Also, 83.8% of the *A. ovatum* plants were correctly grouped, whilst this percentage lowered to 65% in the *A. aristatum*.

A		PREDICTED GROUP				
		1	2	3	4	5
INFRASPECIFIC CLASSIFICATION	1	31(45.6%)	16(23.5%)	21(30.8%)	0(0%)	0(0%)
	2	3(1.5%)	175(88.4%)	19(9.6%)	0(0%)	1(0.6%)
	3	24(19.6%)	52(42.5%)	45(36.9%)	0(0%)	1(0.5%)
	4	4(6.7%)	40(66.6%)	15(25%)	1(1.6%)	0(0%)
	5	0(0%)	0(0%)	0(0%)	0(0%)	100(100%)
B		**PREDICTED GROUP**				
		1	2	3	4	
GEOGRAPHICAL ORIGIN	1	382 (98.2%)	3(0.77%)	4(1.03%)	0(0%)	
	2	7(23.3%)	22(73.3%)	0(0%)	1(3.4%)	
	3	3(10.3%)	0(0%)	25(86.2%)	1(3.5%)	
	4	0	0	0	30	
C		**PREDICTED GROUP**				
		1	2	3		
SPECIFIC CLASSIFICATION	1	223(83.8%)	42(15.8%)	1(0.4%)		
	2	118(64.8%)	63(34.6%)	1(0.6%)		
	3	0(0%)	0(0%)	30(100%)		

Table 4.6. Results of the CDA (cross-validation) performed on quantitative data. A: 1, *A. ovatum var. exertum*; 2, *A. ovatum* var. *ovatum*; 3, *A. aristatum* subsp. *aristatum*; 4, *A. aristatum* subsp.. *macranthum*; 5, *A. alpinum* (outgroup). B: 1, *Anthoxanthum* specimens from Doñana National Park; 2, *A. aristatum* from Monfragüe (outgroup); 3, *A. ovatum* from Montera del Torero (outgroup); 4, *A. alpinum* (outgroup). C: 1, *A. ovatum*; 2, *A. aristatum*; 3, *A. alpinum* (outgroup).

The "character count procedure" (Wilson, 1992) was also applied to quantitative data (ratios excluded). The putative parents selected were the allopatric populations (Montera del Torero and Fuente de Tres Cantos), where the possibility of hybridization can be ruled out and the pair *A. aristatum* subsp. *macranthum* and *A. ovatum* var. *ovatum* from Doñana.

No evidence of hybridization could be drawn from the different crosses tested. Only *A. aristatum* subsp. *macranthum* presented a high, though not significative ($P = 0.342$),

number of intermediate phenotypes between *A. aristatum* and *A. ovatum*. Nevertheless, it must be taken into account that the small differences between putative parents reduces the usefulness of this method (Table 4.7).

	Parents		P. hybrid		P. hybrid	
Traits	**Aa**	**Ao**	**Ax (D)**	**Int?**	**Am (D)**	**Int?**
Plant size	16.442 ±3.96	57.1±15.75	22.1±7.9	Y	29.7±10.5	Y
Spikelet size	0.62 ±0.009	0.72±0.1	0.751±0.009	N	0.72±0.05	N
Upper sterile floret length	0.243±0.005	0.213±0.005	0.267±0.008	N	0.217±0.03	Y
Fertile floret length	0.140±0.003	0.170±0.005	0.185±0.002	N	0.18±0.026	N
Upper glume width	0.3±0.003	0.33±0.004	0.342±0.005	N	0.32±0.034	Y
Spike lower branch length	0.923±0.023	0.92±0.03	1.07±0.03	N	1±0.18	N
Spike lower internode	0.34±0.0125	0.447±0.014	0.264±0.12	N	0.26±0.08	N
N° of long paracladia	9.9±0.468	5.93±0.4	11.18±0.2	N	7.92±0.3	Y
Totals				**7:1**		**4:4**
	Parents		P. hybrid		P. hybrid	
Traits	**Am (D)**	**Av (D)**	**Ar (D)**	**Int?**	**Ax (D)**	**Int?**
Plant size	29.7±10.5	27.83±8.64	25.7±8.8	N	22.1±7.9	N
Spikelet size	0.72±0.05	0.709±0.05	0.72±0.006	N	0.751±0.009	N
Upper sterile floret length	0.217±0.03	0.217±0.02	0.24±0.006	N	0.267±0.008	N
Fertile floret length	0.18±0.026	0.174±0.02	0.17±0.002	N	0.185±0.002	N
Upper glume width	0.32±0.034	0.33±0.04	0.326±0.03	Y	0.342±0.005	N
Spike lower branch length	1±0.18	0.950±0.015	1.03±0.017	N	1.07±0.03	N
Spike lower internode	0.26±0.08	0.216±0.07	0.27±0.009	N	0.264±0.12	N
N° of long paracladia	7.92±0.3	11.18±1.753	9.45±0.2	Y	11.18±0.2	N
Totals				**2:6**		**0:8**

Table 4.7. Character count procedure for quantitative morphological data. Aa, *Anthoxanthum aristatum* subsp. *aristatum* from Monfragüe (outgroup); Ao, *A. ovatum* var. *ovatum* from Montera del Torero (outgroup); Ax (D), *A. ovatum* var. *exertum* from Doñana; Am (D), *A. aristatum* subsp. *macranthum from Doñana*; Av (D), *A. ovatum* var. *ovatum* from Doñana; Av (D), *A. ovatum* var. *ovatum* from Doñana; Ar (D), *A. aristatum* subsp. *aristatum* from Doñana. Int, intermediate. P. hybrid, putative hybrids.

Regarding qualitative traits, the PCoA and the cluster analysis performed showed no clear grouping of specimens (not shown). In addition to this, all Lambda values associated to the different variables were low regardless the arrangement of populations chosen (Table 4.8). The highest values obtained corresponded to leaves pubescence, presence of mucronate glumes, structure of the culm, presence of a bract in the base of the spike and raquis pubescence. The two first characters partially distinguished between *A. ovatum* and *A. aristatum*, whilst all the other features differentiated mainly between Doñana and the allopatric populations.

Character	Infr. taxa Lambda	Doñana *vs* A. P. Lambda	Species Lambda	Sampled localities Lambda
Annual or perennial	0	0	0	0
Culm single or ramose	0.213	0.273	0	0.042
Number of nodes	0	0	0	0
Sheath pubescence	0.042	0.02	0	0
Convolute leaves	0.016	0	0.056	0.014
Leaves pubescence	0.045	0	0.337	0.084
Presence of auricles	0.097	0.124	0	0.084
Colour of auricles	0	0	0	0.112
Ligule shape	0.032	0.02	0	0.031
Presence of a bract in the base of the spike	0.184	0.186	0	0.028
Number of paracladia	0.048	0.116	0	0
Raquis pubescence	0	0.182	0	0
Mucronate glumes	0.03	0	0.337	0.084
Upper glume pubescence	0	0	0	0
Upper glume scabrous or smooth	0	0	0	0
Upper sterile floret erose or entire	0.168	0.120	0	0
Presence of a notch in the upper sterile floret	0.09	0	0	0
Sterile florets pubescence	0	0.035	0.022	0.006
Fertile floret covered by the sterile ones	0.03	0	0	0

Table 4.8. List of Lambda values obtained with qualitative data. Numbers are referred to the different arrangement of specimens and populations. 1, Infraspecific taxa following Valdés (1973); 2, *Anthoxanthum* specimens from Doñana *vs* outgroups; 3, *A. aristatum vs A. ovatum*; 4, Localities. *A. alpinum* excluded from the analysis. A.P., allopatric populations; Infr., infraspecific.

As showed by the PCoA conducted with micromorphological data (Figure 4.7), a clear distinction between the populations from Doñana and the outgroups can be found.

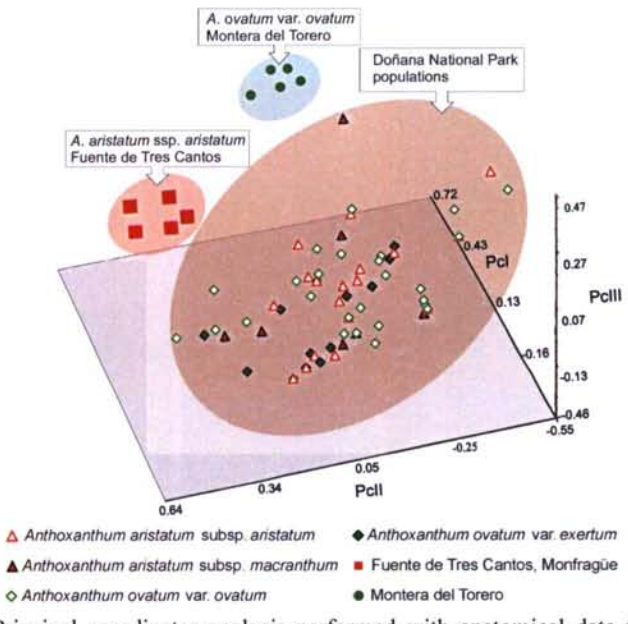

Figure 4.7. Principal coordinates analysis performed with anatomical data from the epidermal surface.

Moreover, high Lambda values were obtained for some of the characters analyzed, particularly the presence of short cells in both costal and intercostal areas ($\lambda = 0.67$ and 0.58, respectively), the presence of macrohairs and prickles in intercostal areas ($\lambda = 0.44$ and 0.55, respectively) and the typoly of long cells in intercostal areas ($\lambda = 0.43$). These traits were useful to characterize one or both allopatric populations, but never groups within Doñana. This behaviour might suggest the influence of the environmental conditions. Such an influence has been already highlighted in the Poaceae by different authors (Böcher, 1979; Aiken, Darbyshire & Lefkovitch, 1985; Ramesar-Fortner *et al.*, 1995).

DISCUSSION

The PCoA-MST performed with AFLP-data showed that all sympatric populations from Doñana but those belonging to *A. aristatum* subsp. *macranthum* were closer to each other than to any of the allopatric populations (Figure 4.3). Specimens belonging to *A. aristatum* subsp. *macranthum* somewhat showed a higher genetic differentiation within the Doñana group. In addition to this, populations belonging to this taxon occupied an independent position between the allopatric *A. ovatum* and *A. aristatum* populations in the NJ dendrogram (Figure 4.4). It is noteworthy that *A. aristatum* subsp. *macranthum* shared a high number of markers with both outgroups and with populations of *A. ovatum* var. *ovatum* and var. *exertum* from Doñana (Table 4.5).

The AFLP bands (Table 4.5) appeared homogeneously distributed accross all populations and taxa, and private fragments were scarce but in *A. alpinum*. Also, most of the bands were shared among more than two groups, indicating a lack of genetic differentiation between *A. aristatum* and *A. ovatum*. This might be explained by extensive introgression between both putative species in sympatry, that blurs the limits between both taxa (Grant, Grant & Petren, 2005).

As showed by the different multivariate analyses conducted with quantitative traits, Doñana populations presented an intermediate phenotype between the outgroups. This pattern was partially consistent with the one obtained with AFLP data (Figure 4.3), and indicated a complete morphological intergradation between both taxa. Nevertheless, some differences can be found. *A. aristatum* subsp. *macranthum* specimens did not cluster independently in the analyses performed with quantitative data. As showed by the CDA (Table 4.6, A), the specimens belonging to this taxon clustered with *A. ovatum* var.

ovatum. Regarding qualitative and micromorphological traits, they failed to reveal any grouping of specimens or populations. However, some characters such as leaves pubescence, mucronate glumes or the presence of short cells in costal and intercostal areas of the abaxial epidermis might have a certain taxonomic interest. However, our data suggest the influence of the environment on these characters.

According to our results, the morphological differentiation of *A. aristatum* and *A. ovatum* and their infraspecific taxa is not possible in sympatry. The CVA performed using the infraspecific classification as explanatory variable (not shown) showed a continuity among the different subspecies and varieties. On the other hand, none of the characters usually employed to distinguish among the different taxa (sterile lemma length, sterile lemma length/sterile lemma width, fertile floret length/sterile lemma length and amount of overlap of fertile floret by the sterile lemmas) was useful to characterize them. Also, the genetic differentiation between both taxa is rather low and the possibility of hybridization has been shown (Borrill, 1963).

According to the CDA (Table 4.6), 41% and 33% of the individuals are misclassified when the infraspecific and specific classifications are employed. Within the infraspecific taxa, the percentage of misclassified individuals varied a lot depending on the taxa considered. Regarding *A. aristatum* subsp. *macranthum*, 67% of its specimens clustered with *A. ovatum* var. *ovatum*. Conversely, only 11.6% of the individuals belonging to the latter taxon were misclassified. The morphological identity between both taxa had been already suggested by López-González (1994). However, it is noteworthy that, according to our results, *A. aristatum* subsp. *macranthum* specimens were slightly differentiated genetically (Figures 4.3 and 4.4). In our opinion, this might be explained by omgoing sympatric speciation. Nevertheless, further research including new populations and areas would be needed to get more conclusive results.

The percentage of misclassified specimens was also high in *A. ovatum* var. *exertum* (54.4%) and in *A. aristatum* subsp. *aristatum* (63.1%). The distinction of these infraspecific taxa is, therefore, doubtful. Regarding the species, the percentage of well classified specimens was much higher in *A. ovatum* (83.8%) than in *A. aristatum* (34.6%). The low percentage observed in the latter species is due to the inclusion of *A. aristatum* subsp. *macranthum*. Conversely, almost 95% of the plants are correctly placed when Doñana populations are compared to the outgroups. This pattern might reflect the environmental influence on different morphological and anatomical traits. This influence has already been showed in different species in the Poaceae and other families (*e.g.*,

Aiken, Darbyshire & Lefkovitch, 1984; Hauenstein, Arriagada & Latsague, 1990; Petit & Thompson, 1997).

In our opinion, the *A. aristatum*/*A. ovatum* complex is an example of ongoing evolution. Within this group, both species represent two quite morphologically differentiated assemblages in allopatry. However, the limits between species are blurred in sympatry due to extensive inbreeding between both taxa and to the lack of ecological separation between them. According to Jones (1964) and Borrill(1963), hybridization between *A. aristatum* and *A. ovatum* involves a certain loss of fertility in the offspring. However, in our opinion, their results are of limited value in natural populations.

Similar situations can be observed in the *Anthoxanthum odoratum* L./*A. alpinum* complex (reviewed in Hedberg, 1990), where, however, ecological barriers between the species exist (Felgrová & Krahulec, 1999). Polyploid complexes, hybridization and recent speciation processes are quite common in the Poaceae due to its recent diversification in geological terms (Jacobs, Kingston & Jacobs, 1999; Kellogg, 2001).

Our results seems to indicate that no infraspecific taxa should be described within the *A. aristatum*/*A. ovatum* complex (Pérez-Lara, 1866; Paunero, 1953; López-González, 1994). Moreover, to decide whether *A. aristatum* and *A. ovatum* should be kept as different species, new populations from different areas should be included in the survey.

CONCLUSIONS

1. According to our results, extensive introgression between *A. aristatum* and *A. ovatum* rather than occasional hybridization between both species explains the morphological variation observed in sympatric populations of the Doñana National Park.

2. The breakdown of species integrity in sympatric areas leads to a continuous morphological and molecular variation that blurs species distinction.Thus, *A. ovatum* and *A. aristatum*, that are quite differentiated in allopatry, can not be distinguished in common areas.

3. Neither morphology nor AFLP-data justify the description of any infraspecific taxa in the *A. aristatum*/*A. ovatum* complex. To determine whether both species can be maintained as different entities, new specimens and populations should be added to the analyses.

5. The taxon *A. aristatum* subsp. *macranthum,* that is morphologically

undistinguishable from *A. ovatum*, is, however, slightly differentiated genetically.

4. A high macro- and micromorphological differentiation was observed between Doñana populations and the outgroups. We suggest an environmental origin for this differentiation.

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Capítulo 5

5

Taxonomy and biogeography of the high polyploid *Anthoxanthum amarum* Brot. (Poaceae; Pooideae) assessed by molecular and morphological methods

Taxonomy and biogeography of the high polyploid *Anthoxanthum amarum* Brot. (Poaceae; Pooideae) assessed by molecular and morphological methods

5

ABSTRACT

A high morphological and ecological diversity has been observed in the NW Iberian Peninsula endemism *Anthoxanthum amarum* Brot., where two different morphotypes (northern and southern type) were described based on qualitative traits and their geographical origin. In the present study, a combined molecular and morphological method was applied to ten populations belonging to these species with the following aims: (i), to assess whether the variation observed was taxonomically meaningful (ii), to assess the influence of the environment on the morphological characters and (iii) to track the possible phylogeographic signal under our data to make inferences about the biogeography of these species in Galicia (NW Iberian Peninsula). To achieve these aims, 22 macromorphological characters were recorded from 279 specimens, and 77 RAPD phenotypes were identified in the 79 studied plants. The association analyses performed with morphological and molecular data showed that no clear separation existed between the morphs, and a strong correlation between qualitative characters and the environment was detected. Moreover, both the multivariate analyses and the assignment test based on RAPD data revealed that genetic variation was hierarchically structured, and three genetically distinct groups could be identified. Two of these clusters might correspond to different expansion routes proposed in the literature for different plant species in Galicia.

ADDITIONAL KEY WORDS: *Anthoxanthum amarum* - Biogeography - ecotypes - endemism - Galicia (NW Iberian Peninsula) - morphology - RAPDs - Taxonomy.

INTRODUCTION

Genus *Anthoxanthum s. str.* (basic number $x = 5$) includes 15-18 species growing mainly in temperate and arctic-alpine regions of Asia, Africa and Europe (Nicora & Rúgolo de Agrassar, 1987; Phillips, 1995). Ploidy level varies largely accross the European species of the genus among which, triploid, tetraploid and up to 18ploid plants can be found (Teppner, 2002). The diploid/tetraploid complex composed by *Anthoxanthum alpinum* Löve & Löve and *Anthoxanthum odoratum* L has been widely studied throughout the last decades (reviewed in Hedberg, 1990). Conversely, few attention has been paid to the high polyploid *Anthoxanthum amarum* Brot. (Poaceae; Pooideae), whose biology and distribution are scarcely known. In the present study, molecular and morphological methods are applied to address different taxonomic, biogeographical and ecological questions in these species.

Anthoxanthum amarum (2n = 86, 88, 90) is an early-flowering perennial aneuploid species (Fernándes & Queirós, 1969; Teppner, 2002) very closely related to *A. odoratum* both morphologically (Pimentel & Sahuquillo, 2003a) and genetically (Pimentel & Sahuquillo, unpublished). Its distribution is restricted to NW Iberian Peninsula, southwards to central Portugal and eastwards to Asturias (Paunero, 1953; Tutin, 1980). Its presence has been also reported in more southern and eastern regions of the Iberian Peninsula like Salamanca, Cuenca and Valencia provinces (Mateo-Sanz & Aguilella-Palasí, 1990; reviewed in Moreno-Saiz & Sainz-Ollero, 1992). However, different *A. amarum* herbarium specimens we have revised from these areas presented sterile florets under 4 mm and lacked bulbils or stolons, being large specimens of the extremely variable *A. odoratum* (Cuenca: XK40, Sierra de Talayuelas, 1300 m, VI/1976, G Mateo; Salamanca: La Alberca, base de la Peña de Francia, VII/1959, S Rivas-Goday). Moreover, in different prospections made in several localities from these regions where the presence of this taxon had been claimed (Mateo-Sanz & Aguilella-Palasí, 1990), such as Sierra de Mira (Cuenca), Sierra del Espadán (Castellón-Valencia) or Peña de Francia (Salamanca) only *A. odoratum* was found. In the light of this, we consider that the occurrence of *A. amarum* in this regions is doubtful. The narrow distribution of *A. amarum* makes it suitable for regional phylogeographical and biogeographical studies, whose importance has been highlighted by different authors (*e.g.*, Soltis *et al.*, 1997; Stehlik, Schneller & Bachmann, 2001).

Anthoxanthum amarum grows mainly in mesic, stable habitats with temperate climate and relatively little seasonal fluctuation in temperature, like forests or riverine areas.

According to Brock & Brown (1969) and Stebbins (1985), many high polyploid grasses are restricted to these habitats, although *A. amarum* can also grow in more disturbed environments like wastelands, ravines or pine plantations.

Despite its narrow distribution area, there is a remarkable morphological variability within *A. amarum*, and two different morphotypes can be distinguished (Pimentel & Sahuquillo, 2003a). A northern one (referred hereafter as N-Type) comprising stoloniferous plants with dark green glabrous leaves and a southern one (referred hereafter as S-Type) characterized by pubescent or glabrous light green leaves and by the presence of bulbils. There are also ecological and reproductive differences between them. Whereas the N-Type usually grows in stable and shadowed habitats, mainly in forests, the S-Type inhabits more disturbed and exposed areas like plantations or damp meadows. Moreover, vegetative reproduction is common in both forms. The two morphotypes are not totally allopatric, as N-Type plants can be found in southern areas and vice versa. Furthermore, intermediate plants and populations between both forms exist.

The combined use of molecular and morphological methods is useful to unravel the taxonomic relationships in complex taxa and to describe the distribution pattern of the genetic and phenotypical variation (Raybould *et al.*, 1991; Tovar-Sánchez & Oyama, 2004; Hagidimitrou *et al.*, 2005). Random Amplified Polymorphic DNA markers (RAPDs) have been widely used to analyse the genetic diversity at low taxonomic levels (*e.g.*, Mehrnia, Zarre & Sokhan-Sanj, 2005; Murray *et al.*, 2004) and to address different biogeographical questions (*e.g.*, Segarra-Moragues & Catalán, 2003). Moreover, detailed analyses of qualitative and quantitative phenotypic variation are still necessary in systematic studies (Hörandl, 2002).

The main goals of the present study were: (i), to determine wheter the two morphs observed are genetically differentiated or if they merely represent end-points of local ecoclines. (ii) To assess the influence of the environment on the morphological characters, specially on those used to distinguish between the N-Type and the S-Type. (iii) To examine the genetic structure and biogeography of *A. amarum* in Galicia (NW Iberian Peninsula), with special emphasis on the existence of possible expansion routes. To achieve these aims, the morphological and molecular relationships among several Galician populations belonging to both morphs of *A. amarum* were analysed.

MATERIALS AND METHODS

PLANT MATERIAL

The collection sites were subjectively chosen in order to embrace most of the morphological and ecological variation observed in *A. amarum* in Galicia. To do so, we examined the specimens of this taxon deposited in different European Herbaria (SANT, BC, MA, LOU, G, FI; Holmgern, Holmgren & Barnett, 1990). Moreover, the selected localities covered most of the habitats generally ocupied by this species in Galicia (NW Iberian Peninsula.

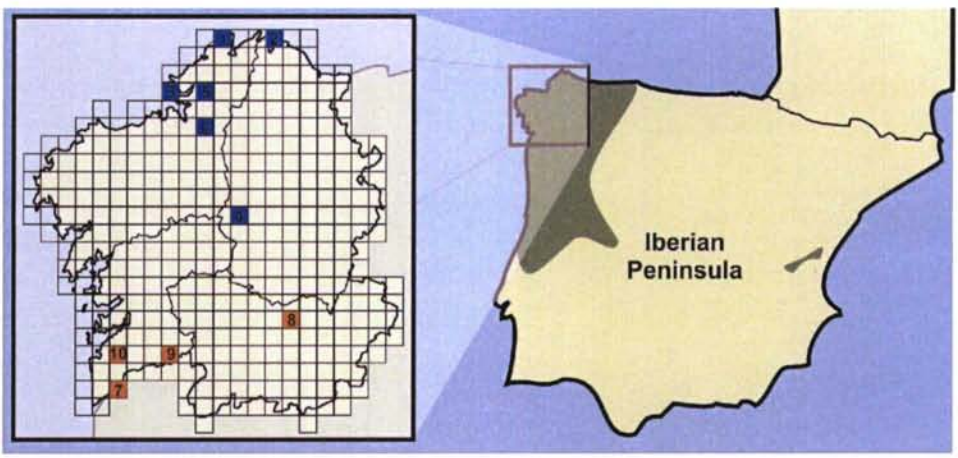

Figure 5.1. Map of the area of distribution of *Anthoxanthum amarum* (in green) and the studied populations. In blue, N-Type; in red, S-Type

N° /TYPE	POPULATION	HABITAT	N° M.	N° L.	V. N°
1 (N-Type)	San Andrés de Teixido, Cedeira, A Coruña	Cliffs by the see, serpentine soil	32	11	SANT-52216
2 (N-Type)	Praia de Esteiro, Xove, Lugo	Coastal dunes	30	9	SANT-52215
3 (N-type)	Valón, Ferrol, A Coruña	Eucalyptus plantation	30	10	SANT-53453
4 (N-Type)	Pambre, Palas de Rei, Lugo	*Quercus robur* L. forest	-	10	SANT-53454
5 (N-Type)	Fraga do Belelle, A Cabana, A Coruña	Sand bank by a river	32	-	SANT-53449
6 (N-type)	Fraga do Mandeo, Coirós, A Coruña	Sand bank by a river	31	-	SANT-53451
7 (S-Type)	Amorín, Tomiño, Pontevedra	Sand bank by a river	30	10	SANT-52217
8 (S-Type)	Gabín, Montederramo, Ourense	Betula alba L. forest	34	9	SANT-52222
9 (S-Type)	As Neves, Pontevedra	Ravine	30	10	SANT-53455
10 (S-Type)	Valadares, Vigo, Pontevedra	Pinewood	30	10	SANT-53448

Table 5.1. Populations studied. In blue, populations belonging to the N-type. In red, populations belonging to the S-Type. N° M., number of specimens included in the morphometric analysis; N° L., number of specimens included in the molecular analysis; V. N°, voucher numbers. For population details see voucher labels.

Ten populations were sampled in Galicia, 4 belonging to the S-Type and 6 to the N-Type (Figure 5.1, Table 5.1). All the collected populations were tentatively assigned to one or the other morph on the basis of the morphological characters highlighted in

Pimentel & Sahuquillo (2003)a, *i.e.*, the presence of pruine, the colour and pubescence of leaves and the presence of stolons/bulbils.

Between forty-five and fifty plants were collected per population. Sampled specimens were at least 5 m apart given the high capacity of *A. amarum* for vegetative spread. Between thirty and thirty-five individuals per population were dried and employed in the morphometric analyses, whereas fresh leaves from the remaining 9-11 individuals were stored in silica gel for molecular studies (Chase & Hills, 1991). According to Hansen, Elven & Brochmann (2000), in populations and species where vegetative spread is common the level of infraspecific variation is expected to be low. For this reason, the analysis of a low number of specimens per population could be enough to obtain taxonomic and biogeographical results. In the present study, a grand total of 358 individuals were considered, 279 in morphological analyses (from 30 to 34 plants per population) and 79 in molecular analyses (between 9 and 10 per population). The survey of studied populations and their habitats, as well as the number of plants used in molecular and morphological tests per population are listed in Table 5.1. Vouchers are deposited in the Santiago de Compostela University Herbarium (SANT) (Holmgren, Holmgren & Barnett, 1990). Voucher numbers are listed in Table 5.1.

RAPD ANALYSIS

DNA was isolated from 25 mg of silica-dried leaves using the CTAB method (Doyle & Doyle, 1990). Subsequently, the quality of DNA was checked on 1% TAE-agarose gel, and its concentration was quantified by spectrometry with the Cary 3E Spectrophotometer (Varian, Palo Alto, USA). Genetic variation among 5 plants including both morphotypes was pre-analyzed with twenty decamer primers from kit A from Operon Technologies (Alameda, USA). Nine arbitrary decamer primers (Table 5.2) that produced the most distinct, reproducible and polymorphic bands were selected for full RAPD analysis of all 79 plants. The PCR program consisted of a melting step, thirty seconds at 94 ºC followed by 40 cycles of thirty seconds at 92 ºC, 1 minute at 35 ºC and 2 minutes at 72 ºC and by a final extension cycle of 7 minutes at 72ºC. Reactions were performed in a 25 µl volume with 1x reaction buffer, 125 µM dNTPs (Roche Pharmaceuticals, Basel, Switzerland), 0.22 µM primer, 5mM $MgCl_2$, 20 ng genomic DNA and 2 U. Amplitaq (Applied Biosystems, Foster City, USA). Amplification products were electrophoresed in 1.5% agarose gel with Tris/Borate/EDTA (TBE) buffer, stained with ethidium bromide and visualized under UV light. A DNA molecular weight

marker (weight marker XIV, Roche Pharmaceuticals, Basel, Switzerland) was included as a reference.

Only clear and evenly distributed bands were selected and scored as presence (1) or absence (0) in a matrix that was manually constructed. Reproducibility of the observed patterns was assessed by conducting the whole RAPD protocol twice for 10 randomly chosen individuals.

Primer id.	Nucleotide sequence (5´to 3´)	Shannon index	Total amp. products	*A. amarum* morphotypes	
				North	South
OPA-2	TGCCGAGCTG	2.108	11(2)	8(4)	11(4)
OPA-3	AGTCAGCCAC	1.951	7(3)	7(3)	7(3)
OPA-4	AATCCGGCTG	2.795	12(1)	9(2)	10(3)
OPA-5	AGGGGTCTTG	2.325	8(4)	8(5)	7(4)
OPA-9	GGGTAACGCC	0.939	10(1)	10(4)	9(1)
OPA-10	GTGATCGCAG	1.503	10(6)	10(5)	8(6)
OPA-11	CAATCGCCGT	0.830	10(3)	9(4)	9(4)
OPA-16	AGCCAGCGAA	0.888	6(1)	6(1)	6(3)
OPA-18	AGGTGACCGT	1.033	10(0)	9(2)	8(1)

Table 5.2. List of decamer oligonucleotids used as random primers, their sequences and number of amplification products.

MORPHOMETRIC ANALYSIS

A total of 28 morphological traits (13 quantitative and 15 qualitative) were considered in the analyis (Table 5.3). In the survey of studied characters, all the traits that are

QUANTITATIVE CHARACTERISTICS	QUALITATIVE CHARACTERISTICS
Plant size (cm)	Leaf blade outline (0 planar; 1 convolute)
Leaf length and width (cm)	Ligule shape (0 round, 1 pointed)
Inflorescence length (cm)	Sheath hairiness (0 glabrous, 1 hairy)
First internode length (cm)	Upper glume hairiness (0 glabrous, 1 hairy)
First branch of the inflorescence length (cm)	Presence of a mucro (0 not mucronate, 1 mucronate)
Spikelet length (cm)	Lower glume hairiness (0 glabrous, 1 hairy)
Upper glume width (cm)	Presence of a forked apex in the upper sterile lemma(*)
Lower glume lengh (cm)	Sterile lemma hairs longer than the sterile lemma(**)
Upper sterile lemma length and width (cm)	Sterile lemma hairiness (0 glabrous, 1 hairy)
Awn length (cm)	Amount of overlap of fertile floret by sterile lemmas (0/1)(***)
Fertile floret length (cm)	Fertile floret scabridity (0 smooth, 1 scabrid)
	Presence of pruine (0 not pruinose, 1 pruinose)
	Colour of the leaves (0 light green, 1 dark green)
	Presence of bulbils (0 absent, 1 present)
	Presence of stolons (0 absent, 1 present)

Table 5.3. Quantitative and qualitative macroscopic morphological traits analysed. (*) Apex of the sterile lemma not forked (0), apex of the sterile lemma forked (1); (**) Sterile lemma hairs not longer than the sterile lemma (0), sterile lemma hairs longer than the sterile lemma; (***) Fertile floret not covered by the sterile lemmas (0), Fertile floret covered by the sterile lemmas (1).

commonly used in *Anthoxanthum* taxonomy, specially in the *A. amarum* diagnosis (Paunero, 1953; Tutin, 1980) have been considered. Moreover, the characters involved in the differentiation of morphotypes (Pimentel & Sahuquillo, 2003a) and other characters of interest in the Poaceae Taxonomy were also included. Different measures have been taken to standardize the data. On the one hand, reproductive characters were gathered in the fourth spikelet of the inflorescence. On the other hand, leaf traits have been measured in the second leaf. Only flowering plants were the second leaf and the fourth spikelet were present have been taken into account. Ratios were not considered to avoid decreasing the power of the statistical methods to discriminate between effect of size from shape (Goldman, van den Berg & Griffith, 2004). Moreover, different statistical analyses were applied to qualitative and quantitative data in order to avoid the process of transformation of quantitative data that, according to different authors, cannot be conducted unambiguously (*e.g.*, Rae, 1998; Greimler, Hermanowski & Jang, 2004).

STATISTICS AND MULTIVARIATE ANALYSIS

RAPD data

The RAPD profile of each individual was expressed as a vector of zeroes and ones. Subsequently, the genetic diversity was calculated by means of the Shannon index and the percentage of polymorphic and private fragments. These parameters were estimated per population, per morphotype and also per different groupings of populations. The Mann-Whitney U-test was used to asess the differences in genetic diversity between groups.

The Euclidean and Jaccard distances as well as the Dice's similarity coefficient were calculated. To assess the equivalence among the different indices, similarity matrices were compared by means of a Mantel correlation whose level of significance was established by a permutation test (1000 replicates) (Mantel, 1967; Legendre & Legendre, 1998). Subsequently, different statistical methods that do not assume any previous genetic structure in the data were applied. (i) A principal coordinates analysis (PCoA) with minimum spanning tree (MST) (Gower & Ross, 1969) was performed to summarize the molecular relationships among samples. (ii) An UPGMA cluster analysis was also conducted to represent the molecular distance among the specimens and populations. The program NTSYS-pc Version 2.1 (Rohlf, 2002) was used to obtain the pairwise distances among samples as well as to perform the PCoA-MST analyses and the Mantel correlation tests. On the other hand, the clusters were constructed with the software MEGA Version

2.1 (Kumar *et al.*, 2001).

In order to identify the population partition most compatible with the genetic diversity observed, the Bayesian clustering method proposed by Pritchard, Stephens & Donnelly (2000) was applied. This method is implemented by a Markov Chain Monte Carlo algorithm (MCMC), and does not assume any prior knowledge of the population structure (Caizergues *et al.*, 2003). All the calculations were performed with the program STRUCTURE Version 2.1 (Pritchard, Stephens & Donnelly, 2000), that assigns individuals into a predefined number of subgroups (K) and returns a likelihood score for each particular partition of the data. For dominant markers, such as RAPDs, the non-admixture model has to be assumed, *i.e.*, any individual genotype belongs purely to one population (Carlsson, Söderberg & Tegelström, 2003; Pritchard & Wen, 2003). We tentatively divided the data into $K = 1$ to $K = 10$ groups, with twenty independent runs for all K values. For each of them, 500,000 MCMC iterations were performed, 50,000 of which were discarded. To select the most appropriate number of subgroups, we followed the method proposed by Evanno, Regnaut & Goudet (2005).

The analysis of molecular variance (AMOVA, Excoffier, Smouse & Quattro, 1992) has been widely used to study the distribution of genetic variance among and within groups (Segarra-Moragues & Catalán, 2003 and references therein). We have performed it at different hierarchical levels: (i), overall analysis of variance (among populations); (ii), between morphotypes and (iii), among the groups derived from the Structure analysis. The significance of the different variance components obtained was assessed by means of a permutation test (1000 replicates). AMOVA tests were conducted using Arlequin 2000 (Schneider *et al.*, 2000).

The genetic differentiation among populations and groups was calculated as the Weir & Cockerham's F_{ST} unbiased estimator (Weir & Cockerham, 1984) using TFGPA Version 1.3 (Miller, 1997). The 95% confidence interval (CI) for the estimator was obtained by bootstrapping 5000 replicates over loci.

Mantel tests were performed to quantify the genetic distance between each pair of individuals as a function of a second inter-individual distance (following Stehlik, Schneller & Bachmann, 2001). For all Mantel tests, the normalized Mantel statistic, R_M, was estimated, and the statistical significance was assessed by means of a permutation test (1000 replicates). To adjust for multiple testing, Bonferroni corrected P values were calculated (Legendre & Legendre, 1998).

Mantel tests were used at different levels. (i) Overall Mantel test; (ii), populational Mantel test and (iii), Mantel test with distance classes (Oden & Sokal, 1986; Legendre & Legendre, 1998; Stehlik, Schneller & Bachmann, 2001). All the calculations were performed with the program NTSYS-pc Version 2.1 (Rohlf, 2002).

Morphological data

A correlation-based principal components analysis (PCA) and the coefficient of variation were calculated to select the most taxonomically interesting quantitative characters and to eliminate outliers. Subsequently, and to test the distinctiveness among groups, we applied a nonmetric multidimensional scaling (NMDS) and a canonical variate analysis (CVA). In the latter test, the morphotypes as well as the groups derived from the molecular analyses were used as categorical variables. Moreover, contingency tables and a PCoA were performed with the qualitative traits.

The similarity among samples was established using the Manhattan distance and the Phi coefficient for quantitative and qualitative data, respectively. The relationships among samples and populations were represented in phenograms obtained using UPGMA clustering methods. All the statistical tests but the PCoA were performed using the program SPSS 11.5 (SPSS Inc., Chicago, USA). The principal coordinates analysis was computed using the NTSYS-pc Version 2.1 (Rohlf, 2002).

Finally, we performed a canonical correspondence analysis (CCA) to assess the influence of the environment on the qualitative characters selected. The environmental parameters considered were soil pH (following Guitián & Carballos, 1976), mean, minimum and maximum annual temperature (ºC), drought period and mean annual rainfall (mm). Climatic data were gathered from Carballeira *et al.* (1983). The meteorological stations nearest to the collection localities were selected. This test was computed with the software CANOCO Version 4.0 (Ter Braak, 1987; Ter Braak & Smilauer, 1998; Lepš & Smilauer, 2003).

RESULTS

RAPD ANALYSIS

The distribution of polymorphism accross the studied populations as well as in both morphotypes is showed in Table 5.4. Overall, 84 reproducible RAPD bands were scored.

GROUP	Nº OF SAMPLES	Nº OF BANDS	POLYM. MARKERS	PRIVATE MARKERS	SHANNON INDEX
San Andrés de Teixido	11	55	11 (20%)	0	5.33
Esteiro	9	59	12 (20.3%)	0	3.15
Valón	10	64	12 (18.7%)	2	3.99
Pambre	10	61	19 (31.1%)	0	6.29
Ov value for N-Type pops.	40	76	45 (59.2%)	2 (4.8%)	11.68
Amorín	10	58	11 (18.9%)		3.55
Montederramo	9	63	16 (25.4%)	3	5.09
As Neves	10	56	6(10.7%)	1	1.55
Valadares	10	59	10(17%)	0	2.92
Ov value for S-Type pops.	39	76	40(60.5%)	8 (10.5%)	11.67
Total	79	84	63(75%)	-	23.88
Ov value for Str. gr. 1	20	63	45(28.6%)		5.13
Ov value for Str. gr. 2	29	68	33(48.5%)		8.11
Ov value for Str. gr. 3	30	81	46(56.8%)	5(10%)	12.76

Table 5.4. Number of bands, degree of polymorphism, number of private fragments and Shannon index values registered per population and morphotype. Str. gr. 1, structure group 1 (Amorín and Valadares populations); Str. gr. 2, structure group 2 (Esteiro, Pambre and As Neves populations); Str. gr. 3, structure group 3 (San Andrés de Teixido, Montederramo and Valón populations). Polym, polymorphic; Ov, overall; pops., populations.

Approximately, 75% of the amplified fragments were polymorphic, allowing the differentiation of 97.5% of the samples on the basis of their RAPD profiles. The Shannon index per population ranged from 1.55 in As Neves (S-Type) to 6.29 in Pambre (N-Type), although there were no significant differences in polymorphism between both morphs (Mann-Whitney U-test; S-Type *vs* N-Type $P = 0.23$). Only 3 of the populations, Montederramo, As Neves and Valón, presented private markers. In overall, 8 amplified products were exclusive to the S-Type populations (10.5%), whereas N-Type populations presented only 2 private bands (4.8%).

Mantel correlation coefficients among the different distance or similarity indices were high and significant (*e.g.*, Euclidean *vs* Jaccard; $R_M = -0.987$, $P = 0.001$; Dice *vs* Jaccard; $R_M = 0.968$, $P = 0.001$). Therefore, only the Euclidean distance was used in the subsequent analyses based on the RAPD dataset.

As showed by the dendrogram (Figure 5.2) and the PCoA-MST (Figure 5.3), the phenetic relationships among populations only partially supported the partition of the samples into the morphotypes. Nevertheless, some common patterns may be observed in both analyses: (i), the southern populations of Amorín and Valadares clustered together and (ii), a mixed group including As Neves (southern), Pambre and Esteiro (northern) was revealed by both analyses. The specimens from Montederramo, Valón and San Andrés de Teixido were not clearly grouped, although the latter populations are linked in both the

MST and the UPGMA dendrogram.

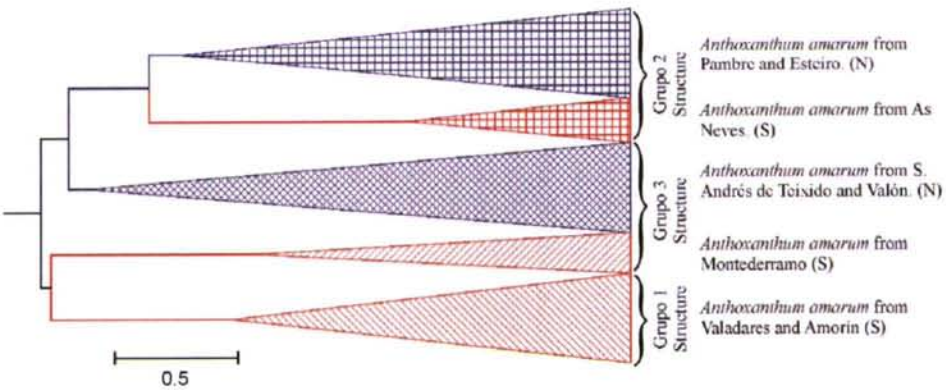

Figure 5.2. UPGMA dendrogram performed with RAPD data. In red, S(southern)-type populations. In blue, N(northern)-type populations.

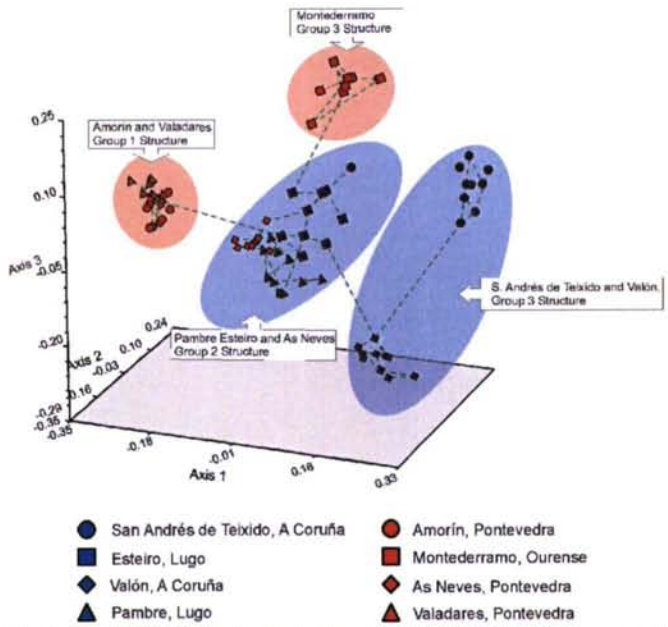

Figure 5.3. Principal Coordinates Analysis based on RAPD data. In red, S(southern)-type populations. In blue, N(northern)-type populations.

In the Structure analysis, the highest likelihood was observed for $K = 8$, *i.e.*, the number of populations included in the molecular assay. However, the modal value of the

Δk was at $K = 3$, which partially portrayed the patterns observed in the PCoA and in the dendrogram. Amorín and Valadares populations were included in the first group (Str. gr. 1), whereas Pambre, Esteiro and As Neves were in the second cluster (Str. gr. 2) and San Andrés de Teixido, Montederramo and Valón composed the third one (Str. gr. 3).

The results of the AMOVA performed are listed in Table 5.5. Non hierarchical AMOVA analysis showed a high genetic differentiation among populations (70.5%), whereas the within-population variation was low (29.5%). The same result was obtained when the unbiased F_{ST} estimator was calculated ($F_{ST} = 0.75$; CI: 0.70-0.80). Moreover, AMOVA tests were performed at two hierarchical levels. On the one hand, only 14% of the genetic variance observed was explained by the differentiation between the morphotypes, whereas 58% was found among populations within groups and 28% within populations. On the other hand, when the 3 Structure groups were considered, the percentage of variance found among groups raised to 27.83%. The F_{ST} estimator was also used to measure the genetic differentiation between morphotypes (0.14; CI: 0.04-0.22) and among Structure groups (0.3; CI: 0.21-0.38).

SOURCE OF VARIATION	df	SS	VARIANCE COMPONENTS	PERCENTAGE OF VARIATION
Global analysis (a).				
Among populations	7	530.36	7.36	70.49%
Whithin populations	71	218.85	3.08	29.51%
Region-wise analysis. Northern *vs* southern populations (b)				
Among regions	1	130.57	1.61	14.47%
Among pops within regions	6	399.78	6.44	57.85%
Whithin populations	71	218.85	3.08	27.68%
Structure-derived analysis				
(Vd and Am) *vs* (Pm Et and An) *vs* (St, Vl, Mt) (d)				
Among groups	2	267.86	3.12	27.83%
Among pops within groups	5	262.50	5.01	44.71%
Whithin populations	71	218.85	3.08	27.46%

Table 5.5. Analyses of molecular variance (AMOVA). Hierarchical partitioning of the overall genetic variation at two levels (within and between population) for *A. amarum* (a); region-wise analysis (b); Structure-derived analyses (c&d). Vd, Valadares; Am, Amorín; St, San Andrés de Teixido; Vl, Valón; Pm, Pambre; Mt, Montederramo; Et, Esteiro; An, As Neves. df, Degrees of freedom; SS, sum of squares. All the estimated values were highly significant.

The overall R_M value calculated with genetic and geographic distance matrices was low but significant (0.16; $P = 0.001$), *i.e.*, pairwise inter-individual genetic distance increased with increasing genetic distance, although the correlation was weak. Mantel tests calculted with distance classes showed a decrease from significantly positive values (up to 80 km) to significantly negative values in distance classes from 110 km on (Figure 5.4, I). Both positive and negative R_M coefficients were observed between 80 and 110 km.

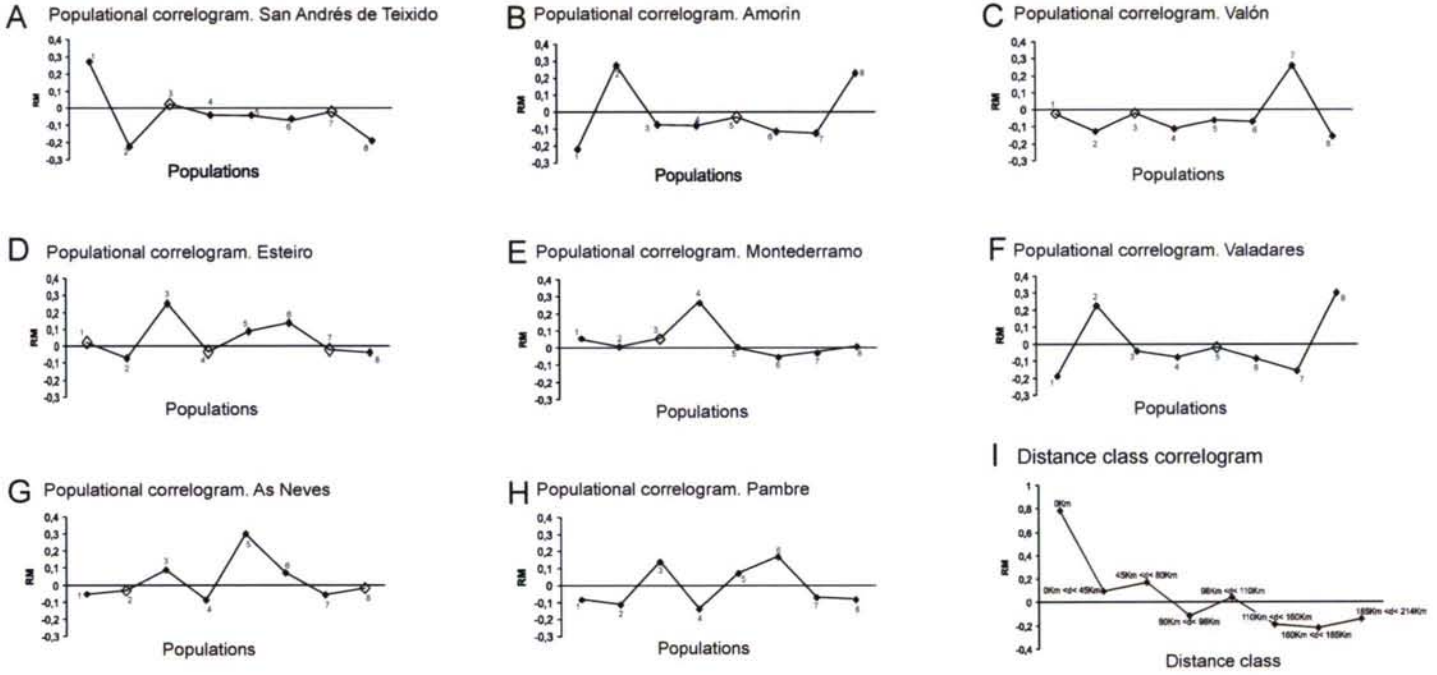

Figure 5.4. Correlograms of Mantel RM coefficients. (A-H) Correlograms of 8 populations individually compared with themselves and all other populations of *A. amarum* studied. (I) Correlogram per distance class among 8 populations of *A. amarum* studied. Empty squares, correlation non significant; filled squares, correlation significant. 1, San Andrés de Teixido; 2, Amorín; 3, Praia de Esteiro; 4, Montederramo; 5, As Neves; 6, Pambre;7, Valón; 8, Valadares.

Populational Mantel correlograms (Figure 5.4, A-H) were partially consistent with the results obtained in the Structure analysis. On the one hand, a positive and significant correlation was observed among the As Neves, Pambre and Esteiro populations (Str. group 1) and between Amorín and Valadares (Str. group 3). On the other hand, San Andrés de Teixido, Valón and Montederramo (Str. group 2) were only correlated to themselves.

MORPHOLOGICAL ANALYSIS

Neither the PCA, nor the NMDS and the cluster analysis based on quantitative traits (not shown) were able to reveal a clear morphological pattern in the dataset, and populations and morphotypes appeared intermingled in the plots. Only the CVA (Figure 5.5) was able to partially distinguish among three groups loosely corresponding to those obtained in the molecular assignment test. The characters responsible for this partial differentiation were mostly vegetative (plant size, leaf length and width and spikelet size).

The PCoA based on qualitative data (Figure 5.6) showed a clear differentiation between morphs, as it was expected. The same result was obtained in the cluster analysis (not shown). As showed by the CCA, a high correlation was observed between the qualitative characters and the environment, specially in those traits used to separate both morphotypes, like the presence of bulbils (related to the availability of water) or the pubescence of leaves (related to temperature). Northern and southern populations are clearly separated in the CCA scatterplot (Figure 5.7). The differentiation between

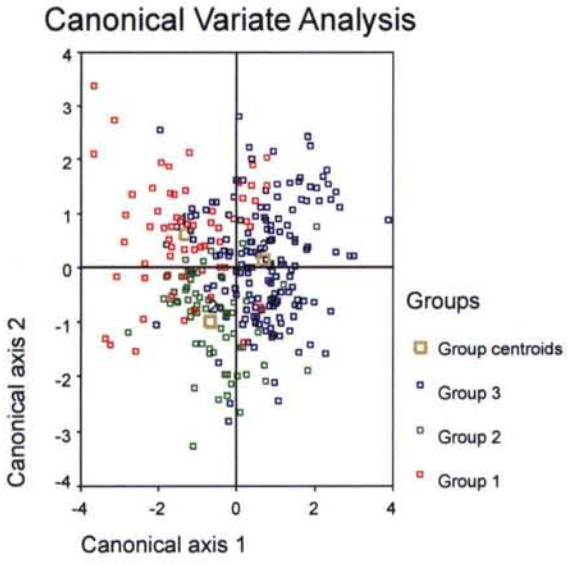

Figure 5.5. Canonical axes one and two of the canonical variate analysis (CVA) performed on the 9 populations of *A. amarum* morphologically analysed. Group 1: Amorín and Valadares populations. Group 2: Esteiro and As Neves populations. Group 3: San Andrés de Teixido, Valón, Belelle, Mandeo and Montederramo populations.

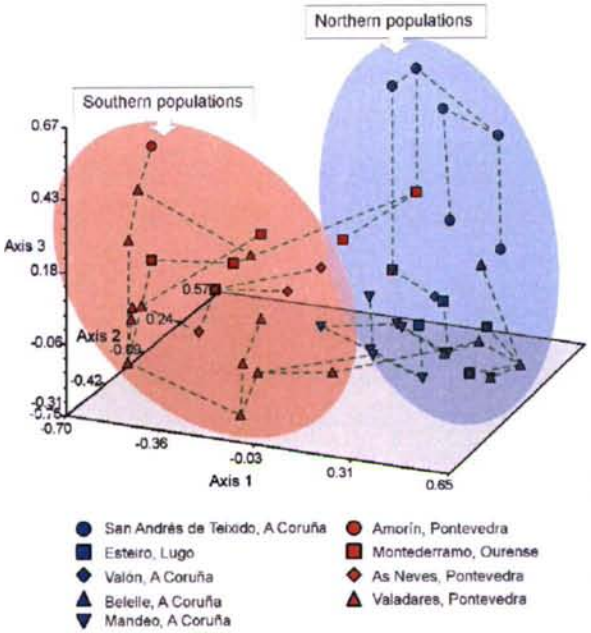

Figure 5.6. Principal coordinates analysis based on qualitative morphological data. In red, S(southern)-type populations. In blue, N(northern)-type populations.

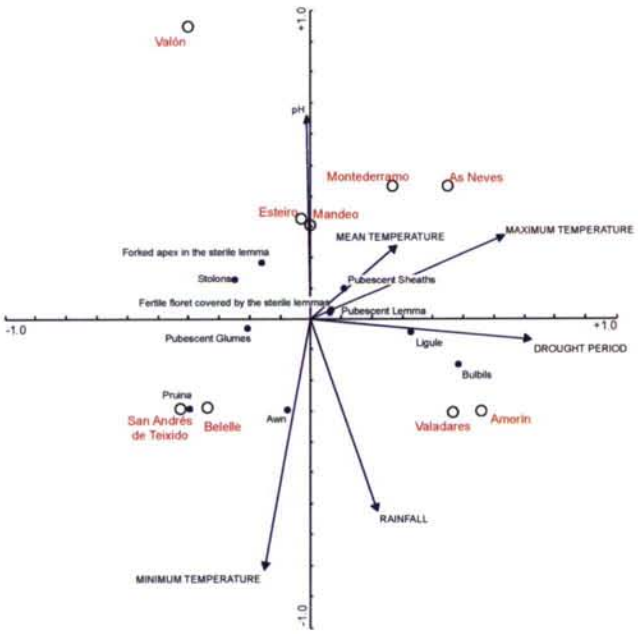

Figure 5.7. Canonical axes one and two of the canonical variate analysis (CVA) performed on the 9 populations of *A. amarum* morphologically analysed. Group 1: Amorín and Valadares populations. Group 2: Esteiro and As Neves populations. Group 3: San Andrés de Teixido, Valón, Belelle, Mandeo and Montederramo populations.

morphotypes was also based on the temperature and the availability of water.

DISCUSSION

TAXONOMY AND ECOLOGY

The differentiation between both morphotypes was not supported by the analyses based on morphological quantitative and molecular data. Specimens and populations from both morphs appeared partially intermingled in the association tests perfomed with molecular data (Figures 5.2 & 5.3). In addition to this, the F_{ST} value obtained (0.14; CI: 0.04-0.22) and the results of the AMOVA (Table 5.5) revealed a low genetic differentiation between morphotypes and a high genetic differentiation among populations. This pattern might be explained by the importance of clonal spread in these species. However, the isolation of some of the populations studied such as Montederramo or San Andrés de Teixido might explain part of the differentiation encountered.

Both morphotypes were separated in the PCoA based on morphological qualitative traits (Figure 5.6). However, a high influence of the environment on qualitative traits was detected (Figure 5.7), specially in those characters responsible for the differentiation between the N-Type and the S-Type. Pubescence and pruine were highly related to mean and maximum temperature, whereas the presence of bulbils or stolons was associated to the water availability. The scarce taxonomic interest of traits such as pubescence was already suggested by Hedberg (1964) for other *Anthoxanthum* species.

Phenotypical plasticity associated to environmental variability has been viewed as an alternative means of adaptation in plants (*e.g.*, Coleman, McConnaughay & Ackerly, 1994), and a high phenotypic plasticity has been already highlighted in other *Anthoxanthum* species (Antonovics, Clay & Schmitt, 1987; Hedberg, 1990; Platenkamp, 1990). The low morphological and genetic differentiation encountered advised against the assignment of any taxonomic differentiation of both morphotypes. According to our results, the variation observed constitutes a phenotypic response to the environmental parameters, specially those related to the water balance (temperature and drought). Nevertheless, performing common garden experiments would be advisable to get more conclusive results.

GENETIC STRUCTURE AND PHYLOGEOGRAPHY

The Structure assignment test based on the RAPD dataset revealed a certain genetic

structure among the studied *A. amarum* populations. Three different groups could be distinguished. A first one composed by Amorín and Tomiño populations, two close coastal localities in SW Galicia, a second one including three inland localities (As Neves, Pambre and Esteiro) and a third group, rather heterogeneous, that comprises Montederramo, San Andrés de Teixido and Valón populations. According to Pritchard and Wen (2003), the results of the assignment test are less reliable when populations are evenly distributed accross the different groups. However, our conclusions are supported by other analyses performed on molecular data, specially the dendrogram (Figure 5.2) and the PCoA (Figure 5.3), that approximately portray the same grouping of populations. Besides, the Mantel correlograms showed a close relationship between populations within the Structure-derived groups 1 and 2 (Figure 5.4). The AMOVA results (Table 5.5) and the F_{ST} value obtained (0.3; 95% CI: 0.21-0.38) also indicated that a certain differentiation among Structure-derived groups existed. Nevertheless, the percentage of genetic variation found among populations within groups was rather high (44.71%). Regarding morphological data, only the CVA performed with quantitative traits loosely reflected separation among Structure-derived groups (Figure 5.5). In this differentiation, the most important traits were plant size, leaf length and width and spikelet length.

To us, the genetic structure observed might be due to gene flow among the populations within the Structure-derived groups 1 and 2. This gene flow would be restricted between these lineages (despite their geographical closeness), or between them and the populations included in the Structure-derived group 3. In our opinion, these restrictions to gene flow might be imposed by the non-existence of populations that could act as a "bridge" between the different regions and by the importance of clonal spread in these species. The frequency of vegetative reproduction might be also involved in the high genetic differentiation observed among the studied populations. If they were originated from one or a few individulas that spread vegetatively, an increase of their genetic differentiation would be expected as a result of the bottleneck and the subsequent processes of genetic drift.

The first Structure-derived group included two coastal populations from SW Galicia (Amorín and Valadares), whereas the second group included three populations along the Miño river basin (As Neves, Esteiro y Pambre). These groups (coastal and inland) might correspond with northwards migration routes proposed in the literature for central and northern Portuguese flora (Izco, 1987) (Figure 5.8). Nevertheless, we can make no inference about the origin of the populations since we have observed no differences in

genetic variability between northern and southern populations, as it would be expected in a colonization process. Regarding the third Structure-derived group, it comprised populations from rather different habitats, which might also explain the high genetic differentiation observed among them. Firstly, specimens from San Andrés de Teixido grow in serpentine soils (IGME, 1977). According to Rajakaruna (2004), these particular edaphic conditions might have led to genetic differentiation of plant populations belonging to different species. Secondly, the Montederramo specimens grow in a relict montane birch forest in an area of Mediterranean climate, being highly isolated and probably affected by genetic drift. The existence of several fixed and private fragments in this population might be a consequence of this process. Nevertheless, the sampling of new specimens and populations would be needed to clarify the origin of this heterogeneous group.

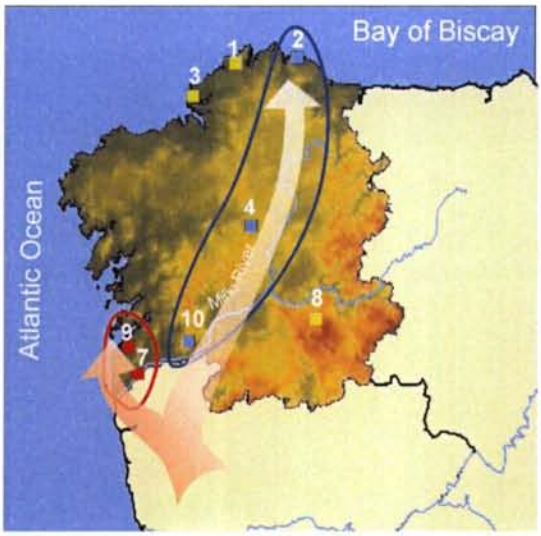

Figure 5.8. Possible expansion routes of *Anthoxanthum amarum* in Galicia (NW Iberian Peninsula). In red, Structure group 1, in blue, Structure group 2, in yellow, Structure group 3. For population numbers, see Table 5.1.

CONCLUSIONS

The lack of genetic and morphological differentiation between the morphotypes of *A. amarum* discourages the assignment of any subspecific rank. Moreover, the influence of the environment on the morphological qualitative characters, specially on those involved on the differentiation between both morphs, indicates that the observed differences might respond to ecotypic variation.

The genetic variation found among some of the populations studied is geographycally structured. To us, gene flow exists among populations within the coastal and inland group

respectively, whereas it is prevented between these groups and between them and the remaining populations.

To understand the origin and diversification of this endemic taxon, the sampling of new populations from northern and central Portugal would be neccesary. On the other hand, a higher number of individuals per population should be analysed from a molecular point of view.

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Capítulo 6

6

Genetic variation in a geographical context inferred from Amplified Fragment Length Polymorphism markers.

The case of European sweet vernal grasses (*Anthoxanthum* L.; Poaceae)

Genetic variation in a geographical context inferred from Amplified Fragment Length Polymorphism markers. The case of European sweet vernal grasses (*Anthoxanthum* L.; Poaceae)

ABSTRACT

Different processes, both present and historical, have contributed to shape the present distribution of plants and animals. Gene flow, genetic drift and Natural History parameters are necessary to explain the genetic structure of species, but not enough. Geological and climatic changes have also had an outstanding importance in the distribution and diversity of the European flora and fauna. In the present study, the Amplified Fragment Length Polymorphisms technique has been used to unravel the phylogeography of the European sweet vernal grasses (*Anthoxanthum* L.: Poaceae: Pooideae).

Forty-six populations belonging to all the European species of *Anthoxanthum* were selected in order to cover a broad geographical and ecological range. Five individuals per population were considered in the analysis. The Shannon Index, the percentage of polymorphism and the number of rare and private fragments per population were calculated to assess the intrapopulational genetic variability. Moreover, the differentiation among taxa and regions was estimated by means of the unbiased F_{ST}. The distribution of the genetic variation obtained was analysed by means of an AMOVA and was summarized in an UPGMA dendrogram. Finally, the relationships between genetics and geography were assessed by means of the Mantel test.

Our results revealed the influence of different past geological and climatic changes on the present distribution and diversity of the European and Macaronesian species of *Anthoxanthum,* specially *A. odoratum* and *A. alpinum.* In addition to this, our results supported the existence of different migration routes and glacial refugia already proposed in the literature.

ADDITIONAL KEYWORDS: AFLP - *Anthoxanthum* - Europe - Glaciations - Mantel test - migration routes - Phylogeography - Sweet vernal grasses.

INTRODUCTION

Both present and historical processes and events must be invoked to explain the distribution and genetic structure of plants and animals (*e.g.*, Hewitt, 1999, 2004). Gene flow among populations and species, genetic drift and natural history parameters are necessary but not enough to explain the present distribution and diversity of European Flora. Geological history and climatic variations have also had a major role in current and past biogeographical patterns (Richards, 1990; Hewitt, 1999). Phylogeographical research has added an overtly evolutionary perspective to biogeographic studies (Taberlet *et al.*, 1998; Schönswetter *et al.*, 2003; Hewitt, 2004), and different molecular methods have been used to analyse genetic variation in a geographical and geological context (*e.g.*, Avise, 1989; Stehlik, Schneller & Bachmann, 2001). In the present study, we have used amplified fragment length polymorphism (AFLP) markers to unravel phylogeographic patterns in European sweet vernal grasses (*Anthoxanthum* L. *s. str.* Poaceae; Pooideae).

Seven closely related *Anthoxanthum* species presenting different levels of ploidy, life cycles and distributions are present in Europe. Among perennial taxa, *Anthoxanthum odoratum* L. (2n = 20) is a highly morphologically polymorphic tetraploid that grows all throughout Europe, although in the northern-most latitudes and in high mountains it is replaced by the diploid *Anthoxanthum alpinum* Á & D Löve. The taxonomic status of this pair of species remains uncertain, as no compelling morphological differentiation between them has been found (Felber, 1986; Felber-Girard, Felber & Buttler, 1996; Hedberg, 1990). *Anthoxanthum amarum* Brot. is a high polyploid (2n = 86, 88, 90) endemic to the north of the Iberian Peninsula. In this taxon, asexual reproduction is quite widespread and it has been often included into *A. odoratum* (Merino, 1909; Fernándes & Queirós, 1969). The recently described diploid *Anthoxanthum maderense* Teppner (2n = 20) grows in the Archipelagos of Madeira and Azores.

The taxonomic differentiation between the quite polymorphic Mediterranean annuals *Anthoxanthum aristatum* Boiss. (2n = 10) and *Anthoxanthum ovatum* Lag. (2n = 10) is rather controversial as well (*e.g.*, Valdés, 1973; López-González, 1994). Lastly, *Anthoxanthum gracile* Biv. is a poorly known annual species whose position inside the genus remains uncertain. This diploid taxon grows mainly in some Mediterranean isles (Malta, Sicily, Sardinia and Crete), although it is also present in the Peloponese and Albania, where it is extremely rare (Fiori, 1969; Tutin, 1980; Pignatti, 1982).

The particular composition and distribution of genus *Anthoxanthum* in Europe makes it specially useful to test different hypotheses on the relationship between speciation and

geological history.

Three geological events have had an specially deep effect on the current distribution of the European Flora.

(i) The period of global climate cooling and drying that began in the Early Eocene, *ca* 55 million years ago (Mya), that produced an extension of grasslands followed by a fast diversification of drought tolerant grasses (Kellogg, 2001). Also, this event led to the differentiation between European and Macaronesian Floras. The formation of the Sahara desert *ca* 3 Mya completed the isolation between both regions (Francisco-Ortega *et al.*, 1999; Sahuquillo & Lumaret, 1999).

(ii) The cycles of desiccation and marine transgression that occurred in the Mediterranean sea in the Late Miocene (Messinian period, 5.5 to 4.5 Mya). Multiple transcontinental pathways between the various Mediterranean basins opened up in this period. This led to an explosion of migrations, favouring specially aggressive species (Bocquet, Widler & Kiefer, 1978; Schüle, 1993).

(iii) The climatic oscillations of the Quaternary had an outstanding influence on species distribution and on speciation processes, although its effect varied with latitude and topography (Hewitt, 1999). During cold (glacial) stages, high latitudes were covered with ice or with permafrost whilst vast periglacial areas were affected by pronounced aridity and ice-cold strong winds (Dawson, 1992; Huijzer & Vandenbergue, 1998). As a result, European temperate environments were reduced and compressed southwards and fauna and flora sheltered at lower latitudes or altitudes. Three different areas acted as main refugia for European biota during the glacial stages: Iberian Peninsula, Italy and the Balkans (Taberlet *et al.*, 1998). In addition to this, different studies support the existence of a fourth refugium in North Africa (reviewed in Vila, 2004). On the other hand, warmer climatic conditions during the interglacials allowed the expansion of some species, whereas others became extinct or shifted in altitude seeking for colder environments (*e.g.*, Comes & Kadereit, 1998; Hewitt, 2004).

Some geographical structuring of the genetic variation would be expected as a consequence of these processes. The succesive events of colonization from peripheral refugia would lead to a loss of genetic diversity in recently deglaciated areas, although variation in natural history parameters of the species involved, specially dispersal and colonizing abilities and breeding systems, could affect these predictions. (Richards, 1990; Hamrick *et al.*, 1991; Taberlet *et al.*, 1998; Hewitt *et al.*, 1999).

AIMS OF THE STUDY

Our main objectives were to unravel the phylogeography of this complex group of species and to help to clarify the taxonomic relationships among these taxa. To achieve these goals, we have used amplified fragment length polymorphism markers (AFLP) (Vos *et al.*, 1995), a molecular technique where the whole genome is analysed and a large number of highly variable fragments are generated (Karp, Seberg & Buiatti, 1996).

AFLP markers have been widely used in the last ten years to address biogeographical and phylogeographical questions in different taxa, most of which present a rather restricted distribution (*e.g.*, Stehlik, Schneller & Bachmann, 2001; Kj¸ lner *et al.*, 2004). These markers proved to be valid in studies both at the intraspecific (Stehlik, Schneller & Bachmann, 2001; Després, Loriot & Gaudeul, 2002; Tribsch, Schönswetter & Stuessy, 2002; Schönswetter *et al.*, 2004) and interespecific levels (Hansen, Elven & Brochmann, 2000; Schönswetter *et al.*, 2003; Stenström *et al.*, 2001; Stuessy *et al.*, 2003; Tremetsberger *et al.*, 2004). However, it has been rarely applied to species groups where such a variation concerning distribution, ecology, life cycles, ploidy and breeding systems occur.

The usefulness of AFLPs on taxonomical studies in closely related taxa have been shown by different authors (*e.g.* Fjellhaim, Elven & Brochmann, 2001; Marhold *et al.*, 2004). In the present study, the consequences of our results for the taxonomy of genus *Anthoxanthum* will be discussed.

MATERIALS AND METHODS

PLANT MATERIAL

Forty-six populations of the European *Anthoxanthum* species were considered in the study. Sampling areas were chosen in order to cover most of the biogeographically interesting areas for this genus in Europe. The selection of populations was based on bibliographical data and on the revision of the *Anthoxanthum* plants deposited in different European Herbaria (SANT, MA, UNEX, BC, LOU, SEV, VAL, UPS, G, FI, CLF). We considered six main geographical areas: Madeira Island, Iberian Peninsula (NW, NE, SW and central areas), the Alps, Scandinavia (N and S), Sudetes Mts (Czech Republic). and Malta Island (Figure 6.1). Between one and fifteen populations belonging to different taxa were sampled in each of the regions (Table 6.1). One population of *Phalaris coerulescens* Desf. was included as outgroup for taxonomical purposes following Trusty