Palynological study of *Ajania* Poljakov and related genera (Asteraceae, Anthemideae)

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ABSTRACT

A morphometrical study of pollen grains using scanning electron microscopy has been performed in seven genera belonging to the subtribe Artemisiinae (Anthemideae). Forty-six populations representing 40 species were considered, mainly from the genus *Ajania* (31 populations studied of 25 species). This work also includes observations in the genera *Brachanthemum*, *Cancrinia*, *Crossostephium*, *Dendranthema*, *Elachanthemum*, *Hippolytia*, *Kaschgaria*, *Poljakovia* and *Stilpnolepis*. Most data here provided constitute the first pollen observation for some species and genera (*Cancrinia* and *Poljakovia*). Two different pollen exine ornamentations are confirmed in the tribe, *Anthemis*-type (echinate) and *Artemisia*-type (microechinate), a result consistent with previous studies. *Artemisia*-type is exclusive of the subtribe whereas *Anthemis*-type is also present outside the Artemisiinae, suggesting that it may represent the ancestral character state for the group. These pollen types appear as clearly differentiated on the basis of their size and exine ornamentation. Their distribution in the phylogeny of Artemisiinae also segregates them: *Anthemis*-type is found in the *Dendranthema* and allied genera, whereas *Artemisia*-type occurs in *Artemisia* and closely related genera. Nevertheless, we found some very rare exceptions to this trend (e.g. *Ajania junnanica*, *Elachanthemum* and *Stilpnolepis*), whose possible origin is discussed.

Additional Keywords: Artemisiinae, Compositae, *Elachanthemum*, exine ornamentation, *Stilpnolepis*, molecular systematics, pollen type.

INTRODUCTION

Pollen forms and structures in the Asteraceae show a great variation, reflected through the numerous pollen types described in the family (Jeffrey, 2007). Pollen characters provide much taxonomically valuable information and have been commonly used as phylogenetic markers. This is the case in the subtribe Artemisiinae, where two pollen-types were described on the basis of the exine ornamentation (Stix, 1960): the *Anthemis*-type, with apparent spines, and the *Artemisia-type*, with spinules (microechinate) instead of spines (echinate). The microechinate pollen was first reported by Wodehouse (1926) as restricted to a group of genera including *Artemisia* L. and some close relatives, which has been confirmed in many studies dealing with *Artemisia* species from different geographical origins (e.g. Monoszon, 1948, 1950a, b; Straka, 1952; Stix, 1960; Singh & Joshi, 1969; Praglowski, 1971; Vallès *et al.*, 1987; Martín *et al.*, 2001, 2003; Grigoreva *et al.*, 2009). Several further studies on pollen exine ornamentation and molecular phylogeny confirmed that each pollen-type characterizes one of the main groups of Artemisiinae: *Dendranthema* (DC.) Des Moul. and relatives present the *Anthemis*-type, whereas *Artemisia* and allied show the *Artemisia*-type (Chen & Zhang, 1991; Rowley *et al.*, 1999; Martín *et al.*, 2001, 2003 for the pollen studies; Vallès *et al.*, 2003; Sanz *et al.*, 2008 for the phylogenies). Exceptions to this trend have been generally considered as the result of taxonomic misplacement (Martín *et al.*, 2001, 2003). In this sense, pollen type has been used to confirm or justify the segregation of several genera from *Artemisia* and their placement in the *Dendranthema* group, and inversely. One of them is the genus *Ajania* Poljakov.

The Asian genus *Ajania* comprises about 30-40 species, depending on the authors (Bremer & Humphries, 1993; Bremer, 1994; Kubitzki, 2007), with a high number of representatives in China and Japan, and also growing in Afghanistan, Kazakhstan, Kyrgyzstan, Mongolia, Northern India, Russia and Tadzhikistan. This genus was segregated from *Artemisia* by Poljakov (1955). Tzvelev (1961), in the Flora of the USSR, accepted the genus *Ajania* with about 25 species, nine of which growing in the USSR, but considered -using pollen ornamentation as one of the main arguments- that Poljakov (1955) had erroneously combined into *Ajania* some species that should be maintained in *Artemisia*. Tzvelev (1961) pointed out that *Ajania* had evolved from ancestral taxa closely related to *Dendranthema*, and that the adaptation of *Ajania* to Middle Asian steppes and deserts originated a high resemblance with the representatives of *Artemisia* occupying these areas. In order to explain the similarities between the three genera, Bremer & Humphries (1993) assumed that independent lines have evolved from the same dendranthemoid ancestor. Three species were removed from *Ajania* to constitute a separate new genus, *Phaeostigma* Muldashev (Muldashev, 1982, 1983). This author justified this change based on pollen characters (spinulose pollen), among others, and also pointed the affinities of this genus with *Artemisia*. He also used palynological features for proposing the combination of *Ajania junnanica* under *Artemisia*, because of its pollen showing “very small spines” (Muldashev, 1983). Two *Ajania* and one *Phaeostigma* species have been recently studied from the palynological point of view (Martín *et al.*, 2001 for *Ajania fastigiata* and *A. fructiculosa;* Martín *et al.*, 2003 for *Phaeostigma salicifolium*). Species of *Ajania* were found to present *Anthemis*-type pollen, which confirms their well-based placement in the *Dendranthema* group, also supported by molecular phylogenies (Masuda & Kondo, 2007; Sanz *et al.*, 2008). Likewise, the representative of *Phaeostigma* studied showed *Artemisia*-type pollen, a result congruent with the hypothesis of its close relationship with *Artemisia*. Because of its complex taxonomic history, with numerous relocations of species between the two main groups of Artemisiinae, *Ajania* and segregate genera represent a particularly interesting group for addressing pollen studies in the subtribe.

The present paper aims to provide new pollen data in the genus *Ajania* and in some other representatives of the Artemisiinae, such as *Brachantemum* DC., *Cancrinia* Kar. & Kir., *Crossostephium* Less., *Dendranthema*, *Elachanthemum* Y. Ling & Y. R. Ling, *Hippolytia* Poljakov, *Kaschgaria* Poljakov, *Poljakovia* Grubov & Filatova and *Stilpnolepis* Krasch. The specific objectives of this study are: (1) to enlarge the palynological data existent for the Artemisiinae; (2) to deepen in the characterization of the two pollen types found in the subtribe through the analysis of new and previous data from our team; (3) to discuss these findings in a phylogenetic framework with a view to contribute to resolve questions related to the systematic and phylogenetic relationships within the subtribe; (4) to consider the possible cause(s) for the transition from one pollen type to another.

MATERIAL AND METHODS

PLANT MATERIAL

Pollen grains from dried specimens of plants collected in field and deposited in the BCN (Universitat de Barcelona), HIMC (Inner Mongolia University, Hohhot) and LE (Botanicheskii Institut im. V.L. Komarova, Sankt Peterburg) herbaria were used to carry out the study (Table 1). Observations using optical and scanning electron microscopy (SEM) were carried out in 46 populations of 40 species of the genera *Ajania* (31 populations of 25 species), *Brachanthemum* (4 sp.), *Cancrinia* (2 sp.), *Crossostephium* (1 sp.), *Dendranthema* (2 sp.), *Elachanthemum* (1 sp.), *Hippolytia* (2 sp.), *Kaschgaria* (1 sp.), *Poljakovia* (1 sp.) and *Stilpnolepis* (1 sp.).

POLLEN OBSERVATIONS AND MEASUREMENTS

Pollen was obtained by dissecting dehydrated anthers with 96 % ethanol. Samples were examined with a scanning electron microscopy after acetolysis following Avetissian's (1950) micromethod. Observation was then carried out after coating with gold using a diode sputtering, and a Hitachi 52300 scanning microscope at l5 kV, at the Serveis Cientificotècnics de la Universitat de Barcelona. For biometrical measurements, pollen samples were acetolysed following the same method, mounted on glycerogelatine and sealed. Measurements were made using a Visopan apparatus (Reichert, Austria). For each specimen, 15 fully developed grains were measured, except in *Ajania aureoglobosa* (seven grains), *A. fruticulosa*-57 (eight grains), *A. junnanica* (10 grains), and *Crossostephium chinense* (five grains). The considered parameters, according to Erdtman (1969), Faegri & Iversen (1964) and Reitsma (1970), were: polar diameter (P), equatorial diameter (E) and sphericity (P/E). For each of them, the arithmetic mean and standard deviation were calculated. In the case of pollen grains with spiny ornamentation, the height of the spine was also measured. The density of supra-tectal spines was calculated from the mesocolpium area. We also proceed to the calculation of an approximation of the pollen volume [V, calculated using the ellipsoid formula: $V = 4/3\Pi(1/2P)(1/2E)^2$], and the counting of the number of spines/spinules in 25 μm^2 of the pollen surface. The pollen terminology used is according to Reitsma (1970).

STATISTICAL ANALYSES

A database grouping the present and previous results (Martín *et al.*, 2001, 2003) in the whole Artemisiinae subtribe was constructed for comparative purposes between the different morphological traits of each pollen type (e.g. volume, spine height and spine density) (Appendix). StatGraphics Plus 5.1 (Statistical Graphic Corp.) was used to carry out the Kruskal-Wallis contrasts. This is a non-parametric test that does not involve any assumption about the frequency of distribution of the variables, and therefore better fits to our data. Some of the 76 representatives of Anthemideae listed are not currently classified in Artemisiinae (as redefined by Oberprieler *et al.*, 2007), and we have not included them for proceeding to the statistical analyses.

MOLECULAR PHYLOGENY

ETS and ITS Genbank sequences were analysed in order to provide a phylogenetic framework for discussing pollen types distribution and evolution in Artemisiinae. Representatives of the genera *Achillea*, *Lepidolopsis* and *Tanacetum* have been chosen as constituting the outgroup for the Artemisiinae on the basis of the Anthemideae phylogeny of Oberprieler *et al.* (2007). Sequences were edited with BioEdit v7.0.9 (Ton Hall, Ibis Biosciences). The alignment was first done using T-COFFEE as implemented by BioX 1.1b1 (E. Lagercrantz [http://www.lagercrantz.name/software/*biox*/]), and next manually-revised with MacClade 4.08 (Maddison & Maddison, 2005). MrModeltest 2.2 (Nylander, 2004) was used to select the best-fit models of nucleotide substitution for our datasets. Bayesian inference analyses performed with MrBayes 3.1.1. (Huelsenbeck *et al.*, 2001) were initiated with random starting trees and run for 10^6 generations. Four Markov chains were run simultaneously, and trees were sampled every 100 generations, which resulted in 10,000 sampled trees. To ensure the Markov chains have become stable, log-likelihood values for sampling trees were plotted against generation time, and those before stationarity were discarded as "burn-in". A majority-rule consensus tree was obtained with PAUP version 4.0b4a (Swofford, 1999). Posterior probability support (PP) $\geq$ 95% was considered statistically significant. We proceeded to separated and also to a combined ETS and ITS analyses, restricting the dataset to individuals with both regions sequenced (ITS of *Phaeostigma salicifolium* AM774423 and EF577281, *P. variifolium* EF577283, and *Stilpnolepis centiflora* AY127695, AY127696 were removed). ETS clones of a same individual that grouped together in the separate analysis were combined in a consensus sequence, and, if this was not the case, they were introduced separately in the combined dataset. In the same way, ETS and ITS sequences of inconsistent positioning in separated analyses were treated independently in combined analysis. We also carried out independent ETS and ITS analyses involving the restricted taxonomic sampling of combined dataset.

RESULTS

Pollen traits of the studied taxa are shown in Table 2, Fig. 1 (A-X) and Appendix. Results from statistical analyses are presented in Fig. 2 (A-C), and of phylogenetic analyses in Fig. 3 (A-C). The studied pollen grains of Artemisiinae share the following features: they are 3-zonocolporate, isopolar, and with radial symmetry. Consistently with the previous palynological works (Chen & Zhang, 1991; Martín *et al.*, 2001, 2003, and references therein), the pollen observed in the present study can be assigned either to *Anthemis*-type or to *Artemisia*-type.

ANTHEMIS POLLEN TYPE (FIG. 1. A, B, D-N, Q, T, U, W, X)

The shape is mainly spherical, but frequently slightly prolate, and slightly oblate in some cases. Mean spine length ranges from 1.92±0.12 µm (*Ajania grubovii*) to 5.50±0.50 µm (*Dendranthema zawadskii*), which corresponds to an echinate pollen. *Dendranthema* presents particularly larger spines than other genera of the group (from 4.50 to 5.50 µm, whereas the maximum spine length found in the remaining genera is 4.40 µm). From one (e.g. *Ajania khartensis, A. pacifica*, *Brachanthemum gobicum*, *Dendranthema* representatives) to four (e.g. *Ajania aureoglobosa, A. fastigiata, A. fruticulosa*) ornamental elements (spines) are found in 25 µm^2 of the pollen surface. Volumes oscillate between 3,966.59 (*Ajania parviflora*) and 23,305.10 µm^3 (*Ajania pacifica*).

ARTEMISIA POLLEN TYPE (FIG. 1. C, O, P, R, S, V)

The shape is spherical, although in some cases slightly prolate or oblate. Concerning ornamentation, the exine is microechinate or microechinate, with spinules measuring less than 1 µm in height. Density of ornamental elements range from 7 [*Elachanthemum intricatum, Phaeostigma salicifolium* (Appendix)] to 44 [*Vesicarpa potentilloides* (Appendix)] for 25 µm^2 of the pollen surface. A small group of taxa shows a particularly high density of spinules (upon 44 spinules for 25 µm^2 of the pollen surface). It is constituted by *Ajaniopsis penicilliformis*, *Chamartemisia compacta*, *Sphaeromeria diversifolia*, and *Vesicarpa potentilloides* (Appendix). Volumes vary between 2,597.13 (*Ajania junnanica*) to 6,608.28 µm^3 (*Crossostephium chinense*).

GENUS *AJANIA* (FIG. 1. A-G)

This genus presents the general morphological traits described for the *Anthemis* pollen-type [with the exception of *A. junnanica* (Fig. 1C)]. The pollen shape is spheroidal, slightly prolate in most cases and sometime slightly oblate. Sometimes perforations of the exine appear between the spines (= ornamental elements). Pollen volumes vary 5.8-fold from 3,966.59 µm^3 (*A. parviflora*) to 23,305.10 µm^3 (*A. pacifica*), but the shape is quite constant in the studied species of this genus (P/E ratio ranges from 1.00 to 1.12).

DISCUSSION

The comparison of the two pollen types highlights some strong differences.The mean of *Anthemis* pollen-type volume (V_1) is significantly larger (almost twice) than that of *Artemisia*-type (V_2) ($V_1 = 8,961.21\ \mu m^3$; $V_2 = 4,574.6\ \mu m^3$; $P < 0.05$), with overlapping values between the volumes 3,966.59 and 6,608.28 μm^3 (Fig. 2A). The exine ultrastructure also clearly discriminates between these pollen types, *Artemisia*-type presenting much smaller ornamentation elements than *Anthemis*-type (Appendix), and significantly more abundant (the mean of ornamental elements found in 25 μm^2 of the pollen surface is 2.36 for *Anthemis* pollen and 14.52 for *Artemisia*-type; $P < 0.05$; Fig. 2B). Both characters – size and density of exine ornamentation – present exclusive rank values for each pollen type. No difference is found regarding the P/E ratio ($P > 0.05$; Fig. 2C), that is to say the shape of both pollen types is quite similar (the mean of P/E values is 1.04 for *Anthemis* pollen type, and 1.06 for *Artemisia*-type).

DISTRIBUTION OF THE POLLEN TYPES THROUGHOUT THE PHYLOGENY OF ARTEMISIINAE: THE SEGREGATION MOSTLY MAINTAINED

As expected, according to previous work (Sanz *et al.*, 2008), pollen types are segregated in the phylogeny and characterize the two main groups of Artemisiinae, the *Artemisia* and *Dentranthema* groups (Fig. 3C). This confirms their great value as phylogenetic marker in the tribe. However, some exceptions to this trend are found:

The molecular evidence locates *Elachanthemum intricatum* in the *Dendranthema* group and *Stilpnolepis centiflora* in the *Artemisia* group, while these species present the pollen type of the other group (Fig. 3C). Both species belong to monotypic genera, segregated from *Artemisia* (Krascheninnikov, 1946 for *Stilpnolepis*; Ling & Ling, 1978 for *Elachanthemum*). Shih (1985) combined the *Elachanthemum* species within *Stilpnolepis*. Ling (1987) argued against this, exine ornamentation being one of the most important differential traits; Kubitzki (2007) followed the same criterion as Ling and kept both genera separated, confirming pollen type as a good taxonomic character. Apart from these systematic considerations, the non-agreement of pollen type with phylogenetic placement in those two genera could constitute the two first cases of reversion in pollen type for the Artemisiinae. Nevertheless, to confirm the reversion event(s), it would be necessary to discard the hypothesis of pollen-type inheritance through hybridization for these species. The case of *Stilpnolepis* raises particular suspicion, because of its undetermined placement in previous ITS analyses (Watson *et al.*, 2002; Oberprieler *et al.*, 2007; Fig. 3A based in the same accessions AY127695, AY127696). This contrasts with the result involving different ITS accessions (AB359695, AB359781), which shows *Stilpnolepis* as sister to the genus *Filifolium* with a very strong support (PP = 100%, Fig. 3A). Regarding *Elachanthemum*, ETS and ITS data do not provide any evidence of hybrid origin. Both regions strongly support the grouping of this taxon with *Dendranthema* and relatives (100% of PP, Fig. 3 A, B), in a clade with exclusively *Anthemis*-pollen species, being *Elachanthemum* the only exception (Fig. 3C). Furthermore, *Elachanthemum* shows a rDNA organization that is different from the rest of Artemisiinae. Most Artemisiinae present a linked rDNA type which holds both 5S and 35S in the same unit (Garcia *et al.*, 2009), however, the only confirmed exception found to this linkage in the subtribe is the case of *Elachanthemum* with the typical, separated arrangement of 5S and 35S found in most angiosperms (Garcia *et al.*, unpubl. res.). Other genera belonging to the same clade, such as *Ajania* or *Brachanthemum*, show the linked arrangement as *Artemisia*; however, results are not still conclusive for *Dendranthema*, in which it seems that linked and unlinked units coexist in some of its species (Garcia *et al.*, unpubl. res.).

Our results also confirm the findings of Muldashev (1983) attributing an *Artemisia*-type pollen to *Ajania junnanica*, while the remaining species of *Ajania* present *Anthemis*-type (Table 2, Appendix). In the molecular phylogeny, the sequenced *Ajania* group along with *Dendranthema* and relatives, in accordance to their pollen affinities (Fig. 3C). In absence of sequence data available for *A. junnanica*, several explanations can be evoked. One hypothesis reconciles the pollen type with the systematic placement, and consists in considering *A. junnanica* misplaced in the genus *Ajania*. This was previously suggested by Muldashev (1983), who combined the species in the genus *Artemisia*, stating that it was "absolutely clear" that, because of its pollen type, it could not stand in *Ajania*. Not considering this trait, Ohashi & Yonekura (2004) combined *Ajania junnanica* in *Chrysanthemum* L., a genus with *Anthemis*-type pollen; in fact, those authors merged within *Chrysanthemum* the complete genera *Ajania*, *Arctanthemum* (Tzvelev) Tzvelev, *Dendranthema* and *Phaeostigma*. Bremer and Humphries (1993) also opted for the misplacement of *A. junnanica*, although in a slightly different way than Muldashev (1983). They considered *Ajania* – or part of the genus – as the sister group of *Artemisia* and allies (those having smooth or with short-spined pollen). The alternative hypothesis consists in maintaining *A. junnanica* in *Ajania*, where it would constitute the unique case of *Artemisia*-pollen. It is interesting to note that *Ajania* presents a certain variability in pollen traits; it exhibits both pollen types (although one only in one species), the greatest range of spine density (for the *Anthemis*-type), and the smaller and the larger pollens of the tribe. However the other genera of Artemisiinae have not been as extensively sampled as *Ajania* in the present study, and consequently we do not know either if such diversity is exceptional or the rule in the tribe or if it traduces in fact the taxonomic heterogeneity of *Ajania*. Anyway, it is to be stated that *Ajania* is basically considered as a genus with *Anthemis*-type pollen grains.

Similar to *Ajania* in some morphological features, but with spinulose pollen grains as a distinctive trait, Shih (1978) described the monospecific genus *Ajaniopsis*, whose *Artemisia*-type pollen was confirmed by Martín *et al.* (2001, 2003). Oberprieler et al. (2007) did not assign *Ajaniopsis* to a subtribe within the Anthemideae because of the lacking molecular framework for the species, but suggested, on the basis of the results from Martín *et al.* (2003), that its pollen features clearly point to the inclusion in the Artemisiinae subtribe.

This study also shows up several inconsistencies concerning the genus *Phaeostigma* (as stated in the introduction, a new genus described in 1981 by Muldashev, constituted by three species previously located in *Ajania*). The analysis of ETS region groups *Phaeostigma quercifolium* with *Achillea schmakovii* (Fig. 3B, C), and the ITS somewhere within early branched genera of Artemisiinae (Fig. 3A). Such a result suggests a possible hybrid origin for this species, from two species belonging to different subtribes of the Anthemideae. ETS firmly locates *Phaeostigma salicifolium* in a clade of the *Artemisia* group (PP = 100%, Fig. 3), in accordance with its *Artemisia*-type pollen (Martín et al., 2003). These results concerning *P. salicifolium* strongly agree with the assumption of a close relationship between *Phaeostigma* and *Artemisia* (Muldashev, 1981). However, this hypothesis is contradicted by the strong positioning of *Phaeostigma varifolium* within the *Dendranthema* group, which is supported by both ETS and ITS markers (PP = 100%, Fig. 3). Therefore, the phylogenetic affinities of the genus *Phaeostigma* remain unresolved, and furthermore, the monophyly of the genus could be questioned. The eventuality of pollen type reversions within Artemisiinae still lacks evidence, none of the cases considered above establishing undoubtedly such a feature.

EVOLUTIONARY TRENDS ON POLLEN FEATURES IN ARTEMISIINAE

The unsupported basal-most nodes of the ingroup impede the determination of the ancestral character state for Artemisiinae pollen type (Fig. 3). Nevertheless, the fact that the species of the outgroup (and most of the tribe) show *Anthemis*-type makes this one the most likely option for the ancestral state, an assumption also supported by the paleogeological record (Wang, 2004). According to this hypothesis, the main tendency in the subtribe would be toward the reduction of global size and size ornamentation of pollen.

Several factors implicated in pollen downsizing events are found in the literature, such as shift to annual life cycle (or more generally to shorter growth cycles), autogamy or a change to extreme environmental conditions (Hidalgo *et al.*, 2008, and references therein). However, none of these factors seem to account for the pollen type distribution pattern observed in Artemisiinae. In fact, the characteristics shown by the two Artemisiinae pollen types fit with the two main pollination syndromes: larger, heavily ornamented pollen grains - as *Anthemis*-type – with much pollenkitt making the pollen sticky, being more likely related to entomophily, and smaller (with also reduced size range variation), less ornamented pollen - as *Artemisia*-type – with almost no pollenkitt making the pollen dry, more likely related to anemophily (Wodehouse, 1935; Friedman & Barrett, 2009). The pollination syndrome is also traduced in terms of floral and inflorescence features, with bigger, showy structures found in insect pollinated plants, and small, not showy structures in wind pollinated plants (Friedman & Barrett, 2009). This trend accords well in Artemisiinae with small, greenish or whitish capitula generally displayed by taxa with *Artemisia*-type pollen, and capitula radiate (e.g. *Dendranthema*), coloured (e.g. *Ajania pacifica*) or arranged in corymbe (e.g. *Stilpnolepis*) in taxa showing *Anthemis*-type pollen. Therefore, a shift in pollination, from entomophily to anemophily may have accounted for the passage from *Anthemis* to *Artemisia* pollen type. Following this assumption, insect pollination would be likely the ancestral state in Artemisiinae, as it is also for the whole Asteraceae whose main apomorphy, the capitulum, is basically designed to draw attention to the display, by making the flowers more noticeable to the pollinator. The Compositae are mostly pollinated by animals, and the few wind-pollinated representatives of the family constitute very interesting exceptions. These are the *Artemisia* group of our present study, the genus *Ambrosia* (Heliantheae), and some species of *Espeletia* (Millerieae/Heliantheae s.l.; Jeffrey, 2007).

There exists, however, one Artemisiinae species which shows pollen and inflorescence pointing to different pollination syndromes: this is *Ajaniopsis penicilliformis*, with *Artemisia* pollen and showy capitula. In addition, several taxa present inflorescences not clearly attributable to one pollination type: *Crossostephium*, *Filifolium* (both with *Artemisia* pollen), and *Brachanthemum*, *Stilpnolepis* (both with *Anthemis* pollen). Such a pattern can indicate mixed pollination. In this sense, frequent insect visits have been also reported in different species of *Artemisia*, suggesting that entomophily could be involved in a certain proportion, even in species showing the anemophilous syndrome (Garnock-Jones, 1986; Vallès, 1989). Probably, some of these cases of incongruent pollen and inflorescence trends may also indicate that secondary shifts in pollination types are ongoing processes. This occurred in the genus *Espeletia*, another Asteraceae in which a shift from animal to wind pollination took place. In this case, the typical reduction of the spine size accompanying anemophily did not follow immediately the shift in pollination type, and was exclusively observed in the more derived species (Rundel et al., 1994, and references therein).

Some other tendencies have been described in the group. One concerns polyploidy, occurring in *Ajania* and considered as one of the main evolutionary factors in plants (Otto & Whitton, 2000, and references therein). This phenomenon presents, *a priori*, the interest, in connection with the data set analysed in the present paper, that the ploidy level may express itself directly through pollen size (Muller, 1979; Julià & Martín, 1994). This trend can not be confirmed in *Ajania*. In fact, the biggest and the smallest pollen have been found in high polyploid species (respectively *A. pacifica*, $2n = 90$, and *A. nematoloba*, $2n = 72$). Nevertheless, the relation between pollen size and ploidy is known to be easily overridden by other factors, and evolutionary short-lived (Muller, 1979; Tate & Simpson, 2004). Another point concerns the group of taxa within the *Artemisia*-type pollen that present a particular high density of ornamental elements (see results), which are all distributed in North America with the exception of *Ajaniopsis*. In fact, some of the species showing this large number of spinules (e.g. *V. potentilloides*, *Ch. compacta*) are nowadays labelled under a single genus, *Sphaeromeria*. Thus, it is more likely that the presence of a high density of spinules in the group might be reflecting a close relationship than an adaptation to particular environmental conditions.

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Figure captions:

Figure 1. Pollen grains of some of the taxa studied at SEM. **A**: *Ajania fruticulosa*. **B**: *A. grubovii*. **C**: *A. junnanica*. **D**: *A. pacifica*. **E**: *A. pacifica* (exine detail from mesocolpium). **F**: *A. nematoloba*. **G**: *A. nubigena*. **H**: *A. roborowskii* (apocolpium). **I**: *Brachanthemum gobicum*. **J**: *B. gobicum* (exine detail from mesocolpium). **K**: *B. kirghisorum*. **L**: *B. pulvinatum*. **M**: *Cancrinia discoidea*. **N**: *C. maximowiczii* (exine detail from apocolpium). **O**: *Crossostephium chinense*. **P**: *C. chinense* (exine detail from apocolpium). **Q**: *Dendranthema zawadskii*. **R**: *Elachanthemum intricatum*. **S**: *E. intricatum* (exine detail, colpus). **T**: *Hippolytia alashanensis*. **U**: *H. trifida*. **V**: *Kaschgaria komarovii*. **W**: *Poljakovia falcatolobata*. **X**: *Stilpnolepis centiflora*. Scale bar = 5 μm.

Figure 2. Box-and-whisker plots of statistical analyses of some pollen traits vs. pollen types. **A**: pollen volume. **B**: spine number. **C**: P/E ratio.

Figure 3. Bayesian phylogenetic inferences. Supported branches (PP ≥ 95%) are indicated in bold. PP values and genbank accessions are provided for the two first trees. **A**: ITS majority rule consensus with SYM+G model (the GTR+G model was also selected by MrModeltest and give similar results; data not shown). **B**: ETS majority rule consensus with GTR+G model. **C**: Combined ITS and ETS phylogram with GTR+I+G model (the GTR+G model, also selected by MrModeltest, gives comparable results; data not shown). Branches independently supported (PP ≥ 95%) by single ETS and ITS analyses involving the restricted taxonomic sampling of combined dataset are indicated

in the combined tree with * (grey) for ETS, and * (black) for ITS. Taxa with known *Artemisia* pollen type are written in yellow, and in red for *Anthemis*-type.

Table 1. Origin of the populations studied, with the indications of the herbaria where the voucher specimens are deposited (BCN: Centre de Documentació de Biodiversitat Vegetal, Universitat de Barcelona; HIMC: Faculty of Life Sciences, Inner Mongolia University, Hohhot; LE: Botanicheskii Institut im. V.L. Komarova, Sankt Peterburg). Asterisks (*) indicate different populations of the same species studied.

Taxa	**Populations**
Ajania achilleoides (Turcz.) Poljakov ex Grubov*	Mongolia, Ubsunur, 60 km SW Under-Khangai, Kheltguin-Ula mountains, 16.VIII.1979, Z. Kapamysheva (LE)
A. achilleoides (Turcz.) Poljakov ex Grubov*	Mongolia, Central Gobi, 16 km NE Erdene-Dalai, 4.IX.2004, Sh. Dariimaa, Sh. Tsooj, J. Vallès (BCN)
A. achilleoides (Turcz.) Poljakov ex Grubov*	Mongolia, Central Gobi, 46 km NE Erdene-Dalai, 4.IX.2004, Sh. Dariimaa, Sh. Tsooj, J. Vallès (BCN)
A. aureoglobosa (W.W. Sm. & Farr.) Muldashev	People's Republic of China, province of Gansu, near Liang Shui, 18.X.1914, E.N. Meyer (LE)
A. fastigiata (Winkl.) Poljakov	People's Republic of China, autonomous region of Xingian-Uigur, Kashgar, 25 km SW Kiushisha, 1,400 m, 19.X.1959, M. Petrov (LE)
A. fruticulosa (Ledeb.) Poljakov*	Kyrgyzstan, mountain pass in the Kurutag mountains, 16.XI.1957, A. Yunatov (LE)
A. fruticulosa (Ledeb.) Poljakov*	Mongolia, Southern Gobi, 10 km S Bulgan, Sh. Dariimaa, Sh. Tsooj, J. Vallès, E. Yatamsuren, 2.IX.2004 (BCN)
A. fruticulosa (Ledeb.) Poljakov*	Mongolia, Southern Gobi, 20 km SW Mandal Oboo, 4.IX.2004, Sh. Dariimaa, Sh. Tsooj, J. Vallès (BCN)
A. gracilis (Hook. f. & Thomson) Poljakov ex Tzvelev	Tadzhikistan, Pamiro-Alai, near Kirakul, 5.VIII.?, A. Kushakevich (LE)
A. grubovii Muldashev	Mongolia, Dzhungar Gobi, Mongolian Altai, 17.VIII.1979, V. Grubov (LE)
A. junnanica Poljakov	People's Republic of China, Northern Yunnan, Pe-Cong-Ching, 3,200 m, 1909-1911, R. Maire (LE)
A. khartensis (Dunn) C. Shih	People's Republic of China, Gansu, 100 km SW Dunkhun, 2.VIII.1958, M. Petrov (LE)
A. kokanica (Krasch.) Tzvelev	Kyrgyzstan, Northern Alai, high river Shakhimaruan river, 12.VIII.1938, A. Mukhamedzhanov (LE)
A. myriantha (Franch.) Y.R. Ling ex C. Shih	People's Republic of China, Northern and Central Yunnan, mountains near Liao-Do, 2,000 m, XI.1910, R. Maire (LE)
A. nana (Krasch.) Muldashev	People's Republic of China, Northern Szetschuan, between Epor and Kanguang, 19.X.1885, G.N. Potanin (LE)
A. nematoloba (Hand.-Mazz.) Ling ex C. Shih	Mongolia, Alaschan mountain, VIII.1880, N.M. Przewalski (LE)
A. nubigena (Wall.) C. Shih	Nepal, Bagmati zone, Kasuwa district, below Khanyyin, 3,650 m, 22.IX.1966, D. Nicholson (LE)
A. pacifica (Nakai) K. Bremer & Humphries	Japan, Honshu prefecture, Chiba, 10 m, 1.XII.1973, M. Togashi (LE)
A. pallasiana (Fisch. ex Besser) Poljakov	People's Republic of China, Kheiluntszyn province, Yaohe district, Hualatszy, 10.IX.1950, Chang Kiang-Cheng (LE)

Ajania parviflora (Grun.) Ling	People's Republic of China, Inner Mongolia, Alxa province, road S128, km 102, near Suhait, sandy and stony soils, 6.IX.2007, J. Vallès, S.W. Zhao (BCN)
A. potaninii (Krasch.) Poljakov	People's Republic of China, Gansu, Fin-Ten-Lin mountain pass, 1885, G.N. Potanin (LE)

Table 1. Cont.

Taxa	Populations
A. przewalskii Poljakov	Mongolia, Alaschan, 9.VIII.1880, N.M. Przewalski (LE)
A. purpurea C. Shih	People's Republic of China, Tibet, Yan-Uzi-Uzyan basin, Nru-Chu canyon, 25.VII.1900, V. Ladyguin (LE)
A. remotipinna (Hand.-Mazz.) Y. Ling & C. Shih	Mongolia, near Kalgans, 1870, A. Lomonossov (LE)
A. roborowskii Muldashev	People's Republic of China, Gansu, 25 km S Lanchisou, 12.VIII.1958, M. Petrov (LE)
A. rupestris (Matsum. & Koidz.) Muldashev*	Japan, Sirano-Asamajama, Happu-Giku, IX.1889, Tschonoski (LE)
A. rupestris (Matsum. & Koidz.) Muldashev*	Japan, Happu-Giku, IX.1889, Tschonoski (LE)
A. scharnhorstii (Regel & Schmalh.) Tzvelev*	People's Republic of China, Tian-Shan, Bogdo-Ola mountains, near Urumqi, 26.VIII.1908, G. Merzbacher (LE)
A. scharnhorstii (Regel & Schmalh.) Tzvelev*	People's Republic of China, Tian-Shan, Bogdo-Ola mountains, 29.VIII.1908, G. Merzbacher (LE)
A. tibetica (Hook. f. & Thomson) Tzvelev	People's Republic of China, Tibet, Peku lake, 4.650 m, 31.VIII.1991 (LE)
A. trilobata Poljakov	Kazakhstan, Semirschen region, Przhevalski district, canyon of river Karakol, 22.VII.1913, V. Saposhnikov (LE)
Brachanthemum gobicum Krasch.	Mongolia, Ubur-Khangai, Arms Bogd mountains, 31.VIII.2004, Sh. Dariimaa, Sh. Tsooj, J. Vallès (BCN)
Brachanthemum kirghisorum Krasch.	Kyrgyzstan, Alatau mountains, Issik-Kul lake basin, 15 km W of Kyzylty, 1,650 m, 20.VII.1970, N.N. Izmailova, S.S. Ikonnikov, D.M. Ladugina (HIMC)
Brachanthemum mongolorum Grubov	Mongolia, Northern region, 15 km W Barun-Matad-Ula, 12.VIII.1989. Ch. Sanchir, V. Khramtsov (LE)
Brachanthemum pulvinatum (Hand.-Mazz.) C. Shih	People's Republic of China, 4.IX.1990 (HIMC)
Cancrinia discoidea (Ledeb.) Poljakov ex Tzvelev	Mongolia, Southern Gobi, 17 km NE Bulgan, 5.IX.1995, A. Bayandzag (BCN)
Cancrinia maximowiczii C. Winkl.	People's Republic of China, 21.VII.1980 (HIMC)
Crossostephium chinense (L.) Makino	People's Republic of China, Chzhchi province, Beijing surroundings, Pokhuashan mountains, 1850-1858, S.M. Vazilievskii (LE)
Dendranthema mongolicum (Y.R. Ling) Tzvelev	Mongolia, Arkhangai, mountain pass Sagan-Davaa, near Tsetserleg, 2,200 m, 25.VIII.2004, Sh. Dariimaa, Sh. Tsooj, J. Vallès (BCN)
Dendranthema zawadskii (Herbich) Tzvelev	Mongolia, Bulgan, Khugunkhaan mountains, 2,000 m, 25.VIII.2004, Sh. Dariimaa, Sh. Tsooj, J. Vallès (BCN)
Elachanthemum intricatum (Franch.) Y. Ling & Y.R. Ling	Mongolia, Suothern Gobi, Gobi Altai, near Gurvan Tes, 5.IX.1979, V.I, Grubov, A. Muldashev, Sh. Dariimaa (BCN)
Hippolytia alashanensis (Ling) C. Shih	People's Republic of China, Inner Mongolia, Alxa province, SW slopes of Helan Shan, Tonguan, 5.IX.2007, J. Vallès, S.W. Zhao (BCN)
Hippolytia trifida (Turcz.) Poljakov	People's Republic of China, 11.VIII.1994 (HIMC)
Kaschgaria komarovii (Krasch. & Rubtzov) Poljakov	Mongolia, Dzhungar Gobi, near Bulgan, 29.VII.1988, I.A. Gubanov, Sh. Dariimaa, R.V. Kamelin (BCN)

Poljakovia falcatolobata (Krasch.) Grubov & Filatova	People's Republic of China, Burkhan-Budda mountains, Khatu canyon, 25.VII.1911, V.N. Ladyguin (LE)
Stilpnolepis centiflora (Maxim.) Krasch.	People's Republic of China, 15.IX.1963 (HIMC)

Table 2. Pollen characteristics of the taxa studied. P: Polar axis [range; X: mean values (standard deviation)]. E: equatorial axis [range; X: mean values (standard deviation)]. P/E: sphericity. Spine height: range; X: mean values (standard deviation). Asterisks (*) indicate different populations of the same species studied (presented in the same order as in Table 1).

Taxa	P (µm)	E (µm)	P/E (µm)	Pollen type	Spine height (µm)
*Ajania achilleoides**	22.91-27.08 X=24.92(1.33)	20.83-27.08 X=23.74(1.63)	1.04	*Anthemis*	3.24-4.28 X=3.60(0.40)
*A. achilleoides**	20.83-27.08 X=22.91(1.92)	17.70-25.00 X=22.14(1.69)	1.03	*Anthemis*	2.75-3.18 X=2.92(0.16)
*A. achilleoides**	20.83-25.00 X=22.91(1.36)	18.75-22.91 X=21.93(1.27)	1.04	*Anthemis*	2.75-3.10 X=2.85(0.15)
A. aureoglobosa	19.79-25.00 X=22.46(1.88)	20.83-22.91 X=21.72(1.11)	1.03	*Anthemis*	2.75-3.10 X=2.96(0.17)
A. fastigiata	22.91-31.25 X=26.31(2.17)	22.91-31.25 X=25.41(1.92)	1.03	*Anthemis*	3.63-4.41 X=3.83(0.33)
*A. fruticulosa**	20.83-33.33 X=25.64(4.41)	20.83-28.12 X=23.34(2.89)	1.09	*Anthemis*	3.76-4.15 X=3.91(0.14)
*A. fruticulosa**	22.91-27.08 X=25.27(1.07)	22.91-29.16 X=25.20(1.58)	1.00	*Anthemis*	3.27-4.31 X=3.75(0.41)
*A. fruticulosa**	20.83-29.16 X=25.41(2.18)	18.75-26.04 X=23.81(1.96)	1.06	*Anthemis*	2.59-4.41 X=3.57(0.74)
A. gracilis	21.87-28.12 X=24.38(1.73)	20.83-25.00 X=22.65(1.42)	1.07	*Anthemis*	1.98-2.84 X=2.30(0.34)
A. grubovii	25.00-29.16 X=26.94(1.51)	22.91-29.16 X=25.20(1.67)	1.06	*Anthemis*	1.72-2.06 X=1.92(0.12)
A. junnanica	14.58-20.83 X=18.33(2.31)	12.5-18.75 X=16.45(2.68)	1.11	*Artemisia*	-
A. khartensis	22.91-29.16 X=24.85(1.61)	20.83-27.08 X=24.30(1.79)	1.02	*Anthemis*	2.75-3.10 X=2.92(0.12)
A. kokanica	25.00-31.25 X=26.73(1.87)	22.91-27.08 X=25.55(1.17)	1.04	*Anthemis*	3.37-4.15 X=3.68(0.28)
A. myriantha	20.83-27.08 X=23.88(1.49)	20.83-26.04 X=23.67(1.39)	1.00	*Anthemis*	2.15-2.32 X=2.21(0.09)
A. nana	20.83-31.25 X=24.51(2.66)	20.83-26.04 X=23.39(1.46)	1.04	*Anthemis*	3.11-3.89 X=3.39(0.39)
A. nematoloba	14.58-23.95 X=21.03(2.36)	14.58-21.87 X=19.64(2.29)	1.07	*Anthemis*	2.06-2.15 X=2.13(0.04)
A. nubigena	25.00-29.16 X=27.77(1.28)	25.00-29.16 X=27.14(1.59)	1.02	*Anthemis*	3.10-4.31 X=3.54(0.45)
A. pacifica	35.41-41.66 X=38.39(2.11)	33.33-40.62 X=34.05(8.37)	1.12	*Anthemis*	3.89-4.93 X=4.43(0.43)
A. pallasiana	20.83-33.33 X=27.56(4.07)	20.83-33.33 X=26.80(3.57)	1.02	*Anthemis*	3.62-4.48 X=4.03(0.35)
A. parviflora	16.00-24.00 X=19.72(2.09)	18.00-22.00 X=19.60(1.20)	1.00	*Anthemis*	3.28-3.88 X=3.67(0.22)
A. potaninii	21.87-25.00	18.75-25.00	1.05	*Anthemis*	2.58-3.01

	X=23.18(1.21)	X=21.94(2.06)			X=2.84(0.17)

Table 2. Cont.

Taxa	P (µm)	E (µm)	P/E (µm)	Pollen type	Spine height (µm)
A. przewalskii	22.91-25.00 X=23.60(0.85)	17.70-25.00 X=22.28(1.79)	1.05	*Anthemis*	3.11-3.89 X=3.26(0.34)
A. purpurea	25.00-29.16 X=25.62(1.23)	22.91-29.16 X=24.64(1.70)	1.03	*Anthemis*	2.58-3.10 X=2.87(0.22)
A. remotipinna	22.91-29.16 X=25.62(1.56)	20.83-28.12 X=24.99(1.71)	1.02	*Anthemis*	3.76-4.15 X=3.96(0.17)
A. roborowskii	22.91-31.25 X=26.31(2.85)	18.75-31.25 X=24.16(3.03)	1.08	*Anthemis*	2.58-2.75 X=2.63(0.07)
*A. rupestris**	22.91-28.12 X=24.37(1.61)	22.91-27.08 X=24.02(1.44	1.01	*Anthemis*	3.01-3.62 X=3.30(0.29)
*A. rupestris**	22.91-29.16 X=26.31(1.98)	20.83-29.16 X=24.85(2.29	1.05	*Anthemis*	3.10-3.62 X=3.37(0.23)
*A. scharnhorstii**	22.91-35.41 X=26.38(3.09)	20.83-27.08 X=24.16(2.16	1.09	*Anthemis*	2.59-3.63 X=3.21(0.43)
*A. scharnhorstii**	22.91-31.25 X=26.31(2.37)	20.83-29.16 X=23.95(2.55	1.09	*Anthemis*	2.84-3.62 X=3.08(0.31)
A. tibetica	25.00-29.16 X=25.69(1.28)	20.83-27.08 X=23.60(1.70	1.08	*Anthemis*	3.89-4.67 X=4.30(0.29)
A. trilobata	22.91-27.0 X=25.20(1.37)	22.91-26.04 X=24.65(1.01	1.02	*Anthemis*	3.11-3.63 X=3.31(0.21)
Brachanthemum gobicum	33.33-37.5 X=33.81(1.17)	32.29-35.41 X=33.67(1.01	1.00	*Anthemis*	3.62-4.56 X=4.03(0.41)
B. kirghisorum	24.80-34.00 X=29.16(2.55)	24.80-30.00 X=27.56(1.70	1.05	*Anthemis*	3.2-4.02 X=3.65(0.32)
B. mongolorum	22.91-33.33 X=29.02(3.47)	22.91-31.25 X=28.33(3.03)	1.02	*Anthemis*	2.93-4.13 X=3.56(0.49)
B. pulvinatum	24.00-26.00 X=24.9(0.55)	22.00-25.33 X=23.27(0.84)	1.07	*Anthemis*	2.83-3.2 X=2.99(0.13)
Cancrinia discoidea	22.91-27.08 X=24.16(1.53)	20.83-25.00 X=22.63(1.54)	1.06	*Anthemis*	2.75-3.18 X=3.01(0.17)
C. maximowiczii	23.60-28.65 X=25.98(2.62)	20.00-25.07 X=23.52(1.56)	1.10	*Anthemis*	4.02-4.62 X=4.26(0.22)
Crossostephium chinense	19.48-27.08 X=24.13(3.18)	20.83-27.08 X=22.87(2.58)	1.05	*Artemisia*	-
Dendranthema mongolicum	31.25-35.41 X=33.60(0.99)	29.16-35.41 X=32.01(2.24)	1.04	*Anthemis*	4.15-5.71 X=4.72(0.64)
D. zawadskii	29.16-35.41 X=32.42(2.11)	27.08-33.33 X=31.45(2.01)	1.03	*Anthemis*	4.93-6.49 X=5.50(0.59)
Elachanthemum intricatum	22.91-25.00 X=23.32(0.95)	20.83-23.95 X=22.63(0.91)	1.03	*Artemisia*	-
Hippolytia alashanensis	24.00-28.80 X=26.96(1.55)	23.20-30.00 X=26.6(1.83)	1.01	*Anthemis*	3.73-4.17 X=3.97(0.16)
H. trifida	27.20-34.00 X=30.94(1.74)	24.80-34.00 X=29.82(2.41)	1.03	*Anthemis*	3.58-4.44 X=4.07(0.39)
Kaschgaria komarovii	20.83-22.91 X=21.80(0.99)	20.83-25.00 X=21.94(1.27)	0.99	*Artemisia*	-
Poljakovia falcatolobata	25.00-33.33 X=29.64(2.11)	20.83-33.33 X=27.42(3.96)	1.08	*Anthemis*	3.27-4.31 X=3.75(0.37)
Stilpnolepis centiflora	24.00-28.00 X=26.00(0.89)	22.00-26.00 X=24.00(1.26)	1.08	*Anthemis*	2.38-2.83 X=2.58(0.21)