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Natural hybridization between *Aster ageratoides* var. *scaberulus* and *Kalimeris indica* (Asteraceae): evidence from morphology, karyotype, and ITS sequences

Wei-Ping LI *

College of Life Sciences, Hunan Normal University, Changsha 410081, P. R. China

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ABSTRACT. A new natural hybrid from the Hengshan Mountain in Hunan Province, China, was found. Comparisons of morphology, karyotype, and ITS sequences among the hybrid and its parental species show that the hybrid represents F_1 progeny from hybridization between *Aster ageratoides* var. *scaberulus* and *Kalimeris indica*. Based on morphological and karyotypical observations of the hybrid, this study suggests that natural hybridization between *Aster* and *Kalimeris* could have lead to the shortening of the pappus and a special karyotype (LS-type) and makes the delimiting of *Aster* and *Kalimeris* difficult. This study also indicates that karyotype investigations may be particularly useful in resolving some of the taxonomic confusion between *Aster* and *Kalimeris*.

Keywords: *Aster*; *Kalimeris*; Karyotype; Natural hybridization; Pappus.

INTRODUCTION

Natural hybridization is a frequent phenomenon in plants and plays an important role in plant evolution, leading in at least some cases to formation of new species. In addition, hybridization often generates considerable taxonomic confusion by facilitating introgression of plant traits. Therefore, confirmation of the hybrid nature and parentage is important in studying plant evolution, and may help to resolve certain taxonomic problems. Unfortunately, many hybrids described in the botanical literature (e.g. Hsieh and Wang, 1989; Chang, 1991; Sun and Huang, 1994; Zhang et al., 1995; Zhang and Zhao, 1995; Qian and Sun, 1998; Ren, 1996; Zhou and Wu, 2001; Zhang et al., 2003) were determined based only on their morphological intermediacy between their putative parents. However, morphological intermediacy may result from continuous variation and the high plasticity of many traits (Xu, 1998; Craft et al., 2002). In recent years, molecular data have played an increasing role in the detection and verification of putative hybrids (Smith et al., 1996; Morrell and Rieseberg, 1998; Milne, 1999; Durand, 2002; Denda and Yokota, 2003).

Tara (1972, 1977, 1978a, 1978b, 1979) first described intergeneric hybridization between *Aster* and *Kalimeris* (Asteraceae, Astereae, Asterinae). Based on cytological analyses, Tara suggested that hybridization had occurred between *Aster ovatus* (Franch. et Sav.) Soejima et Mot. Ito (*A. ageratoides* subsp. *ovatus* (Franch. et Sav.) Katam.) and *Kalimeris incisa* (Fisch.) DC., and that in fact *A. ovatus* was an amphidiploid that originated following intergeneric hybridization between *Aster* and *Kalimeris*. However, many subsequent authors have overlooked the morphological characters of hybrids and the potential impact of intergeneric hybridization on the complex and uncertain relationship between these two genera. This study provides the first report of hybridization between *Aster ageratoides* var. *scaberulus* (Miq) Ling and *Kalimeris indica* (L.) Sch.-Bip. with verification of the parentage. This study demonstrates the use of karyotyping and of molecular sequencing as a means to detecting hybrids and discusses the implication of these results for the phylogeny of *Aster* and *Kalimeris*.

MATERIALS AND METHODS

Study site and materials

Plant materials for this study were collected from Hengshan Mountain, located in the middle-subtropical monsoon zone in central Hunan Province, in China, at 112°34’ to 112°44’E and 27°10’ to 27°20’N. The putative hybrid plants were found at a sunny deforested site near a subtropical coniferous forest at 300 m alt. These hybrids appeared to have leaves and inflorescences intermediate between two sympatric taxa, *Aster ageratoides* var.

*Corresponding author: E-mail: lwp@hunnu.edu.cn; Tel: 86-731-8871052; Fax: 86-731-8883310.

scaberulus and *Kalimeris indica*, which were common at the site. *Aster ageratoides* var. *laticorymbus* (Vant.) Hand.-Mazz. also occurs at Hengshan Mountain, but the population was found to be a hexaploid (Li, unpublished) and was spatially distant from the putative hybrid, suggesting that it could not be one of the parents of the putative hybrid. It is thus excluded from this study.

Twelve putative hybrid individuals were collected and prepared as specimens for morphological analyses, and rhizomes of three individuals were transplanted to Hunan Normal University for cytological and molecular investigation. Thirty plants each of *A. ageratoides* var. *scaberulus* and *K. indica* that grew near the putative hybrids were prepared as specimens for morphological analyses, and five each were transplanted for cytological and molecular investigation. All specimens were identified according to Ling and Chen (1985) and were deposited as herbarium vouchers at Hunan Normal University (HNNU).

Methods

After preliminary observation, 16 morphological characters—including features of branches, middle leaves, phyllaries, florets, and achenes—were found to differ between the two putative parental species. All field collections were scored for these 16 characters. For quantitative features, such as size of middle leaves and number of florets, the measurements are presented as a range of variation.

Actively growing root tips for chromosome observations were cut from living plants, pretreated with 0.1% colchicine at 8-12°C for 4 h, fixed in Carnoy I (glacial acetic acid: 95% ethanol=1:3) at room temperature for 12 h, then macerated in 1 mol/L hydrochloric acid at 60°C for 8 min, stained in 5% $NH_4Fe(SO_4)_2$, at room temperature for 3 h, stained in 0.75% hematoxylin for 3 h, washed in distilled water for 30 min, and finally depigmented and squashed in 45% acetic acid.

Four samples for ITS analysis, one each from *A. ageratoides* var. *scaberulus* and *K. indica*, and two, respectively, from putative hybrid individuals were obtained from plants cultivated at Hunan Normal University. Total genomic DNA was isolated from fresh leaf tissue using the CTAB method of Roger and Bendish (1988). The two internal transcribed spacers (ITS-1, ITS-2) and 5.8S nrDNA were amplified using the primers "ITS1" (5´ AGT CGT AAC AAG GTT TCC GTA G 3´) and "ITS4" (5´ TCC TCC GCT TAT TGA TAT GC 3´). The amplification reaction was performed in 50 µL volumes using 10-20 ng of genomic DNA, 5 µl $MgCl_2$ (25 mmol/L), 4 µl dNTP (2.5 mmol/L), 1 µl (6.25 pmol) of each primer, and 0.25 µl (5 U/µl) Taq DNA polymerase. The following temperature and time profile was used in the amplification of the Polymerase Chain Reaction (PCR): an initial denaturation step of 94ºC for 5 min; 36 cycles of 94ºC for 1 min, 57ºC for 1 min, and 72ºC for 2 min, and a final extension of 10 min at 72ºC. The amplified double stranded DNA fragments were purified and then directly sequenced on an ABI 377 automated sequencer (PE Applied Biosystems, Inc.). Primers were the same as those of the initial PCR. For each sample, both forward and reverse sequences of ITS1, 5.8S and ITS2 were obtained. Each base position was examined for agreement between complementary strands.

RESULTS

Morphology

A morphological comparison among the three taxa is summarized in Table 1. The two putative parents differ significantly in all 16 characters. By contrast, the presumed hybrids have intermediate character states in eight characters, including distribution of stomata, number of florets, size of achenes, and especially length of pappus. The hybrids resemble *A. ageratoides* var. *scaberulus*, in five characters, such as lack of stomata on upper leaves, shape of inner phyllaries, width of middle phyllaries, and lack of glands on phyllaries and ovaries, and resemble *K. indica* in the other three, including number of branches above, size of middle leaves, and color of ray florets.

Karyotype

The metaphase chromosomes and the karyotype of the three taxa are shown in Figure 1. All three taxa are tetraploid with 2n=4x=36. However, there are significant differences in karyotype among them. All 36 chromosomes in *A. ageratoides* var. *scaberulus* are L-type (large chromosomes), with the length varying from 2.51-4.35 µm while all 36 chromosomes of *K. indica* are S-type (short chromosomes), ranging in length from 1.4-1.93 µm. The longest chromosome pair of *K. indica*, 1.93 in length, is shorter than the shortest pair of *A. ageratoides* var. *scaberulus*. The putative hybrid is intermediate in karyotype, with 18 L-type chromosomes (2.49-4.1 µm) and 18 S-type chromosomes (1.35-1.85 µm).

ITS sequences

The boundaries of the ITS region (ITS1, 5.8S, and ITS2) were determined by comparison to published ITS sequences of *Aster amellus* L. and *Kalimeris integrifolia* (L.) Less (Noyes and Rieseberg, 1999). The length of the ITS sequence of all the materials studied is 630 bp. The sequences of *A. geratoides* var. *scaberulus* and *K. indica* differ from one another at 13 sites (2%; Table 2). At each of these 13 sites the sequence spectrum of the putative hybrid individuals shows two overlapped bands with the peak about half as high as those of neighboring sites, indicating that two different base pairs are at each of these sites that correspond with those of *A. geratoides* var. *scaberulus* and *K. indica*, respectively (Table 2), exhibiting a combination of the ITS sequences of the two putative parent species. The forward and reverse sequences agree with each other for complementary strands. The ITS sequences from the two hybrids were identical. Therefore, the overlapped bands, as a polymorphism, can be attributed to the fact that there are

Table 1. A morphological comparison among *Aster ageratoides* var. *scaberulus*, *Kalimeris indica* and their putative hybrid.

Features	*Aster ageratoides* var. *scaberulus*	The putative hybrid	*Kalimeris indica*
Branches above	None or rarely 1-3	Many (>5)	Many (>5)
Size of middle leaves (Length × Width) (cm)	6-15×2-6	6-9×2-3.5	6-9.5×2-4
Stomata on the upper surface of middle leaves	None	None	Present, but rare
Size of leave on branches (Length × Width) (cm)	3-8×1.4-3	2-4×0.5-1.5	1-2.5×0.5-1
Length of peduncles (cm)	0.3-2.1	0.3-9	1.2-10
No. of layers of phyllaries	4-5	3-4	2-3
Shape of inner phyllaries	Linear oblanceolate	Linear oblanceolate	Ovate in the upper 2/3, and constricted and lanceolate in the lower 1/3
Glands on middle phyllaries	None	None	Present
Width of middle phyllaries	0.8-1	0.85-1	1.3-1.6
No. of florets per head	24-40	54-72	100-130
No. of ray florets per head	12-20	19-22	25-35
No. of disc florets per head	12-20	34-51	90-100
Coloure of ray florets	White	Blue-violet	Blue-violet
Glands on ovary	None	None	Present
Length of pappus (mm)	4.75-5.5	0.5-2.25	0.1-0.2
Size of achenes (Length × Width) (mm)	2-2.5×0.65-0.95	1.95-2.25×0.9-1	1.75-2.1×0.95-1.15

two kinds of ITS sequences in each hybrid plant instead of to a test error.

DISCUSSION

Confirmation of the hybrid

Sympatry of parents is one of preconditions for hybridization. The putative hybrid, *A. ageratoides* var. *scaberulus*, and *K. indica* co-occurred at a sunny deforested site of the Hengshan Mountain with no other *Kalimeris* or *Aster* species nearby. Their spatial distribution indicates the possibility that the putative hybrid is derived from *A. ageratoides* var. *scaberulus* and *K. indica*. A hybrid is usually morphologically intermediate between its parents (Stebbins, 1950), and may also share some features with them (Rieseberg and Ellstrand, 1993). According to Table 1, the putative hybrid had eight intermediate characters between *A. ageratoides* var. *scaberulus* and *K. indica*, three similar to *K. indica*, and five similar to *A. ageratoides* var. *scaberulus*, supporting the notion that the putative hybrid was the product of *A. ageratoides* var. *scaberulus* and *K. indica*.

Even though both *Aster ageratoides* var. *scaberulus* and *K. indica* at this locality are tetrapolid with 2n=4x=36, their karyotypes are fortunately quite different (Figure 1), which makes it easy to test the hybrid cytologically. Since the karyotype of the two taxa was investigated simultaneously with the same method and in the same reagents, length of the chromosomes was comparable. The shortest chromosome pair (L-type) of *A. ageratoides* var. *scaberulus* was found to be longer than the longest of *K. indica* (S-type), and the putative hybrid had 18 chromosomes each of L- and S-Type, both resembling in size and morphology the presumed parental species.

Tara (1972, 1978a, 1978b, 1979) studied intergeneric hybridization between *Aster* and *Kalimeris* based on karyotypical comparisons among hybrids and their parents as well as observations of meiosis. Tara reported that there were several hybrid types, such as 2n=72=18 L-chromosomes + 54 S-chromosomes, 2n=72=9 L-chromosomes + 63 S-chromosomes, 2n=81=27 L-chromosomes + 54 S-chromosomes, and 2n=108=18 L-chromosomes + 90 S-chromosomes, which resulted from the natural hybridization of *A. ovatus* and *K. incisa* and the backcross

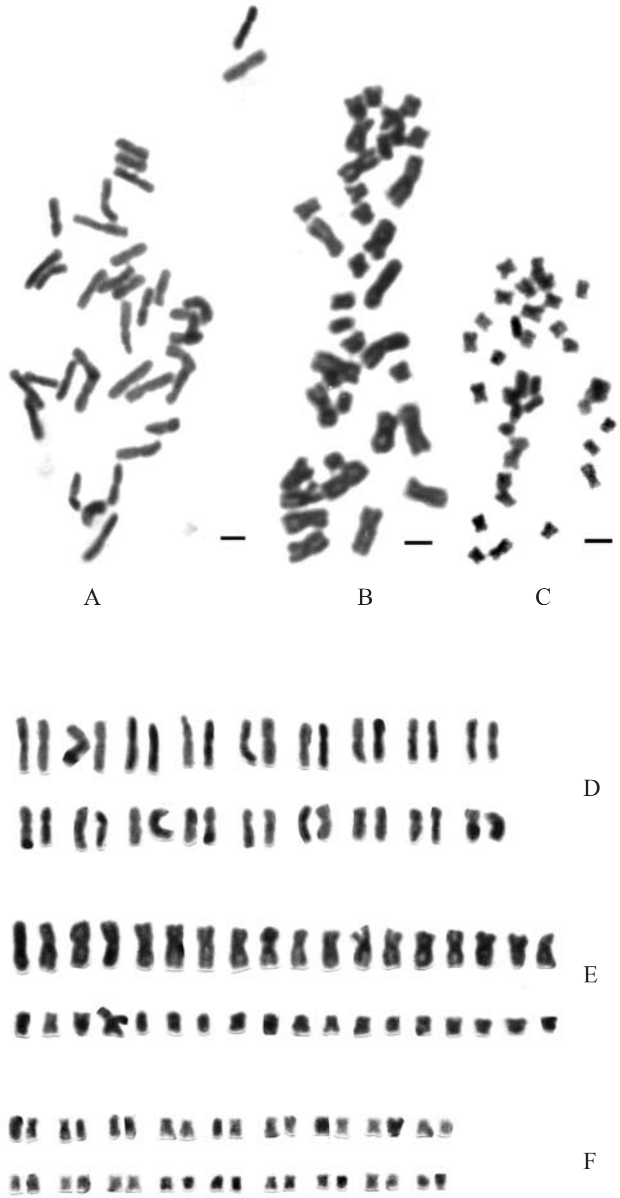

Figure 1. Photomicrographs of the metaphase chromosomes and the karyotype of *Aster ageratoides* var. *scaberulus*, the putative hybrid, and *Kalimeris indica* (Scale bar=2 μm). A, D: *Aster ageratoides* var. *scaberulus*; B, E: the putative hybrid; C, F: *Kalimeris indica*.

between the F_1 and *K. incisa*. Moreover, the hybrid chromosome behavior at meiosis confirmed the origin of the two types of chromosomes. On the other hand, *A. ovatus* was verified to be an amphiploid (2n=4x=36=18 L-chromosomes + 18 S-chromosomes), with 18 L-chromosomes and 18 S-chromosomes derived from *Aster* and *Kalimeris*, respectively (Tara, 1977). Since L-chromosomes are one of the important traits of *Aster* and S-chromosomes are a specific character of *Kalimeris* (Huziwara, 1957a, b, 1958; Chen et al., 1992a, b; Soejima and Peng, 1998; Li, 2002, 2003; Li and Zhang, 2004), karyotypical investigation is useful in testing intergeneric hybridization between *Aster* and *Kalimeris*.

The sequence of the nrDNA of the hybrid should possess ITS sequences of both parental species, and as expected, the results (Table 2) showed this to be the case. Generally, it is expected that concerted evolution in hybrids should result in a homogeneous nrDNA with segments from one or both parents. The fact that concerted evolution in the hybrid studied had not begun indicates that this is a recent F1 hybrid.

The taxonomic relationship between *Kalimeris* and *Aster*

Kalimeris, native to East Asia, is a difficult taxon. This is reflected mainly in its complicated relationship with *Aster*. Since the name *Kalimeris* was first published as a subgenus of *Aster* by Cassini in 1822, the species group has changed many times in its taxonomic position, being treated as part of *Asteromoea* Blume, a genus (*Calimeris* Ness), a section of *Aster* L. (sect. *Calimeris* Hoffmann or sect. *Asteromoea* Makino), a section of *Boltonia* (sect. *Asteromoea* Benth. et Hook. F.), or the genus *Kalimeris* Cass (see Ling and Chen, 1985; Nesom, 1994, 2000; Ito and Soejima, 1995; Gu and Hoch, 1997). Most Asian botanists consider the types of *Kalimeris* and *Asteromoea* to be congeneric (Gu and Hoch, 1997), so *Asteromoea* is regarded as a synonym of *Kalimeris*. Although *Boltonia* shares several floral and achene characters with *Kalimeris*, the long-discussed possibility of a close relationship between the New World *Boltonia* and Old World *Kalimeris* was rejected by Ling and Chen (1985) and Gu and Hoch (1997). However, the relationship between *Kalimeris* and *Aster* is still controversial. Some botanists treated *Kalimeris* as

Table 2. ITS comparison among *Aster ageratoides* var. *scaberulus*, *Kalimeris indica* and their putative hybrid.

Positions	43	73	74	117	136	142	179	236	419	488	492	555	583
AST	5′.....A	T	A	T	T	A	A	G	G	G	T	G	G.............3′
HYB	5′...A & C	T & C	A & G	T & C	T & A	A & G	A & T	G & T	G & A	G & C	T & C	G & T	G & T......3′
KAL	5′.....C	C	G	C	A	G	T	T	A	C	C	T	T…...3′

AST: *Aster ageratoides* var. *scaberulus*; HYB: The putative hybrid; KAL: *Kalimeris indica.*

a genus (Ling and Chen, 1985; Nesom, 2000), and some maintained that it should be a section (sect. *Callimeris*) of *Aster* (Ito and Soejima, 1995). Still others thought that its taxonomic position was pending (Gu and Hoch, 1997).

The primary morphological character that differentiates *Aster* from *Kalimeris* is length of pappus. The pappus of *Kalimeris* is less than 2 mm long while that of *Aster* usually exceeds 3 mm (Ling and Chen, 1985; Gu and Hoch, 1997). Over-dependence upon the generic diagnostic character is what has caused taxonomic confusion. Some species were classified into *Kalimeris* based on their short pappus, but other evidence suggested different taxonomic positions.

Ling and Chen (1985) thought that some species with a short pappus such as *Aster prorerus* Hemsl., blur differences between *Aster* and *Kalimeris*. *Aster prorerus* (*Kalimeris prorerus* (Hemsl.) S. Y. Hu) is controversial in taxonomic position (Hu, 1967; Ling and Chen, 1985). *Aster giraldii* Diels was placed in *Aster* sect. *Calimeris* Ness by Diels in 1905 (see Ling and Chen, 1985). *Aster prorerus* and *A. giraldii*, as well as *A. smithianus* Hand.-Mazz., *A. dolichopodus* Ling, *A. hunanensis* Hand.-Mazz., and *A. menelii* Levl., were thought to resemble *Kalimeris* because all of them have a short pappus (Ling and Chen, 1985). According to the description of Ling and Chen (1985), these six species are also similar to *Kalimeris* in other features. For instance, their heads are terminal and solitary on leafy peduncles with few to numerous heads forming a loosely corymboid inflorescence. Wang et al. (1999) reported that *A. procerus* and *A. smithianus* share the same characters of pollen grains with *Kalimeris*. Of course, these six species also possess features of *Aster*, hence their placement in *Aster* (Ling and Chen, 1985). Based on the fact that these species are similar to *Aster* in some characters and to *Kalimeris* in others, it is necessary to provide karyotypical and molecular data to determine their taxonomic positions.

By contrast with pappus, karyotype is more reliable in discriminating between *Aster* and *Kalimeris*. As shown here, the karyotype is one character that can verify that a putative hybrid is a true hybrid. *Aster ovatus* possesses a mixed LS karyotype (2n=18 long chromosomes + 18 short chromosomes) and appears to be derived from hybridization between *Aster* and *Kalimeris* (Tara, 1977). *Aster miquelianus* Hara was treated as a species of *Kalimeris* (namely *K. miquelianus*) because it has short pappus britles (Kitamura, 1937; Ling and Chen, 1985). However, Ito and Soejima (1995) and Gu and Hoch (1997) excluded it from *Kalimeris* and left it as part of *Aster*, a move which is supported strongly by cytological evidence (Gu, 1989). Gu (1989) reported that 18 chromosomes of *A. miquelianus* (2n=2x=18) are much longer than those of *Kalimeris* and like those of *Aster*. Therefore, karyotypical studies may be particularly useful in clarifying the taxonomic confusion produced by the six species such as *A. procerus*, for which cytological study has not been reported.

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微糙三脈紫菀和馬蘭（菊科）的天然雜交：來自形態學、核型和 ITS 序列分析的證據

黎維平

中國湖南師範大學生命科學學院

一個天然雜種在中國湖南省衡山被發現。對該雜種及其二親本種的形態、核型和 ITS 序列進行比較後發現，該雜種是微糙三脈紫菀（*Aster ageratoides* var. *scaberulus*）和馬蘭（*Kalimeris indica*）之間雜交的子一代。基於對雜種形態和核型的觀察，本研究表明紫菀屬（*Aster*）和馬蘭屬（*Kalimeris*）之間的雜交是造成冠毛變短和形成特殊核型（LS 型）的一個途徑，也可能是造成紫菀屬和馬蘭屬之間界線難於劃分的原因之一。而且本研究還顯示核型研究對於澄清紫菀屬和馬蘭屬之間的某些分類學混亂可能是特別有效的。

關鍵詞：紫菀屬；天然雜交；馬蘭屬；冠毛；核型。