Female reproductive success decreases with display size in monkshood, Aconitum kusnezoffii (Ranunculaceae)

Wan-Jin Liao, Yi Hu, Bi-Ru Zhu, Xia-Qing Zhao, Yan-Fei Zeng and Da-Yong Zhang*

State Key Laboratory of Earth Surface Processes and Resource Ecology and MOE Key Laboratory for Biodiversity Science and Ecological Engineering, Beijing Normal University, Beijing 100875, China

Received: 1 February 2009 Returned for revision: 30 March 2009 Accepted: 7 August 2009 Published electronically: 18 September 2009

Background and Aims Reduction in female fitness in large clones can occur as a result of increased geitonogamous self-fertilization and its influence through inbreeding depression. This possibility was investigated in the self-compatible, bee-pollinated perennial herb Aconitum kusnezoffii which varies in clone size.

Methods Field investigations were conducted on pollinator behaviour, flowering phenology and variation in seed set. The effects of self-pollination following controlled self- and cross-pollination were also examined. Selling rates of differently sized clones were assessed using allozyme markers.

Key Results High rates of geitonogamous pollination were associated with large display size. Female fitness at the ramet level decreased with clone size. Fruit and seed set under cross-pollination were significantly higher than those under self-pollination. The pre-dispersal inbreeding depression was estimated as 0.502 based on the difference in seed set per flower between self- and cross-pollinated flowers. Selling rates of differently sized clones did not differ.

Conclusions It is concluded that in A. kusnezoffii the negative effects of self-pollination causing reduced female fertility with clone size arise primarily from a strong early-acting inbreeding depression leading to the abortion of selfed embryos prior to seed maturation.

Key words: Early-acting inbreeding depression, Aconitum kusnezoffii, clone size, female reproductive success, geitonogamy.

INTRODUCTION

Clonal growth can influence the number and spatial distribution of flowers and this can have profound consequences for pollen dispersal and mating opportunities (Charpentier, 2002; Barrett, 2003). Large genets with clumped architecture can incur a high rate of pollen transfer between flowers of the same plant, referred to as geitonogamous self-pollination (Handel, 1985; De Jong et al., 1993; Harder and Barrett, 1995). In self-compatible species geitonogamy can result in mating costs associated with selfing and inbreeding depression, and in self-incompatible plants it can reduce fitness through pollen discounting (Harder and Barrett, 1995).

The high rate of geitonogamy expected for large clones leads to the prediction that maternal success should be affected by clone size, especially by the number of flowering ramets. Unfortunately, as mentioned by Routley et al. (2004), the relationship between clone size and geitonogamy is poorly understood. To date, only a handful of studies have addressed the influence of clone size on maternal (Handel, 1985; Wilcock and Jennings, 1999; Wolf et al., 2000; Charpentier, 2002; Routley et al., 2004; Tarasjev, 2005) and/or paternal success (Routley et al., 2004). Most of these studies observed negative relationships between female reproductive success by ramets and clone size, with the exception of domesticated apple Malus × domestica (Routley et al., 2004), for which self-incompatibility prevents self-fertilization.

Several explanations have been invoked to explain the observed reduction in female reproductive success in large clones. First, large clones with larger floral display may receive more pollinator visits at the genet level, but the number of visits per flower and stigmatic pollen load may be lower because of a large number of flowers, reducing receipt of pollen (De Jong et al., 1992; Wilcock and Jennings, 1999; Wolf et al., 2000; Wang et al., 2005). Secondly, resource competition among ramets might be more intense in large than in small clones, especially in plants with a clumped architecture. Because female reproduction is often limited by available resources (Burd, 1994; Liao et al., 2006), reproductive success per flower might decline with flower number. Thirdly, because larger clones display more flowers, individual flowers are more likely to be surrounded by inflorescences of the same genet, resulting in proportionally more geitonogamous pollination. In self-compatible species, this should result in a higher proportion of selfed zygotes and greater risk of reductions in maternal fitness through inbreeding depression (Charlesworth and Charlesworth, 1987; Husband and Schemske, 1996; Eckert, 2000; Charpentier, 2002). Variations in the magnitude of inbreeding depression have been observed among life stages and populations (Husband and Schemske, 1996; Goodwillie and Knight, 2006). Early-acting inbreeding depression resulting from abortion of homozygous offspring during embryo development because of the presence of deleterious recessive alleles (Seavey and Bawa, 1986; Krebs and Hancock, 1990; Husband and Schemske, 1996) could be an important factor reducing
fruit/seed set (Charlesworth and Charlesworth, 1987; Guillaume and Jacquemart, 1999; Mahy and Jacquemart, 1999; Araujo et al., 2007). The incidence of early-acting inbreeding depression can be evaluated by comparing embryo abortion following controlled selfing and crossing.

Fourthly, in self-incompatible species large clones may be more susceptible to ‘pollen clogging’, whereby self-pollen restricts access of cross-pollen to the stigma surface or self-pollen tubes interfere with cross-pollen tube growth in the style or ovary (Barrett, 2002). Several investigations have provided experimental evidence for the inhibitory effects of self-pollen on female fertility (Bertin and Sullivan, 1988; Galen et al., 1989; Waser and Price, 1991; Lloyd and Wells, 1992; Broyles and Wyatt, 1993; Barrett, 2002; Kawagoe and Suzuki, 2005).

Despite the importance of clonal growth in perennial plants, few studies have assessed the causes and consequences of clone size on maternal reproductive success. Here, these issues are investigated in the perennial herb *Aconitum kusnezoffii* (Ranunculaceae), which has a clumped clonal architecture. First, patterns of fruit and seed set were correlated with the number of flowering ramets per genet in four natural populations. The role of pollen limitation was then assessed based on per-flower pollinator visitation and stigmatic pollen loads. The resource limitation hypothesis was evaluated by quantifying raceme size and ramet size in large and small clones. The effects of self- and cross-pollination on maternal success were also examined to test the self-incompatibility. Lastly, to determine the role of early-acting inbreeding depression in maternal success, we conducted seed set following crossing and selfing, and estimated the selfing rates of clones of different sizes using allozyme markers.

### MATERIALS AND METHODS

#### Study species and sites

*Aconitum kusnezoffii* Rchb. is a bee-pollinated protandrous herb. It grows clonally via root tubers, resulting in a clumped architecture. Clones occupy different local patches, as confirmed by allozyme analysis. We used six polymorphic loci (*Aat*, *Skd-1*, *Skd-2*, *Pgd*, *Esr* and *Gdh*) in five enzyme systems to estimate the clonal architecture. By randomly choosing ten patches each with >20 ramets and scoring the genotypes, it was found that all ramets within a patch had the same multilocus genotypes across the six polymorphic loci, so genets are easily distinguished under field conditions. Here, we use the number of flowering ramets of a genet as a reasonably accurate proxy for clone size.

In studies in four populations in Xiaolongmen National Forest Park, west Beijing, China (Table 1). Few fruit matured in the lateral racemes during either 2006 or 2007, so we focused on terminal racemes in this study.

#### Flowering phenology

In 2006, one ramet from each of nine putative genets was sampled to quantify the flowering phenology in population 4. Every sampled ramet had a terminal raceme with more than eight flowers. The flowers on the nine terminal racemes were checked daily from 12 August to early September. We measured the duration of male function of each open flower based on pollen exposure and of female function based on stigma receptivity. Male and female functions of a flower are considered as overlapping if the flower presented some pollen exposure while its stigmas were receptive. Finally, floral longevity was defined as the duration from first anther dehiscence to the end of stigma receptivity. Stigma receptivity was determined using the MTT method (Dafni, 1992). During 2008, five genets were chosen to monitor flower phenology in population 4. We checked all flowers on terminal and lateral racemes for each genet from 11 August to early September.

#### Pollinator observations

Observations of pollinators were recorded to determine whether their behaviour could cause geitonogamy. *Bombus ignitus* Smith was the principal pollinator and foraged primarily on nectar. Voucher specimens of pollinators are stored at Beijing Normal University. On 27 August, 2006, two vicinal clones, the larger with eight flowering ramets and the smaller with only one ramet, were chosen for the observation of pollinators in population 1. Pollinators were observed from 8:00 to 17:00. Visit frequency is presented as the number of events per 30-min period. On 27 August, 2007, pollinators were observed for eight clones of different sizes from 9:00 to 11:00 in population 1 and for eight clones from 14:00 to 16:00 in population 4. Visit frequency is presented as the total number of events. The number of opening flowers was counted, and the visiting time of *Bombus ignitus* and the numbers of ramets and flowers visited per foraging bout were recorded. Stigmas were also collected from two flowers in each of the 43 clones with different numbers of flowering ramets in population 1, and counted the stigmatic pollen load to determine whether maternal success was limited by the number of pollen grains on stigmas.

<table>
<thead>
<tr>
<th>Population ID</th>
<th>Location</th>
<th>Altitude (m)</th>
<th>Community type</th>
<th>Investigation performed*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>39°57′32.1″N, 115°27′03.8″E</td>
<td>1034</td>
<td>Grassland dominated by <em>Artemisia dubia</em></td>
<td>E, P, R, S, Po</td>
</tr>
<tr>
<td>2</td>
<td>39°57′43.7″N, 115°26′32.1″E</td>
<td>1091</td>
<td>Deciduous forest dominated by <em>Juglans mandshurica</em></td>
<td>E</td>
</tr>
<tr>
<td>3</td>
<td>39°57′18.6″N, 115°25′15.7″E</td>
<td>1368</td>
<td>Deciduous forest dominated by <em>Populus cathayana</em></td>
<td>E</td>
</tr>
<tr>
<td>4</td>
<td>39°58′05.5″N, 115°25′48.0″E</td>
<td>1188</td>
<td>Deciduous forest dominated by <em>Populus cathayana</em></td>
<td>E, H, Po</td>
</tr>
</tbody>
</table>

*E, effects of clone size on maternal success; P, pollen limitation test; R, resource limitation test; S, selfing rate variation; H, hand-pollination experiments; Po, pollinator observations.*
Clone size and female reproductive success

The number of flowering ramets per clone was recorded to estimate clone size in four populations, with 60 clones in each population. For each flowering ramet of a sampled clone, the flowers of the terminal raceme were counted. Four weeks after the flowering season, fruits were collected from the terminal inflorescences of all marked ramets and the seeds were counted. Three fruits were selected from the top, middle, and bottom of each inflorescence. For each fruit, we counted the number of developed and undeveloped seeds (aborting embryos and unfertilized ovules are visually discernible) to determine the seed set of each raceme. Each of these values was then averaged across all ramets within a clone for statistical analysis.

Female reproductive success was estimated by: fruit set, the number of fruits produced divided by the number of flowers originally present in the terminal raceme; seed set, the number of seeds per fruit divided by the sum of seeds, aborted embryos and undeveloped ovules; and seed set per flower, calculated as fruit set × seed set. The influence of clone size on female reproductive success was analysed by regressing fruit set, seed set, and seed set per flower against the number of flowering ramets for individual populations, respectively, using a linear regression model in SPSS (Release 13.0).

Resource availability related to clone size

Sixty differently sized clones were randomly sampled from population 1. For each clone the number of flowers in the terminal raceme and the height of all the ramets were measured and these values were averaged at the ramet level within clones. These values were then used to test whether the resources available to each ramet decreased with increasing clone size.

Effects of self pollination

To assess the effects of self-pollination on fruit and seed production, hand-pollination experiments were performed in population 4. We randomly selected 90 terminal racemes and randomly classified them into three groups (30 per group) to conduct the following three pollination treatments: cross-pollination with cross-pollen from at least three other genets; self-pollination, hand-pollinated and then rebagged. For each treatment, one flower from each was removed 2, 4, 8, 12, 24 and 48 h after pollination, and its pistil was fixed in FAA for 24 h and then stored in 70% ethanol in 1.5 mL microcentrifuge tubes at 4 °C. Pistils were cleared in 8 mol/L NaOH for 12 h and rinsed with tap water for 1–2 h, after which they were stained with 0.1% (w/v) aniline blue in 0.1 mol/L potassium acetate as described in Dafni (1992). The growth rate of pollen tubes (v) was measured by the length of the pollen tube (L\text{pt}), divided by the length of the style (L\text{s}), i.e. v = L_{pt}/L_{s}.

Selfing rate variation among genets and inbreeding depression

At the end of the growth season, 22 clones from population 1 were sampled. We randomly sampled 72 out of all matured seeds from each maternal plant to assess the selfing rate at the genet level. The seeds were placed separately into a cooled mortar and homogenized with a pestle in 150 μL of 0.1 M Tris–HCl extraction buffer consisting of 1 mM tetrasodium salt (EDTA), 10 mM potassium chloride (KCl), 10 mM magnesium chloride (MgCl2), 5% (w/v) sucrose and 14% (w/v) polyvinylpyrrolidone-40 (PVP-40), all dissolved overnight in 0.1 M Tris–HCl buffer (pH 7.0), with 1% (v/v) β-mercaptoethanol added just before use (Solit et al., 1983). Using a Tris–glycine buffer system (pH 8.3), vertical slab polyacrylamide gel electrophoresis was used to separate allozyme bands. Gels were run under constant current (200 V) until a bromophenol blue marker reached the bottom of the gel. Then gels were stained for enzyme activity following recipes described by Wendel and Weeden (1989) and Wang (1996). Four variable loci were resolved for three enzyme systems: aspartate aminotransferase (Aat; EC 2.6.1.1), shikimate dehydrogenase (Skd; EC 1.1.1.25) and 6-phosphogluconate dehydrogenase (PgD; EC 1.1.1.44), and genotypes were inferred based on the segregation patterns characteristic of either dimeric or monomeric co-dominant enzymes. We detected two alleles at the Aat locus (with relative frequencies of 0.962 and 0.038), two alleles at Skd-1 (0.974 and 0.026), three alleles at Skd-2 (0.938, 0.061 and 0.001) and three alleles at PgD (0.253, 0.629 and 0.119).

We jointly estimated single- and multilocus selfing rates for population 1, with the expectation–maximization method, using the program MLTRWIN (Ritland, 2002). Post-dispersal inbreeding depression (\textit{d}) was estimated based on the inbreeding coefficient of parents and offspring and the selfing rate of maternal families following Ritland (1990). The single- and multilocus selfing rates of each genet were then estimated.
specifying the outcrossing rate at the population level as the initial \( t_m \) value that allowed iterations to start. Standard deviations were based on 1000 bootstrap values, using individual seeds within families as the unit of resampling.

Pre-dispersal inbreeding depression was estimated as \( 1 - (w_s / w_c) \), where \( w_s \) was the product of fruit and seed set for self-pollinated flowers and \( w_c \) was the same for cross-pollinated flowers. Given estimates of pre-dispersal inbreeding depression (\( E \)) and post-dispersal inbreeding depression (\( L \)), lifetime inbreeding depression equals \( 1 - (1 - E)(1 - L) \).

**RESULTS**

**Flowering phenology**

During 2006 flowering commenced on 12 August and ceased in early September. Every flowering ramet had a terminal raceme with 2–34 hermaphrodite flowers. The mean (± s.e.) floral longevity was 6.3 ± 0.09 d. Flowers are protandrous, with 4.8 ± 0.09 d of pollen exposure, followed by 1.7 ± 0.06 d of stigma receptivity. Sixteen of the 136 flowers investigated had a 1 d overlap between male and female function. During 2008, the flowering time is almost the same as the observation in 2006, beginning on 11 August and ceasing in early September. The mean floral longevity was 6.6 ± 0.74 d, first with 5.3 ± 0.7 d of pollen exposure, and then 1.3 ± 0.54 d of stigma receptivity. Only eight of the 344 flowers investigated had a 1 d overlap between male and female function. The flowering period for each genet involved two phases; flowers on terminal racemes bloomed first and those on lateral racemes flowered after the flowers on terminal racemes withered. Flowering progressed acropetally within inflorescences.

**Pollinator visitation**

*Bombus ignites* was the principal pollinator: while foraging primarily on nectar it contacted the sexual organs and carried pollen. Other flower visitors included *Apis mellifera* and *Episyrphus balteatus*; both only collected pollen and are unlikely to function as pollinators because they did not contact stigmas.

The rate of pollinator visitation varied positively with display size of *A. kusnezoffii*. *Bombus ignites* visited large clones more frequently (6.0 ± 0.62 visits per 30 min) than small clones with a single flowering ramet (1.7 ± 0.27 visits per 30 min), during 2006. In the large clones with eight flowering ramets, bees visited 2.4 ± 0.16 ramets and 7.7 ± 0.67 flowers per foraging bout, whereas they visited only 3.0 ± 0.32 flowers in the small clones. During 2007, pollinator visit frequency, the numbers of flowering ramets and flowers visited by *B. ignites* per foraging bout varied positively with the number of flowering ramets (Fig. 1). However, per-flower pollinator visits did not differ between large and small clones (\( P > 0.05 \)).

**Effects of clone size on maternal success**

The estimated clone size (the number of flowering ramets) varied from one to 28 across the four populations. A total of 182 of 240 marked clones (76 %) were harvested, and the rest were excluded from analysis because of senescence or herbivory. Fruit set and seed set per flower varied negatively with clone size in all four populations. Seed set was not consistent across the four populations, decreasing with increase of clone size in populations 2, 3 and 4, while not varying significantly with clone size in population 1. Generally, larger clones had lower fruit set, seed set, and seed set per flower (Fig. 2).

**Pollen and resource limitation**

The stigmatic pollen loads collected from 43 clones ranged from one to 111 and did not vary significantly with the number of flowering ramets (\( P > 0.05 \)). If female reproductive success per flower is limited by resource availability, then both the ramet height and the number of flowers in a ramet should decline in large clones as a result of more intense competition among ramets. However, the results indicate that neither raceme size (\( P > 0.05 \)) nor ramet height (\( P > 0.05 \)) varied significantly with the number of flowering ramets.

**Effects of self-pollination**

Because of the senescence of the inflorescence or possibly herbivory, only 55 (61 %) of the initial 90 hand pollination experimental ramets were sampled. The results indicate seed set was 0.465 ± 0.029 in self-pollination and 0.810 ± 0.020 in cross-pollination. Self-pollen significantly reduced the seed set by 43 % after cross-pollination (\( P < 0.05 \)). Results of pollen tube growth from controlled pollinations demonstrate that *A. kusnezoffii* is self-compatible. Both self- and cross-pollen grains germinated quickly upon arrival on stigmas and all can grow to the ovary after 12 h, with similar pollen tube growth rates (Fig. 3).

**Selfing rates and inbreeding depression**

The estimated mean multilocus selfing rate was 0.145 ± 0.016 for population 1. At the genet level, the selfing rates varied from 0.028 ± 0.000 to 0.204 ± 0.031; however, they did not vary significantly with clone size (\( P > 0.05 \)), so genets with more flowering ramets did not exhibit higher selfing rates (Fig. 4). However, we can note that selfing rates <0.05 occur only in plants with <15 flowering ramets, suggesting a role for clone size and geitonogamy in the mating system of *A. kusnezoffii*.

Both pre- and post-dispersal inbreeding depression were estimated. Based on the product of fruit set and seed set, pre-dispersal inbreeding depression was 0.502. Post-dispersal inbreeding depression was 0.876 ± 0.299. As a result, lifetime inbreeding depression was estimated to be 0.938.

**DISCUSSION**

Our aim was to estimate the effects of the number of flowering ramets on maternal success in clonal *A. kusnezoffii* and to determine the mechanisms underlying this pattern. The negative effects of floral display size on female reproductive success were apparent, with reductions in fruit set, seed set, and seed set per flower in large clones as measured by the number of flowering ramets. Our pollinator visitation results (Fig. 1) indicate that larger clones may suffer higher geitonogamous pollination. Seed set following self-pollination (0.465 ± 0.029) was
significantly lower than after crossing (0.810 ± 0.020). As A. kusnezoffii is self-compatible, such results suggest early-acting inbreeding depression. This depression was estimated as 0.502, so most of the selfed embryos were aborted during the stage of seed maturation. As a consequence, the selfing rates of differently sized clones estimated at the seed stage would be much lower than the primary selfing rate, ranging from 0.028 ± 0.000 to 0.204 ± 0.031, and higher selfing rates in genets with more flowering ramets were not exhibited.

Effect of pollen and/or resource limitation on maternal success

Reduced female success by large clones has been reported previously (Eriksson and Bremer, 1993; Free, 1993; Wilcock and Jennings, 1999; Wolf et al., 2000; Tarasjev, 2005). Our results also show such a reduction, with fruit set, seed set, and seed set per flower decreasing with increasing clone size across all four populations of A. kusnezoffii (Fig. 2).

Pollen limitation is often proposed to account for reduced female fitness with increasing clone size (Haig and Westoby, 1988; Burd, 1994). In animal-pollinated plants, pollen quantity may be reduced as a result of fewer pollinator visits or less pollen delivered per visit (Ashman et al., 2004). Since neither per-flower pollinator visits nor stigmatic pollen loads changed with the number of flowering ramets, pollen limitation appears unlikely for A. kusnezoffii.

Resource competition among ramets may also lead to reduced maternal success per flower with increasing clone size. Larger clones, especially in clonal plants with a clumped architecture, would be expected to allocate fewer resources per ramet, and this effect may reduce female reproductive success per flower by resource limitation. If so, larger clones should have fewer flowers at the ramet level and smaller ramets than smaller clones if the amount of available resources does not increase proportionally with the increase of the number of flowering ramets. In fact, neither the flower number per ramet nor the ramet height differed with clone size, suggesting that the resource limitation hypothesis may not be relevant. A plausible explanation for the absence of resource limitation is that larger clones may be able to

![Graphs showing the effects of the number of flowering ramets on pollinator visit frequencies, the number of flowering ramets visited by pollinators per foraging bout and the flowers visited per bout for populations 1 and 4.]

**Fig. 1** The effects of the number of flowering ramets on pollinator visit frequencies, the number of flowering ramets visited by pollinators per foraging bout and the flowers visited per bout for populations 1 and 4.
‘forage’ in a wider area (Magyar et al., 2007), thus reducing competition among the ramets.

Negative effects of self-pollination and early-acting inbreeding depression

Inbreeding depression is another potential consequence of geitonogamous pollination within clones. This trend depends on flowering phenology and pollinator foraging behaviour (Goulson, 2000; Ishii and Sakai, 2001). Aconitum kusnezoffii is dichogamous, but the flowers on terminal racemes bloom asynchronously within ramets, and synchronously among different ramets. These factors allow the possibility of geitonogamous pollination within and among ramets, although autogamy is uncommon because there is little overlap between the male and female functions within a single hermaphroditic flower. Pollinators usually visited more ramets and more flowers per foraging bout within larger clones (Fig. 1), which is not only indirect evidence of the occurrence of geitonogamy within a clone but also supports the view that larger
clones may have a higher rate of geitonogamy. As a consequence, the stigmas in larger clones may receive pollen grains containing a larger percentage of self-pollen than do those in smaller clones.

Geitonogamy is considered to be a negative but unavoidable consequence of cross-pollination when more than one flower opens simultaneously on individual plants (Lloyd, 1992; Jurne and Charlesworth, 1993). It is expected to cause severe male and female fitness reduction through higher selfing rates and consequent inbreeding depression (Charlesworth and Charlesworth, 1987; Eckert and Barrett, 1994; Husband and Schemske, 1996; Charpentier, 2002). Our results demonstrate the impairment of fruit and seed production under self-pollination compared with that under cross-pollination in *A. kusnezoffii*. Similar results were observed for clonal *Iris pumila* (Tarasjev, 2005) and *Asclepias speciosa* (Finer and Morgan, 2003).

Such negative effects of self-pollination on female function might be attributed to the consequences of geitonogamous pollination, the selfing rate and the subsequent inbreeding depression (Charlesworth and Charlesworth, 1987; Husband and Schemske, 1996; Charpentier, 2002). In self-compatible species, larger clones with proportionally more geitonogamous pollination may exhibit higher embryo selfing rates and result in a reduction in fruit and/or seed production through the expression of deleterious recessive alleles under mixed mating systems (Eckert and Barrett, 1994). We calculated the selfing rates of 22 genets by detecting the genotypic variations (Eckert and Barrett, 2000). In *A. gymnandrum*, including *A. kusnezoffii*, both self- and cross-pollen tubes could enter into ovary, with a similar pollen tube growth speed (Fig. 3), suggesting that stylar inhibition is absent. Moreover, self-compatibility was found in some congeneric species, including *A. lycocotonum* (Utelli and Roy, 2000) and *A. gymnandrum* (Zhang et al., 2006). Taken together, we may reach a tentative conclusion that early-acting inbreeding depression, rather than OSI, is the most probable explanation for the reduction in female function within large clones in *A. kusnezoffii*. More studies are clearly needed to obtain a definite resolution of this issue.

**ACKNOWLEDGEMENTS**

We thank Spencer Barrett and Lawrence Harder for their useful comments and guidance in the planning of this study and linguistic help with the English of this manuscript, anonymous reviewers for their comments, and Chuan Ni for his assistance in the field. This work was supported by the National Natural Science Foundation of China (30430160).

**LITERATURE CITED**


