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Self-compatible sympatric *Chamaecrista* (Leguminosae-Caesalpinioideae) species present different interspecific isolation mechanisms depending on their phylogenetic proximity

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Abstract The mating system of seven sympatric taxa of *Chamaecrista* occurring in the Chapada Diamantina Mountains, northeastern Brazil, was studied to determine the occurrence of self-incompatibility and interspecific genetic isolation mechanisms within the group. Self- and cross-pollination experiments and inter-taxon bidirectional crosses were performed. All of the populations were self-compatible and showed high percentages of spontaneous seed abortion on both self- and cross-pollinated fruits. The inter-incompatibility among the taxa of *Chamaecrista* is directed by different mechanisms depending on their degrees of phylogenetic proximity. In the crosses between closely related taxa (same clade), seed inviability was observed in the crossing pairs *C. desvauxii* var. *graminea* × *C. desvauxii* var. *latistipula*, and *C. chapadae* × *C. glaucofilix*. Inter-compatibility between species of the same clade occurred in *C. blanchetii* and *C. confertiformis*, with the formation of viable seeds. Pre-zygotic gametophytic reproductive isolation was observed among taxa of different clades, without penetration of the pollen tube into the ovule, or post-zygotic isolation through embryo or endosperm inviability, with abortion of the seeds. Inter-incompatibility represents an important factor in reproductive isolation and thus in the maintenance of the genetic identity of sympatric taxa that flower in synchrony and share pollinators.

Introduction

Studies of plant reproductive systems aid our understanding of the taxonomic complexity of certain species and patterns of gene flow, and are of basic importance in studies of the genetic evolution of those populations, in the elucidation of speciation processes, and the genetic relationships between species (Stace 1989; Richards 1997). A full understanding of the processes of speciation will involve determining what reproductive barriers are involved in reducing gene flow between populations and understanding the evolutionary forces that produced them (Nosil and Crespi 2006). Barriers to gene flow between species are frequently characterized as pre- or post-zygotic isolation mechanisms (Grant 1981; Stace 1989; Tiffin et al. 2001; Coyne and Orr 2004). Evolutionary biologists have undertaken many studies focusing on biogeography, ecology, and speciation in attempts to better understand the evolution of reproductive isolation mechanisms acting between sympatric species (Coyne and Orr 2004; Borba et al. 2001; Vieira 2002), but detailed studies examining reproductive isolation involving both pre- and post-zygotic mechanisms are still needed (Widmer et al. 2009).

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The genus *Chamaecrista* (Leguminosae, "Caesalpinioideae") belongs to the subtribe Cassiinae and comprises approximately 330 species distributed into seven sections (Conceição et al. 2009), of which approximately 230 species are found in Brazil (Irwin and Barneby 1982). This genus is quite common in the Brazilian *campos rupestres* (open rocky field vegetation) (Irwin and Barneby 1982; Conceição et al. 2008b) and is represented in the municipality of Mucugê in the Chapada Diamantina Range, northeastern Brazil, by eight taxa that occur sympatrically. Some hybrids between *Chameacrista* species have been recorded in this locality (Conceição et al. 2008a), a situation that is apparently facilitated by overlapping guilds of floral visitors and flowering periods (Costa 2007). There have been few published works focusing on the mating systems of *Chamaecrista*, and although it is known that *C. fasciculata* (Fenster 1995), *C. keyensis* (Liu and Koptur 2003), and *C. desvauxii* (Costa et al. 2007) are self-compatible, only a single study has been published concerning compatibility between taxa of this genus (Costa et al. 2007).

Costa (2007) analyzed the phenology and floral biology, and the behavior and visiting frequencies of floral visitors to the sympatric taxa of *Chamaecrista* occurring near Mucugê to evaluate the pre-zygotic and post-zygotic reproductive isolation mechanisms acting in these taxa.

Different flowering strategies have been noted in sympatric species of *Chamaecrista*, but there are large flowering overlaps among most of them (Madeira and Fernandes 1999; Costa 2007). In addition, differences in floral morphology and pollination mechanisms create pre-zygotic barriers-forming a system of filters that diminish interspecific gene flow between species of *Chamaecrista* (Costa 2007).

To better understand evolutionary strategies involving pre-zygotic, post-pollination, and post-zygotic mechanisms of reproductive isolation, we examined the reproductive systems of sympatric taxa of *Chamaecrista* and their potential for hybridization using experimental pollinations. We tested whether: (1) pre-zygotic, post-pollination, and post-zygotic mechanisms of reproductive isolation act as reproductive barriers between sympatric taxa of *Chamaecrista*, preventing the formation of hybrids; (2) more closely related taxa (belonging to the same clade) show the same reproductive barriers as less closely related taxa (belonging to different clades); (3) pre-zygotic and post-zygotic reproductive barriers act together in the reproductive isolation of sympatric taxa.

Materials and methods

Study area

The study was undertaken in the Mucugê Municipal Park in the municipality of Mucugê, in the Chapada Diamantina Mountains that make up the northern portion of the Cadeia do Espinhaço Range (12°59′02″S to 13°00′18″S and 41°20′41″W to 41°20′33″W) in Northeastern Brazil. The reserve occupies approximately 16 ha at an altitude of approximately 1,000 m. The predominant vegetation in the area is *campo rupestre*, which is principally composed of herbaceous and sub-shrub plants growing on and between rock outcrops (Giulietti and Pirani 1988). The regional climate is type Cwb in the classification system of Koppen (1948)—mesothermic with average annual temperatures of 19.8 °C. The average annual rainfall is between about 830 and 1,192 mm. Vouchers specimens of the populations studied were deposited in the herbarium at the Universidade Estadual de Feira de Santana (HUEFS): *C.B.N.Costa 109-Chamaecrista desvauxii* var. *latistipula*; *C.B.N.Costa 110-C. confertiformis*; *C.B.N.Costa 111-C. blanchetii*; *C.B.N.Costa 112-C. desvauxii* var. *graminea*; *C.B.N.Costa 113-C. glaucofilix*; *C.B.N.Costa 115-C. chapadae*; *C.B.N.Costa 128-C. rotundifolia* var. *grandiflora*.

Taxa studied

To examine the reproductive mechanisms of closely related and sympatric lineages the species were analyzed from the perspective of phylogenetic proximity, based on a study of the sections undertaken by Irwin and Barneby (1982). The phylogeny of *Chamaecrista* was studied by Conceição et al. (2009) based on the data from the ITS/5.8S nuclear region and plastid *trn*L-F. This study revealed four major lineages in *Chamaecrista*, denominated here as the Apoucouita clade (*C.* sect. *Apoucouita*, sensu Irwin and Barneby 1982), the Absus clade (*C.* sect. *Absus* subsect. *Absus* and *C.* sect. *Grimaldia*), the Baseophyllum clade (*C.* sect. *Absus* subsect. *Baseophyllum*), and the Chamaecrista clade (*C.* sect. *Chamaecrista*, *C.* sect. *Calyciopsis* and *C.* sect. *Xerocalyx*). In the latter clade, the section *Xerocalyx* was sustained as monophyletic (denominated here as the Xerocalyx clade) and nested within the section *Chamaecrista* (which appears to be paraphyletic).

A total of seven taxa were studied including six species of *Chamaecrista* belonging to four clades of the genus occurring sympatrically: (1) the Absus clade (*C. glaucofilix*, *C. chapadae*); (2) the Baseophyllum clade (*C. confertiformis*, *C. blanchetii*); (3) the Xerocalyx clade (*C. desvauxii* var. *graminea*, *C. desvauxii* var. *latistipula*); and (4) the Chamaecrista clade (section "*Chamaecrista*", *C. rotundifolia* var. *grandiflora*). The natural hybrid *C. × blanchetiformis* (Baseophyllum clade) was not included in this study (Costa 2007).

The populations studied were subjectively analyzed by direct observation of the frequencies and distributions one hundred individuals of the most common taxa (*C. blanchetii*, *C. confertiformis*, and *C. desvauxii* var. *graminea*) in

an area of approximately 140,000 m². *Chamaecrista blanchetii* was the most frequent species, with ample and roughly uniform distribution within the study area (except along river margins), co-occurring with all of the other taxa of the genus. *C. confertiformis* only occurred along river margins within the park, forming dense groups of individuals. *C. desvauxii* var. *graminea* was also amply distributed in the study area on well-drained soils, especially in areas with white sands. Fifty individuals of *C. glaucofilix* were found in the study area in small clumps with small numbers of individuals in rock crevices and other localities with thin soils. *C. desvauxii* var. *latistipula* (23 individuals encountered) formed small and very sparse groups, preferentially on clayey soils, while *C. rotundifolia* var. *grandiflora* (57 individuals) occurred only as a small clump of individuals growing on sandy soils. *C. chapadae* was also rare in the area, occurring preferentially in humid and shaded environments, with grouped individuals divided into two subpopulations of six (Subpop "Trilha") and 52 individuals (Subpop "Morro Fervido"), situated about 1 km from each other. Owing to the close proximity of the individuals within each subpopulation and the low flowering frequency of this species, all of the cross-pollinations were performed between individuals from different subpopulations. The procedures of cross-pollination in the other taxa were very similar, with mixed pollen being collected from different individuals and immediately used in pollination.

Observations on phenology, floral biology and floral visitors were made in the taxa studied to detect pre-zygotic reproductive isolation mechanisms (Table 1), for more information refer to Costa (2007).

Mating systems

The procedures of experimental pollination followed the methodology described by Costa et al. (2007). Experimental self- and cross-, spontaneous self-, and open-pollinations were conducted with each taxon in the field to examine the formation of fruits (under otherwise natural conditions). Intra-taxon cross-pollinations were performed using pollen from individuals growing at distances greater than 100 m from each other. Inter-taxon cross-pollinations were performed in a bidirectional manner among all of the taxa showing overlapping flowering (and having available flowers). At least 25 pollinations were performed per treatment, although the numbers of flowers of individuals used in each treatment varied widely due to differences in

Table 1 General characters of the *Chamaecrista* taxa growing in the Mucugê Municipal Park, Bahia State, Brazil

Taxa	Flower diameter (cm)	Shape of internal petal	Duration of the flowers (days)	Flowering period	Main pollinator	Pollen deposition onto the pollinator	Chromosomal data[a]
Absus clade (*C.* sect. *Absus*)							28
C. chapadae	1.6	Tubular	3	Jan–Dec (Jul–Aug)	*Centris fuscata*	Pleurotribic	
C. glaucofilix	2.5	Tubular	1	Jan–Dec (May–Sep)	*Bombus brevivillus*	Pleurotribic	
Baseophyllum clade (*C.* sect. *Absus* subsect. *Baseophyllum*)							?
C. blanchetii	2.0	Falcate	3	May–Oct (Jun–Jul)	*Bombus brevivillus*	Nototribic	
C. confertiformis	1.8	Falcate	2	Sep–Feb (Oct–Dec)	*Bombus brevivillus*	Nototribic	
Chamaecrista clade (*C.* sect. "*Chamaecrista*")							16
C. rotundifolia var. *grandiflora*	2.3	Flat	1	Apr–Jun (May)	*Pseudaugochlora* spp.	Esternotribic	
Xerocalyx clade (*C.* sect. *Xerocalyx*)							14
C. desvauxii var. *graminea*	2.5	Curved	1	Jun–Nov (Jul–Aug)	*Bombus brevivillus*	Entire body	
C. desvauxii var. *latistipula*	2.0	Curved	1/2	Jan–Dec (Jul–Sep)	*Bombus brevivillus*	Entire body	

Flowering periods (flowering peak) (Data from Costa 2007)
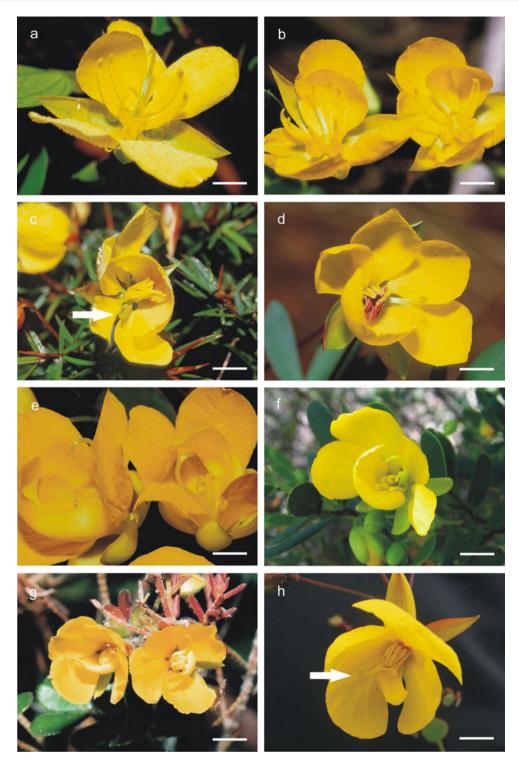
[a] Data from Conceição et al. (2009)

Fig. 1 *Chamaecrista* flowers. **a**, **b** *C. rotundifolia* var. *grandiflora* flowers with flat internal petals; **c** *C. desvauxii* var. *graminea* flower with a curved internal petal and lateral gynoecium (*arrow*); **d** *C. desvauxii* var. *latistipula* flower with reddish anthers facing the curved internal petal; **e** *C. blanchetii* flower with a falcate internal petal; **f** *C. confertiformis* flower with a falcate internal petal; **g** *C. chapadae* flower with its internal petal forming a tube; **h** *C. glaucofilix* flower with its internal petal differentiated into a tube, with its base surrounding the apices of the anthers and its terminal section opposite the stigma (*arrow*). *Scale bars* **a** 0.4 cm; **b**, **e** 0.57 cm; **c**, **d**, **f**, **g** 0.5 cm; **h** 0.64 cm

the availability of flowers on the different individuals. We used only flowers on the first day of anthesis that had been bagged as buds to exclude pollinators; they were bagged

again immediately after manual pollination. The flowers remained covered until the fruits mature and their seeds were liberated.

Table 2 Fruit set, seed formation and germination under natural conditions and in pollination experiments in taxa of *Chamaecrista* in the Mucugê Municipal Park, northeastern Brazil

Treatment	Fruiting individuals % (N^1)	Fruiting (fruit/flower) (%)	Seeds/fruit average (min–max)	Germination % (seeds)	GVI
Absus clade (*C*. sect. *Absus*)					
C. chapadae					
Open-pollination	100 (11)	12.5 (4/32)	2.3 (0–7)	75 (8)	6.9
Spontaneous self-pollination	0 (7)	0 (0/17)	–	–	–
Manual self-pollination	100 (9)	57.1 (24/42)	1.7 (0–6)	95.6 (25)	9.3
Manual cross-pollination	77 (13)	18.8 (12/64)	1.9 (0–7)	100 (10)	9.0
C. glaucofilix					
Open-pollination	100 (12)	13.3 (12/90)	3.1 (0–8)	73.3 (30)	1.8
Spontaneous self-pollination	0 (16)	0 (0/28)	–	–	–
Manual self-pollination	100 (8)	64.4 (29/45)	2.6 (0–5)	82 (50)	3.4
Manual cross-pollination	100 (10)	63.2 (24/38)	3.6 (0–8)	92 (50)	3.9
Baseophyllum clade (*C*. sect. *Absus* subsect. *Baseophyllum*)					
C. blanchetii					
Open-pollination	100 (9)	11.8 (12/102)	3.5 (0–8)	100 (30)	5.0
Spontaneous self-pollination	0 (3)	0 (0/28)	–	–	–
Manual self-pollination	81.8 (11)	39.6 (21/53)	2.6 (0–8)	90 (50)	7.6
Manual cross-pollination	81.8 (11)	53.2 (33/62)	4 (0–9)	95 (60)	6.0
C. confertiformis					
Open-pollination	100 (4)	15.4 (6/39)	2.5 (0–8)	95 (10)	5.6
Spontaneous self-pollination	0 (4)	0 (0/37)	–	–	–
Manual self-pollination	36.4 (11)	21.4 (9/42)	2.7 (0–5)	100 (17)	8.9
Manual cross-pollination	55.5 (9)	25 (9/36)	2.5 (0–8)	100 (15)	8.5
Chamaecrista clade (*C*. sect. "*Chamaecrista*")					
C. rotundifolia var. *grandiflora*					
Open-pollination	100 (6)	17.1 (6/35)	1.3 (0–6)	72 (8)	6.0
Spontaneous self-pollination	0 (17)	0 (0/50)	–	–	–
Manual self-pollination	66.7 (9)	15 (7/47)	0.43 (0–3)	100 (3)	4.5
Manual cross-pollination	45.5 (11)	11 (5/46)	2 (0–5)	100 (10)	7.5
Xerocalyx clade (*C*. sect. *Xerocalyx*)					
C. desvauxii var. *graminea*					
Open-pollination	86.7 (15)	44.4 (20/45)	1.5 (0–100)	71 (20)	–
Spontaneous self-pollination	0 (5)	0 (0/26)	–	–	–
Manual self-pollination	100 (8)	67 (21/31)	0.8 (0–6)	92.3 (11)	6.03
Manual cross-pollination	100 (11)	69 (25/36)	2.3 (0–7)	82 (39)	6.15
C. desvauxii var. *latistipula*					
Open-pollination	100 (10)	63.3 (19/30)	6.4 (0–13)	100 (75)	–
Spontaneous self-pollination	0 (10)	0 (0/27)	–	–	
Manual self-pollination	100 (7)	59 (19/32)	6 (0–11)	92 (75)	21.0
Manual cross-pollination	100 (9)	70 (21/30)	7.6 (0–13)	100 (75)	22.8

GVI germination velocity index of seeds, N^1 number of individuals used in the treatment

Two to four pollinated flowers in each treatment were collected between 6 and 72 h after pollination and fixed in 50 % FAA to examine pollen tube growth and penetration into the ovules. The pistiles were bleached with 2.5 % NaClO for 15–30 min (until completely cleared) and then stained with 0.25 % aniline blue and examined under an epifluorescence microscope (modified from Martin 1959).

Seed germination

The seeds collected from the fruits formed during the experiments were weighed and sown into germination trays. Dormancy was broken by mechanical scarification followed by disinfection in 2.5 % NaClO for 5 min and subsequent rinsing in distilled water (modified from Gomes et al. 2001). Different numbers of seeds were planted from

each treatment depending on their availability from the experiments. The seeds were sown into germination trays lined with filter paper wet with distilled water, and subsequently kept in germination chambers under constant light conditions and temperature of 27 °C. Germination was observed daily for 30 days to obtain their Germination Velocity Index (GVI) (Maguire 1962) and germination percentages.

The analysis of variance (ANOVA) and the Tukey's test were used to compare sample averages, seed weights, and the number of seeds formed in the different treatments, utilizing the BIOESTAT 3.0 software program (Ayres et al. 2003).

Results

Floral morphology

All of the taxa studied had yellow flowers that showed only small differences in terms of their floral diameters; *C. chapadae* and *C. desvauxii* var. *graminea*/*C. glaucofilix* had the smallest and largest floral diameters respectively (Table 1; Fig. 1). The principal morphological differences between the taxa were related to modifications of the most internal petal (most visible in the floral bud stage). Four basic morphological modifications were seen in the most internal petal (Fig. 1): flat (*Chamaecrista rotundifolia* var. *grandiflora* (Fig. 1a, b); curved (*C. desvauxii* var. *graminea* and *C. desvauxii* var. *latistipula*) with reddish pigmentation at its base (*C. desvauxii* var. *graminea* (Fig. 1c, d); falcate (*C. blanchetii* and *C. confertiformis*) (Fig. 1e, f); and tubular (*C. chapadae* and *C. glaucofilix*), with the petal forming a tube whose base covered the apices of the poricide anthers (Fig. 1g, h).

The gynoecium was greenish-yellow and elongated in all the taxa examined. The style was curved in its most distal region, with the apical stigma facing the floral axis. The stigma was covered by simple trichomes and formed a stigmatic chamber in all of the taxa examined (see Costa et al. 2007). All of the taxa demonstrated pollen viability levels greater than 80 %.

The stigma remained receptive throughout the duration of the flower (Table 1), and most of the pollen was collected during the first visits by pollinators. The flowers themselves lasted for different periods of time, excising in the first half of the first day (in *C. desvauxii* var. *latistipula*) or lasting up to three days (*C. chapadae*) (Table 1).

The principal pollinators of all of the *Chamaecrista* taxa were bees that buzzed the anthers to collect pollen (Table 1). *Bombus brevivillus* Franklin, 1913 (Apidae) visited all of the taxa and was the principal pollinator of *C. blanchetii*, *C. confertiformis*, *C. glaucofilix*, *C. desvauxii* var. *graminea*,

and *C. desvauxii* var. *latistipula*. Other pollinators, such as *Centris fuscata* Lepeletier, 1841 (Apidae) and *Pseudaugochlora* sp2 frequently visited *C. chapadae* and *C. rotundifolia var. grandiflora*, respectively.

Intra-taxon pollinations

Fruit set in open pollinations varied between 12 and 17 % in all of the taxa, with the exception of the two varieties of *C. desvauxii* (with 44 and 63 %). No fruits were formed by spontaneous self-pollination or apomixis in any of the taxa (Table 2). Similar rates of fruit and seed formation were observed by self-pollination and experimental cross-pollination, indicating self-compatibility in all populations. High variation in fruit set was seen, however, among the different taxa: the success of experimental self-pollination varied from 15 % (*C. rotundifolia* var. *grandiflora*) to 67 % (*C. desvauxii* var. *graminea*), while cross-pollination success varied from 11 % (*C. rotundifolia* var. *grandiflora*) to 70 % (*C. desvauxii* var. *latistipula*).

The only exception was *C. chapadae*, in which fruit set by cross-pollination was quite inferior to that observed by self-pollination (Table 2). The results were basically the same even if the data was separated according to subpopulations; the small differences seen were probably related to the small sample sizes (cross × self: 16 × 61 %, subpop. "Trilha"; 22 × 44 %, subpop. "Morro"). Most individuals had only small number of pollinated flowers, although two individuals in the subpopulation "Trilha" varied from nine to 18 pollinations per treatment. The same general pattern was observed among these two individuals as in the population as a whole, with fruiting being three to five times greater by self-pollination (removal of those two individuals from the analysis did not alter the general results).

The seed set per fruit was quite low under both experimental self- and cross-pollination regimes in terms of the maximum number of ovules seem in all of the taxa. Significant difference in the number of seeds formed in these treatments was only observed in *C. desvauxii* var. *graminea*, which produced greater seed set by cross-pollination ($F = 5.5194$, $p < 0.05$). There was large number of aborted seeds in both experiments among all the taxa, although no pattern was observed in terms of the position of the aborted seeds in the fruit (being closer or more distant to the base of the style).

Seeds derived from cross-pollination were heaviest in *C. blanchetii* ($F = 9.0333$, $p < 0.01$), *C. glaucofilix* ($F = 19.974$, $p < 0.01$) and *C. chapadae* ($F = 4.8924$, $p < 0.01$), with no significant differences being observed among the other taxa. All of the seeds produced by experimental self- and cross-pollination demonstrated high germination percentages (above 80 %) in all the taxa

(Table 2). The germination velocity index (GVI) was similar in all of the experiments with all of the taxa; the greatest difference was seen with *C. rotundifolia* var. *grandiflora*, which demonstrated greater germination velocity in cross-pollinated seeds (Table 2).

The growth velocities of the pollen tubes were similar among all the taxa, reaching the ovules approximately 6 h after pollination. There were no differences in growth velocity among pollen tubes derived from self- or cross-pollination, with the exception of *C. desvauxii* var. *latistipula*. This taxon showed faster growth of the pollen tubes in cross-pollinated flowers, with tubes reaching all the ovules 6 h after pollination, while in self-pollination tubes had reached only the ovules closest to the style. No morphological differences were observed between pollen tubes formed by self-pollination or cross-pollination, with normal development being uniformly seen until penetration of the micropyle.

Inter-taxon intra-clade crosses

Low fruit set was observed in crosses between *C. glaucofilix* and *C. chapadae* in the Absus clade, in quantities inferior to those observed in intra-taxa pollinations (Table 3); fruit production was unidirectional, occurring only with *C. glaucofilix* as the pollen donor, but only two malformed seeds were produced without germinative potential (Table 4).

Crosses between *C. blanchetii* and *C. confertiformis* in the Baseophyllum clade formed fruits and seeds bidirectionally, although these were inferior in number to those

formed through intra-taxon pollinations (Tables 3, 4). Malformed fruits and fruit with aborted seeds were common, and only two seeds were formed when *C. confertiformis* was the pollen receptor. There was no significant difference in the number of seeds per fruit. Larger numbers of morphologically well-formed seeds were produced when *C. blanchetii* was the pollen receptor ($N = 5$). None of the seeds from these crosses germinated, however, while the two seeds formed by *C. confertiformis* did germinate.

Crosses within the Xerocalyx clade (varieties of *C. desvauxii*) resulted in high fruit set that was similar to, or greater than, that observed in intra-taxon pollinations. These inter-taxon crosses, however, all resulted in aborted seeds (Tables 3, 4).

Inter-clade crosses

All of the inter-clade crosses demonstrated fruiting rates inferior to those observed in intra-taxa cross-pollinations. *Chamaecrista blanchetii* (Baseophyllum clade) demonstrated high potential as a pollen donor, with the formation of pollen tubes and penetration of the micropyle of the ovules. In most cases, these crosses resulted in fruit set (Table 2), with up to 24 % fruiting in *C. desvauxii* var. *graminea* (Xerocalyx clade). The fruits in all cases, however, had undeveloped ovules or aborted seeds (Table 4). In those crosses, in which *C. blanchetii* was the pollen receptor, fruits were only formed with *C. glaucofilix* (Absus clade).

In crosses between *C. chapadae* (Absus clade) as the pollen receptor and *C. desvauxii* var. *latistipula* (Xerocalyx

Table 3 Fruiting percentages (fruit/flower) in experimental pollinations between taxa of *Chamaecrista* in the Mucugê Municipal Park, Northeastern Brazil

Receptor	Donor						
	C. chapadae	*C. glaucofilix*	*C. blanchetii*	*C. confertiformis*	*C. rotund.* var. *grandiflora*	*C. desv.* var. *graminea*	*C. desv.* var. *latistipula*
Absus clade (*C.* sect. *Absus*)							
C. chapadae	<u>18.8 (12/64)</u>	11.5 (3/26)	16 (4/25)	0 (0/8)	0 (0/13)	0 (0/24)	3.5 (1/29)
C. glaucofilix	0 (0/33)	<u>63.2 (24/28)</u>	3.1 (1/32)	0 (0/11)	0 (0/8)	0 (0/33)	0 (0/34)
Baseophyllum clade (*C.* sect. *Absus* subsect. *Baseophyllum*)							
C. blanchetii	0 (0/34)	5.9 (2/34)	<u>53.2 (33/62)</u>	18.7 (9/48)	0 (0/12)	0 (0/39)	0 (0/33)
C. confertiformis	0 (0/11)	0 (0/14)	13 (6/46)	<u>25 (9/36)</u>	–	11 (1/9)	0 (0/8)
Chamaecrista clade (*C.* sect. "*Chamaecrista*")							
C. rotundifolia var. *grandiflora*	0 (0/9)	0 (0/7)	0 (0/18)	–	<u>11 (5/46)</u>	–	0 (0/13)
Xerocalyx clade (*C.* sect. *Xerocalyx*)							
C. desvauxii var. *graminea*	0 (0/35)	0 (0/36)	24 (9/38)	0 (0/4)	–	<u>69 (25/36)</u>	83.3 (35/42)
C. desvauxii var. *latistipula*	0 (0/32)	0 (0/33)	3.5 (1/29)	0 (0/12)	0 (0/12)	52.4 (22/42)	<u>70 (21/30)</u>

Data is also presented showing the results of intra-taxa cross-pollinations for comparison (underlined). Sample sizes in parentheses

Table 4 Average number of seeds per fruit and percentage germination of seeds produced from crosses between taxa of *Chamaecrista* in the Mucugê Municipal Park, Northeastern Brazil

Receptor	Donor						
	C. chapadae	C. glaucofilix	C. blanchetii	C. confertiformis	C. rotundifolia var. grandiflora	C. desvauxii var. graminea	C. desvauxii var. latistipula
Absus clade *(C.* sect. *Absus)*							
C. chapadae	1.9 (0–7)/100 %	0.7 (0–1)/0 %	0 (0–0)	–	–	–	0 (0–0)
C. glaucofilix	–	3.6 (0–8)/92 %	0 (0–0)	–	–	–	–
Baseophyllum clade *(C.* sect. *Absus* subsect. *Baseophyllum)*							
C. blanchetii	–	0 (0–0)	4 (0–9)/95 %	1.1 (0–5)/0 %	–	–	–
C. confertiformis	–	–	0.3 (0–2)/100 %	2.5 (0–8)/100 %	x	0 (0–0)	–
Chamaecrista clade *(C.* sect. *"Chamaecrista")*							
C. rotundifolia var. grandiflora	–	–	–	x	2 (0–5)/100 %	x	–
Xerocalyx clade *(C.* sect. *Xerocalyx)*							
C. desvauxii var. graminea	–	–	0 (0–0)	–	x	2.3 (0–7)/ 82 %	0 (0–0)
C. desvauxii var. Latistipula	–	–	0 (0–0)	–	–	0 (0–0)	7.6 (0–13)/ 100 %

Data are also presented showing the results of intra-taxa cross-pollinations for comparison (underlined). Seed formation values are averages (minimum–maximum)

– indicates no fruit set (see Table 3), *x* unrealized treatment

clade) as the donor, the pollen tubes penetrated into the micropyle with subsequent fruit development, although viable seeds were not produced (Tables 3, 4). The same situation occurred with *C. confertiformis* (Baseophyllum clade) as the pollen receptor and *C. desvauxii* var. *graminea* (Xerocalyx clade) as the donor.

Chamaecrista rotundifolia var. *grandiflora* (Chamaecrista clade) did not produce fruits when crossed with any of the other taxa, whether as the pollen donor or receptor (Table 3). In the other crosses among the other taxa with no fruit formation, the pollen grains germinated and the pollen tubes developed normally to the ovaries, although there was no penetration.

Discussion

The pre-zygotic isolation mechanisms seen in the sympatric taxa of *Chamaecrista* do not appear to be sufficient to impede genetic flux among them. The phenological patterns observed for the various taxa of *Chamaecrista* revealed overlapping flowering periods, with a total absence of overlapping only with *C. confertiformis* and *C. rotundifolia* var. *grandiflora*. In the same way, the bee *Bombus brevivillus* visited all of the taxa and could facilitate genetic flux between them. One pre-zygotic mechanisms that could diminish genetic flux between these taxa was floral morphology—more specifically, the morphology

of the internal petal. Among species of the clade Absus, the positioning of pollen deposition on the pollinator's body (Pleurotribic) by the tubular petal could diminish inter-taxa pollen transfer unidirectionally among species of the clade Baseophyllum as their stigmas enter into contact only with the dorsal region of the pollinator's body that experienced nototribic pollen deposition. On the other hand, a falcate petal (encountered among the species of the clade Baseophyllum studied here) has a more open morphology than the tubular petal, and would allow at least part of the pollen carried on the dorsal region of the bees body to be spread onto its lateral region, thus favoring pollen flux from the Baseophyllum to the Absus clade.

The internal petals of *Chamaecrista* have been widely examined in terms of their forms and functions. Wolfe and Estes (1992) noted the importance of this petal in attracting pollinators to *C. fasciculata*, with fruit formation occurring in only 5 % of the flowers that had this pedal removed—in contrast to 45 % fruiting in non-manipulated flowers. These results do not, however, take into consideration another function attribute of this petal, which involves spreading pollen over most of the pollinator's body (a ricochet type mechanism, sensu Westerkamp 2004), so that the observed reductions in fruiting could have been influenced by a less efficient deposition of pollen on the pollinator. Westerkamp (2004) noted that flowers that have ricochet-type mechanisms can deposit pollen on body parts of the pollinator that cannot be easily reached while

grooming, resulting in greater pollination efficiency. Among taxa of the clade Xerocalyx (*C. desvauxii* var. *graminea* and *C. desvauxii* var. *latistipula*), a curved petal appears to determine where the bees will land, and functions as a type of attractant (or guide) for pollinators (in the former variety) as it has pigments that absorb ultraviolet light. According to Costa et al. (2007), there are no pre-zygotic barriers between these taxa that have otherwise similar floral morphologies, overlapping flowering periods, and share pollinators. The curved petals seen in these taxa do not appear to represent barriers to genetic flux as they serve to spread pollen over the bodies of the pollinating bees. This pattern of pollen deposition also favors pollen transfer to be flowers of sympatric taxa of *Chamaecrista* that flower synchronously, as the bees cannot clean off all of the pollen covering their bodies.

The capacity to produce fruits and seeds by self-pollination and cross-pollination enables the taxa of *Chamaecrista* to use mixed strategies of sexual reproduction that favor the colonization of new areas in heterogeneous environments (such as the *campos rupestres*). The self-compatibility observed in these species appears to be a common characteristic in this genus (e.g., Fenster 1995; Liu and Koptur 2003; Costa et al. 2007). Although self compatibility occurs in other genera of "Caesalpinioideae", such as *Bauhinia* (Ramirez et al. 1984), *Senna* (Simpson 1977), and *Caesalpinia* (Lewis et al. 2000), self-incompatibility appears to more common in this particular subfamily, even in many species of these same three genera (Simpson 1977; Ramirez et al. 1984; Lewis et al. 2000; Carvalho and Oliveira 2003). Late-acting self-incompatibility has been frequently encountered in this subfamily (Lewis and Gibbs 1999; Freitas and Oliveira 2002; Carvalho and Oliveira 2003), and *Chamaecrista* is currently the only genera of Caesalpinioideae known to present self-compatibility among all the examined species.

Among the taxa of *Chamaecrista* studied, the occurrence of aborted seeds in intraspecific pollinations indicates the presence of lethal alleles as the most probable cause of low fruiting, occasioned by inbreeding depression. The high number of fruits formed by cross-pollination containing few developed seeds, or none at all, in some of the species indicates the possibility of inbreeding depression occurring in crossings of related individuals (biparental endogamy). This may be due to the small population size of most of the taxa studied. Large differences between the number of seeds per fruit resulting from self-pollination and cross-pollination was only present in *C. rotundifolia* var. *grandiflora* and in *C. desvauxii* var. *graminea* (the latter being well-represented in the area in terms of population size). Inbreeding depression was also observed in *C. keyensis* (Liu and Koptur 2003) which presented low levels of seed formation and low germination rates with self-pollination. Inter-population crosses can be used to test this hypothesis in the future as lower seed abortion rates would be expected than in inter-population pollinations. Low fruit set (Gibbs et al. 1999; Freitas and Oliveira 2002) and high rates of aborted seeds (Rodriguez-Riaño et al. 2004, Costa et al. 2007) seem to be common characteristics in the Leguminosae. Rarely, however, have there been reports of reductions in seed development due to the lack of fertilization or to post-zygotic abortions resulting from inbreeding depression or by insufficient nutritional resources (Gomes and Zamora 2003).

The results encountered with *C. chapadae* indicated that the two subpopulations appear to be in the process of genetic separation due to their low fruiting indices when crossed. The maintenance of the fruiting values when the subpopulations and some individuals are analyzed separately also indicates that these results are not merely experimental artifacts or sampling effects. This reproductive isolation between subpopulations may be due to a high genetic divergence between nearby populations, caused by the isolation, as found in *Chamaecrista fasciculata* for short distances (up to 200 m) (Fenster et al. 2003). Sletvold et al. (2012) noted that fragmentation and increased isolation may also limit gene flow among populations and promote inter-populational genetic differentiation. These authors documented 47 % reductions in fitness among orchids (*Gymnadenia conopsea*) in crosses between two subpopulations when separated by 1.6 km. This isolation may be caused by structural chromosome alterations, or even numerical alterations such as polyploidization, which would result in genomic incongruence that would prevent gene flow (Grant 1981; Rieseberg 2001). More frequently, these alterations involve reproductive (not developmental) problems in inter-populational hybrids, at least in the initial stages of divergence. However, the occurrence of a strong sub-dominance in heterozygotic chromosomal rearrangements results in very reduced fitness of the hybrids. In addition, as these rearrangements are adaptive in homozygotes, strong reproductive isolation can occur within the population, as predicted by the stasipatric speciation model (Grant 1981; Rieseberg 2001). As such, cytogenetic studies designed to determine chromosome numbers and structures, and the genetic structures of the populations to determine the occurrence of gene flow as well as detailed morphological studies including morphometric analysis of *C. chapadae* are necessary to determine if these two sub-populations correspond to incipient biological species.

Intra-clade reproductive isolation

Overlapping reproductive barriers appear to be acting as filters that isolate *C. glaucofilix* and *C. chapadae* in the Absus clade. The differences in their micro-habitats and in

the frequencies of their principal pollinators, and the absence of syntopy all act as pre-pollination barriers and the production of malformed seeds in *C. chapadae* functions as a post-zygotic barrier when it is the pollen receptor. The lack of viability of the hybrid seeds seems to be the most efficient mechanism maintaining reproductive isolation between these species. This type of intrinsic post-zygotic isolation is apparently more common than hybrid sterility (Coyne and Orr 2004) and may be caused by alleles that reduce hybrid fitness through deleterious epistatic interactions (Dobzhansky–Muller incompatibility) (Orr and Turelli 2001).

The formation of viable seeds as seen in crosses between *C. blanchetii* and *C. confertiformis* (Baseophyllum clade) are in consonance with the formation of natural hybrids between these two species (*C.* × *blanchetiformis*; Conceição et al. 2008a). These hybrids are fertile individuals that are capable of inter-crossing with their parents, thus resulting in introgression (Costa 2007). Despite this potential for hybrid formation (Conceição et al. 2008a), their occurrence in the area is quite rare (perhaps a dozen individual plants encountered among thousands of parental individuals), reflecting the presence of partial barriers that are insufficient to ensure complete isolation between the species under natural conditions. The displacement of the flowering periods of *C. blanchetii* and *C. confertiformis* seems to represent the principal reproductive barrier between these taxa, but when overlapping flowering does occasionally occur (with the extension of this phenophases by *C. blanchetii* or its anticipation in *C. confertiformis*) other mechanisms do not seem to be sufficient to maintain full reproductive isolation. The absence of records of allopatry among populations of *C. blanchetii* and *C. confertiformis* in the Baseophyllum clade (Conceição et al. 2008b) does not allow for accurate inferences concerning the evolution of shifting flowering periods as a possible mechanism reinforcing reproductive isolation between them.

The formation of natural hybrids is a relatively frequent event among angiosperms and has had an important role in the evolution of this group (Ellstrand et al. 1996). Hybridization occurs in approximately 34 % of the angiosperm families (Rieseberg 1997) and seems to be quite common in groups such as the Orchidaceae (Steiner et al. 1994; Borba and Semir 1998), Onagraceae (Zardini et al. 1991; Vieira 2002), and Asteraceae (Ellstrand et al. 1996). Natural hybrids have been identified in various genera of the Leguminosae, such as *Bauhinia* (Lau et al. 2005), *Parkinsonia* and *Cercidium* (Hawkins et al. 1999) and *Lathyrus* (Hammett et al. 1994), as well as in other species of *Chamaecrista* sect. *Baseophyllum* (Conceição et al. 2008a).

The taxa examined in the Xerocalyx clade (*C. desvauxii* var. *graminea* and *C. desvauxii* var. *latistipula*) do not

appear to have pre-zygotic barriers (Costa et al. 2007), and high rates of fruit formation were observed in inter-taxa crosses. The absence of viable seeds, however, indicates inter-incompatibility among these plants. A total lack of gene flow between these taxa has been shown in studies with molecular markers, suggesting that they should be considered distinct species, and a taxonomic revision of the entire complex is required (Costa et al. 2007).

The potential for crossing between taxa of the same clade in *Chamaecrista* seems to be regulated by isolation mechanisms that prevent or reduce the possibility of forming natural hybrids. Unexpectedly, pre-zygotic reproductive isolation barriers appear to be weak or even non-existent between the closest taxa, principally in the clades Absus and Xerocalyx, and post-zygotic mechanisms act to maintain reproductive isolation in these cases. However, there is an inversion in the relative importance of these mechanisms in the Baseophyllum clade, with pre-zygotic reproductive barriers acting as the principal isolation mechanisms, with no effective impediments to genetic flux through post-zygotic mechanisms.

The roles of pre- and post-zygotic reproductive barriers have been widely discussed in the literature. Widmer et al. (2009) noted that while flowering phenology and the specificities and behaviors of pollinating agents act as filters in the isolation of various plant species, these pre-zygotic barriers may be insufficient to impede genetic flux between different species unless there are also active post-zygotic barriers. A literature review conducted by Lowry et al. (2008) to quantify the strengths of reproductive isolation barriers between neighboring species observed that any single mechanism is rarely sufficient by itself for complete isolation, and that pre-zygotic barriers are stronger than post-zygotic barriers. These observations corroborate the results encountered for *Chamaecrista* that show both pre- and post-zygotic barriers acting between pairs of species from different clades.

Inter-clade reproductive isolation

Crosses carried out between the different clades indicated the presence of different forms of reproductive isolation between the taxa. Large numbers of aborted seeds were encountered in all of the crosses in which fruits were formed, suggesting the occurrence of post-zygotic isolation through seed inviability. In the inter-taxon crosses that did not set fruits, the principal reproductive barrier apparently acted through pre-zygotic post-pollination mechanisms with gametophyte isolation, with the pollen tubes reaching the ovary but not penetrating into the micropyle. This type of isolation includes barriers that act in the interval between pollination and fertilization, and the inability of the gametophyte to penetrate into the micropyle is

apparently based on the differences in chemical attractants that promote fertilization (Coyne and Orr 2004). Gametophytic isolation seems to be common among angiosperms, and is observed in other Leguminosae such as the genera *Dahlstedtia* (Teixeira and Ranga 2004) and *Glicine* (Moyle et al. 2004).

Gametophytic isolation among *Chamaecrista* does not, in principle, compromise the integrity of the ovules as there is no penetration into the micropyle (although it could lead to the loss of the flowers because of the excessive quantity of inter-specific pollen deposited on the stigma). The stigmatic chambers of the *Chamaecrista* examined so far are small (~ 0.3 mm) and of difficult access (Owens and Lewis 1989; Costa et al. 2007)—thus limiting the numbers of pollen grains that can be held by the trichomes in a stigmatic chamber. As the average polar diameter of the pollen grains studied was 47.27 ± 10.9 μm and their equatorial diameter was 31.67 ± 5.26 μm (unpublished data), it would be possible to saturate the stigma with approximately 60 pollen grains. As such, inter-specific pollination could lead to pollen-saturation of the stigma, making it more difficult to retain intra-specific pollen. Small stigmas are common in the subtribe Cassiinae (Owens and Lewis 1989; Dulberger et al. 1994), with the exception of some species such as *Senna sylvestris* that have elongated and crateriform stigmas that allow the retention of large amounts of pollen (Carvalho and Oliveira 2003).

Pre- and post-zygotic reproductive isolation mechanisms were seen to be efficient among most of the sympatric taxa of *Chamaecrista* and they functioned as overlapping filters to make gene flow difficult or impossible and such overlapping reproductive barriers acting as filters were also encountered in other groups of plants such as the Onagraceae (Vieira 2002), Orchidaceae (Borba et al. 2001), and Solanaceae (Bedinger et al. 2011), indicating that the mechanism of overlapping filters seems to be a common strategy in sympatric taxa with phylogenetic proximity. According to Widmer et al. (2009), it is improbable that speciation could result from a process mediated by a single reproductive isolation barrier.

Different from observations in taxa of the same clade, where pre-zygotic barriers were less efficient as reproductive isolation mechanisms, these strategies are quite variable among taxa of different clades, showing not only pre-zygotic pre-pollination barriers (Costa 2007) and post-pollination barriers, but also post-zygotic isolation mechanisms. These observations indicate the existence of uniform patterns of reproductive isolation mechanisms among these taxa and the importance of partial, but overlapping barriers that can impede or diminish gene flow.

Studies of the chromosome numbers in the genus *Chamaecrista* have revealed large variations among the clades, with values of $2n = 32$, 28, 16 and 14 chromosomes generated by polyploidy, disploidy, and aneuploidy events (Goldblatt 1981; Biondo et al. 2005). Comparisons of chromosomal data with phylogenetic analyses (Conceição et al. 2009) supports $2n = 28$ as the plesiomorphic position of the genus, with reduced values of $2n = 16$ and 14 being considered synapomorphies for more derived clades such as Chamaecrista and Xerocalyx, respectively. The chromosomal variations encountered in *Chamaecrista* are probably responsible, at least in part, for the inter-incompatibility observed between the clades, generating genomic incongruities and reinforcing the maintenance of the genetic identities of sympatric taxa. A similar situation was reported by Moyle et al. (2004), in which the effectiveness of post-zygotic barriers increased with increasing genetic differences between the taxa. Widmer et al. (2009) observed that the effectiveness of post-zygotic isolation barriers can vary considerably, even among taxa that diverged only recently—indicating that these barriers must evolve relatively rapidly in some groups.

The Chapada Diamantina has unique environmental characteristics that have favored the diversification of the taxa of *Chamaecrista*, and the genus demonstrates elevated richness in the region (Conceição et al. 2008b). The different reproductive isolation mechanisms encountered among the taxa of *Chamaecrista* appear to reflect the heterogeneity of their environments and aid in maintaining their genetic identity. Sympatric speciation events originating in disruptive selection in the occupation of new micro-habitats (see Coyne and Orr 2004) or by hybridization, especially in the Baseophyllum clade (*Conceição* et al. 2008a; Costa 2007), may be common events in the evolution of history of the *Chamaecrista* in the Chapada Diamantina.

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