Seasonal isolation as a reproductive barrier among sympatric *Salix* species

A. MOSSELER

Forestry Canada, Newfoundland and Labrador Region, P.O. Box 6028, St. John's, Nfld., Canada A1C 5X8

AND

C. S. PAPADOPOL

Ontario Tree Improvement and Forest Biomass Institute, Maple, Ont., Canada L0J 1E0

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Differences in flowering phenology may be an important premating reproductive barrier between willow species. The seven sympatric species studied fall into two flowering groups within which there is substantial seasonal overlap: an early flowering group that includes *Salix bebbiana* Sarg., *Salix discolor* Muhl., *Salix eriocephala* Michx., and *Salix petiolaris* Smith, and a later flowering group that includes *Salix amygdaloides* Anderss., *Salix exigua* Nutt., and *Salix lucida* Muhl. The species flowering sequence was reasonably consistent over the 3-year period from 1984 to 1986. Thermal control over flowering phenology was related to cumulative growing degree-days. The inheritance of phenological responses in artificially produced F_1 interspecific hybrids was intermediate between that of the parental species.

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Des différences dans la phénologie de la floraison pourraient constituer une barrière importante aux croisements interspécifiques chez les saules. Les sept espèces sympatriques étudiées se répartissent en deux groupes de floraison entre lesquels existe un chevauchement saisonnier important : un groupe à floraison précoce incluant *Salix bebbiana* Sarg, *Salix discolor* Muhl., *Salix eriocephala* Michx. et *Salix petiolaris* Smith, et un groupe à floraison tardive qui inclut *Salix amygdaloides* Anderss., *Salix exigua*. Nutt. et *Salix lucida* Muhl. La séquence de floraison des espèces fut cohérente au cours de la période de 3 ans de 1984 à 1986. Le contrôle thermique de la phénologie de la floraison est relié au cumul des degrés-jours de croissance. L'hérédité des réponses phénologiques chez des hybrides interspécifiques F_1 produits artificiellement est intermédiaire entre celle des espèces parentales.

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Introduction

The potential exists for natural interspecific hybridization and gene flow to influence genetic variation in sympatric willows (*Salix* spp.). Earlier European literature, culminating with the artificial hybridization studies of Nilsson (1918), maintained that hybridization among willows was widespread and had a substantial effect on intraspecific variability. However, more recent studies of willows native to North America (Argus 1974; Dorn 1976) and Asia (Skvortsov 1968) contest the view that such hybridization has had an important effect on intraspecific variability, since very little evidence of natural hybridization exists.

Willows provide a unique opportunity to investigate the reproductive barriers that control interspecific gene flow because different combinations of up to six species often occupy the same wetland habitats (Mosseler 1987). Reproductive barriers between closely related species have been classified as either premating or postmating (Stebbins 1950; Levin 1978). The premating barriers that might restrict or prevent hybridization among sympatric species may result from spatial separation due to different habitat preferences (Bateman 1947), differences in seasonal timing of flower receptivity or anthesis (Pauley 1956; Ronald 1982), or differences in pollinator specificity as promoted by differences in floral mechanisms such as taste, colour, or fragrance (Levin and Kerster 1974). Spatial separation due to habitat preferences can be discounted as an important isolating mechanism in willows, since the species studied were observed in virtually all species combinations within multispecies associations in the populations that were sampled for genotypes in composing a common garden. In a

related study, Mosseler (1987) observed a broad spectrum of pollinators, including flies (*Diptera*), wasps (*Hymenoptera*), honeybees (*Apis*), bumblebees (*Bombus*), and beetles (*Coleoptera*) harvesting pollen and (or) nectar, but no host-specific pollinator relationships were evident. In a study of effective pollinators in willows, Van der Werf (1983) also recorded a number of insect visitors to *Salix caprea* L. To our knowledge, no evidence for pollinator specificity in willows has been presented.

Argus (1974) suggested that temporal isolation imposed by seasonal differences in flowering times may be an important breeding barrier between willows. In comparative phenological studies, it must be assumed that, other things being equal, each species reacts in a definite and constant way to a certain thermal regime (Sanderson 1954). Growing degree-days (GDD) are the degrees of temperature above 5°C for each day that the daily mean temperature exceeds a 5°C threshold (Atmospheric Environment Service 1984). The concept of GDD provides a reference system for relating the stages of development in plants to the variable daily thermal regime. In this study we attempted to relate GDD to the phenology of flowering in the following *Salix* species: *S. amygdaloides* Anderss., *S. bebbiana* Sarg., *S. discolor* Muhl., *S. eriocephala* Michx., *S. exigua* Nutt., *S. lucida* Muhl., and *S. petiolaris* Smith. These species are sympatric over much of their ranges in eastern North America (Fernald 1950).

The specific objectives of this study were (*i*) to document the seasonal flowering periods of seven willow species in successive years, (*ii*) to determine the importance of seasonal isolation as a potential reproductive barrier, (*iii*) to relate

TABLE 1. Length of the flowering period (in days) in 1984, 1985, and 1986 for six willow species in a common garden

Species	1984		1985		1986	
	n^a	Mean ± SD (range)	n	Mean ± SD (range)	n	Mean ± SD (range)
S. amygdaloides	1	7	5	8.8±1.3 (7−10)	5	9.6±1.5 (8−11)
S. bebbiana	1	9	4	9.8±1.0 (9−11)	4	12.2±2.8 (9−15)
S. discolor	11	na	14	6.4±1.3 (4−8)	15	12.7±3.5 (6−16)
S. eriocephala	11	6.8±2.2 (4−10)	13	6.3±1.6 (5−9)	13	11.0±2.5 (5−16)
S. lucida	16	7.3±1.2 (5−9)	18	9.4±1.7 (8−11)	22	10.1±1.6 (8−14)
S. petiolaris	8	6.0±1.8 (5−9)	10	5.6±1.6 (4−7)	11	8.6±1.0 (7−10)

[a]Number of clones observed.

flowering phenology in willows to abiotic factors of the environment, and (iv) to determine the pattern of inheritance for flowering phenology in artificially produced F_1 (first filial generation) interspecific hybrids.

Materials and methods

The common garden

Clones of the seven willow species were propagated from stem cuttings collected from plants found in natural populations located within a 100-km radius of Toronto, Ont., and were established in a common garden near Maple, Ont. (latitude 43°52′, longitude 79°29′). The natural populations sampled for genotypes were well isolated from one another and often existed as multispecies associations consisting of up to six different willow species. Each ortet was represented in the common garden by four ramets spaced at a 1 × 2 m spacing on a well-prepared silty clay loam nursery site.

Observations on female flower receptivity and pollen shedding were made daily during the flowering period beginning in mid-April and ending early in June from 1984 to 1986. For each male clone (genotype), the first and last day of pollen shedding (anthesis) were recorded. For each pistillate clone, the initiation of flower receptivity was recorded on the first day on which the stigmas became fully reflexed on the first female catkin. The last day of female receptivity was recorded as the date on which the last flower stigmas began to show the withering and brown discolouration that signals a loss of receptivity (Helenurm and Barrett 1987). Observations on the flowering periods of F_1 interspecific hybrids produced in 1985 were carried out on plants that had been growing under field conditions for one full growing season. Some of the smaller F_1 hybrid plants had not reached sexual maturity at the time of observation; other interspecific hybrids were subsequently found to suffer from hybrid sterility. Observations on F_1 hybrids in 1986 were carried out on plants that had been growing under field conditions for approximately 1½ growing seasons (established in August 1984) at which time all species with the exception of S. amygdaloides flowered abundantly. Salix amygdaloides is a tree-forming willow and may require more time to reach full sexual maturity. The flowering periods for parent clones and their F_1 progeny (which were established in adjacent plantations) were recorded to obtain the average first and last date of flowering and the range in the flowering period.

Climatological data

Daily measurements of temperature (maximum, minimum, and average) were obtained from the Richmond Hill, Ont., weather station from the electronic archive of the Atmospheric Environment Ser-

vice of Environment Canada. This station is located approximately 5 km from the site of the common garden where phenological observations were made. The electronic archive for this station contains continuous daily observations from March 1959 to the present. To obtain a sound basis for comparison of the thermal regime of individual years, we calculated the daily normal temperatures (maximum, minimum, and average) from 1960 to 1986. The normal daily temperatures were then subjected to a smoothing operation and compared with the monthly normals for the same station (Atmospheric Environment Service 1982).

Results

The flowering interval

With the exception of S. exigua, seasonal flowering periods were relatively short in duration, with an average length of 6−10 days in 1984 and 1985, and 8−13 days in 1986, depending on species (Table 1). Many plants of S. exigua continued to flower sporadically over the whole growing season following a peak flowering period in May of each year. Certain clones of this species were observed releasing seeds until the end of October in 1984. Under the heavy insect activity of the common garden, flowers were usually pollinated shortly after becoming receptive, and stigmas generally withered soon after pollination. We considered the fresh appearance of the stigmas to accurately reflect their state of receptivity.

The average first and last date of female flower receptivity and pollen shedding and the range (in days) of flowering from 1984 to 1986 for the parent species established in the common garden are presented in Figs. 1−3. The same information for the parent species and the F_1 interspecific hybrids is presented in Figs. 4 and 5. The seven willow species can be classified into two phenological groups: an early flowering group, which includes S. discolor, S. eriocephala, S. bebbiana, and S. petiolaris, and a late flowering group, which includes S. amygdaloides, S. exigua, and S. lucida. Among species within each flowering group, there was a large degree of overlap in seasonal flowering period. The early and late flowering species groups were seasonally isolated in 1984 and 1985 (Figs. 1−3). In 1986, there was a slight overlap in the flowering periods of the two flowering groups, demonstrating that annual weather patterns can influence opportunities for natural cross-pollination to some extent.
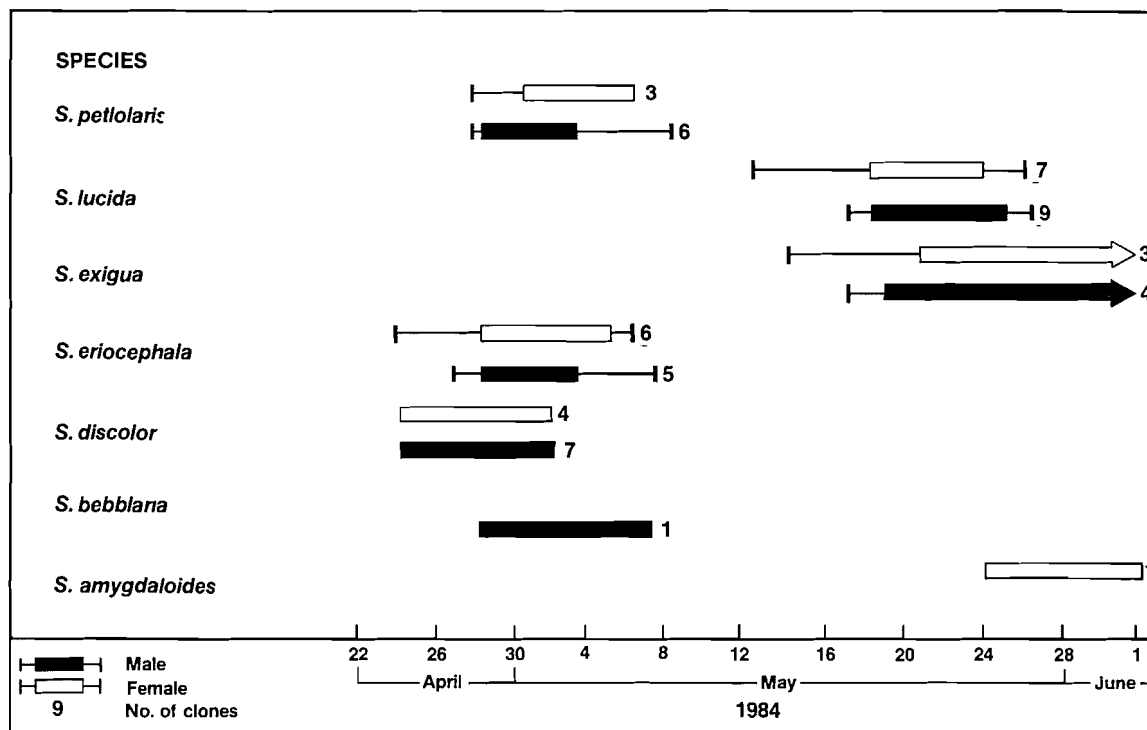
FIG. 1. Average first and last date (heavy bar) and range (thin bar) of flower receptivity — anthesis and number of genotypes observed in 1984 in seven *Salix* species.
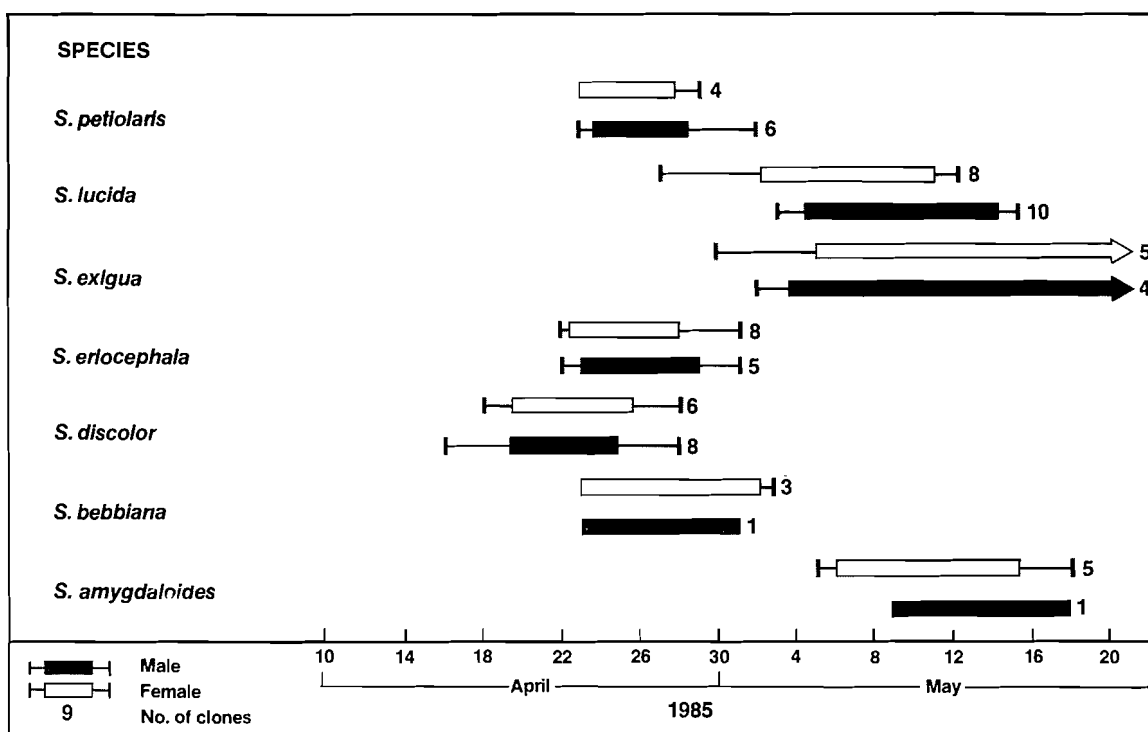

FIG. 2. Average first and last date (heavy bar) and range (thin bar) of flower receptivity — anthesis and number of genotypes observed in 1985 in seven *Salix* species.

The atmospheric environment and phenology

During the flowering period, unusually warm or cold periods modified the evolution of phenological events (Figs. 6, 7). The smoothing operation applied to daily values of the average temperatures of 1984 to 1986 and their comparison with the daily normal average temperature facilitates the identification of these warm and cold intervals (Figs. 6A–6C). A comparison of the smoothed daily temperatures with the daily normal
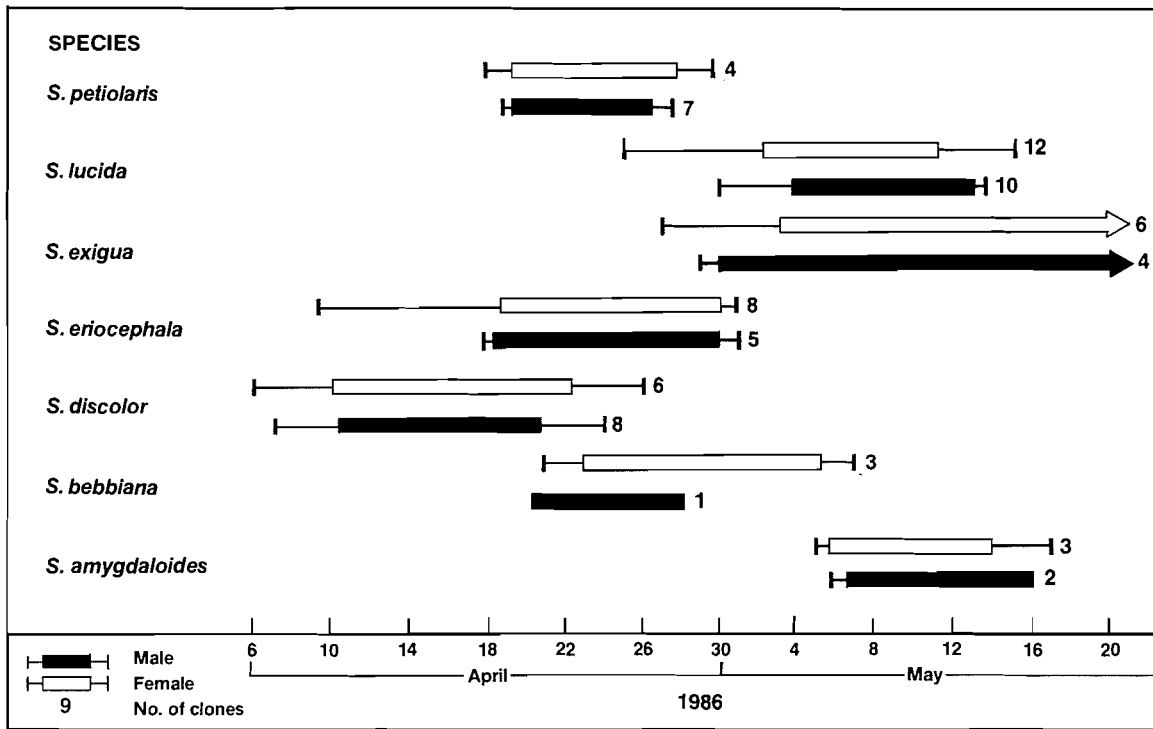
FIG. 3. Average first and last date (heavy bar) and range (thin bar) of flower receptivity — anthesis and number of genotypes observed in 1986 in seven *Salix* species.
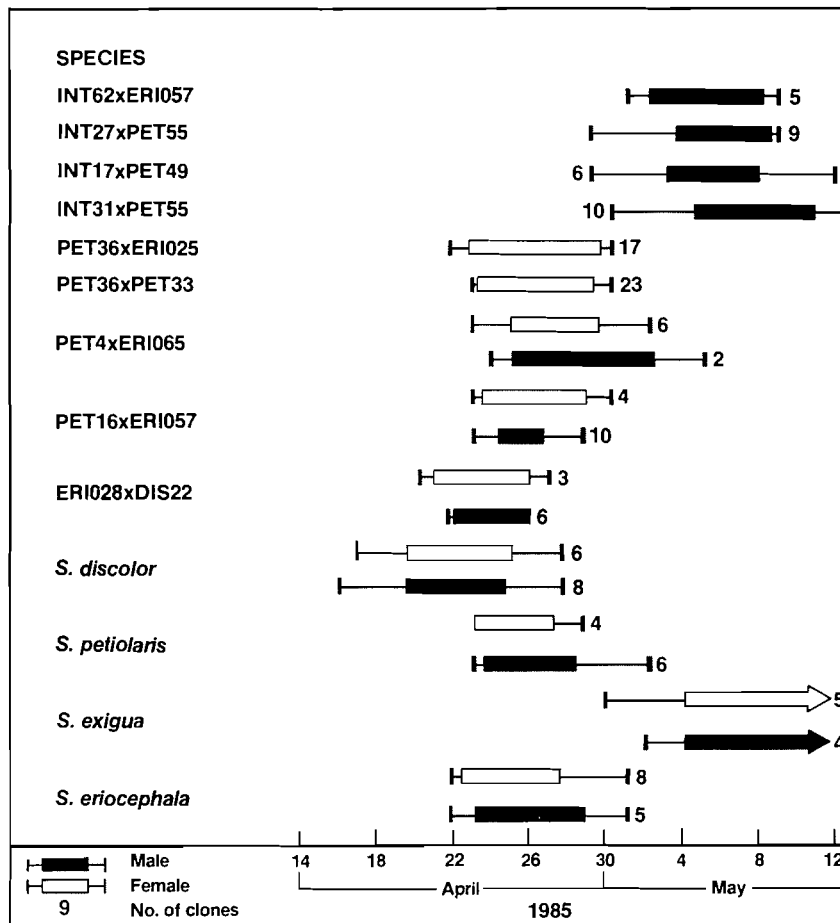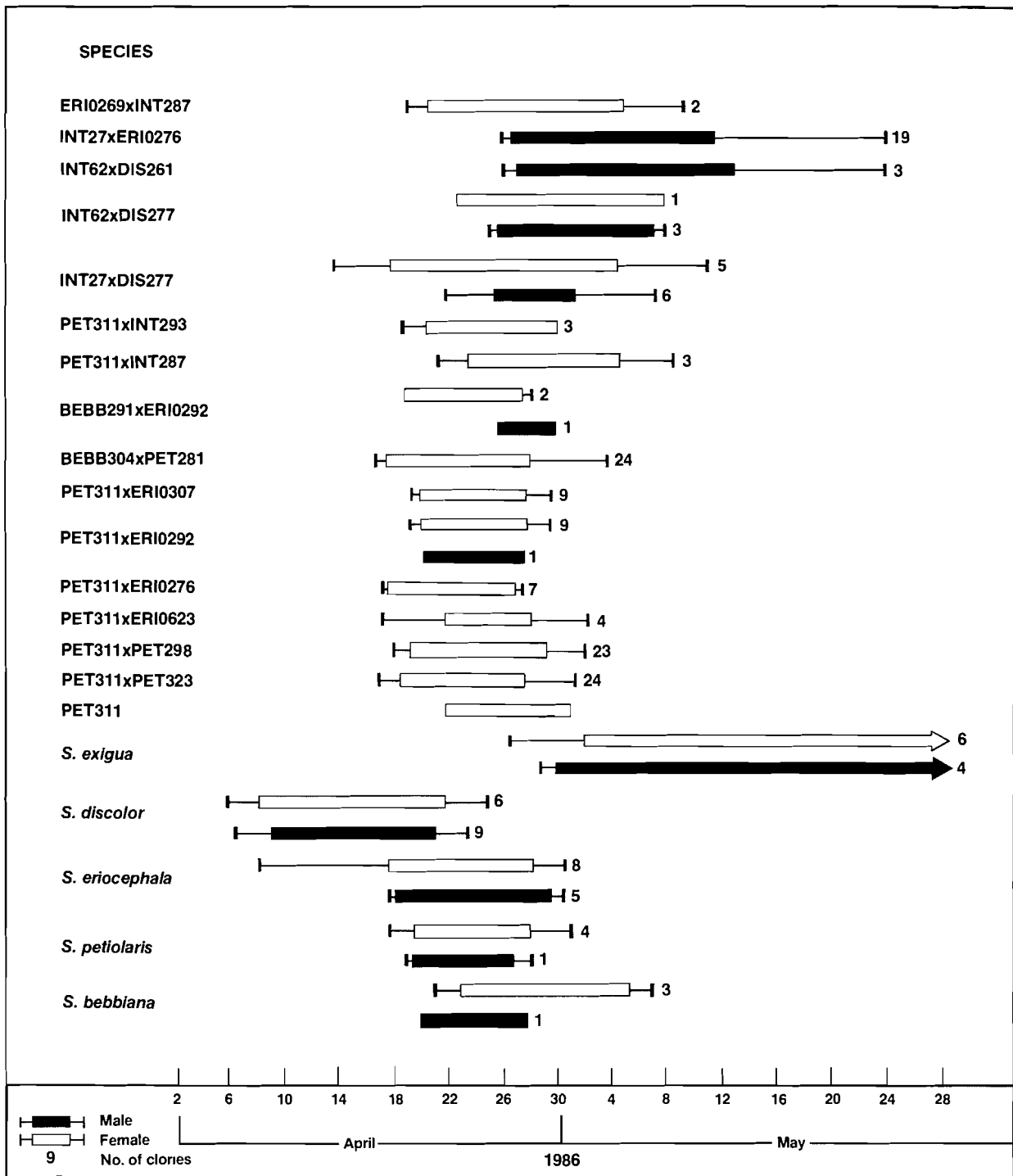

FIG. 4. Average first and last date (heavy bar) and range (thin bar) of flower receptivity — anthesis and number of genotypes observed in 1985 for four *Salix* species and their interspecific hybrids. DIS, *S. discolor*; ERIO, *S. eriocephala*; INT, *S. exigua*; PET, *S. petioloris*.

SPECIES

Fig. 5. Average first and last date (heavy bar) and range (thin bar) of flower receptivity – anthesis and number of genotypes observed in 1986 for five *Salix* species and their interspecific hybrids. BEBB, *S. bebbiana*; DIS, *S. discolor*; ERIO, *S. eriocephala*; INT, *S. exigua*; PET, *S. petiolaris*.

for the months of April and May (days 91 – 152 of each year) shows that (*i*) 1984 displayed two unusually warm periods in this interval, but the deviation from normal was moderate (Fig. 6A); (*ii*) 1985 had two extended, intense, and well-defined heat pulses in this interval (Fig. 6B); and (*iii*) the whole spring of 1986 was warmer than the normal (Fig. 6C). The differences in the thermal requirements for flowering in these two groups were illustrated by their bahaviour in 1984, when the flowering of the two groups was coincidental with two heat pulses that were clearly separated by a cold spell. In

1985, an extended and intense heat pulse between days 103 and 122 of that year caused the early flowering group to flower 4 – 6 days earlier than in 1984 and advanced flowering in the later flowering group by 14 – 16 days, probably as a result of the intensity of this heat pulse. In 1986, a much warmer than average spring advanced flowering in both groups. While the species flowering sequence was generally maintained during this unusual spring, some modification in the flowering sequence was observed with some clones of *S. exigua* flowering before *S. lucida*.
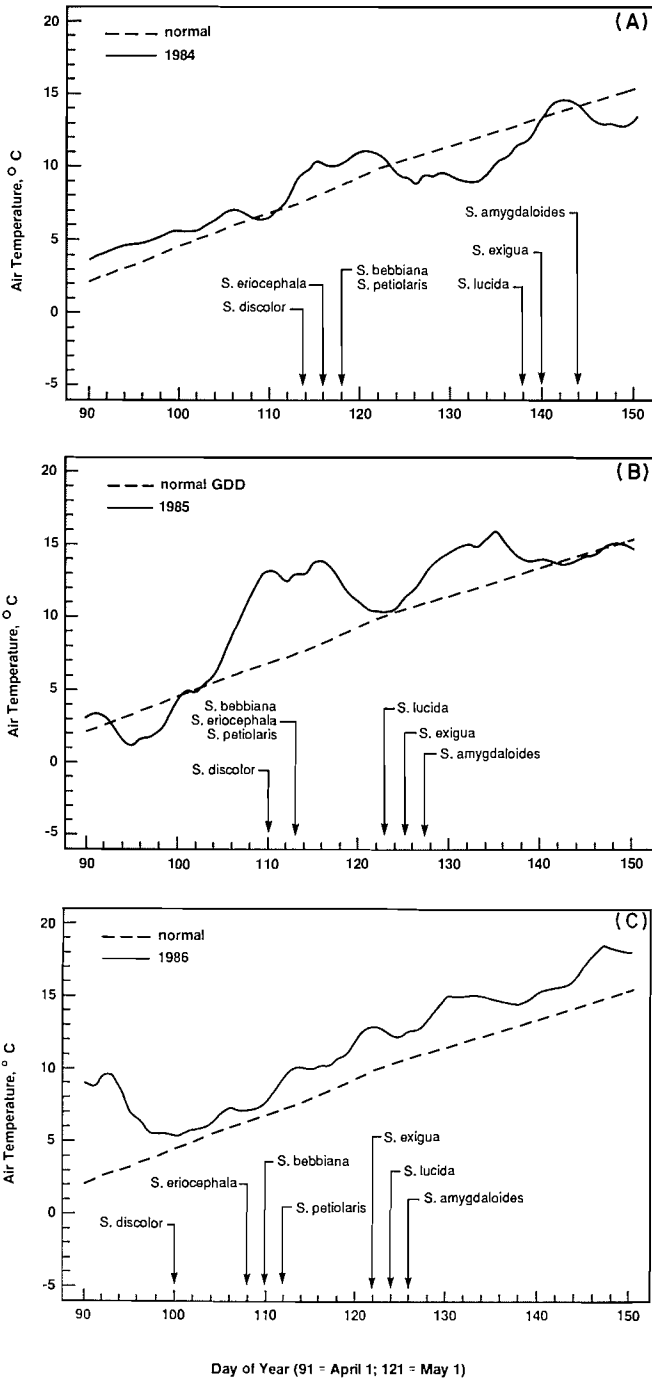
FIG. 6. Willow flowering dates and the daily and long-term average (1960–1986) air temperature. (A) 1984; (B) 1985; (C) 1986.
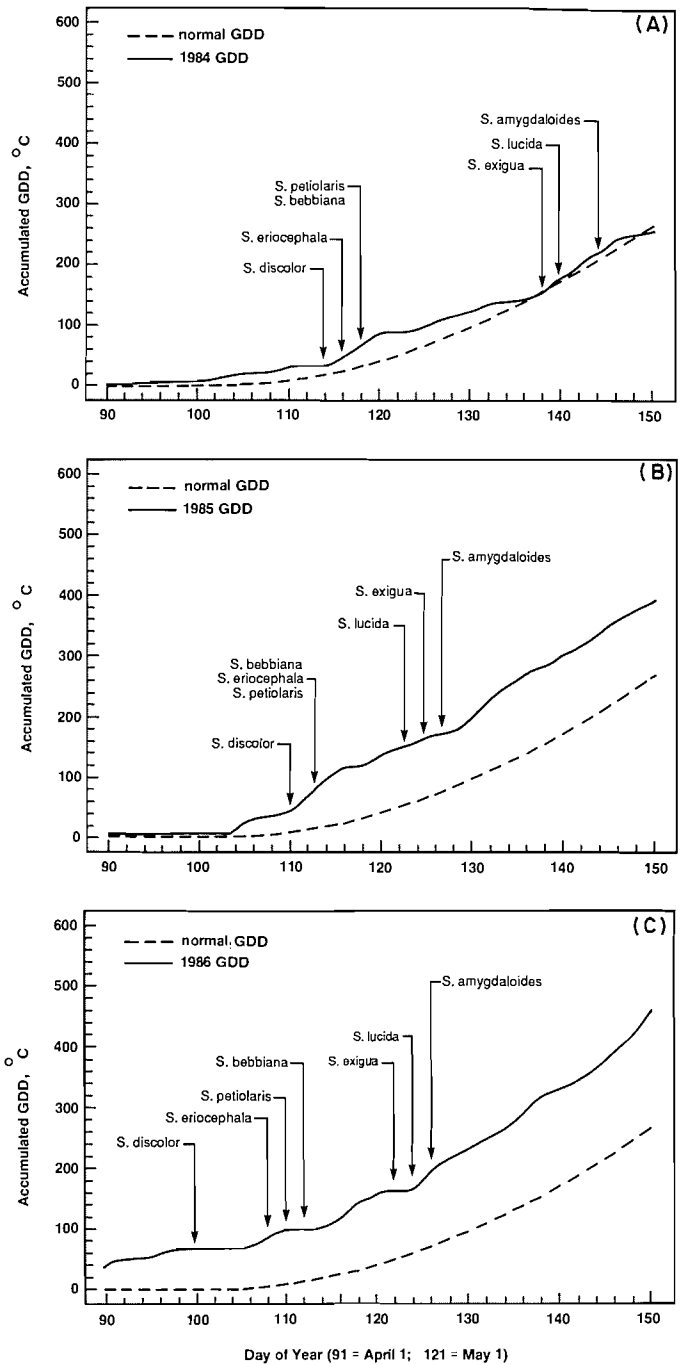

FIG. 7. Willow flowering dates with the cumulative growing degree-days (GDD) and the long-term average GDD (1960–1986). (A) 1984; (B) 1985; (C) 1986.

To characterize the seasonality of flowering, the first flowering date of each species is shown together with the accumulated GDD (Figs. 7A–7C). Despite the short duration of observations, we can define the thresholds for the first flowering date as 30–60 accumulated GDD for S. discolor, 60–80 for S. eriocephala, 70–80 for S. bebbiana, 70–90 for S. petiolaris, 150–160 for S. lucida, 160–180 for S. exigua, and 190–210 for S. amygdaloides. For the year 1986, in which all the species flowered at an earlier date, it appeared that these thresholds were too low and that the actual flowering required an additional 10–30 GDD. This observation suggests that day length also has an influence on flowering, as observed

by Garner and Allard (1920), and should be considered in future attempts to model the seasonality of flowering.

Discussion

Observations on the species composition of natural wetland populations native to southern Ontario showed the following species associations to be the most common: (i) S. amygdaloides, S. lucida, and S. petiolaris along the edges of hardwood swamps, seasonally inundated fields, or poorly drained meadowlands; (ii) S. eriocephala and S. exigua along sandy

streambanks; (*iii*) *S. bebbiana*, *S. discolor*, and *S. eriocephala* on poorly drained or seasonally inundated upland meadows and better drained midslopes. However, any and all possible species combinations can be found on the same site and within close spatial proximity. Therefore, ecological or spatial isolation imposed by habitat preference must be considered a weak barrier to cross-pollination between these species within the study area. As Argus (1986) pointed out, the primary ecological determinants for the establishment and growth of most willows are a moist substrate for seed germination and ample sunlight for subsequent growth. These conditions are met on most of the disturbed, wetland sites colonized by willows.

Willows are predominantly insect pollinated (Kevan 1972; Sacchi and Price 1988). The morphological differences between flowers of different willow species do not limit accessibility by pollinator species. Unless flower scent or colour encourage flower constancy in willows (Meeuse 1979), the existence of host-specific pollinators appears unlikely. Insect pollinators are essentially opportunistic in their foraging habits, and there is a cost in becoming too specialized on individual plant species (Heinrich 1976; Schmitt 1983), particularly in a group of species that flowers over such short periods.

Flower buds in willows are formed during the season prior to flowering and are probably activated by specific temperature thresholds or accumulated heat units. However, *S. exigua* also initiates flowers at the tips of current shoots throughout the growing season. The timing of the flowering period in most temperate woody species is regulated by spring temperature (Wareing 1963; Smithberg and Weiser 1968; Reader 1975, 1983; Rathcke and Lacey 1985). Willows demonstrate a strong thermal control over flowering, which occurs in response to heat pulses and may be delayed by cold spells. The concept of accumulated GDD can be used to predict phenological events in willow, and the consistency of the annual flowering cycle of each species with respect to GDD demonstrates the genetic control over phenological response.

The strong seasonal differences observed between willows from the two different flowering groups suggests that flowering phenology may be an important premating barrier to interspecific pollination and potential gene flow between the early and late flowering species. The ease with which controlled hybridization can be carried out between the early flowering species and the late flowering *S. exigua* and the absence of naturally occurring hybrids between these species suggests that seasonal isolation provides a reproductive barrier between these species. Whether or not seasonal isolation evolved in response to negative selection against hybridization between willow species, the Wallace effect (Grant 1971), is unclear. Although the genetic basis of inheritance of flowering phenology in willows has not been determined, the relatively simple genetic inheritance of flowering phenology in other plants (Murfet 1977; Waser 1978) and its potentially important adaptive function in preventing hybridization probably exposes the seasonal timing of flowering to natural selection for reproductive isolation. The possibility of phenological separation as an evolved response to selection against hybridization was raised as a possibility by Rathke and Lacey (1985).

The inheritance of flowering phenology in F_1 hybrids such as *S. bebbiana* × *eriocephala*, *S. bebbiana* × *petiolaris*, *S. petiolaris* × *eriocephala*, and *S. eriocephala* × *discolor* was difficult to characterize because of the phenological similarity of the parental species. However, the inheritance of flowering

phenology in the F_1 interspecific hybrids between the late flowering *S. exigua* and early flowering *S. bebbiana*, *S. discolor*, *S. eriocephala*, and *S. petiolaris* (Figs. 4, 5) was generally intermediate between the parental species. The F_1 interspecific hybrids in which *S. exigua* was used as the pistillate parent (Fig. 4) began flowering later than would have been expected, based on the assumption of a strictly intermediate inheritance of flowering. These hybrids also flowered over a distinct and much shorter seasonal period in comparison with the continuous or indeterminate flowering habit of the pistillate parent, *S. exigua*. In the absence of strong postmating reproductive barriers, natural interspecific hybrids would most likely backcross with their pistillate parent, based on the extent of overlap in the flowering period (Figs. 4, 5).

In the course of this study, only one naturally occurring interspecific hybrid was found in the area in which natural populations were sampled. This hybrid resembled artificial crosses between *S. petiolaris* × *eriocephala*. This general absence of naturally occurring interspecific hybrids and the absence of obvious premating barriers within the two flowering groups suggest that stronger postmating barriers limit natural hybridization between these species.

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