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Floral development, stigma receptivity and pollen viability in eight *Nolana* (Solanaceae) species

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Abstract *Nolana* L.f. is a large, diverse genus in the Solanaceae endemic to the coastal deserts of Peru and Chile. Floral development studies were conducted in eight species (*Nolana adansonii, N. aticoana, N. elegans, N. humifusa, N. ivaniana, N. laxa, N. plicata,* and *N. rupicola*) as a precursor to breeding efforts and studies of interspecific sexual compatibility. Levels of stigma receptivity and pollen viability were evaluated at different stages during flower development. Species were found to be receptive to pollination over a wide range of floral developmental stages, including stages prior to anthesis. Pollen was found to remain viable throughout the open flower period and into senescence. Floral development keys were developed which provide a visual reference correlating morphological appearance of buds and flowers of each species at each developmental stage, and their corresponding durations and levels of stigma receptivity.

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Introduction

Nolana is the fourth largest genus in the Solanaceae, with 89 species currently recognized. It is endemic to the coastal desert in Peru (43 species) and the Atacama Desert in central and northern Chile (49 species). Four species are recorded in both countries, and one species is endemic to the Galapagos Islands, Ecuador (Dillon et al. 2007). The majority of species are found between 7°59′ and 33°21′S latitude, at 50–600 m altitude, and within a few kilometers of the Pacific coast (Mesa 1981, 1986; Dillon et al. 2003). Most species are found in fog-dependent isolated patches of vegetation called *lomas* formations, and flourish during El Niño years when the *lomas* experience high rainfall and humidity (Dillon and Rundel 1989; Tago-Nakawaza and Dillon 1999). The genus *Nolana* was considered to belong to a distinct family, Nolanaceae, due to its 5-carpelled gyonecium, but is now included in Solanaceae based on chloroplast DNA analysis (Olmstead and Palmer 1992). *Nolana* is considered monophyletic and is diagnosed by possessing unusual sclerified fruits called mericarps, a unique character in the Solanaceae (Knapp 2002). The ovary develops into a dry fruit of 2–30 normally one-seeded mericarps (Bondeson 1986; Tago-Nakawaza and Dillon 1999).

As far as we are aware, field studies on *Nolana* floral biology are non-existent. Furthermore, research on hybridization and compatibility between *Nolana* species is also very limited. Artificial hybridization has been documented between *N. prostrata* × *N. atriplicifolia* (currently named *N. humifusa* and *N. paradoxa*) (Saunders 1934), and between five Chilean species (*N. acuminata, N. aplocaryoides, N. elegans, N. paradoxa* and *N. rupicola*) (Freyre et al. 2005). We selected eight *Nolana* species collected from four *lomas* locations in Peru (*N. adansonii, N. aticoana, N. humifusa, N. ivaniana, N. laxa,* and *N. plicata*) and one in Chile (*N. elegans* and *N. rupicola*) for studies on hybridization and compatibility studies. The goal of the present research was to characterize the floral development of these eight *Nolana* species and to associate timing of corolla growth and anthesis with stigma receptivity and pollen dehiscence and viability. Manual pollinations were used to identify timing of stigma receptivity and pollen viability as determined by fruit set. Floral development keys were constructed for each *Nolana* species illustrating developmental stages, their corresponding durations and levels of pollination success for use as visual reference in artificial hybridizations for compatibility studies and breeding.

Materials and methods

Plant material

Eight *Nolana* species were selected for these studies (*N. adansonii, N. aticoana, N. elegans, N. humifusa, N. ivaniana, N. laxa, N. plicata*, and *N. rupicola*). Detailed collection information for plant material is reported in Table 1. A map of the *lomas* collection areas has been published elsewhere (Dillon et al. 2003). Mericarps were germinated in seed trays with bottom heat and intermittent mist. Stock plant material included three to five accessions for each *Nolana* species, which were then vegetatively propagated and grown to maturity. Herbarium vouchers for all accessions are housed at the Hodgdon Herbarium, University of New Hampshire, and at the Field Museum of Natural History, Chicago, IL.

Plant material was maintained in an insect exclusion, double-poly hoop house at Woodman Research Farm, University of New Hampshire, Durham, NH. Plants were grown in 20-cm pots with Sunshine LA4 aggregate mix (SunGro Horticulture Inc., Bellevue, WA, USA). Fertilization was constant with a 20N–4.3P–16.7K fertilizer at a maximum 150 mg l^{-1} N. No additional lighting was utilized. Average air temperature was

Table 1 Collection information and growth habit for *Nolana* accessions of eight species used in floral development studies

Species	UNH accession code[a]	Collection location	Habit[b]
N. laxa	La1-2, La1-4, La1-5, La3-1, La3-2	11°58′S, 76°46′W, 670–700 masl, Peru, Lima, Los Condores	Erect herbaceous perennial
N. humifusa	H28, Hu1-2	12°11′S, 76°48′W, 170 masl, Peru, Lima, Lomas de Pachacamac	Herbaceous annual
	Hu9-4	11°58′S, 76°46′W, 675 masl, Peru, Lima, Los Condores	
N. plicata	P5, P7, P11	15°47′S, 74°21′W, 400 masl, Peru, Arequipa, Lomas de Atiquipa	Herbaceous perennial
N. aticoana	A2, A3, A13	15°47′S, 74°21′W, 450–480 masl, Peru, Arequipa, Lomas de Atiquipa	Herbaceous perennial
N. adansonii	Ad2-2, Ad2-3, Ad4-1, Ad4-11, Ad4-14	17°01′S, 72°02′W, 0–5 masl, Peru, Arequipa, Catarindo Beach, west of Mollendo, few meters from sea shore	Erect herbaceous perennial
N. ivaniana	Iv2-1, Iv2-3, Iv2-5	17°01′S, 72°02′W, 5–10 masl, Peru, Arequipa, Catarindo Beach, west of Mollendo, 20–30 m from sea shore	Erect herbaceous annual
N. elegans	Ele1, Ele2, Ele3, 051-3, 051-5	25°26′S, 70°26′W, 890 masl, Chile, Region II, Prov. Antofagasta, Cerro Perales, near Taltal	Procumbent herbaceous annual
N. rupicola	Rup1, Rup2, Rup3	26°01′S, 70°36′W, 720–780 masl, Chile, Region III, Atacama, Prov. Chanaral, Parque Nacional Pan de Azucar. Las Lomitas	Herbaceous perennial

[a] Herbarium vouchers housed at UNH Hodgdon Herbarium, Durham, NH, USA and Field Museum of Natural History, Chicago, IL, USA

[b] Adapted from Tago-Nakawaza and Dillon (1999)

recorded with a HOBO temperature logger (H08-001-02, Onset Corp., Bourne, MA, USA). Mean daily air temperatures were 21.1 and 25.8°C for the periods of December 23, 2004 through January 13, 2005 and July 7, 2005 through August 15, 2005 respectively.

Study of floral development

Floral development was studied on seven *Nolana* species between December 23, 2004 and January 13, 2005. *N. rupicola* was omitted in this study due to insufficient flowering. Three to five accessions per species and one plant per accession were used. Five buds per plant were tagged with an identifying number at an early developmental stage when the length of the bud's corolla was shorter than its calyx. Each bud was observed daily between 8 and 10 am. Data collection was initiated on the day when the tip of the corolla was even in length with the tips of the calyx (designated *Even* stage), or when the length of the corolla surpassed that of the calyx if the preceding stage occurred between daily observations.

Based on the observational data collected in this study, floral ontogeny of *Nolana* was subdivided into seven developmental stages: (1) *Calyx:* Immature bud stage in which length of the corolla is shorter than that of the calyx. Depending on the species, the corolla may be enclosed within a sealed calyx or may be visible within an open-ended calyx. This stage includes all bud development occurring prior to the start of data collection. (2) *Even:* The point at which the length of the corolla is even with that of the calyx. This is the point designated as the starting point of data collection. (3) *Closed corolla:* The corolla remains tightly closed, but has exceeded the length of the calyx and is continuing to elongate. (4) *Partially open:* The corolla is loosening and its tips are slightly apart. This stage includes the time during which the corolla continues to unfold and expand. (5) *Anthesis* (*Open flower*): Begins when the corolla has fully unfolded. (6) *Wilted:* Flowers have lost their vibrant color and have taken on a washed-out appearance. Corolla tissue becomes limp and often reflexes. (7) *Senescent:* Corolla tissue collapses and the flower loses its open shape. Corolla tissue may hang loosely from the calyx or may begin to dry and shrivel, and eventually drops.

Average stage durations were rounded to the nearest whole number to remain consistent with 24 h observation intervals. Analysis of variance was performed to compare the number of days at each stage of development between and within species. Differences in duration of stages indicated by ANOVA were analyzed using Tukey's HSD at the $P = 0.05$ level (Systat 10, SPSS Inc, 2000). Measurements of the depths and diameters of fully expanded corollas at *Anthesis* were made with digital calipers (MarCal 16EX) using five flower per accession for each species. Corolla colors were documented using the RHS colour charts (Royal Horticultural Society 1995).

Determination of stigma receptivity, pollen viability and pollination success during floral development

Experiment 1

One accession was chosen for *N. humifusa* (Hu9-4), *N. laxa* (La1-2) and *N. adansonii* (Ad4-11) and studied between July 7, 2005 and August 5, 2005. Three plants per accession were used. Pollinations were performed daily between 8 and 9 am. For each day of development starting from *Even* to *Senescent* stage, stigmas of three buds (or flowers) were pollinated with fresh pollen from a compatible individual of the same species. Compatible individuals (Hu9-4 × Hu1-2; La1-2 × La1-4; Ad4-11 × Ad4-14) were identified in pilot studies by complete fruiting success after performing manual pollinations. Additionally, three buds each of *N. humifusa* and *N. laxa* were pollinated each day with pollen from a different species (Hu9-4 × *N. aticoana* A2; La1-2 × *N. plicata* P5). Success or failure of fruit set was recorded for each pollination. A final set of three stigmas per day of *N. humifusa* and *N. laxa* were tested for the presence of dehydrogenases as an indicator of receptivity, by treatment with 3-4, 5-dimethylthiazol-2-yl-2,5-diphenyl-tetrazolium bromide (MTT). Stigmas were treated with 20 g l^{-1} MTT in 5% sucrose at 28°C as described (Dafni et al. 2005) and observed by microscopy within 30 min of initiation of staining.

Pollen viability was determined for flowers of *N. humifusa* and *N. laxa* representing each day of development starting on the day the bud reached or surpassed *Even* stage. For each day of development, pollen from three buds of Hu9-4 and La1-2 was applied to stigmas of freshly open flowers of a compatible individual of the same species (Hu1-2 × Hu9-4;

La1-4 × La1-2). Pollen from a second set of three buds from Hu9-4 and La1-2 was applied to stigmas of a compatible individual of a different species (*N. aticoana* A2 × Hu9-4; *N. plicata* P5 × La1-2). All flowers to be pollinated were emasculated at the *Even* stage to avoid possible interference by self pollen. Success or failure of fruit set was recorded for each pollination. Pollen from a final set of three buds per day was tested for the presence of dehydrogenases by treatment with MTT as described above.

Experiment 2

Plant material consisted of one accession of each of five *Nolana* species. Data collection took place between August 4, 2005 and August 15, 2005. On each accession, five buds or flowers per each of the seven floral developmental stages were pollinated with pollen obtained from freshly opened flowers of a compatible individual of the same species, as identified by pilot studies (*N. aticoana* A2 × A3, *N. humifusa* Hu9-4 × Hu1-2, *N. ivaniana* IV2-1 × IV2-2, *N. plicata* P5 × P7, and *N. rupicola* Rup1 × Rup2). Success or failure of fruit formation was recorded for each pollination.

Construction of floral development keys

Data was compiled to create graphical representations of floral development for each *Nolana* species studied. Buds and flowers representative of each stage of floral development were photographed, and pollination success data for each developmental stage was included as bar graphs. Timelines above the photographs illustrate durations (in days) of each developmental stage under our study conditions. Divisions in the timelines represent days of observation, with '1' being the first day of data collection and corresponding to the *Calyx* stage.

Results

Description of *Nolana* species

Detailed morphological descriptions for *N. elegans* and *N. rupicola* have been published elsewhere (Freyre et al. 2005). Photographs of plants grown in 20-cm pots and a close up of a flower from each of the eight *Nolana* species studied are shown in Fig. 1. Measurements of corolla diameters, depths, and colors are reported in Table 2. Flower morphology ranged from shallowly to deeply infundibular (funnelform). Flower size ranged from 2.6 cm diameter and 1.3 cm depth in *N. humifusa* to 5.4 cm diameter and 3.3 cm depth in *N. rupicola*. Colors varied from bright blue in *N. elegans* and *N. rupicola*, to pale blue in *N. aticoana, N. humifusa*, and *N. plicata*, bright purple in *N. adansonii* and *N. laxa*, and pale purple in *N. ivaniana*. Conspicuous dark feathered nectar guides and/or bicolored throats were apparent in all species with the exception of *N. ivaniana*.

Study of floral development

Each *Nolana* species studied is unique in flower and bud morphology. Thus, a consistent starting point in the early stages of bud development was designated which could be easily identified in each species by visual inspection, corresponding to the stage at which the tips of the immature closed corolla were even in length with the tips of the calyx. This point may not be physiologically comparable between species. Therefore, data may only be interpreted as durations between developmental events with this identified stage being a key reference point.

Duration of key stages (*Calyx, Even, Closed corolla, Partially open, Anthesis, Wilted* and *Senescent*) in each of the studied species is summarized in Table 3. Not all designated stages were observed in each bud. For example, buds in *N. humifusa* developed from the *Calyx* stage to the *Partially open* stage within 1 day, with the *Even* and *Closed corolla* stages occurring between observations. In *N. humifusa* and *N. ivaniana* flowers progressed from *Anthesis* to *Senescent* in one day, and the *Wilted* stage was not observed. Analysis of variance was used to determine whether floral development durations differ between accessions and between species. Statistically significant differences in the duration of one or more floral stages were found among accessions of *N. adansonii, N. humifusa, N. ivaniana* and *N. plicata* (data not shown). Duration of each stage of development was compared between species, indicating highly significant differences between species in the duration of floral developmental stages ($p < 0.001$ in all cases). Differences between species in regards to a single stage of development, *Closed corolla*, are shown in

Fig. 1 Greenhouse-grown plants and close-up of flowers of eight *Nolana* species used in floral development studies. *Top row* (*left* to *right*): *N. humifusa*, *N. laxa*, *N. aticoana*, *N. plicata*. *Bottom row* (*left* to *right*): *N. adansonii*, *N. ivaniana*, *N. elegans*, *N. rupicola*. Scale in bottom right of images is 1 cm

Table 3, which are important because this is the preferred stage for controlled hybridizations to prevent contamination from foreign pollen.

Buds were examined in all plants by visual inspection for the presence of dehiscent anthers at the earliest stage possible without disruption of the corolla. In all cases, pollen was dehiscent upon first inspection of the anthers, except in two buds (in *N. elegans* accession Ele1 and in *N. aticoana* A2), where pollen was dehiscent one day after corolla opening.

Determination of stigma receptivity and pollen viability

N. humifusa and *N. laxa* were determined to have extreme differences in duration of floral development (with total average duration of developmental stages lasting 4 and 14 days, respectively, Table 3). These two species were selected for further study ("Experiment 1") as to the timing of stigma receptivity and pollen viability. One accession of *N. adansonii* was also included in the study of stigma receptivity in "Experiment 1". These studies were performed during summer months of July and August. Temperature-dependent and/or light-dependent differences in the duration of developmental stages were evident as compared to the previous floral development study. For example, *N. laxa* (La1-2) and *N. adansonii* (Ad4-11) took an average of 14 and 15 days respectively to complete floral development in the winter study (at mean daily temperature of 21.1°C), and only 8 and 7 days in the stigma receptivity study performed during summer months (mean 25.8°C).

An attempt was made to evaluate stigma receptivity and pollen viability by chemical enzyme staining with MTT. Unfortunately, the MTT testing technique proved to be an unreliable indicator of both receptivity and pollen viability in the two *Nolana* species tested. All stigmas tested positive for the presence of dehydrogenase enzymes regardless of the

Table 2 Corolla colors and average corolla dimensions at *Anthesis* for *Nolana* accessions used in floral development studies

Accession	Corolla color[a]			Corolla size	
	Outer corolla	Throat	Veins	Average diameter[b] (cm ± st.dev.)	Average depth[c] (cm ± st.dev.)
N. adansonii					
Ad2-2	92A	94B	–	2.0 ± 0.6	2.7 ± 0.3
Ad2-3	85C	85B	–		
Ad4-1	87D	–	93C		
Ad4-11	92B	92A	–		
Ad4-14	87D	–	93C		
N. aticoana					
A2	94B	155C	89A	3.6 ± 0.4	2.2 ± 0.4
A3	91A	155C	89A		
A13	94B	155C	89A		
N. elegans					
Ele2	89B	155C	151A	4.5 ± 0.5	2.4 ± 0.5
Ele3	95B	155C	151A		
051-3	96B	155C	151A		
051-5	95B	155C	151A		
N. humifusa					
H28	92D	–	88A	2.6 ± 0.6	1.3 ± 0.2
Hu1-2	92C	–	88A		
Hu9-4	92B	–	88A		
N. ivaniana					
Iv2-1	91B	–	–	2.9 ± 0.5	1.9 ± 0.2
Iv2-3	91B	–	–		
Iv2-5	91B	–	–		
N. laxa					
La1-2	88C	–	88A	3.3 ± 0.4	2.7 ± 0.3
La1-4	88C	–	88A		
La1-5	87C	–	87A		
La3-1	87D	–	88A		
La3-2	87C	–	88A		
N. plicata					
P5	94C	155C	89A	4.5 ± 0.5	2.9 ± 0.4
P7	94C	–	89A		
P11	94C	155C	89B		
N. rupicola					
Rup1	96A	155C	146D	5.4 ± 0.7	3.3 ± 0.3
Rup2	94A	155C	–		
Rup3	96A	155C	146D		

[a] Corolla colors determined by RHS colour charts

[b] Average diameter (at the widest point) of five fully expanded corollas per accession

[c] Average depth (from the base of the receptacle to the highest point of the corolla) of five fully expanded corollas per accession

age of the stigma. Similarly, all pollen grains in all samples tested positive for the presence of dehydrogenase enzymes.

Subsequently, stigma receptivity and pollen viability were evaluated indirectly using manual pollinations and recording fruit set. To determine stigma

Table 3 Average duration (in days rounded to the nearest whole number) for five designated stages of floral development for seven *Nolana* species

Species	*Even*	*Closed corolla*[z]	*Partially open*	*Anthesis*	*Wilted*	Total duration
N. laxa	1	5[d]	1	5	2	14
N. humifusa	0	0[b]	1	2	0	3
N. plicata	0	1[bc]	0	3	1	5
N. aticoana	0	0[b]	1	3	1	4
N. adansoni	1	9[a]	2	2	1	15
N. ivaniana	0	1[bc]	0	3	0	5
N. elegans	1	2[c]	1	3	2	9

N. rupicola was not included due to insufficient flowering

[z] Different superscripts indicate significant differences between species in duration of in the *Closed corolla* stage using Tukey's HSD at the 95% confidence level

receptivity, stigmas from flowers representing a full range of developmental stages were pollinated using either intraspecific pollen (pollen from an accession of the same species) or interspecific pollen (pollen from an accession of a different species). Fruiting success by developmental stage of each flower at time of pollination is shown in Table 4. Fruiting success was similar between intraspecific and interspecific pollinations for both *N. humifusa* and *N. laxa.* Fruiting success was complete for *N. humifusa* when flowers in *Anthesis* were pollinated (6 fruits/6 pollinations) whereas *Wilted* or *Senescent* flowers were not receptive. *N. laxa* also had complete fruiting success during *Anthesis* (5/5 and 7/7), and varying success during other floral stages. Flowers were not receptive during *Even* stage, and fruiting success was low during *Senescence* (1/10), medium during *Closed corolla* and *Wilted* (6/12 and 7/11), and high during *Partially open* (7/8). Timing of receptivity in *N. adansonii* was analyzed using only intraspecific pollen because a compatible individual of a different species could not be identified in our pilot studies. Fruiting success was limited at *Closed corolla* stage (3/9), but was complete for flowers at *Partially open* and *Anthesis* stages.

Table 4 Stigma receptivity for flowers at a range development stages for *N. humifusa*, *N. laxa,* and *N. adansonii* as measured by successful fruit set ("Experiment 1")

	Floral development stage					
	Even	*Closed corolla*	*Partially open*	*Anthesis*	*Wilted*	*Senescent*
N. humifusa						
Intraspecific (Hu9-4 × Hu1-2)	–	–	–	6/6[a]	0/6	0/6
Interspecific (Hu9-4 × A2)	–	–	–	6/6	0/6	0/6
Total				12/12	0/12	0/12
N. laxa						
Intraspecific (La1-2 × La1-4)	0/3	3/6	3/4	5/5	4/6	0/5
Interspecific (La1-2 × P5)	0/3	3/6	4/4	7/7	3/5	1/5
Total	0	6/12	7/8	12/12	7/11	1/10
N. adansonii						
Intraspecific (Ad4-11 × Ad4-14)	0/3	3/9	3/3	6/6	1/1	0/2

N. humifusa and *N. laxa* stigmas were pollinated with pollen from flowers of a sexually compatible accession of the same species (intraspecific) or from flowers of a sexually compatible accession of a different species (interspecific). Accession codes are described in Table 1

[a] Number of successful pollinations/number of attempted pollinations per developmental stage

Table 5 Determination of pollen viability in *N. humifusa* and *N. laxa* measured by successful fruit set in "Experiment 1"

	Floral development stage					
	Even	*Closed corolla*	*Partially open*	*Anthesis*	*Wilted*	*Senescent*
N. humifusa						
Intraspecific (Hu1-2 × Hu9-4)	–	–	–	6/6[a]	3/3	6/9
Interspecific (A2 × Hu9-4)	–	–	–	5/5	4/6	1/7
Total	–	–	–	11/11	7/9	7/16
N. laxa						
Intraspecific (La1-4 × La1-2)	0/3	0/6	–	5/6	1/2	4/8
Interspecific (P5 × La1-2)	0/3	1/6	–	6/7	1/1	2/8
Total	0/6	1/12	–	11/13	2/3	6/16

Pollen from flowers of different developmental stages was harvested and was applied to stigmas at the *Anthesis* stage from a sexually compatible accession of the same species (intraspecific) or from a sexually compatible accession of a different species (interspecific)

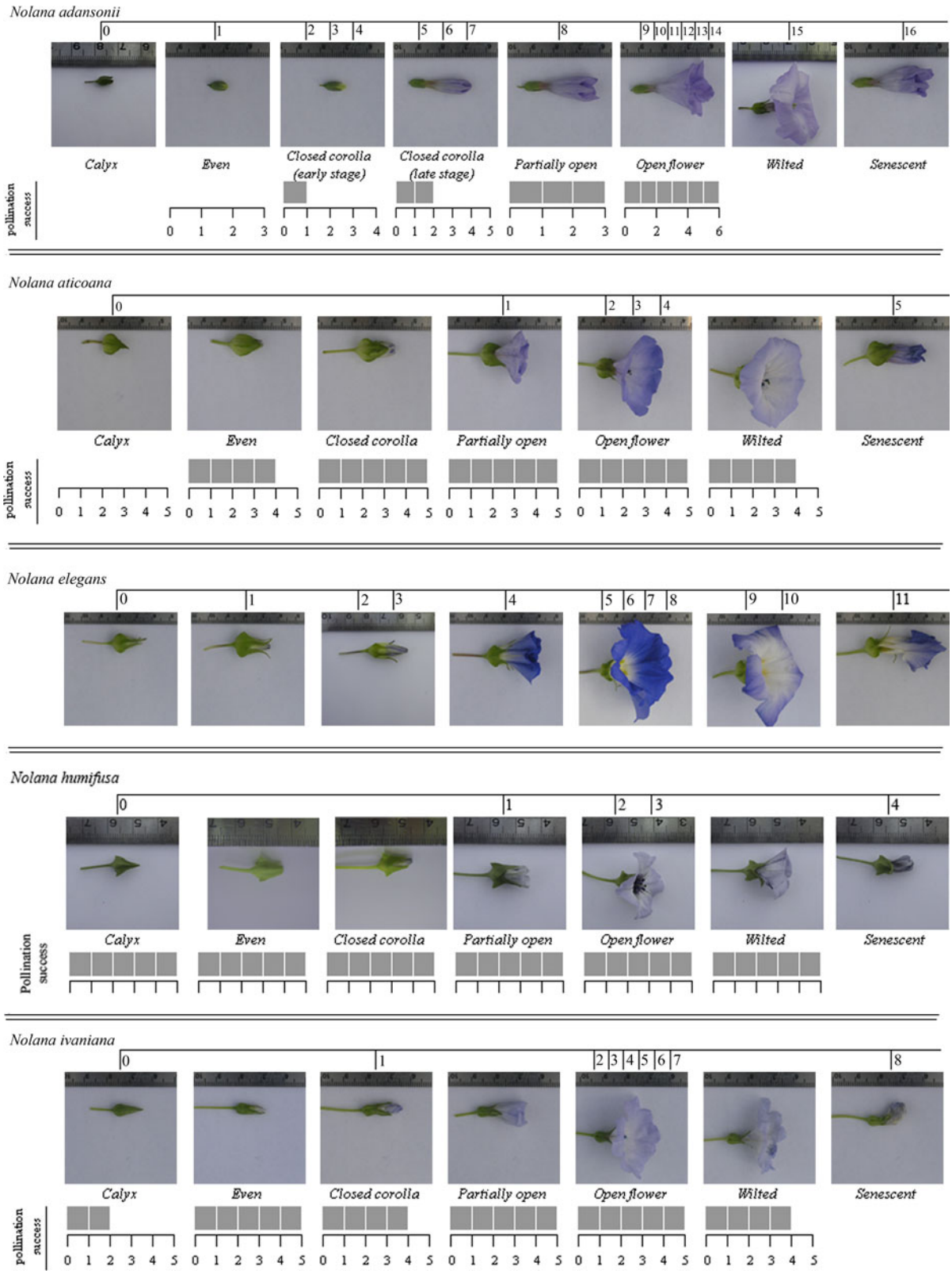
[a] Number of successful pollinations/number of attempted pollinations per developmental stage

Similar techniques were used to evaluate pollen viability in *N. humifusa* and *N. laxa* whereby pollen harvested from flowers at different stages were used to pollinate stigmas from flowers at *Anthesis* (Table 5). For *N. humifusa*, total fruiting success was higher in intraspecific (15/18) versus interspecific (10/18) crosses. Fruiting success was complete for both types of crosses when using pollen from flowers in *Anthesis*. For intraspecific crosses, pollen from *Wilted* flowers was also completely successful, and pollen from *Senescent* flowers resulted in medium success (6/9). For interspecific crosses, pollen from *Wilted* and *Senescent* flowers had medium (4/6) and limited success (1/7), respectively. For *N. laxa*, both intraspecific and interspecific pollinations had medium success (10/25). High success was obtained using pollen from flowers in *Anthesis* (5/6 and 6/7), and success declined when using pollen from *Wilted* and *Senescent* flowers, respectively. It was not possible to determine the viability of pollen from partially open flowers in these two species because flowers progressed from *Closed corolla* to *Anthesis* in a single day.

In "Experiment 2", levels of stigma receptivity as measured by successful fruit set were evaluated in flowers for each visual stage of floral development in five *Nolana* species (*N. aticoana, N. humifusa, N. plicata, N. ivaniana* and *N. rupicola*). Timing of receptivity was collected in the previous study for *N. laxa* and *N. adansonii*. Because of insufficient flowering, pollination success and stage duration data were unavailable for *N. elegans* and *N. rupicola*, respectively.

Fig. 2 Floral development keys for *N. adansonii, N. aticoana, N. elegans, N. humifusa, N. ivaniana, N. laxa, N. plicata* and *N. rupicola*. Photos represent visual appearance of buds or flowers at each designated floral developmental stage. Timeline above photos indicates the average stage of buds/flowers observed at 24 h intervals. Bar graphs indicate fruiting success for between three and ten pollinations performed at each stage with a compatible individual from the same species. Due to insufficient flowering during the studies, fruiting success and timeline are not available for *N. elegans* and *N. rupicola*, respectively ▶

Data obtained were compiled to create floral development keys for each species and are shown in Fig. 2. Each key has three components. Photographs of representative buds or flowers from each developmental stage are displayed with corresponding graphs of fertilization success at each stage. Timelines above the photographs illustrate durations (in days) of each developmental stage under our study conditions. Divisions in the timelines represent days of observation, with '1' being the first day of data collection and corresponding to the *Calyx* stage. Bar charts in Fig. 2 summarize stigma receptivity at different stages as measured by fruiting success. For the five species tested in "Experiment 2", complete fruiting success was obtained when flowers were pollinated during *Anthesis*. However, there was fruiting success at most other floral stages, ranging from 11/25 at *Calyx*, 22/25 at *Even*, 24/25 at *Closed Corolla*, 24/25 at *Partially Open*, 25/25 at *Anthesis*, and 21/25 at *Wilted*.


Nolana adansonii
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Calyx
Even
Closed corolla (early stage)
Closed corolla (late stage)
Partially open
Open flower
Wilted
Senescent
pollination success
0 1 2 3
0 1 2 3 4
0 1 2 3 4 5
0 1 2 3
0 2 4 6
Nolana aticoana
0 1 2 3 4 5
Calyx
Even
Closed corolla
Partially open
Open flower
Wilted
Senescent
pollination success
0 1 2 3 4 5
Nolana elegans
0 1 2 3 4 5 6 7 8 9 10 11
Nolana humifusa
0 1 2 3 4
Calyx
Even
Closed corolla
Partially open
Open flower
Wilted
Senescent
Pollination success
Nolana ivaniana
0 1 2 3 4 5 6 7 8
Calyx
Even
Closed corolla
Partially open
Open flower
Wilted
Senescent
pollination success
0 1 2 3 4 5

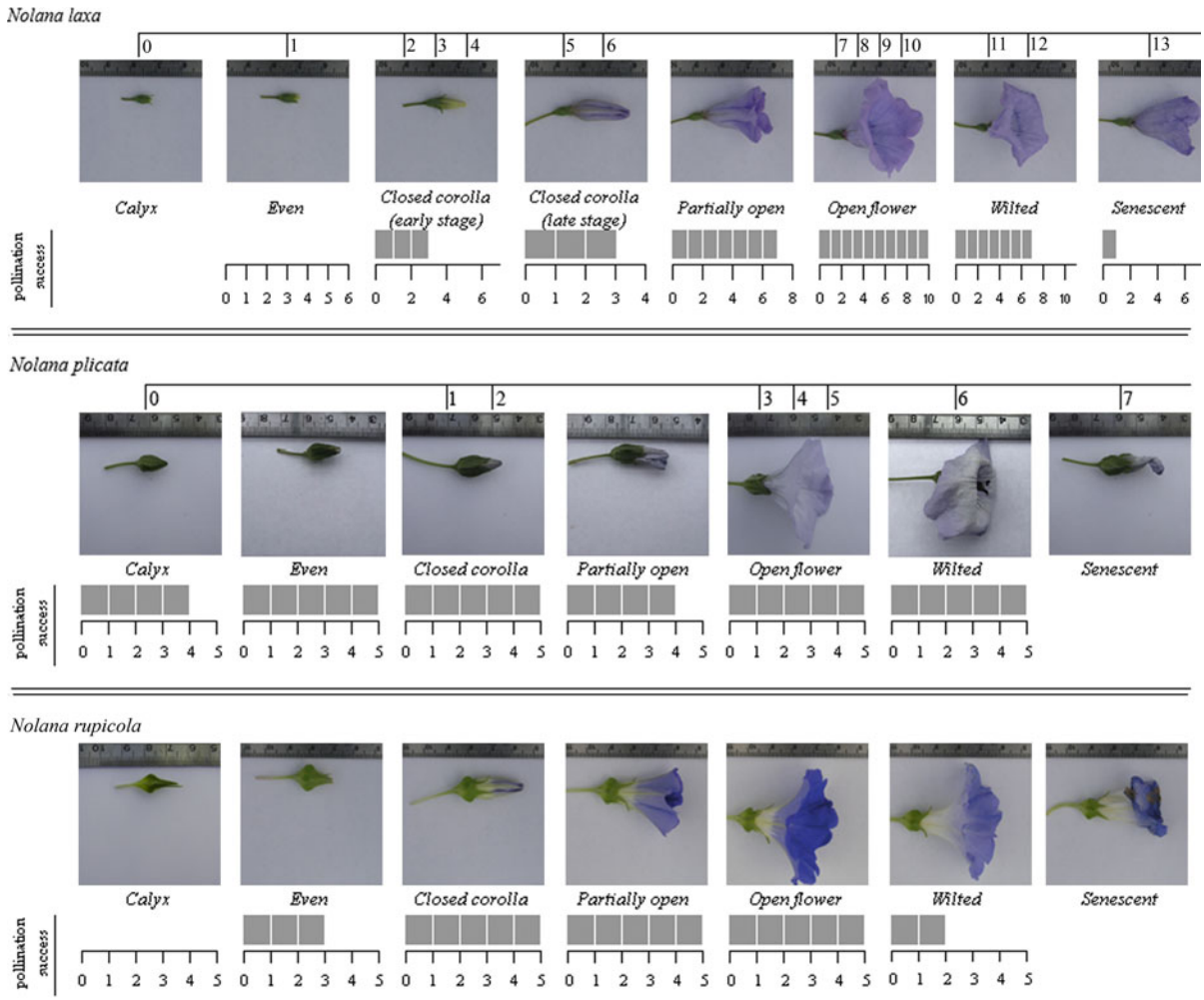


Fig. 2 continued

Discussion

Nolana is a large and diverse genus in the Solanaceae. Recently, phylogenetic relationships for 63 species of *Nolana* were constructed using partial sequences of the GBSSI or *waxy* gene (Dillon et al. 2007) and sequences of four plastid markers and the nuclear LEAFY second intron (Tu et al. 2008). The eight *Nolana* species selected for the present study were all included in the phylogenetic analysis based on combined sequences of four plastid markers. This plastid DNA tree detected two large clades for *Nolana*, one containing taxa from Chile (cp-I) and consisting of two subclades, and a sister clade with taxa from Chile and Peru (cp-II), consisting of five subclades. *N. elegans* and *N. rupicola* are included in one subclade in cp-I. The other six species in the present study are included in three different subclades in cp-II, with *N. aticoana, N. humifusa* and *N. laxa* in one subclade, *N. ivaniana* and *N. plicata* in another subclade, and *N. adansonii* in a third. It is problematic to obtain mericarps for different *Nolana* species, and furthermore germination rates are extremely low, therefore the availability of a live germplasm collection of *Nolana* is limited. The eight species selected are not representative of the whole diversity in the genus *Nolana*, however the present study provides a valuable insight into floral ontogeny from a subset of species from Peru and northern Chile, and is useful for subsequent research on compatibility between these species and breeding efforts.

Nolana species have diverse growth habit, and range from herbaceous annuals to soft wooded shrubs (Carlquist 1987; Tago-Nakawaza and Dillon 1999). Most species display showy flowers borne singly in leaf axils, ranging in color from lavender to deep blue, sometimes with bicolored white, yellow or purple throats. Although their ornamental potential is high, very few commercial cultivars of *Nolana* are available. Seed propagated 'Bluebird', 'Snowbird', 'Cliff Hanger Blue' and 'Cliff Hanger White' are all selections of *N. paradoxa* or "Chilean bellflower". Currently only two vegetatively propagated cultivars exist: 'Nolgold' (U.S. PP14,141), a hybrid of *N. paradoxa* × *N. humifusa*; and 'Loma Blanca' (U.S. PP19,450), selected by one of the authors from open pollinated progeny from an interspecific *N. paradoxa* × *N. aplocaryoides* maternal parent. There is potential to use the wide diversity in *Nolana* to breed new ornamental cultivars. Desirable traits such as the large showy flowers, compact growth habit and drought tolerance can be found throughout the genus, but cannot be found all within a single species. In order to develop ideal ornamental cultivars, it appears necessary to combine characteristics of multiple *Nolana* species through creation of interspecific hybrids. Moreover, our preliminary observations indicate that certain interspecific hybrids are sterile, which is ideal to avoid potential problems due to invasiveness.

Research on compatibility between *Nolana* species is scarce (Saunders 1934; Freyre et al. 2005). A prerequisite for studies of sexual compatibility between species and for breeding is an understanding of species' floral development. Erroneous conclusions are possible in experiments involving hand-pollinations if care is not taken to ensure pollen viability and stigmatic receptivity (Stone et al. 1995). Using only stigmas and pollen that are at the appropriate developmental stage for maximum fertilization potential allows for increased confidence that failure of a manual hybridization to produce viable seed is truly caused by sexual incompatibility rather than incorrect timing.

In the present study, floral development of eight *Nolana* species were described and subdivided into seven stages based on morphology. The duration of each stage of floral development was found to be significantly different between species. The average longevity of flowers from bud to senescent ranged from 3 and 4 days in *N. humifusa* and *N. aticoana*, to 14 and 15 days in *N. laxa* and *N. adansonii*, respectively. The first two species have rotate flowers and the duration prior to anthesis is very short, as opposed to a long period during which the corolla elongates in *N. laxa* and *N. adansonii* which have tubular-salveform flowers. Additionally, flowers remain open for 5 days in *N. laxa*, considerably longer than other species. This species is particular in that it is endemic to an extremely arid inland location in Lima, Peru. The sandy and rocky hillsides had very sparse vegetation, and the few *Nolana* plants collected were not found in close proximity to one another. A long duration of anthesis may be an evolutionary adaptation to maximize pollinator visitation and allogamy.

Timing of anther dehiscence was examined by visual inspection of buds at the earliest stage possible without disruption of the corolla. With only two exceptions dehiscent pollen was observed upon first inspection of the anthers and prior to anthesis. This indicates that emasculation at an early stage of bud development is necessary to avoid the presence of self pollen on stigmas. Our future studies of self-fertilization will identify whether *Nolana* species are self-compatible and whether emasculation is needed when performing manual pollinations.

Attempts were made to use chemical staining to determine timing of stigma receptivity and pollen viability. MTT changes color from yellow to purple in presence of dehydrogenase enzymes in stigmas and pollen, indicating that stigmas are mature and will be receptive to pollination, and that pollen is mature and viable (Rodriguez-Riano and Dafni 2000). MTT has been successfully used to identify stigma receptivity and pollen viability in diverse genera such as *Caesalpinia, Iris, Caulokaempferia* and *Oxalis* (Li et al. 2004; Sapir et al. 2005; Wang et al. 2005; Luo et al. 2006). In this study, use of MTT was unsuccessful for *Nolana* since samples from flowers at different developmental stages had similar color changes. Therefore, manual pollinations and fruit set were utilized to identify stigmas and pollen of the appropriate developmental stage for use in hybridization in this genus.

Previous female receptivity studies in *Capsicum annuum* (as judged by seed set) indicated that maximum fertility occurred on the day of anthesis, however the receptivity period lasted from 5 days

prior, to 3 days after anthesis (Aleemullah et al. 2000). Our results with *Nolana* are similar, with maximum fertilization success for all species obtained during *Anthesis*. However, all species exhibited a wide window of pollination success. Pollination success when using buds at *Calyx* stage was variable between species, failing in *N. rupicola* and *N. aticoana*. Pollination at *Closed corolla* and *Partially open* resulted in nearly complete fruit set in all species, and *Even* stage was also highly successful. In practical terms, corollas and attached anthers may be pulled from the bud at *Closed corolla* or *Partially open* stage and pollination can be performed immediately. This technique allows for efficiency in performing hybridizations while avoiding risk of self-pollination or contamination by pollinators.

Pollen viability was studied in two species with very different floral ontogeny, *N. humifusa* and *N. laxa*. Results indicated that pollen from open flowers provides maximum hybridization potential, but high levels of fruiting success were still obtained when using pollen from wilted flowers, and declined when using pollen from senescent flowers. In the two studied species it was not possible to determine the viability of pollen from partially open flowers because flowers progressed from *Closed corolla* to *Anthesis* in a single day. Pollinating with pollen from partially open flowers would be preferable to using pollen from open flowers to decrease potential contamination by pollinator activity. Additional studies are needed with other *Nolana* species to evaluate pollen performance at the *Partially open* stage.

Our floral development studies conducted on *Nolana* have successfully provided the necessary information for future breeding and hybridization studies in these species with confidence that manual hybridizations are performed at the time of highest fertilization potential. Data were compiled to create floral development keys for each species which can be used as quick visual reference to obtain species-specific information regarding durations of developmental stages and effective timing for manual pollinations. These keys provide a valuable tool for use in breeding and in studies of sexual compatibility in *Nolana*. While results may be dependant on geographic location and weather conditions, they provide a visual reference for researchers to quickly identify flowers of an appropriate stage for use in hybridizations to ensure maximum fertilization potential.

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