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Evidence of functional specialization and pollination syndrome in *Amomum subulatum* Roxb. (Zingiberaceae)

Kundan Kishore[1,*], H. Kalita[2], D. Rinchen[2] and Boniface Lepcha[2]

[1]Directorate of Research on Women in Agriculture, Baramunda, Bhubaneswar 751 003, India
[2]ICAR Research Complex for NEH Region, Sikkim Centre, Tadong, Gangtok 737 102, India

Here we study functional specialization in *Amomum subulatum* in recruiting specific pollinators and in exhibiting pollination syndrome. Among diverse assemblages of animals, only native bumble-bees (*Bombus braviceps* Smith and *Bombus haemorrhoidalis* Smith) acted as effective pollinators in terms of visitation frequency, pollination efficiency, pollination potential index, pollen delivery and fruit set, whereas *Udaspes folus* and *Macroglossum* sp. acted as nectar robbers and *Apis cerena* and *Episyrphus balteatus* were pollen-resource wasters. Native bumble-bee were the sole functional group that increased the plant's fitness by being the 'most effective pollinators'. Foraging behaviour is the most crucial factor to bring about pollination in *A. subulatum*. Medium tongue length and proficient nectar-foraging behaviour make bumble-bees the most effective pollen vectors. Low secretion rate of nectar during morning hours could be the strategy of plants to bring about pollination effectively by instigating medium-tongued nectar foragers to move deep inside the labellum and the anther–stigma column. *A. subulatum* may be categorized as an obligate specialist as it recruits only the bumble-bee as the most effective pollinator, thereby giving evidence of pollination syndrome.

Keywords: *Amomum subulatum*, functional specialization, nectar robber, pollination syndrome.

KÖLREUTER[1] and Darwin[2] had elaborated the views of plant–pollinator interaction, and gave an indication of specialization and recruitment of specific groups of pollinators by plants. Plant guilds with similar suites of floral traits might have evolved in order to attract and utilize specific functional groups of pollinators[3–5]. The markedly similar plants within these guilds are often only distantly related, suggesting independent and often convergent evolution of floral traits to match the traits of their common pollinators – one of the most visual testimonies to natural selection[6–8]. However, different pollinators promote selection for diverse floral forms giving rise to 'pollination syndrome', which is defined as a suite of floral traits, including rewards, associated with the attraction and utilization of specific 'functional groups' of pollina-

*For correspondence. (e-mail: kkhort2003@yahoo.com)

tors. Moreover, it provides a mechanistic explanation for floral diversity, i.e. convergent adaptation for specific types of pollinating agents[5].

Contrarily, most plants show moderate to substantial generalization in their pollination system and in fact visited by diverse assemblages of flower visitors that could be equally or more effective pollinators[9,10]. Studies indicate the evolution of floral traits in response to selection imposed by pollinators involving more complex adaptive pathways due to the visitation of a broader spectrum of visitors based on pollination syndrome[5,11,12]. Floral traits may function not only to facilitate pollination by the primary pollinator, but also to restrict other potential pollinators. Such traits may represent adaptations to pevent ineffective pollinators from 'wasting' pollen that would be better transferred by the primary pollinator[13]. Moreover, traits that restrict pollinators may also represent adaptive trade-offs by facilitating pollination by one type of pollinator and sacrificing pollination by another[11]. The overall fitness of a plant is actually a function of all its pollinators – most effective and less effective. This allows for complex adaptive landscapes with fitness peaks corresponding to evolutionary outcomes spanning the continuum from generalization to specialization[14–16].

Many species of the family Zingiberaceae are pollinated exclusively by long-tongued floral visitors and consequently display similar traits, such as elongated tubular flower, nectar and pollen as rewards, similar colouring and making of the flower[17]. *Amomum subulatum* Roxb. (large cardamom), a member of the family Zingiberaceae, is endemic to the eastern Himalayan region of India and is one of the important cash crops of the region. It is an allogamous sciophytic perennial shrub cultivated in the altitudes ranging from 600 to 2000 m amsl under temperate humid climate[18,19]. The reproductive biology of *A. subulatum* has been studied in detail and results have confirmed pollination by long-tongued bumble-bees[20–23]. However, besides a hint of floral specialization and pollination syndrome in *A. subulatum*[22,23], detailed studies on pollination syndrome have not been carried out. Considering the role of functional specialization of flowers in recruiting specific pollinators and exhibiting pollination syndrome, a detailed study was carried out to answer the following questions: (i) How do floral traits and rewards recruit pollinators? (ii) Whether specific bees are the only pollinators? (iii) Does functional specialization lead to pollination syndrome in *A. subulatum*?

Studies were carried out at two locations, viz. the research farm of ICAR Sikkim Centre Tadong, Gangtok (altitude 1300 m amsl; lat. 27°20′N; long. 88°04′E) and Dzongu, North Sikkim (altitude 820 m amsl; lat. 27°40′N; long. 88°44′E). The experiment was conducted during 2009–2011 on Sawney cultivar of large cardamom. The clump of Sawney produces 8–10 tillers each bearing 2–3 spikes (inflorescence) and each spike produces 30–40 bisexual flowers.

Flower morphology in terms of flower length and width, basal corolla (labellum) length (length from corolla base to end of corolla lobe), upper corolla length, corolla tube (nectar tube) length, corolla tube width, outer aperture width (distance between distal end of dorsal corolla lobes) and inner aperture width (width at the split of corolla lobes) was studied in 40 randomly selected flowers of 5 different healthy and productive clumps (a clump represents a plant) spaced at a distance of 10 m. Flower phenology (anthesis, pollen dehiscence and flower senescence) was studied on the oldest flower buds (that would open the next morning), which were bagged in the evening and observed from 0500 h till their senescence. Pollen production/flower was quantified by a hemocytometer. The number of pollen grains in each line traverse of the hemocytometer was counted using a microscope (objective magnification 10×; eyepiece magnification 10×). The grid lines of the counting graticule fitted the field of the microscope and allowed majority of the total pollen load to be counted and finally the pollen production per flower was calculated using the formula

$$N = \frac{a \times v \times 10^4}{n},$$

where N is the number of pollen grains/flower, a the mean number of pollen grains counted/corner square, v the volume of suspension made with the pollen grains, and n is the number of anthers/flower.

Pollen removal was quantified in two ways: the number of pollen grains removed per anther and proportion of available pollen removal. The number of pollen grains removed by flower visitors was worked out separately by trapping the flower visitors after a visit in polybags containing ethyl acetate. Then, the pollen grains suspended in ethyl acetate were centrifuged (RM-12C micro centrifuge) for 3 min at 10,000 rpm; the supernatant was removed and pollen grains (pallet) counted by a hemocytometer (as described above). The proportion of available pollen removal was calculated by dividing the number of pollen grains removed and total pollen available on the anther. Pollen deposition on the stigma by each visitor was estimated by collecting pollen of 15 excised flowers, which were counted by the hemocytometric method.

Flower visitors were trapped in a net and taxonomically identified. Tongue lengths were measured after relaxing the pollinators in a humid jar for 2 days, which allows the tissue to soften and the tongue to be pulled out. Quantitative studies – visitation frequency and foraging time of floral visitors – were carried out by selecting 40 flowers from 5 clumps of large cardamom which were clearly visible from an observation site and observations were recorded from 0600 h (soon after anthesis) until 1700 h (when visitations ceased) for 12 days (6 days each in April and May). The total monitoring duration was about 120 h. The foraging habit of visitors was studied on the basis of their landing pattern, approach towards floral rewards (pollen and nectar) and probability of pollinating the stigma. The approximate pollination potential index (score 0–1) was calculated for each visitor group following the method of Jacob *et al*.[24]. It was assumed that a score closer to 1 reflected greater relative contribution of visitors to pollination.

To ascertain pollination efficiency for all the visitors, the flowers were divided into seven groups on the basis of six visitors and an open-pollinated group. Based on solitary visit by animals onto flowers, pollination efficiency was worked out considering the number of pollinated stigma. The frequency of visitors was monitored by allowing them to land only once on a flower. Stigmas of 40 flowers in each group were examined for the presence of pollen grains under stereo-zoom microscope (Leica), whereas in open-field condition, 100 stigmas were randomly examined for the presence of pollen at 1600 h.

Nectar content was measured by a micropipette (Ependorf 1–100 μl) at hourly intervals (0600–1700 h) by excising ten flowers and leaving the nectar tube intact ($N = 100$). The nectar sugar content was estimated by hand refractometer (ERMA, 28-50°Brix). Hourly change in temperature and relative humidity (RH) during the study period was recorded using portable weather tracker (Kestrel 4000). Data were subjected to analysis of variance (ANOVA) and Duncan multiple range test (DMRT) at $P \leq 0.05$ to compare the means of variables using SPSS statistical package (11.5 version). The correlation test among variables was done by Pearson's correlation (r).

Floral phenology showed tempospatial variation[20–23]. The study indicated that at both the sites flowering seasons lasted from early April to late May, with peak flowering occurring between mid-April and mid-May. Anthesis began early in the morning with simultaneous dehiscence of the anther. The outer corolla aperture showed sequential outward movement and attained its maximum size (44.5 ± 3.8 mm) after 4 h of anthesis, whereas the inner corolla aperture did not show any temporal variation. The corolla tube which contains nectar and nectaries at its base was more than 30 mm long. Polleniferous flower had significantly more pollen-bearing area than the stigma and the plant produced a large number of pollen grains (51,780 ± 8,982). Pollen grains were spherical, large, echinate and easily accessible to visitors[22]. A small cup-shaped stigma located just above the anther exhibited peak receptivity between 0700 and 1000 h. Large cardamom secreted ample nectar (64.5 ± 9.6 μl/flower) for the visitors with significant temporal variation (one-way ANOVA, $F_{6,28} = 0.285$). However, nectar production was significantly low in the early morning hours at both the sites, which increased gradually and reached maxima at 1400 h (Figure 1).

Flowers of *A. subulatum* attract six different visitors belonging to different groups: bumble-bee (*Bombus braviceps* Smith and *Bombus haemorrhoidalis* Smith), honey bee (*Apis cerena*), hover fly (*Episyrphus balteatus*) moth (*Macroglossum* sp.) and butterfly (*Udaspes folus*) that varied morphometrically[21,23]. There was no variation in the composition of visitors at the two sites; however, their visitation frequency varied significantly. *B. braviceps* and *B. haemorrhoidalis* showed temporal variation in their prevalence[21]. *U. folus* and *Macroglossum* sp. had relatively large body size and long tongue compared to *B. braviceps* and *B. haemorrhoidalis*, whereas *A. cerena* and *E. balteatus* had a significantly small body and tongue length (Table 1).

Visitation frequency and foraging time of visitors varied significantly with time slots (one-way ANOVA, $F_{6,72} = 0.346$). However, foraging behaviour differed. Bumble-bees started visiting flowers in the early morning and continued till 1700 h (Figure 2). Their frequency of visits gradually increased with the advancement of the day, reached a maximum between 0900 and 1000 h and decreased thereafter. The average visitation frequency and foraging time of bumble-bees were significantly more than those of other visitors (Figures 2 and 3). Each flower received more than 40 bumble-bees with the cumulative foraging time of 124.63 s. Visitation frequency and foraging time of *U. folus* and *Macroglossum* sp. was minimum. *A. cerena* exhibited a tendency of high foraging in the early morning, which decreased thereafter and no bee was observed after 1300 h. Visitation frequency and foraging time of visitors did not show significant correlation with nectar secretion. However, a positive and significant correlation was observed between nectar secretion and day temperature ($r = 0.607^*$ $P \leq 0.05$).

Interestingly, foraging habit of flower visitors was different. *B. braviceps* (Figure 4 *a*) and *B. haemorrhoidailis* were primarily nectar foragers; in the course of foraging they first landed on the labellum and moved deep inside the flower column to forage nectar. Whereas *A. cerena* (Figure 4 *b*) and *E. balteatus* (Figure 4 *c*) were pollen collectors and their foraging behaviours were different than that of bumble-bees. *A. cerena* collected more pollen than *E. balteatus* because of the pollen basket. *U. folus* (Figure 4 *d*) and *Macroglossum* sp. were also nectar foragers, but they collected nectar from their long tongue without moving inside the flower column.

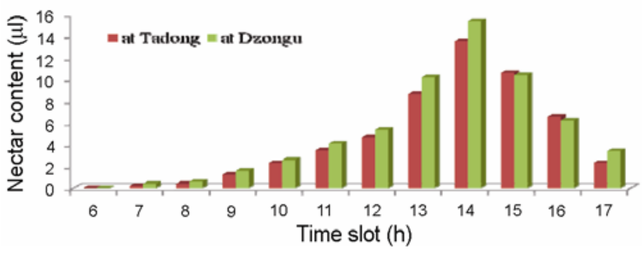

Figure 1. Temporal variation in quantity of nectar at two sites in *Amomum subulatum*.

Table 1. Comparative morphometry of flower visitors of *Amomum subulatum*

Flower visitors	Body length (mm)	Body width (mm)	Tongue length (mm)	Pollination efficiency (%)
Bombus braviceps	31.6 ± 3.9[c]	14.4 ± 2.6[b]	14.9 ± 1.4[c]	78.5[a]
Bombus haemorrhoidalis	28.7 ± 2.6[d]	13.8 ± 1.9[b]	14.2 ± 1.1[c]	74.8[b]
Apis cerena	21.4 ± 1.3[e]	5.4 ± 0.5[c]	5.8 ± 1.2[d]	4.6[c]
Udaspes folus	41.2 ± 2.8[a]	25.4 ± 3.6[a]	44.8 ± 3.4[a]	3.4[c]
Macroglossum sp.	36.6 ± 2.1[b]	14.5 ± 2.4[b]	34.4 ± 2.8[b]	2.9[cd]
Episyrphus balteatus	15.4 ± 1.3[f]	3.7 ± 0.3[c]	4.9 ± 0.8[d]	2.2[d]

Mean values in each column with the same letter are not significantly different by Duncan's multiple range test at $P \leq 0.05$.

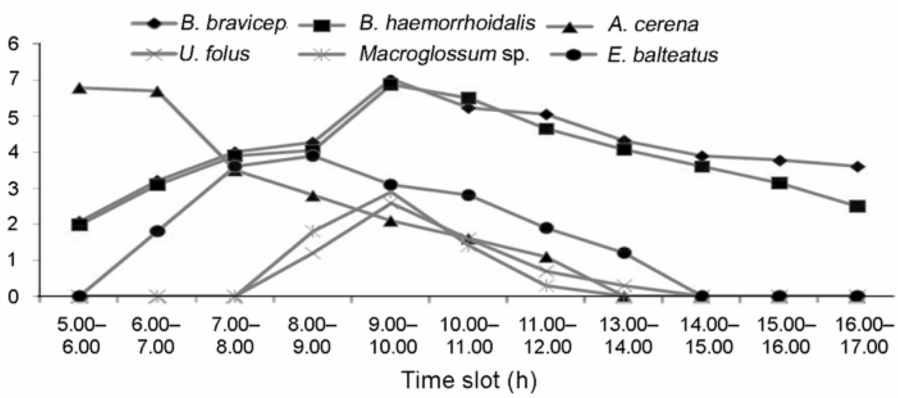

Figure 2. Temporal variation in visitation frequency of flower visitors of *A. subulatum*. Data are the mean of two sites.

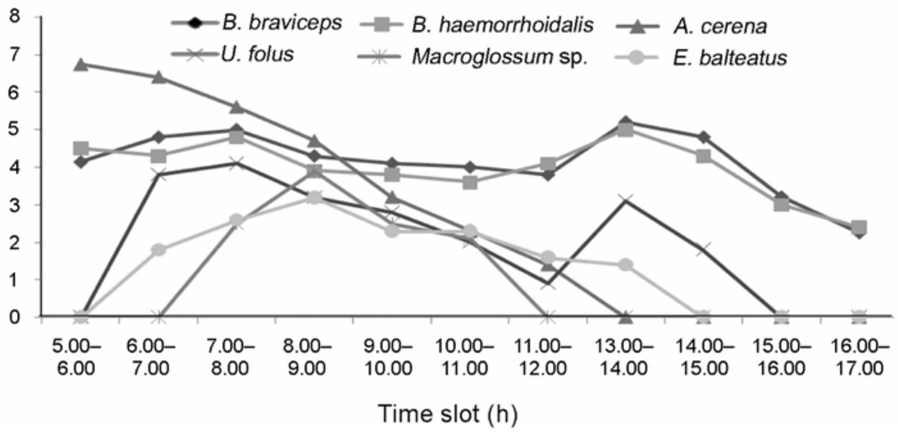

Figure 3. Temporal course of foraging time of flower visitors of *A. subulatum*. Data are the mean of two sites.

Data showed significant variation in the pollination efficiency of flower visitors (one-way ANOVA, $F_{6,78}$ = 0.367). Bumble-bees were found to be highly efficient in carrying out pollination in *A. subulatum* with an efficiency of about 75% (Table 1). However, the efficiency increased with subsequent visits and all the flowers were pollinated with three visits. On the contrary, the pollination efficiency of other visitors, including *A. cerena* was less than 5%. Under open pollinated condition the pollination rate was more than 50%, which gives an indication of bumble-bee pollination. The findings agree with the results of Sinu and Shivanna[20]. Pollination efficiency was not significantly correlated with body size (r = 0.386) and tongue length (r = 0.376) of the visitors.

Bumble-bees and honey bees tended to remove high quantities of pollen, whereas the pollen removal rate was significantly low for the other visitors (Table 2). Bumble-bees removed more than 50% of the pollen in a visit, whereas honey bees removed more than 40% of the pollen. In spite of high pollen removal, the honey bees could hardly deposit pollen on the stigma. On the contrary, bumble-bees showed high pollen removal and deposition rate. Hover fly, butterfly and moth neither removed pollen in considerable amounts nor deposited substantial

Table 2. Pollen removal, deposition and pollination potential (PP) index of flower visitors of *A. subulatum*

Flower visitors	Pollen removal/visit	Proportion of pollen removal (%)	Pollen deposition on stigma	Proportion of removed pollen deposited on stigma (%)	Number of visits during an observation period	PP index score
B. braviceps	31,545 ± 3524[a]	58.9 ± 4.9[a]	2,758 ± 196[a]	8.7 ± 0.9[a]	443.5 ± 54.6[a]	0.84 ± 0.08[a]
B. haemorrhoidalis	28,856 ± 3146[b]	53.6 ± 4.1[b]	2,246 ± 158[b]	7.6 ± 0.8[b]	386.4 ± 43.8[b]	0.76 ± 0.06[b]
A. cerena	21,124 ± 2465[c]	41.7 ± 2.8[c]	89 ± 12.3[c]	0.4 ± 0.03[d]	169.7 ± 11.4[d]	0.08 ± 0.01[c]
U. folus	2,039 ± 242[e]	4.8 ± 0.42[d]	22 ± 3.4[d]	0.8 ± 0.07[d]	39.7 ± 5.7[e]	0.01[d]
Macroglossum sp.	2,954 ± 292[d]	6.1 ± 0.53[d]	78 ± 11.6[c]	2.3 ± 0.02[c]	47.8 ± 6.8[e]	0.02[d]
E. balteatus	1,219 ± 193[f]	2.2 ± 0.21[e]	32 ± 3.9[d]	2.8 ± 0.03[c]	117.6 ± 32.4[c]	0.01[d]

Mean values in each column with the same letter are not significantly different by DMRT at $P \leq 0.05$.

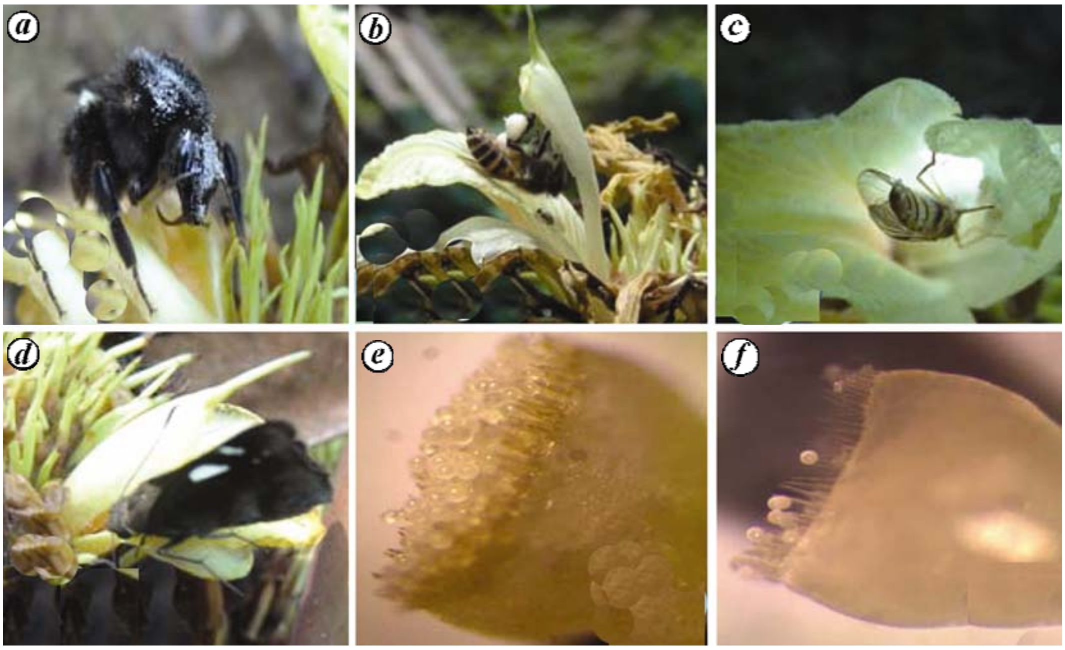

Figure 4. Foraging behaviour of visitors. ***a***, *Bombus braviceps* moving inside a flower to forage nectar. ***b***, *Apis cerena* engaged in pollen collection. ***c***, *Episyrphus balteatus* collecting pollen. ***d***, *Udaspes folus* forages nectar using its long tongue. ***e***, Stigma cup filled with pollen in *B. braviceps* and *Bombus haemorrhoidalis*-visited flowers. ***f***, Few pollen grains adhered onto non-receptive stigma hairs in *A. cerena*, *E. balteatus*, *U. folus* and *Macroglossum* sp.-visited flowers.

number of pollen grains on the stigma. Bumble-bees were found to be effective pollinators by virtue of delivering relatively higher number of pollen grains on the stigma even though the percentage deposition was less than 10%. The mode of pollen delivery showed spatial difference with the pollinators. In bumble-bee-visited flowers, pollen grains were delivered inside the receptive cup of the stigma, which will in turn affect fertilization (Figure 4 *e*), whereas other visitors deposited pollen around the non-receptive stigma hairs present on the margin of the stigma cup (Figure 4 *f*). Bumble-bees exhibited the highest pollination potential (PP) index score due to high pollen load and visitation rate (Table 2). The significantly low PP index score of *A. cerena* in spite of high pollen load was attributed to the low visitation rate. The low PP index of hover fly, butterfly and moth has been ascribed to significantly low pollen load and visitation rate. The contribution of visitors to fruit set clearly indicates the indispensible role of native bumble-bee by ensuring high fruit set (Table 3). However, spatial variation was also observed.

Differences in foraging behaviour and morphometry often result only in a few functional groups actually visiting a flower to pollinate it and they exert selective pressure on the floral form[13]. Given such variation between pollinators, it is understandable that selective pressure would favour floral specialization on only one functional group out of all the animals that visit a flower[25]. In *A. subulatum* bumble-bees are the sole functional groups that increases the plant's fitness by being the most effective pollinators[20–23]. Moreover, pollination in *A. subulatum* seems to be the function of floral form (tubular), foraging behaviour and body size of the bumble-bees. We adhere to the view that many floral traits reflect adaptive responses to selection by the pollinators, and that the direction of selection is a function of the properties of pollinator morphology and behaviour.

Table 3. Contribution of visitors to fruit set (capsule) in large cardamom at two sites

Flower visitors	Fruit set (%)	
	At Tadong	At Dzongu
B. braviceps	38.3 ± 2.4[a]	42.9 ± 3.4[a]
B. haemorrhoidalis	34.6 ± 2.8[b]	37.7 ± 2.3[b]
A. cerena	1.2 ± 0.3[c]	0.9 ± 0.2[c]
U. folus	0.0[c]	0.0[c]
Macroglossum sp.	0.0[c]	0.0[c]
E. balteatus	0.0[c]	0.0[c]

Mean values in each column with the same letter are not significantly different by DMRT at $P \le 0.05$.

Generalists attract a number of animal species for pollination, whereas specialists use a few or just one for pollination[15]. Large cardamom attracts diverse assemblages of animals; however, only native bumble-bees act as effective pollinators in terms of visitation frequency, pollination efficiency, PP index and pollen delivery others act either as a pollen robbers[20] (*A. cerena*) or nectar robbers (*U. folus* and *Macroglossum* sp.).

Studies clearly indicated that tongue length and body size of pollinators were not the most crucial factors for being the most effective pollinators, but the foraging behaviour. Visitors with long tongue (*U. folus* and *Macroglossum* sp.) were unable to pollinate as their long tongues do not allow them to move inside the corolla column; it restricts them to forage nectar simply by landing on the labellum, thereby minimizing the chance of body–stigma contact. Additionally, the visit of long-tongued animals (*U. folus* and *Macroglossum* sp.) was correlated with the movement of the upper corolla aperture and they prefer to visit after 0800 h when the labellum and anther–stigma column is wide enough to land properly with minimal chance of body–stigma contact. On the other hand, the foraging behaviour of short-tongued animals (*A. cerena* and *E. balteatus*) increases the probability of pollination due to their pollen-collection behaviour. But they deliver pollen mostly on the non-receptive stigma hairs and thus waste pollen resource. Bumble-bees were nectar foragers and on the basis of their tongue length they may be grouped under medium-tongued animal. In our view, the medium tongue and nectar foraging habit of bumble-bees instigate them to push their large body deep inside the labellum and anther–stigma column to forage nectar and consequently, pollen grains adhered on thoracic region fill the stigma cup. Moreover, bumble-bees start their search for nectar right from anthesis when the labellum and anther–stigma column are narrow enough to ensure maximum probability of brushing of stigma with the pollen-adhered thoracic region of the bees. Had bumble-bees possessed a long tongue, they would have behaved like *U. folus* and *Macroglossum* sp. and would not have been the most effective pollinators of large cardamom. It seems that flower architect and reward in the form of nectar make flowers of large cardamom specialized[23].

Large cardamom exhibits temporal variation in nectar secretion with the advancement of the day. We perceive that low secretion rate of nectar during morning hours could be the strategy of plants to compel medium-tongued nectar foragers to move deep inside the labellum and anther–stigma column to bring about pollination effectively.

Floral traits of *A. subulatum* show obligate specialization[26] by filtering only bumble-bees as the most effective pollinators and therefore, clearly indicate pollination syndrome for large, medium-tongued, nectar foragers. Except bumble-bees, no visitor contributed differentially to the selective pressure exerted via the reproductive success of the plant. Moreover, floral traits respond differentially to selective pressure and contribute more to functional specialization and in turn pollination syndrome. *A. subulatum* follows the most effective pollinator principle[27], since it is specialized on the most effective and most abundant pollinator, when pollinator availability is reliable.

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Genetic diversity in unique indigenous mango accessions (Appemidi) of the Western Ghats for certain fruit characteristics

C. Vasugi[1,]*, M. R. Dinesh[1], K. Sekar[2], K. S. Shivashankara[1], B. Padmakar[1] and K. V. Ravishankar[1]

[1]Indian Institute of Horticultural Research, Hessaraghatta Lake Post, Bangalore 560 089, India
[2]Department of Horticulture, Annamalai University, Annamalai Nagar 608 002, India

Mango is one of the choicest fruit crops of the tropical and subtropical regions in the world. Utilization of the conserved germplasm in breeding programmes requires precise information on the genetic relationships between the accessions. Considering the difficulties involved in the traditional divergence studies based on morphological characterization, microsatellites were successfully used for genetic diversity analysis of the indigenous 'Appemidi' type. Also, the major compounds that contribute to the unique aroma of these types were estimated. The materials used in the study consisted of 43 accessions and 14 SSRs developed at the Indian Institute of Horticultural Research, Bangalore. Analysis of sap volatiles was done using GCMS fitted with a DB-5 MS column using helium as the carrier gas. The analysis of 211 bands detected by the 14 Simple Sequence Repeats (SSRs) markers showed unambiguous discrimination of the 43 mango genotypes. The dendrogram resulted in the grouping of accessions into two major clusters, viz. cluster I with highly acidic types and cluster II with less acidic and high TSS group. The aroma of pickle type of mangoes is due to totally different type of terpenes as well as a completely different combination of monoterpenes.

Keywords: Appemidi, aroma compounds, characterization, diversity, mango.

MANGO (*Mangifera indica* L.) is one of the choicest fruit crops of the tropical and subtropical regions in the world. Its popularity and importance can easily be realized by the fact that it is referred to as the 'king of fruits' in the tropical world. Utilization of the conserved germplasm in the breeding programme requires precise information on the genetic relationships among the accessions. Information on the genetic distance among the germplasm accessions will also help avoiding duplicates, thus clearing the nomenclature ambiguity, widening the genetic base of the core collections and ultimately helping in preserving the valuable diversity. Considering the difficulties involved

*For correspondence. (e-mail: vasuc@iihr.ernet.in)