Conservation of *Beclardia macrostachya*

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Abstract

Beclardia macrostachya is a critically endangered orchid species in Mauritius. Very low level of fruit set has been attributed to contribute to the decline in its population. Surveys carried out in the different forest regions reveal the occurrence of this orchid at only one location, Pigeon Wood, with a present population of only 75 individuals. Biological studies at Reunion Island reveal preferential adaptation to higher altitudes, evidenced by observed differences in fertility rates at different forest areas. Taxonomic studies using morphological characters suggest the placement of *Beclardia macrostachya* within a group that includes *Cryptopus elatus* and *Aerides lawrenceae*. When cultured in vitro, leaf explants developed nodular mass which failed to develop into protocorm-like bodies (PLBs). However, embryo rescue proved to be an efficient way for micropropagation of this orchid with a high regeneration capacity. Breeding programs carried out in Mauritius yielded capsules and the latter were successfully cultured to generate PLBs which would subsequently grow into plantlets.

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1. INTRODUCTION

Due to encroachment of the natural reserves and forests it has become important to conserve our genetic wealth before it is irretrievably lost. Most of the countries rich in

biodiversity have gained rights to regulate access to their germplasm and prevent bio-piracy. With present ecological concerns pertaining to plant protection rights, more emphasis need to be placed on conservation and also protection of plants from poaching. Effective management of germplasm posits assessment and monitoring of the existing genetic diversity, formulation of what should be conserved on a priority basis and, evaluation of the genetic relationships between different taxa. The conservation of any taxon requires information about the ecogeographic structure of the target taxon and such data should include accepted taxon name (taxonomic and vernacular), target area where reported, flowering and fruiting timing, habitat preference, topographical preference, soil performance, geological preferences, climate and micro-climate preferences, breeding system, genotypic and phenotypic variation, biotic interactions, archeological evidence, ethnobotanical evidence and conservation status (Maxted & Guarino, 1997).

In Mauritius, 89 orchids have been described of which nine are endemic. Threatened by habitat destruction and alterations some orchid species have been considerably reduced in number. *Beclardia macrostachya* is one such orchid, which was estimated to just 15 individuals in the wild (Roberts, 2001). This orchid is only found in Mauritius, Réunion Island and Madagascar. The flowers of this plant are very showy with a short spur, white perianths and the labellum has a green-yellowish tinch, which turns yellowish orange as the flower develops. Racemes are produced with 8-12 flowers and these flowers persist on the axis for a month. The inflorescence axis is persistent and remains green for up to 3 months. This orchid can serve as an ornamental and the inflorescence can be marketed as cut flowers.

Two known individuals were reported from the wild (per. Comm., National Parks and Conservation Services). Such a low population of *Beclardia macrostachya* required urgent measures for the development of conservation strategies for propagation of this orchid. Very low level of fruit set was recorded, and hence *in vitro* techniques were opted for its propagation. Along with *Beclardia macrostachya*, *Angraecum cadetti*, *Angraecum eberneum* and *Hederorkis scadens* are the other rare orchids found in Mauritius. Very little research was done on the biology of these orchids in Mauritius (Roberts, 2001). Therefore, the biology of *Beclardia macrostachya* was studied and consequently, appropriate strategies would be devised for its conservation.

There is only one known species under the genus *Beclardia* which is *Beclardia macrostachya*, and several synonyms were attributed to this species by earlier taxonomists which were *Aeranthes brachystachys, Aeranthes macrostachys, Aerides macrostachyum, Beclardia brachystachya, Beclardia erostris, Epidendrum brachystachyum,Epidendrum macrostachys, Oeonia erostris, Oeonia macrostachya* and *Rhaphidorhynchus macrostachys.* The synonyms were attributed to at least seven different genera and most of these genera were distributed in the subtribes Angraecinae and Sarcantinae of the tribe Vandeae. Hence, the taxonomic placement of this orchid within the tribe Vandeae had to be re-defined.

Tissue culture is an essential tool for *ex situ* conservation. Instead of growing plants in the field which will require vast space, tissue culture provides a much more efficient way

of conserving the species. Seeds can be stored, or propagules (embryogenic callus, somatic embryos) can be maintained in labs. Thus, distinct genotypes can be maintained as distinct clones using *in vitro* techniques. Newer techniques such as cryopreservation have been developed such that the clones can be maintained for even longer periods and these preserved clones can be grown again whenever needed. Cryopreservation protocols have been developed for a few orchids e.g. such as *Doritaenopsis* (Tsukazaki, 2000) and *Bletilla striata* (Ishikawa, 1997). *In vitro* culture also provides plausible solutions to propagation of plant species that show low fruiting success, those which have non-viable seeds, and as a means to overcome seed dormancy. The use of vegetative parts (leaves, shoots, roots, nodal segments) to propagate plants defines the applicability of this technique to conservation and propagation.

2. MATERIALS AND METHODS

2.1 Criteria for Biological studies

The main aspects considered for understanding the biology of this orchid were the study of the population dynamics, spatial distribution, performance of the species and the phenology and breeding system of this orchid. Each plant found was tagged and its proper location was defined on a map. Surveys were carried out in the different forest areas of the country and also in Reunion Island. At Reunion Island surveys were carried out (in transects) at Redalliers, Tacamaca, Eden and Bebours. Performance of the species at the different forest areas was assessed based on leaf lengths and fertility rates. Breeding system was evaluated through manual pollination.

2.2 Analysis of morphological markers.

The choice of related species to be studied were based on the synonyms that were attributed to *Beclardia macrostachya* by the earlier taxonomists and also, from morphological similarities that the different orchid exhibited with other orchids. However, the choices were quite biassed towards angraecoid orchids because *Beclardia* showed much more affinities to these orchids. Foreign species like *Aerides lawrenceae, Aeranthes grandiflora, Epidendrum radicans* were kindly provided by orchid growers from their personal collection. The *Angraecums , Jumellea sp., Aeranthes arachnites and Beclardia macrostachya* were all obtained from the forest areas that were managed by the National Parks and Conservation Services. *Oeniella aphrodites* and *Cryptopus elatus* were obtained from the Forestry Department of Mauritius (Ministry of Agro-Industry and Fisheries). Most of the macromorphological characters were assessed and noted down in the field itself but the flowers and inflorescence were preserved in 60% alcohol and brought to the lab for microscopic observations. The flowers were carefully dissected and observed under the binocular microscope. A list of all the characters has been given in table 2. However, the foreign species *Oeonia* and *Rhyncostylis*, which should have been included in this study, could not be obtained. All the characters were treated as independent, unordered and of equal weight. A binary data matrix was constructed and phylogenetic analysis was performed using PAUP Version 4.0b10. A dendrogram generated by UPGMA option of the PAUP. *Epidendrum radicans* was used as outgroup

2.3 Culture media and Plant growth regulators (PGRs)

The culture media that was optimized was a modified Murasige and Skoog's medium (1962). The macro elements concentrations were equivalent to MS, but the microelements was reduced to half of the actual MS concentration. The medium was supplemented with glycine, myo inositol, thiamine HCl, Nicotinic acid, pyridoxine HCl. Other additives to this modified MS included Sodium di-hydrogen phosphate, peptone, and coconut water. The pH was kept according to the different explants being used. The solidifying medium used was phytagel. The different plant growth regulators (TDZ, BAP and NAA) were used singly or in combinations at different concentrations.

2.4 Explants

With the limited number of plants available in the field, the experiments were carried out very meticulously, avoiding any risks of depleting the available plant material. It should be noted that at the beginning of the work only two plants were available in the field, and it only later after thorough field surveys that 36 plants were discovered and finally more were found at Pigeon Wood. Each plant had only four to six leaves, and the orchid being monopodium, no branching was present. Care was taken so that the available plant materials were not depleted, and only few plants were used for tissue culture. From each plant a maximum of only 2 leaves were used for tissue culture, and shoot tip culture was not used because of the risk of losing one whole plant when only so little were available. Also it is the leaves that keep track of the flowering season of a plant and hence minimum explants available for tissue culture experiments. At least two replicates of each experiment were also carried out, so that after each experiment, the results could be better analysed. Immature capsules were much later obtained from Reunion Island and, these were then used.

3. RESULTS

3.1 General Distribution of the orchid

The preserved specimen of *Beclardia macrostachya* (MSIRI national herbarium) was analysed. It has been reported that the plant is herbaceous with an epiphytic habit, and were commonly occurring on the trunk of *Labourdonnasia sp.* They were quite common in the areas near Grand Bassin, Bois Sec and Chemin Cheval. They were also reported from Plaines Paul and Pigeon Wood. Since all the specimens bearing flowers were collected in the months of March and April, it could be said that these were the months when this orchid flower. None of the Mauritian dried specimens at the herbarium showed capsules but specimens from Reunion Island present at the herbarium showed capsules. The field surveys were carried out at Pigeon Wood, Bois Sec, Les Mares, Combo, Bassin Blanc, Mont Verts and Bel Ombre but no other individuals were found.

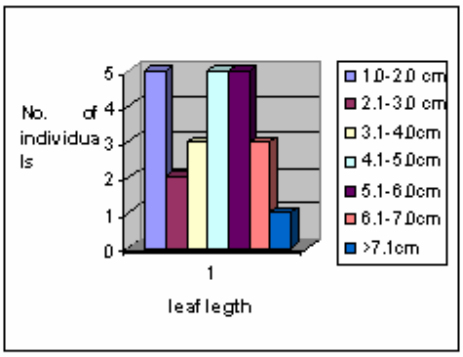


Fig. 1.1
bar chart to show leaf length of the different individuals at Pigeon wood

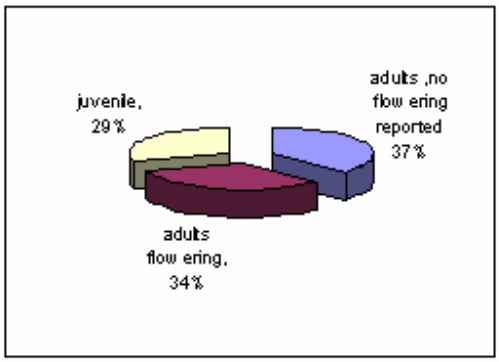


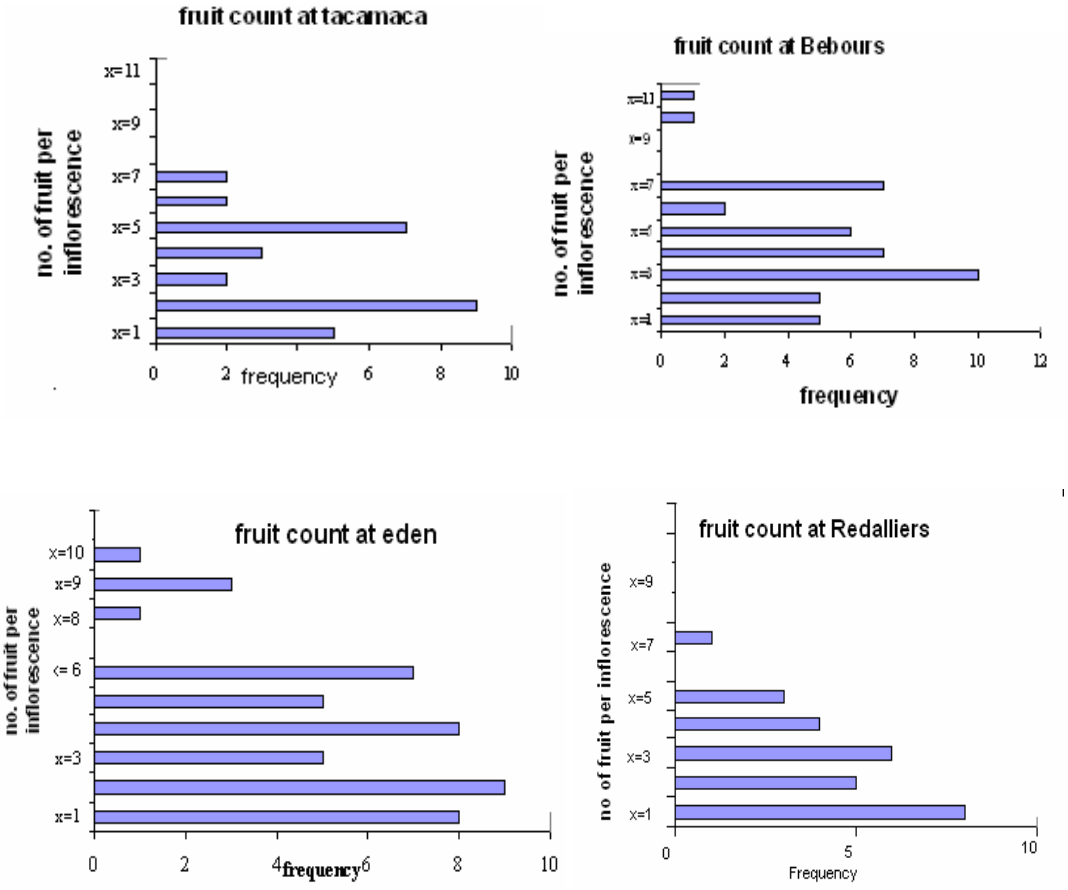
Fig 1.2
pie chart to show number of adult and juvenile individuals

Pigeon wood represents the only forest area in Mauritius where this orchid is found, and the present population consist mainly of adult individuals (71%), indicating a disturbed regeneration capacity in the past years. The population of *Beclardia macrostachya* at the different Forests in Réunion Island was not uniform. Very abundant and dense populations were observed at Bebours and Eden and more scattered populations at Redalliers and Tacamaca. Surveys were carried out at these 4 forest areas and detailed observations made. The individuals from the higher altitude regions of Reunion Island were quite robust compared to the Mauritian type. The length of the main axis was also much longer, and the fertility rate extremely high. It was astonishing to see such flourishing populations. The dominant host plant for *Beclardia macrostachya* were woody tree species, and moss and thallose lichens were very common near the roots of *Beclardia.*

3.2 Performance of Beclardia macrostachya at Réunion Island

Site	Redalliers	Tacamaca	Eden	Bebours
Altitude	540- 600m	600-800m	1400m	1800m
Fertility rate (%)	36.45	44.1	62.4	62.5
Leaf length (cm)	9.86 ± 2.34	10.2 ± 3.55	8.2 ± 2.44	10.2 ± 2.73

Compared to Mauritius where no fruiting was recorded for the past years, higher fertility rates were obtained at Réunion Island. Higher fertility rates were observed at the higher altitude forests (Bebours and Eden) while, the lower altitude forests areas at Redalliers and Tacamaca revealed lower fertility rates.



Histograms to show fruit counts at the four different forest areas in Reunion Island. Following a Kruskal–Wallis test, the fruit counts in the different areas were found to be significantly different. Fruit count will be dependant on pollination success and available resources of the plant to bear the developing capsules. However, for the floral count no significant differences were observed indicating this character was more dependant on the genotype, rather than environmental effects.

3.3 Taxonomic status

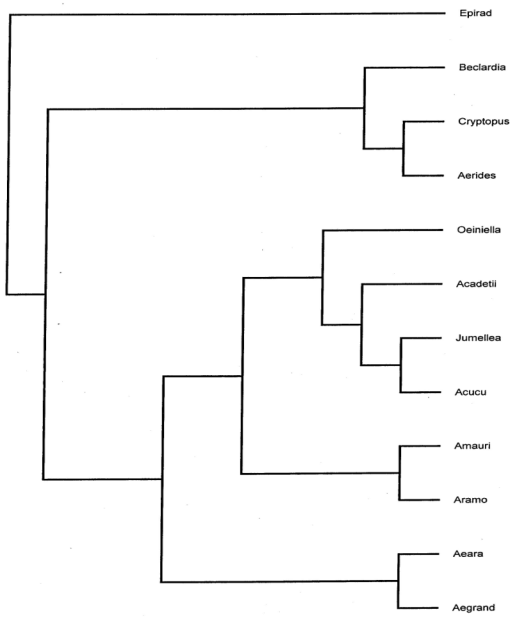
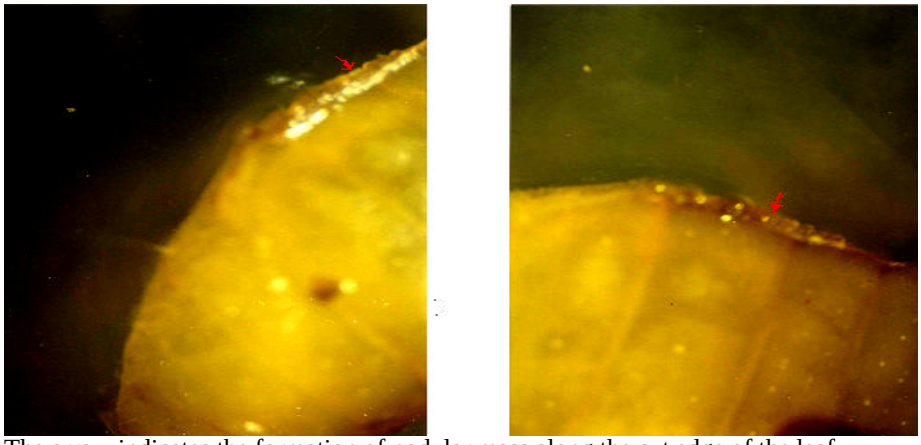


Fig. 1.4

From the data matrix obtained from morphological characters, a dendrogram with 4 clades was generated. Three distinct subgroups were obtained, one that clustered *Beclardia macrostachya*, *Cryptopus elatus* and *Aerides lawrenceae* together. A second group clustered the 2 species of *Aeranthes* together and a third group clustered all the other taxa, excluding *Epidendrum radicans* which was used as outgroup. The four *Angraecum* species did not cluster together and the placement of *Jumellea fragrans* and *Oeniella aphrodites* is debatable.

Fig.1.4 represents a Dendrogram generated using 32 morphological markers (listed in table 2). Key names used: Epirad: *Epidendrum radicans*, Beclardia (*Beclardia macrostachya*), Cryptopus: *Cryptopus elatus*, Aerides: *Aerides lawrenceae*, Oeniella: *Oeniella aphrodites*, Acadetti: *Angraecum cadetti*, Jumellea: *Jumellea fragrans*, Acucu: *Angraecum cuculatum*, Amauri: *Angraecum mauritianum*, Aramo: *Angraecum ramosum*, Aera: *Aeranthes arachnites*, Aegrand: *Aeranthes grandiflora*.

3.4 Tissue culture response



The arrow indicates the formation of nodular mass along the cut edge of the leaf

PLB formed from immature embryos

Culture media with and without activated charcoal

Leaf explants from the field when sterilised and cultured in solid and liquid media, showed difference in infection rates. While the rate of infection was comparatively very low in solid medium, the leaf explants showed higher level of infections with the liquid medium turning turbid within a week. TDZ at a concentration of 1.0mg/L gave the best response but only after the same leaf explants were transferred to 0.3mg/L TDZ concentration. Level of TDZ above 1.5mg/L showed higher necrosis and below 1.0 showed slightly higher survival rates but the explants eventually turned brown. Out of the NAA and BAP concentrations that were tried out, combination of 0.5 mg/L BAP and 1.0 mg/L NAA gave the best response, i.e. production of nodular mass. Tissues with

nodular mass from solid media were also transferred to liquid media with same PGR combinations, 1.0 mg/L TDZ and combination of BAP (0.5mg/L) and NAA (1.0mg/L), but the explants browned within a week. The globular structures (nodular mass) were obtained and only after this stage that, development of protocorm like bodies (PLBs) occurs, which later give rise to plantlets.

The leaf explants taken from the fields gave response within 25 days after inoculation. But not all segments from the leaves showed the response. Only the segments closer to the leaf base produced nodular mass. Segments taken from the leaf blade and leaf tips necrosed within a week after inoculation. Older leaves taken from the plants necrosed , and only the leaf closest to the leaf primordia showed response , i.e. the very young leaf that are newly formed. Nodular mass arose from the cut edges of the leaf explants. A few leaf explants did not necrose for more than 30 days, but slowly the green colour disappeared turning the explants greyish and these explants remained dormant.

Immature capsules from Mauritius and Reunion Island have been successfully used to generate PLBs. The initial PLB formation from the immature embryos were obtained in half MS (1962) supplied with a low dose of TDZ (0.1mg/L) and present work is being carried out for the development of plantlets from these PLBs.

4. DISCUSSION

It is often assumed that healthy populations follow a J shaped distribution where adults are outnumbered by saplings, while populations with few juveniles relative to adults are in decline. In Mauritius, 29 % of the individuals are juvenile and in the past two years no fruiting success has been recorded. This clearly indicates that the population is tending towards a rapid decline, and unless conservation strategies are developed this orchid will be extinct from Mauritius. *Beclardia macrostachya* was found to be self and cross pollinating, but requires a suitable vector for pollination**.** The low fruiting success can be attributed to absence of a suitable pollinator. The first breeding experiment carried out in Mauritius resulted in shedding of flowers within a week after pollination which could be accounted by the following reasons(i) the climatic conditions prevailing were not favourable for capsule development (ii)the plant was competent to bear flowers but not physiologically competent to bear fruits (iii) the removal of pollinia from the flowers might be triggering a cascade that leads to shedding of flowers (iv) fertilizing most of the flowers from an inflorescence somehow created a stress condition. Thus, the second breeding experiments were carried out with the more robust and mature plants which were pollinated in bright sunny days and only 3 flowers per inflorescence were considered and the remaining flowers left intact. Selfed and cross pollinated flowers yielded fertile fruits, and these capsules after 3 months of maturity were used for *'in vitro'* culture.

The effects of the varying altitude could be clearly observed through the abundance and fertility rates, the orchid showing higher fertility rates and abundance at high altitudes region like Bebours and Plaines des Fougeres at Reunion Island. However, so far it

cannot be concluded whether altitude is directly responsible for the stability of this orchid, but the presence of the pollinating agents (birds) and prevailing climatic conditions need to be studied. The size of the plants were different among the populations in Reunion Island itself, the more robust individuals recorded at Plaines des Fougeres and Bebours, and least robust individuals found in Mauritius. This can be reflected as the performance of the species at varying altitudes or climatic conditions. The leaf length somewhat varies with age of the plant and a much reliable source to account for the productivity rates was considering adult plants that had flowered.

Tissue culture has been the major part of successful ecorestoration stories of endangered endemic orchids like *Vanda spathula* (Decruse *et al*., 2003) and *Ipsea malabarica* (Martin & Paradeep, 2003). Our initial experiments were solely based on leaf explants because of unavailability of any other propagules. Using leaf as explants imposed constraints, because the plant bear rarely more than six leaves, of which only the two youngest leaves could be used for *in vitro* culture because of lower level of tissue differentiation, a prerequisite for *in vitro* response. And, of the available Mauritian population only few plants were considered for this experiment, leaving most of other individuals intact so that they could be considered for breeding experiments. Half MS supplied with a low dose of TDZ proved to be the proper condition for *in vitro* response of this orchid. Presently, a successful regeneration protocol has been established based on embryo rescue with propagules obtained from Réunion Island. The same media composition has been successfully used for the capsules that have been developed in Mauritius also.

Most of the Mauritian orchids belong to the subtribe Angracinae, and *Beclardia* was often considered under this tribe because of its white flowers and monopodial nature by most of the earlier taxonomists. However, the results obtained based on morphological suggest the contrary and support its placement within a different subtribe. The initial bias to Angraecinae, resulted in consideration of more members from the latter for taxonomic studies and therefore, the future experiments will consider more members from other sub-tribes of the tribe Vandae. Present results based on molecular studies also suggest that the genus *Angraecum* (includes most of the Mauritian orchid species) to be a much more synthetic taxon, which implies that some members have been wrongly placed within this genus and their taxonomical status need to be reviewed. However, it is too early to derive conclusions on the taxonomic status of *Beclardia* because these preliminary results are solely based on morphological characters and present emphasis is being given on the use of molecular markers through Randomly Amplified DNA polymorphism (RAPD) and finer techniques such as microsatellites and Amplified Fragment Length Polymorphism.
Differences with respect to performance based on robustness, leaf length measures and fertility rates at different populations from Reunion Island revealed that, there could be two varieties of this orchid or the differences encountered could be environmental effects, suggesting ecotypes instead of varieties. Thus, for future studies DNA samples from 60 different individuals including four populations from Reunion Island and the single population from Mauritius have been collected. Hence, the further work in this aspect will include use of sensitive molecular markers and a wider range of morphological

character and data from biological studies, to define the evolutionary trend of this orchid and thus provide a model for the evolution of orchid within this phytogeographic region.

5. ACKNOWLEDGEMENTS

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Table 2: Characters used for classification of *Beclardia macrostachya*

character	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
high altitude	1	1	1	0	0	0	0	0	0		0	0
Epiphyte	1	1	1	1	1	1	1	1	1	1	0	1
Lithophyte	0	1	0	0	0	1	1	0	0	1	0	1
Terrestrial	0	0	0	0	0	1	0	0	0	0	1	0
stem erect	1	1	1	1	1	0	0	1	1	1	1	1
Monopodial	1	1	1	1	1	1	1	1	1	1	0	1
Stem>25 cm	0	1	0	0	0	1	1	0	0	0	1	1
leaf coriaceous	1	1	1	1	1	1	1	1	1	1	1	1
leaf imbricate	1	0	0	0	1	0	1	1	1	1	0	0
unequally bilobed	1	1	1	1	1	1	1	1	1	1	0	1
leaf alternate	1	1	1	1	1	1	1	1	1	1	1	1
Simple raceme	1	1	0	1	0	0	0	0	0	0	1	1
Spike	0	0	0	0	1	0	0	0	1	1	0	0
solitary cyme	0	0	1	0	0	1	1	1	0	0	0	0
flowers white	1	1	1	1	1	1	1	1	0	0	0	1
tinched flowers	1	1	0	0	0	0	0	0	0	0	0	1
transluscent tepals	0	0	0	0	0	0	0	0	1	1	0	0
Spur	1	1	1	1	1	1	1	1	1	1	0	1
gland present	1	1	1	1	1	1	1	1	1	1	0	1
Pollen masses 2	1	1	1	1	1	1	1	1	1	1	1	1
Caudicle 2	1	1	0	0	0	0	0	0	1	1	0	1
Flower fragrant	1	0	0	0	1	0	1	0	1	1	0	1
Inflo axillary	1	1	1	1	1	1	1	1	1	1	0	1
tetals entire	1	0	1	1	1	1	1	1	0	0	1	1
Labellum entire	0	0	1	1	1	1	1	1	1	1	0	0
Flower stalk wirelike	0	0	0	1	0	0	0	0	1	1	0	1
Flower no.>20	0	0	0	1	0	0	0	0	0	0	0	1
lip complex	0	1	0	0	0	0	0	0	1	0	0	1
Flower no.5-14	1	1	0	1	1	0	0	0	1	0	1	0
Aerial roots prominent	1	1	0	0	0	1	0	0	0	0	1	1
Spur>4 cm	0	0	1	0	0	1	1	1	1	1	0	0
Branched stem	0	1	0	0	0	1	1	0	0	0	0	1

1 indicates occurrence of character

0 indicates that character does not occur in that particular state

I : *Beclardia macrostachya*, II *Cryptpus elatus*, III: *Jumellea sp*, IV : *Oenialla sp*, V: *Angraecum cadetii*, VI: *A .mauritianum*, VII: *A.ramosum*, VII: *A.cuculatum*, IX: *Aeranthes arachnites*, X: *Aeranthes grandiflora*, XI *Epidendrum radicans*, XIV: *Aerides lawrenciae*