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Diversity and molecular systematics of orchids in Mount Cameroon

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A survey was carried out in Mount Cameroon to document the distribution and diversity of the Orchidaceae which is the most abundant plant family worldwide and the second most abundant in this study area. The study area was divided into 5 different zones made up of 4 ecotypes in the wild and some cultivated gardens. The ecotypes in the wild were further divided into different macro-habitats based on altitudinal gradients and the sides of the mountain (leeward and windward). A total of 11 macro-habitats were surveyed and an inventory of all species present was made, samples were collected and identified using morphological techniques. Their identities were confirmed using molecular techniques and phylogenetic analyses established. A total of 4,528 orchids belonging to 86 species and 26 genera were observed. The most abundant genus (25 species) was *Bulbophyllum*, with *Habenaria procera* (1,155 individuals) being the most abundant species. The macro-habitat with the least number of individuals (855 individuals) was the montane rainforest, while the lava outcrop had the highest number of individuals (3,238). The windward side had 2,673 individuals, the leeward side had 1,559 individuals and the cultivated gardens had 296 individuals. Based on molecular phylogeny, the orchids were grouped into three subfamilies; the Orchidiodeae, and Vanilloideae with one species each, while the Epidendroideae had 84 species. *Bulbophyllum dayanum* and *Bulbophyllum bequartii* were recorded for the first time in the Mount Cameroon. A single stand of *Ansellia africana* which is considered as being vulnerable by IUCN 2010 occurred only in the 1995 lava flow. The diversity and distribution of orchids in Mount Cameroon is high, but there is need for conservation through domestication.

Key words: Diversity, Mount Cameroon, orchids, *Bulbophyllum*.

INTRODUCTION

Orchidaceae represent a large and diverse taxon of flowering plants and include over 800 genera and 26,000

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species (Chase et al., 2015; Govaerts et al., 2017). They account for approximately 10% of world's seed plants (Fay, 2018). Orchids survive in a variety of ecological conditions and generally occur in four forms based on their habitat (terrestrial, epiphytic, lithophytic and saprophytic).

The terrestrial species account for approximately one-third of the family (Gale et al., 2018) and tend to live in small isolated populations, placing them at risk of extinction (Keppel et al., 2016). The establishment and survival of these plants do not only depend on environmental conditions of the forest, but on the presence of some species of fungi which help them acquire nutrients for seed germination. This is so because their seeds are very minute and contain few stored reserves (Shao et al., 2020).

The Mount Cameroon Region (MRC) has a complex ecosystem, subjected to natural (volcanic activities, lava flow and landslides) and anthropogenic drivers (disturbances). The volcanic nature of this region has attracted large agricultural plantation companies like the Cameroon Development Corporation (CDC). Cable and Cheek (1998) reported that orchids were the second largest group of plant communities in the Mount Cameroon region. The huge plantations of rubber, palms and banana negatively affect orchid communities in this region. The main threats to orchid population are associated to habitat destruction and unsustainable collection. Any effective management plan for orchids in this habitat can only be drawn following a proper inventory of the species that occur therein. It is for this reason that this study was conducted to evaluate the diversity and distribution of orchids in Mount Cameroon Region.

This complex family presents a considerable challenge to taxonomists interested in classification based on morphological and phylogenetic reconstruction. Pre-DNA era classifications of Orchidaceae were based on a relatively small set of morphological aspects and features, particularly on the column and pollinarium as well as on the cladistic analyses of the morphological data. This however, showed limited resolution at lower taxonomic levels (Freudenstein and Chase, 2015). DNA analyses of orchids (Chase et al., 2015) have provided surprising findings for taxonomists and supported the monophyly of the orchid family, including the apostasioids and cypripedioids. Molecular phylogeny has been shown to be more reliable than the use of morphological characteristics only.

In the study reported here, the aim was to evaluate the diversity of orchids found in the different macro-habitats of MCR, where they are threatened by habitat destruction and to determine whether there exists a phylogenetic relationship amongst the species that occurred in each of these macro-habitats.

MATERIALS AND METHODS

Study area and plot layout

Mount Cameroon is the highest mountain in West and Central Africa, with an elevation of 4,095 m located in the South West region in the town of Buea. It is an active volcano which last erupted in 2000 (Britannica, 2016). The study site was divided into 5 different zones made up of 4 ecotypes in the wild and one cultivated garden. The ecotypes in the wild were: forest, lava, grassland and plantation. The altitudinal gradients and the sides of the mountain (leeward and windward), were taken into consideration, since a previous study (Focho et al., 2010) in Mount Cameroon had indicated a sharp difference in the climatic and vegetation patterns of the leeward and windward sides. Thus the lava zone was further divided into 4 macro-habitats: low altitude of leeward lava (lwla), high altitude of leeward lava (lwha), low altitude of windward lava (wwla), and high altitude of windward lava (wwha). The plantations which were found at the low altitudes in the mountain were partitioned into the leeward and the windward sides. The forest was divided into the leeward and windward sides. The grassland was also partitioned into the leeward and the windward sides. In all, there were 11 macro-habitats surveyed. In each macro-habitat of the respective ecotypes in the wild, 30 plots of 5 × 5 m were established, giving a total of 300 plots sampled, plus the cultivated garden.

Survey and collection of orchid samples

During the survey in the field plots, just like in the cultivated garden, all orchids were counted, photographed *in situ* and recorded following the protocol reported in Johnson (2012). Samples were collected, codes assigned to them and recorded on a pre-data sheet. The information included the GPS location, species name and number of individuals encountered. Voucher specimens of species collected were pressed and dried at 70°C to constant weight. Dried samples and photographs of the species were taken to the Yaoundé National Herbarium (YA) for identification.

Morphological identification

This was done using identification manuals (Rolfe, 1898, Hyde et al., 2004, La Croix and Cribb, 1998; Droissart et al., 2020). The voucher specimens were further examined at the Yaoundé National Herbarium (YA) to validate the field identification.

Molecular identification

DNA extraction and amplification

Genomic DNA was extracted from fresh leaves, collected from orchid plants in the wild and from cultivated gardens during the survey in the MCR, using the method described by Gilbertson et al. (1991). A leaf from each sample was frozen at -80°C and a small portion cut and ground in 500 µl of extraction buffer (400 mM Tris-HCL (pH 8), 60 mM EDTA-pH 8.0, 150 mM NaCl and 1% sodium dodecyl sulphate), to a smooth paste using a mortar and pestle. The paste was transferred to a 1.5 mL Eppendorf tube and 33 µL Sodium Dodecyl Sulphate (SDS) was added and vortexed. The mixture was then incubated at 65°C for 10 min. 160 µL of potassium acetate was then added vortexed and centrifuged at

10,000 × g for 10 min. The supernatant was transferred into a sterile 1.5 mL Eppendorf tube and 225 µL of isopropanol was added, vortexed and centrifuged again for 10 min at 10,000 × g. The supernatant was discarded and 300 µL of 70% alcohol was added to the DNA pellets and centrifuged for 5 min at 10,000 × g. The supernatant was discarded and pellets were air dried and re-suspended in Tris EDTA (TE) buffer. Extracted DNA was stored at -20°C.

Agarose Gel Electrophoresis on a 1.5% agarose gel was used to determine if genomic DNA extraction was successful. The gel was placed in an electrophoretic tank containing 1 × TAE buffer, the genomic DNA was loaded onto the wells and run for about 15 min at 100 V in an electrophoretic tank connected to a power pack (Bio-Rad, Belgium). The bands were visualized in a UV trans-illuminator and imaged using a molecular imager (Bio-Rad, Belgium).

PCR analysis method described by Cuénoud et al. (2002) was used during the extraction. The PCR primers used were matK 390F (5'-CGATCTATTCATTCAATATTTC-3') and matK 1326R (5'-TCTAGCACACGAAAGTCGAAGT-3'). The total reaction volume of 25 µL consisted of 12.5 µL One Taq 2 × Quick load Master mix (New England Biolabs, Beverly, MA), 0.5 µL of the forward and reverse primers, 1 µL template DNA and 10.5 µL of nuclease-free water. The thermocycling program used was a pre-denaturation (95°C for 1 min), 35 cycles of denaturation (95°C for 30 s), annealing (46°C for 30 s) and extension (68°C for 1 min), then a final extension (68°C for 5 min). The amplification products were separated on a 2% agarose gel along with negative controls and imaged using Molecular imager (Gel Doc^{TM} XR+). The tubes containing the PCR amplicons were sealed with parafilm and stored at -20°C pending sequencing.

Sequencing of PCR amplicons of orchids

Sequencing of the PCR amplicons was performed by Inqaba Biotechnical Industries (Pty) Ltd, Pretoria – South Africa using BigDye® Terminator V3.1 Cycle Sequencing on an ABI3500XL sequencer. PCR products were cleaned using Exo/SAP. The Exo/SAP master mix was prepared by adding 50 µL of Exonuclease I (NEB M0293) 20 U/µL and 200 µL of Shrimp Alkaline Phosphatase (NEB M0371) 1 U/µL to a 0.6 mL micro-centrifuge tube. The reaction mixture was properly mixed and incubated at 37°C for 30 min. The reaction was then stopped by heating the mixture at 95°C for 5 min. Sequencing was done with the ABI V3.1 Big dye kit according to the manufacturer's instructions. The labelled products were then cleaned with the Zymo Seq Clean-up Kit following the manufacturer's protocol. The cleaned products were injected on an ABI3500XL Genetic Analyzer with a 50 cm Capillary Array, using POP-7 polymer. Sequence chromatograms were viewed using FinchTV Version 1.4.0 (Geospiza Inc).

DNA barcode analysis of the different orchid species

Sequence data was analyzed using the NCBI algorithms comprising Nucleotide Basic Alignment Search Tool of GenBank (BLASTN) and Multiple Sequence Comparison by Log-Expectation (MUSCLE). The matK partial gene sequences were compared with similar existing sequences in the database of NCBI using BLASTN algorithm. Accession numbers of species found in the GenBank and equally present in the MCR, which were difficult to amplify were obtained from NCBI data base. The DNA sequences were aligned and a phylogenetic tree was constructed by neighbour joining method using MUSCLE.

Phylogenetic analysis

Phylogenetic tree of orchids in the MCR

An orchid phylogenetic tree was constructed to show the evolutionary relationship between orchid species found in the MCR based on the available DNA data obtained during the study. The tree was drawn using the Neighbour Joining Method.

Data analyses

Diversity index

To estimate the diversity index of orchids in the MCR with respect to the different macro-habitats of the different ecotypes and cultivated gardens, Shannon-Wiener index (H) (Babour et al., 1987) was used as represented in Equation 1:

$$H' = \Sigma\ (pi)\ (lognpi) \quad (1)$$

where pi = ni/n; ni = number of individuals of a species and n= total number of individuals.

Similarity index

Sorensen's similarity index (Barbour et al., 1987) was used to investigate the similarity of the different ecotypes and the cultivated gardens. It was obtained using the formula in Equation 2.

$$\text{Sorensen's weighted by cover (SS)} = \frac{2MC \quad X \quad 100}{MA \quad X \quad MB} \quad (2)$$

MA is total % cover of species in stand A, MB is total % cover of species in stand B and MC is total % cover in both stand A and B using the lower % cover figure for each species.

RESULTS

Diversity and distribution of species

Overall, a total of 86 orchid species distributed in 26 genera were encountered in the MCR (Table 1). *Bulbophyllum* was the most represented genus (25 species), followed by *Polystachya* with 21 species. A total of 4,528 individual orchids were counted within the 300 plots sampled in the wild and cultivated gardens. Of this number, 3,238 (71.51%) occurred in the lava outcrops, 913 (20.16%) in forest, 81 (1.79%) in plantations and 296 (6.54%) in cultivated orchid gardens. Of the 86 species, 70 occurred on the windward side and 39 on the leeward side of the study area (Table 1). *Habenaria procera* dominated the lava out crops and accounted for 25.51% (1,155) of individual orchids in the survey. *Angraecum birrimense* was the only species that existed in all the different macro habitats surveyed. It was interesting to note that species of orchids found in cultivated gardens were not found in the wild during the survey.

Table 1. Distribution and abundance (number of individuals) of orchid species in different macro-habitats of the MCR.

S/N	Species	Life forms	Lava outcrop				Plantation		Montane rain forest		Montane grassland		Cultivated
			lwla	lwha	wwla	wwha	lw	ww	lw	ww	lw	ww	
1	*Aerangis biloba*(Lindl.) Schltr.	E	-	-	-	-	10	02	02	01	02	-	-
2	*Angraecopsis parviflora* (Thouars) Schltr.	E	-	-	-	03	-	-	03	05	-	-	-
3	*Angraecum augustipetallum* Rendle	E	-	-	20	-	-	-	-	06	-	-	-
4	*Angraecum bancoense* Burg	L	-	-	-	03	-	-	-	-	-	-	-
5	*Angraecum birrimense* Rolfe	E/L	06	-	36	02	04	30	02	20	-	-	-
6	*Angraecum distichum* Lindl.	E	-	-	-	01	-	-	-	-	-	-	-
7	*Angraecum eichlerianum* Kraenzl.	E	-	-	16	03	-	-	-	05	-	-	-
8	*Ancistrorhynchus capitatus* (Lindl.) Summerh	E	-	-	19	-	-	-	-	06	-	-	-
9	*Ancistrochilus rothschildianus* O'Brien	E	-	02	05	01	-	-	-	02	-	-	-
10	*Ancistrorhynchus seratus* Summerh	E	-	-	09	02	-	-	-	03	-	-	-
11	*Ansellia africana* Lindl.	L	01	-	-	-	-	-	-	-	-	-	-
12	*Arachnis* Maggie Oei (yel. rib)	T	-	-	-	-	-	-	-	-	-	-	28
13	*Arachnis* Maggie Oei (red rib)	T	-		-	-	-	-	-	-	-	-	12
14	*Bletilla striata* (Thunb.) Rchb.f.	T	-	-	-	-	-	-	-	-	-	-	220
15	*Bletilla sp*	T	-	-	-	-	-	-	-	-	-	-	26
16	*Bolusiella fractiflexa* Droissart, Stevart & Verlynde	E/L	-	-	-	02	-	-	-	02	-	-	-
17	*Bulbophyllum acutibracteatum* (De Wild) J.J. Verm.	E	-	-	06	05	-	-	-	10	-	-	-
18	*Bulbophyllum barbigerum* Lindl.	E/L	10	03	02	-	-	-	15	06	-	-	-
19	*Bulbophyllum bidenticulatum* J.J. Verm.	E	-	-	26	-	-	-	02	12	-	-	-
20	*Bulbophyllum bifarium* Hook.f.	E	-	-	10	-	-	-	02	18	-	-	-
21	*Bulbophyllum buntingii* Rendle	E	-	-	18	-	-	-	-	35	-	-	-
22	*Bulbophyllum calvum* Summerh	E	02	-	12	-	-	-	06	10	-	-	-
23	*Bulbophyllum calyptratum* Kraenzl.	E/L	06	-	36	02	-	-	03	22	-	-	-
24	*Bulbophyllum falcatum* (Lindl.) Rchb.f.	E/L	22	-	18	-	-	-	04	06	-	-	-
25	*Bulbophyllum flavidium* Schltr.	E/L	-	-	06	6	-	-	02	02	-	-	-
26	*Bulbophyllum fuscum* Lind	E/L	10	02	-	-	-	-	03	06	-	-	-
27	*Bulbophyllum intertextum* Lindl.	E/L	-	-	12	18	-	-	06	25	-	-	-
28	*Bulbophyllum josephii* (Kuntze) Summerh	E	-	-	40	08	-	-	-	18	-	-	-
29	*Bulbophyllum lupulinum* Lindl.	L	280	-	-	-	-	-	06	-	-	-	-
30	*Bulbophyllum melinostachyum* Schltr.	E	-	-	25	10	-	-	-	30	-	-	-
31	*Bulbophyllum oreonastes* Rchb.f.	E/L	02	02	66	02	-	-	-	28	-	-	-
32	*Bulbophyllum porphyrostachys* Summerh	E	-	-	102	-	-	-	-	45	-	-	-
33	*Bulbophyllum pumilum* (Sw.) Lindl.	E	-	-	15	-	-	-	-	10	-	-	-

Table 1. Contd.

34	*Bulbophyllum resupinatum* (Kraenzl.) J.J. Verm.	E	-	-	25	-	-	-	-	08	-	-	-
35	*Bulbophyllum sandersonii* (Kraenzl.) J.J. Verm.	E	-	-	03	-	-	-	-	-	-	-	-
36	*Bulbophyllum scaberulum* (Rolfe) Bolus	L	15	-	-	-	-	-	-	03	-	-	-
37	*Bulbophyllum schimperianum* Kraenzle	E	-	-	10	02	-	-	-	05	-	-	-
38	*Bulbophyllum simonii* Summerh	E	-	-	46	24	-	-	-	22	-	-	-
39	*Bulbophyllum unifoliatum* (J. Will.) J.J. Verm.	E	-	-	04	03	-	-	-	04	-	-	-
40	*Bulbophyllum* sp_1	E	26	-	-	-	-	-	-	-	-	-	-
41	*Bulbophyllum* sp_2	E	17	-	-	-	-	-	-	-	-	-	-
42	*Calyptrochilum christyanum* (Rchb.f.) Summerh	E	-	-	-	-	03	-	-	-	-	-	-
43	*Calyptrochilum emarginatum* (Afzel.ex Sw.) Schltr.	E/L	03	-	-	-	12	-	08	-	-	-	-
44	*Chamaeangis ichneumonea* (Lindl.) Schltr.	E	-	-	06	-	-	-	-	03	-	-	-
45	*Chamaeangis lanceolata* Summerh	E	-	-	03	-	-	-	-	-	-	-	-
46	*Chamaeangis odoratissima* (Rchb.f.) Schltr.	E/L	05	02	16	02	-	-	02	01	-	-	-
47	*Cyrtorchis arcuata* (Lindl.) Schltr.	E/L	03	01	04	02	-	-	-	05	-	-	-
48	*Cyrtorchis chailluana* (Hook.f.) Schltr.	E/L	06	02	10	-	-	-	05	02	-	-	-
49	*Diaphananthe bidens* (Afzel.ex Sw.) Schltr.	E	10	-	-	-	08	02	-	05	-	-	-
50	*Diaphananthe bueae* (Schltr.) Schltr.	E	-	-	-	01	-	-	01	-	-	-	-
51	*Graphorkis laurida* (Sw.) Kuntze	E/L	16	-	19	-	-	-	10	12	-	-	-
52	*Habenaria procera* (Afzel.ex Sw.) Lindl.	L	600	02	550	03	-	-	-	-	-	-	-
53	*Liparis epiphytica* Schltr.	E/L	-	-	03	-	-	-	-	03	-	-	-
54	*Liparis nervosa* (Thunb.) Lindl.	E/L	-	-	129	-	-	-	12	10	-	-	-
55	*Listrostachys pertusa* (Lindl.) Rchb.f.	E	03	06	20	25	-	-	-	48	-	-	-
56	*Papilionanthe teres* (Roxb.) Schltr.	T	-	-	-	-	-	-	-	-	-	-	01
57	*Polystachya adansoniae* Rchb.f.	E/L	02	-	06	-	-	-	03	16	-	-	-
58	*Polystachya affinis* Lindl.	E	-	-	02	16	-	-	-	21	-	-	-
59	*Polystachya albescens* Ridl.	E	48	-	10	-	-	-	-	-	-	-	-
60	*Polystachya bamendae* Szlach. & Mytnic	E	-	-	05	-	-	-	-	03	-	-	-
61	*Polystachya bifida* Lindl.	E	-	-	-	-	-	-	08	60	08	16	-
62	*Polystachya caloglossa* Rchb.f.	E	02	-	27	04	-	-	05	12	-	-	-
63	*Polystachya concreta* (Jacq.) Garay & Sw.	E/L	03	-	08	-	-	-	02	18	-	-	-
64	*Polystachya cultriformis* (Thouars) Spreng.	L	26	-	-	-	-	-	09	05	-	-	-
65	*Polystachya dolichophylla* Schltr.	E /L	02	-	03	-	-	-	-	-	-	-	-
66	*Polystachya elegans* Rchb.f.	L	30	-	-	-	-	-	-	-	-	-	-
67	*Polystachya fulvilabia* Schltr.	E	-	-	05	-	-	-	-	11	-	-	-
68	*Polystachya galeata* (Sw.) Rchb.f.	E	-	-	-	-	-	-	-	02	-	-	-

Table 1. Contd.

69	*Polystachya laxiflora* Lindl.	E	-	-	45	10	-	-	-	30	-	-	-
70	*Polystachya letouzeyana* Szlach.	E	-	-	05	40	-	-	-	12	-	-	-
71	*Polystachya ramulosa* Lindl.	E	-	-	52	-	-	-	-	22	-	-	-
72	*Polystachya subuluta* Finet	E	-	-	12	-	-	-	-	04	-	-	-
73	*Polystachya supfiana* Schltr.	E	-	-	03	16	-	-	-	10	-	-	-
74	*Polystachya odorata* Lindl.	L	52	-	31	-	-	-	05	02	-	-	-
75	*Polystachya paniculata* (Sw.) Rolfe	E/L	100	-	24	-	-	-	04	09	-	-	-
76	*Polystachya polychaete* Kraenzl.	E/L	18	-	20	-	-	-	02	02	-	-	-
77	*Polystachya tessallata* Lindl.	E	-	-	02	-	-	-	-	03	-	-	-
78	*Plectrelminthus caudatus* (Lindl.) Summerh.	E	-	-	-	-	-	-	-	01	-	-	-
79	*Rhipidoglossum rutilum* (Rchb.f.) Schltr.	E	-	-	-	-	-	-	02	-	-	-	-
80	*Rhipidoglossum kamerunense* (Schltr.) Garay	E	-	-	-	-	-	-	02	01	-	-	-
81	*Solenangis clavata* (Rolfe) Schltr.	E	-	-	29	02	-	-	-	04	-	-	-
82	*Solenangis scandens* (Schltr.) Schltr.	E	-	-	46	05	05	05	-	32	-	02	-
83	*Tridactyle tridactylites* (Rolfe) Schltr.	E/L	-	-	19	-	-	-	-	01	-	-	-
84	*Tridactyle bicaudata* (Lindl.) Schltr.	E/L	-	-	02	-	-	-	-	03	-	-	-
85	*Vanda* Miss Joaqium	T	-	-	-	-	-	-	-	-	-	-	10
86	*Vanilla planifolia* Jacks. ex Andrews	E	-	-	-	-	-	-	-	01	-	-	-
	Total number of individuals		1349	22	1700	167	42	39	136	749	10	18	296

E= Epiphytes , L= Lithophytes, T= Terrestrial , E/L = species exist as both epiphytes and lithophytes. Absence (-), lwla = leeward side at low altitude, lwha = leeward side at high altitude, wwla = windward side at low altitude, wwha = windward side a high altitude.

The most abundant orchid species observed during the survey included: *H. procera* (1,155), *Bulbophyllum lupulinum* (286), *Liparis nervosa* (151), and *Bulbophyllum porphyrostachys* (147) (Figure 1a-d).

The rare orchid species, with only one individual each, encountered in the study area were; *Ansellia africana*, *Angraecum distichum*, *Plectrelminthus caudatus* and *Vanilla planiflora* (Figure 2a-d).

Some orchid species like *Bletilla striata*, *Arachnis* Maggie Oei and *Vanda* Miss Joaqium (Table 1 and Figure 3a-c) were in cultivation in floral gardens in the study area. However, these cultivated species were not encountered in the wild during the survey.

Life forms of orchids found in the survey

All the three life forms of orchids: epiphytes, lithophytes and terrestrial were encountered in the MCR. Most (49%) of the orchids encountered were epiphytes (Figure 4) growing on trees and they had long aerial roots covered with velamen. Others (8%) grew on rock boulders where their roots penetrated into the crevices and were firmly attached to the substrate. Similar species (23%) were also present both in the lithophytic and epiphytic forms. The least (5%) were the terrestrial life form that grew on soil (Figure 4).

Diversity of orchids in the different macro-habitats of the MCR

The most diverse macro habitats were the windward and leeward sides of the montane rainforest with diversity indices of 3.69 and 3.14,

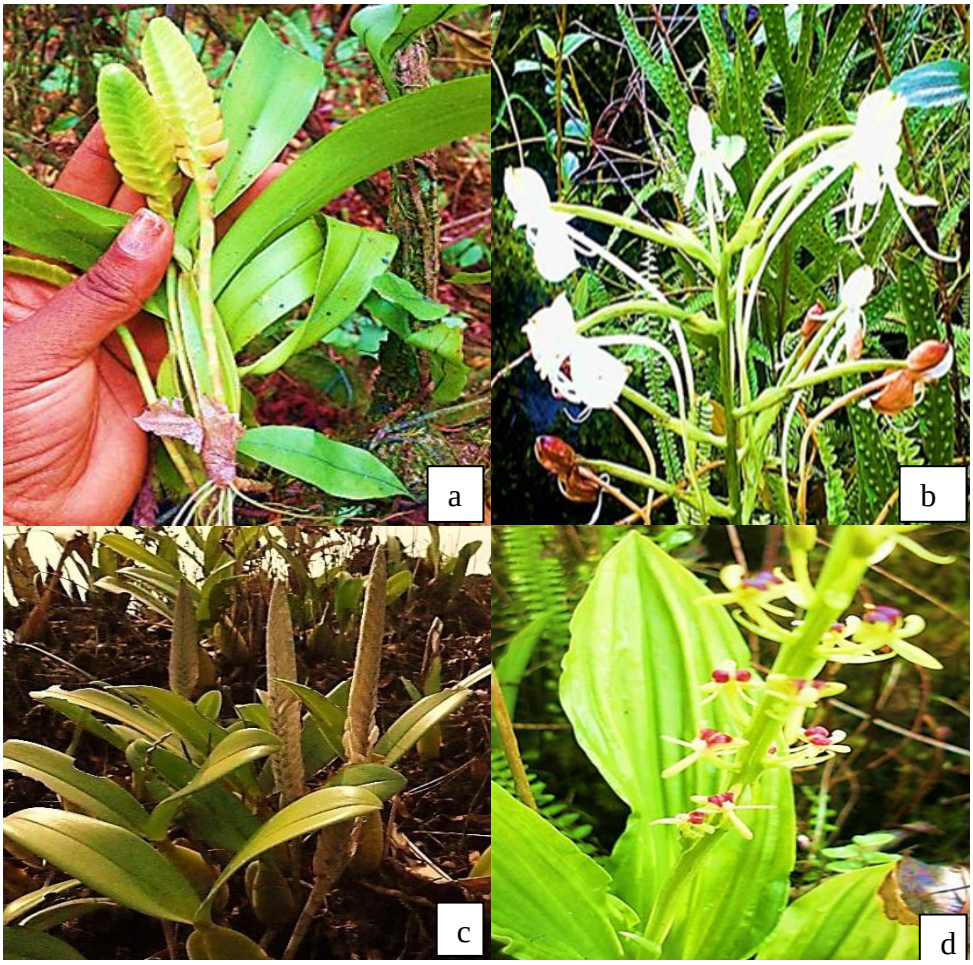

Figure 1. The most abundant orchid species found in the wild in Mount Cameroon. (a) *Bulbophyllum lupulinum,* (b) *Habenaria procera,* (c) *Bulbophyllum porphyrostachys,* (d) *Liparis nervosa*.

respectively. This was followed by the low (3.03) and high (2.86) altitudes of the windward side of the lava outcrops (Table 2).

The least diverse zone was the montane grassland on both the leeward (0.50) and the windward (0.35) sides. Low diversity indices were observed in the plantation vegetation which was nonetheless greater than those of the montane grasslands. Diversity decreased from the montane rainforest > lava outcrops > plantations > cultivated flower gardens > montane grasslands (Table 2).

Similarities of the different vegetational zones

It was observed that the most similar zones were the low altitude of windward side of lava outcrop and the windward side of montane rainforest with a similarity index of 0.87, followed by low altitude of leeward side of lava outcrop and leeward side of montane rainforest with a similarity index of 0.62. It clearly indicated that, vegetational zones found at same side of the mountain had high values of similarities (Table 3). Likewise, relatively high similarities were recorded between Lwwla and Lwha (high altitude of leeward side of lava) (0.58), Lwwha and MRww (windward side of montane rainforest) (0.56), Pww (windward side of plantations) and MRlw (0.50) and by MGlw (leeward side of montane grassland) and MGww (windward side of montane grassland) (0.5). Moreover, zones with lowest similarities were Lwwla and Plw (leeward side of plantation) (0.001) and MRlw and MRww (0.005). The cultivated flower gardens had no similarity at all with other vegetational zones and vice versa.

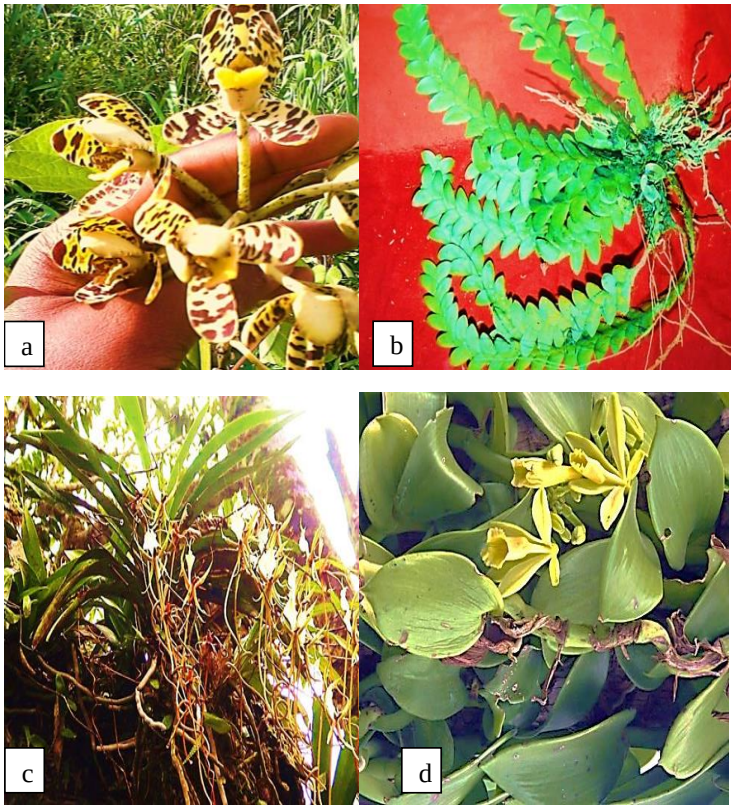

Figure 2. Rare orchid species found in the wild in Mount Cameroon (a) *Ansellia africana* (b) *Angraecum distichum* (c) *Plectrelminthus caudatus* (d) *Vanilla planifolia*.

Molecular systematics of orchids

DNA of orchids was successfully extracted from all species encountered, however, only 13 were effectively amplified using matK gene primers (Figure 5).

The thirteen species whose DNA were amplified included: *Angraecum birrimense*, *Ansellia africana*, *Bulbophyllum dayanum*, *Bulbophyllum bequaertii*, *Cyrtorchis chailluana*, *Polystachya adansoniae*, *Polystachya dolichophylla*, *Polystachya mauritiana*, *Polystachya henrici*, *Aerangis macrocentra*, *Vanda cristata*, *Aerides odorata* and *Papilionanthe teres*.

Molecular taxonomic analysis of Orchidaceae of the MCR

After the BLAST, a taxonomic classification of the Orchidaceae of MCR was drawn up as presented in Table 4. The orchids belonged to three main subfamilies: Epidendroideae (82 species), Orchidioideae (1 species) and Vanilloideae (1 species). The Epidendroideae were represented in 05 tribes with the Vandeae being the most represented with 17 out of the 25 genera. It is worth mentioning that the *Polystachya* which is the second in terms of species abundance of the Orchidaceae falls under the Vandeae. After the Vandeae is the Malaxideae which is composed of 03 genera, but contains *Bulbophyllum* which is the most represented genus in the entire MCR. From the sequencing and the BLAST analysis *B. dayanum* and *B. bequaertii* were recorded for the first time from the Mount Cameroon Region.

It was also observed that matches were not found within the GenBAnk for most species of *Bulbophyllum* using FASTA for Mat K genes (Table 4).

Phylogenic tree of orchids in the MCR

The orchid phylogenetic tree (Figure 6) was generated from the species for which matches were found in GenBank. The tree could be divided into four parts, corresponding to four monophyletic clades with good support (BP 98-100%), numbered I to IV. There was

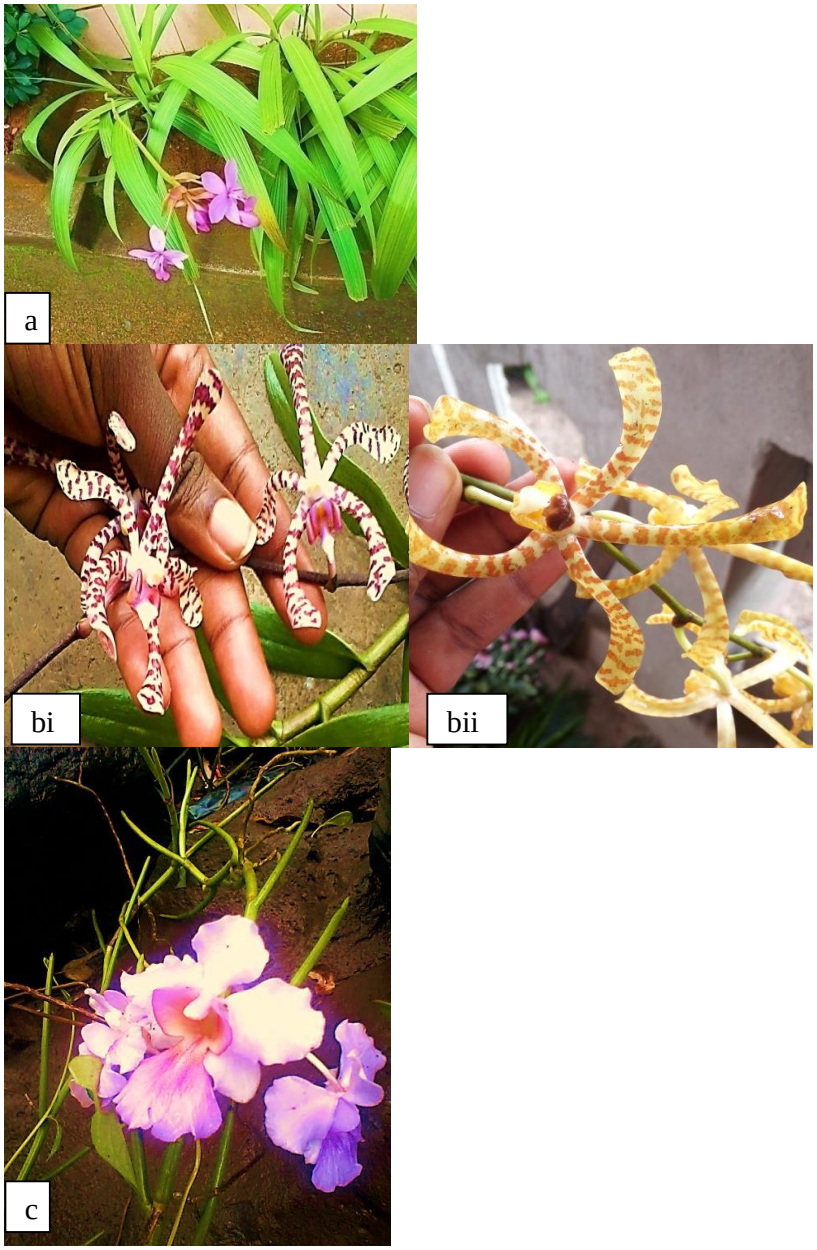

Figure 3. Cultivated orchid species in Mount Cameroon Region (a) *Bletilla striata*, (bi) *Arachnis* Maggie Oei red ribbon, (bii) *Arachnis* Maggie Oei yellow ribbon, and (c) *Vanda* Miss Joaqium.

good support (BP 100%) that *Liparis nervosa* was the earliest extant lineage to diverge from the rest of the species of clade I. *Polystachya* represented by clade II forms a monophyletic group with good support (BP > 90%). *Polystachya affinis* was the earliest extant lineage to diverge from the rest of the *Polystachya* (clade II), followed by lineages represented here by clade III while *Polystachya bifida* represented the earliest extant lineage to diverge from clade III. Clade IV forms a sub clade within clade III in the *Polystachya* with good support (BP 100%). *Polystachya caloglossa* and *Polystachya laxiflora* form a sub clade within clade IV with good support (BP 100%), while *Polystachya galeata, Polystachya supfiana* and *Polystachya fulvilabia* form another sub clade within clade IV with good support (BP > 90%) (Figure 6).

Furthermore, still within the *Polystachya*, *Polystachya*

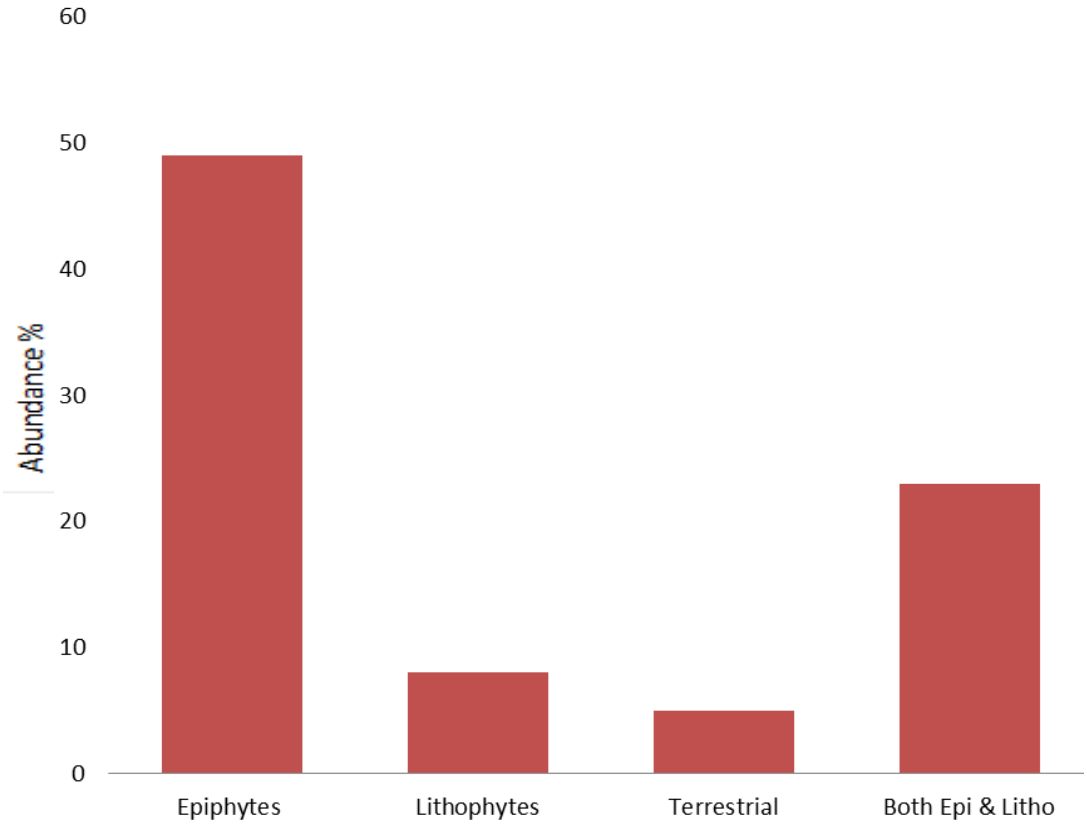

Figure 4. Life forms of orchids encountered in the Mount Cameroon Region.

Table 2. Shannon Wiener diversity index of orchid habitats in the MCR.

Variable	Lava outcrop				Plantation		Montane rain forest		Montane grassland		Cultivated
	lwla	lwha	wwla	wwha	lw	ww	Lw	ww	lw	ww	
H’	1.96	2.07	3.03	2.86	1.68	0.77	3.14	3.69	0.50	0.35	0.87

ramulusa and *Polystachya albescens* formed a clade with good support (BP > 90%), *Polystachya cultriformis* and *Polystachya melliodora* formed another clade with good support (BP > 90%) while *Polystachya henrici, Polystachya odorata* and *Polystachya concreta* formed a sub clade (BP 100%) in which *P. odorata* and *Polystachya concreta* further clustered into a smaller clade (BP > 90%). A total of eight subclades with good support (BP > 90%) were identified within the *Polystachya*.

There was good support (BP 100%) for the clade represented by *Aerides odorata* and *Vanda tessallata* and the larger clade represented by all the species from *Angraecopsi parviflora* to *Solenangis clavata.* Within the said larger clade, there was good support (BP 100%) for the sub clades represented by *Angraecum bancoense* and *Angraecum distichum* and by *Angraecum eichlerianum* and *Angraecum birrimense* and good support (BP > 90) for the sub clades represented by *Cyrtorchis arcuata* and *Crytorchis chailluana;* and by *Rhipidoglossum kamerunense* and *Rhipidoglossum rutilum.*

Species of the *Bulbophyllum* also formed a clade (BP 78%) with *B. bequaertii* representing the earliest extant lineage to diverge from the rest of the genus while *Bulbophyllum andersonii* and *B. dayanum* formed a clade with good support (BP 96%) and one sub-clade occurred within the genus.

Table 3. Similarity of different vegetational zones habouring orchids in the MCR.

Correlation	LLwla	LLwha	Lwwla	Lwwha	Plw	Pww	MRlw	MRww	MGlw	MGww	Cul
Llwla	-	0.42	0.45	0.27	0.17	0.12	0.62	0.46	0	0	0
Llwha		-	0.25	0.26	0	0	0.21	0.22	0	0	0
Lwwla			-	0.58	0	0.07	0.38	0.87	0	0.03	0
Lwwha				-	0.11	0.12	0.27	0.56	0	0.06	0
Plw					-	0.80	0.17	0.12	0.25	0.25	0
Pww						-	0.50	0.12	0.33	0.33	0
MRlw							-	0.01	0.13	0.13	0
MRww								-	0.06	0.06	0
MGlw									-	0.50	0
MGww										-	0
Cul											-

Llwla= low altitude of leeward side of lava outcrop; Llwha = high altitude of leeward side of lava outcrop; Lwwla = low altitude of windward side of lava outcrop; Lwwha = high altitude of windward side of lava outcrop; Plw = leeward side of plantation; Pww = windward side of plantations; MRlw = leeward side of montane rainforest; MRww = windward side of montane rainforest; MGlw = leeward side of montane grassland; MGww = windward side of montane grassland; Cul. = cultivated flower gardens.

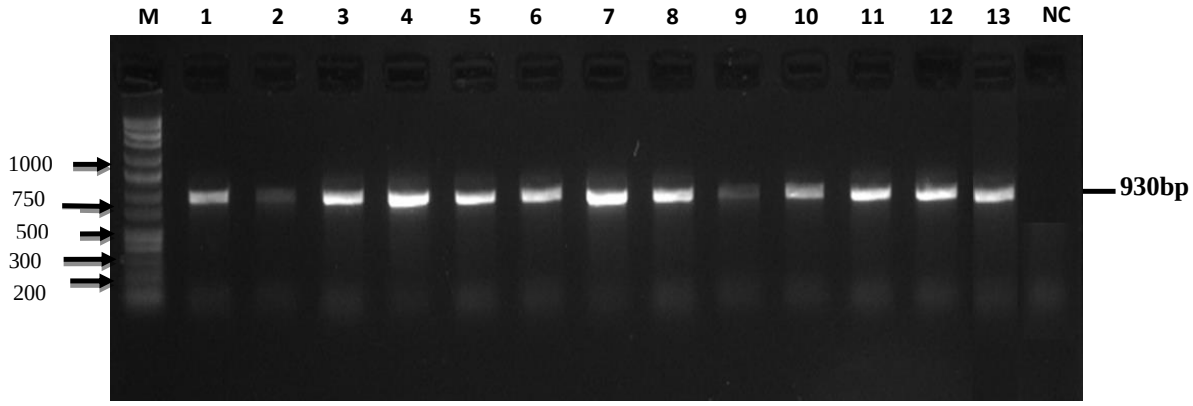

Figure 5. Two % Agarose Gel Electropherogram of the PCR Products of orchid samples. Lane M: Molecular weight markers (bp), N: Negative control, Lane 1 – 13: bands.

DISCUSSION

Diversity and distribution

Orchids were observed more abundant in the low altitude than in the high altitude of the study area. They were also many more individual plants in the windward than on the leeward side. The high diversity of orchids in the windward side than the leeward side was equally reported by Focho et al. (2010), who studied orchid distribution and diversity on selected lava flows of Mount Cameroon. They concluded in their study that high rainfall and high relative humidity led to high diversity of orchids. They also mentioned the die back phenomena usually frequent in the leeward side of the mountain, whereby natural forest fires clear up existing population of orchids leading to decrease in diversity. The Montane rainforest both on the leeward and windward sides had relatively higher diversity levels as a result of persistent cloud cover and mist which resulted to lowest amount of annual sunshine thus conducive for orchids growth and establishment. The abundance decreased from the lava outcrop to the montane forest, then the plantation and grassland. This indicated that habitat destruction would lead to reduction in the plant's population. It also re-enforced the need for the elaboration of a sustainable management strategy for these plants in this habitat. The fact that orchids encountered in the wild did not exist in

Table 4. Taxonomic analysis of Orchids in Mount Cameroon.

Subfamily	Tribe	Sub-Tribe	Genus	Species	Accession numbers
Epindendroideae	Vandeae	Aerangidinae	*Aerangis*	*Aerangis biloba* (Lindl.) Schltr.	KF557946.1
			Angraecopsis	*Angraecopsis parviflora* (Thouars) Schltr.	KF557967.1
			Bulusiella	*Bolusiella fractiflexa* Droissart, Stevart & Ver.	-
			Diaphananthe	*Diphananthe bidens* (Afzel ex Sw.) Schltr.	-
				Diphananthe bueae (Schltr.) Schltr.	-
			Plectrelminthus	*Plectrelminthus caudatus* (Lindl.) Summer	EF079269.1
			Listrostachys	*Listrostachys pertusa* (Lindl.) Rchb.f.	DQ091384.1
			Rhipidoglossum	*Rhipidoglossum rutilum* (Rchb.f.) Schltr	DQ091369.1
				R. kamerunense (Lindl.) Schltr.	DQ091367.1
			Solenangis	*Solenangis clavata* (Rolfe) Schltr	DQ091409.1
				Solenangis scandens (Schltr.) Schltr	-
			Chamaeangis	*Chamaeangis ichneumonea* (Lindl.) Schltr	DQ091373.1
				Chamaeangis lanceolata Summer.	-
				C. odoratissimum (Lindl.) Schltr	-
			Tridactyle	*Tridactyle tridactylites* (Rolfe) Schltr	-
				Tridactyle bicaudata (Lindl.) Schltr.	DQ091388.1
			Arachnis	*Arachnis* Maggie Oei	-
		Aeridinae	*Aerides*	*Aerides odorata*	KF557954.1
			Papilionanthe	*Papilionanthe teres* (Roxb.) Schltr.	KC823036.1
			Vanda	*Vanda* Miss Joaquim	-
		Angraecinae	*Angraecum*	*Angraecum augustipetallum* Rendle	-
				Angraecum bancoense Berg	-
				Angraecum birrimense Rolf	KF557930.1
				Angraecum distichum Lindl	KF672265.1
				Angraecum eichlerianum Kraenzl.	AF506365.1

Table 4. Contd.

			Calyptrochilum	*Calyptrochilum chistyanum* (Rchb.f.) Summer	DQ091325.1
				Calyptrochilum emarginatum (Afzel.ex Sw) Schltr.	-
			Cyrtorchis	*Cyrtorchis chailluana* (Hook.f.) Schltr.	DQ091381.1
				Cyrtorchis arcuata (Lindl.) Schltr.	DQ091380.1
			Polystachya	*Polystachya adansoniae* Rchb.f	GQ145098.1
				Polystachya affinis Lindl.	GQ145090.1
				Polystachya abescens Ridl	KF672259.1
				Polystachya bamendae Szlach., Baranow & Mytnic	-
				Polystachya bifida Lindl	GQ145101.1
				Polystachya calaglossa Rchb.f	GQ145105.1
				Polystachya concreta (Jacq.) Garay & H.R. Sweet	GU556925.1
				Polystachya cultriformis (Thouars) Lindl. ex Speng	GQ145125.1
				Polystachya elegans Rchb.f	GQ145129.1
				Polystachya fulvilabia Schltr.	GQ145137.1
				Polystachya galeata (Sw.) Rchb.f	GU556928.1
	Vandeae	Polystachyinae		*Polystachya henrici*	KF557919.1
				Polystachya laxiflora Lindl.	GQ145153.1
Epindendroideae				*Polystachya letouzeyana* Szlach & Olszezski	-
				Polystachya mauritiana	KF558012.1
				Polystachya melliodora	KF557922.1
				Polystachya ramulosa Lindl.	GQ145182.1
				Polystachya subuluta Finet	-
				Polystachya supfiana Schltr	GQ145191.1
				Polystachya odorata Lindl	GQ145165.1
				Polystachya paniculata (Sw.) Rolfe	GQ145171.1
				Polystachya polychaete Kraenzl.	GQ145178.1
				Polystachya tessallata	-
	Cymbodieae	Cymbidiinae	*Graphorkis*	*Graphorkis laurida* (Sw.) Kuntze	-
		Cyrtopodiinae	*Ansellia*	*Ansellia africana* Lindl.	KF358098.1
	Epidendreae	Laeliinae	*Ancistrochilus*	*Ancistrochilus rothschildianus* O'Brien	AY121729.1
	Malaxideae	Dendrobiinae	*Bulbophyllum*	*Bulbophyllum acutibracteatum* (De Wild) J.J. Verm	-
				Bulbophyllum adersonii	KF361642.1
				Bulbophyllum barbigerum Lindl.	-
				Bulbophyllum bequaetii	EF065597.1

Table 4. Contd.

				Bulbophyllum bidenticulatum J.J. Verm.	-
				Bulbophyllum bifarium Hook.f.	-
				Bulbophyllum buntingii Rendle	-
				Bulbophyllum calvum Summer.	-
				Bulbophyllum calyptratum Kraenzl	-
				Bulbophyllum dayanum	KJ462092.1
				Bulbophyllum falcatum (Lindl.) Rchb.f.	-
				Bulbophyllum flavidium Lindl.	-
				Bulbophyllum fuscum Lindl.	-
				Bulbophyllum intertextum Lindl	-
				Bulbophyllum josephii (Kuntze) Summerh	-
		Dendrobiinae	*Bulbophyllum*	*Bulbophyllum lupulinum* Lindl.	-
	Malaxideae			*B. melinostachyum* Schltr.	-
				Bulbophyllum oreonastes Rchb.f.	-
Epindendroideae				*B. porphyrostachys* Summer.	-
				Bulbophyllum pumilum (Swartz) Lindl.	-
				Bulbophyllum resupinatum (Kraenzl.)J.J. Verm.	-
				Bulbophyllum sandersonii (Hook.f.)Rchb.f	-
				Bulbophyllum scaberulum (Rolfe)Bolus	-
				B. schimperianum Kraenzl	-
				Bulbophyllum simonii Summerh	-
				Bulbophyllum unifoliatum De Wild	-
		Malaxidinae	*Liparis*	*Liparis epiphytica* Schltr	-
				Liparis nervosa (Thunb.) Lindl.	AY907158.1
	Arethuseae	Coelogyninae	*Bletillia*	*Bletilla striata* (Thunb.) Rchb.f	AF263630.1
				Bletilla sp	-
Orchidoideae	Orchideae	Habenariinae	*Habenaria*	*Habenaria procera* (Afzel. ex Sw.) Lindl.	-
Vanilloideae	Vanillaeae	Vanillinae	*Vanilla*	*Vanilla planifolia*	JN004635.1

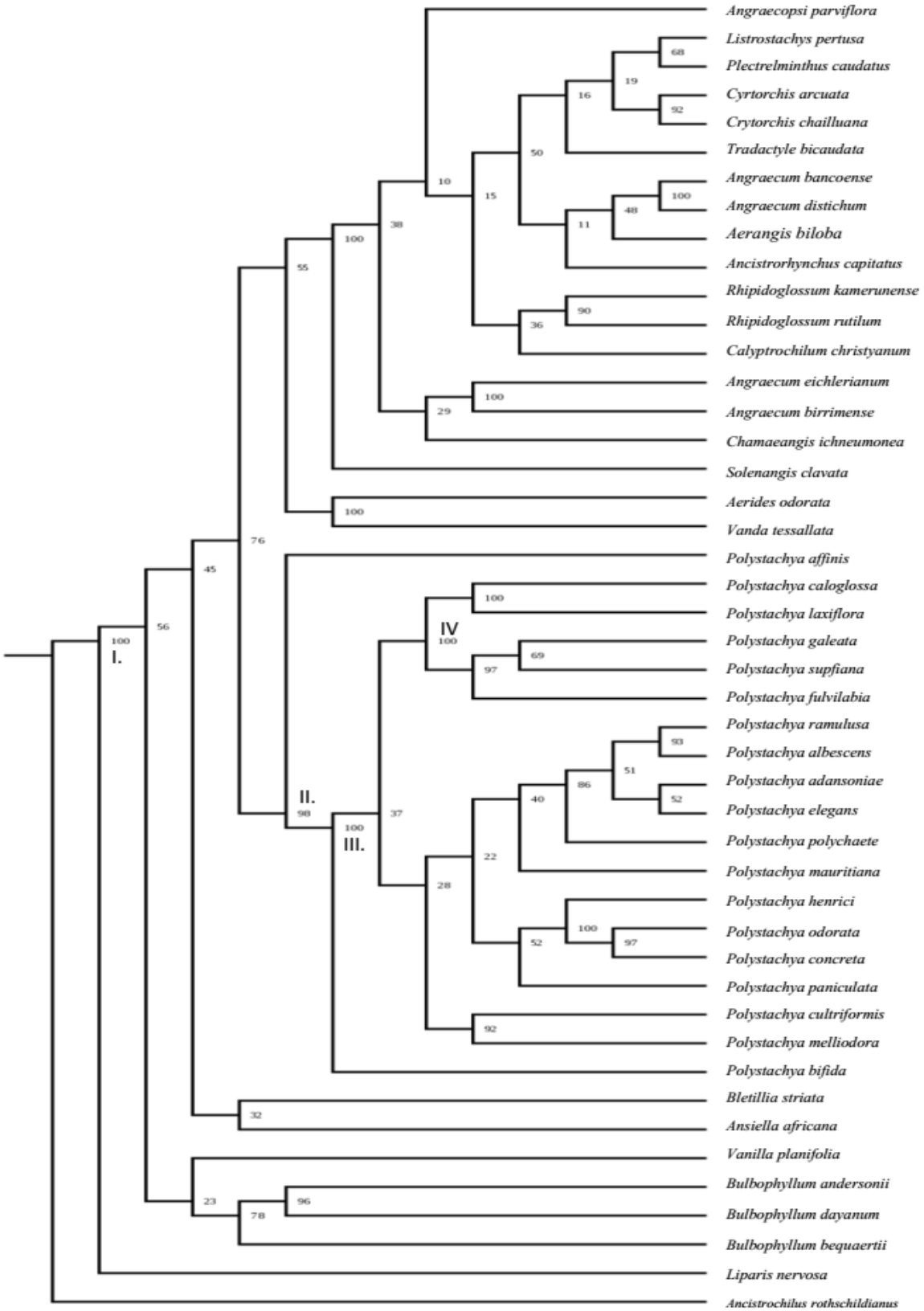

Figure 6. Phylogenetic tree of Orchidaceae in the MCR using Neighbour Joining method.

cultivated gardens was also a call for concern. Conservation through domestication must therefore be encouraged.

Bulbophyllum was the most represented genus in the MCR and was of the Subfamily: Epidendroideae, Tribe: Dendrobieae, Subtribe: Bulbophyllinae. Besides being the largest genus of orchids with over 1500 species, it is also the most geographically diverse in all tropical areas (Gamisch and Comes, 2019). The flowers have the foot or the column which is hinged attached to the labellum such that they have a moving part which bobs, weaves, jiggles or jumps in the slightest breeze. It was described by Thouars in 1822 and the type species being *Bulbophyllum nutans*. Although most bulbos grow well in wooden slat baskets with some tree fern and sphagnum as potting media (Dustin, 2019), it was interesting that in the study reported here no *Bulbophyllum* was encountered in cultivated gardens. *Bulbophylum barbigierum* which was one of the species observed in the study area is listed as endangered, in the IUCN Red list of endangered species (IUCN, 2010). *Bulbophyllum porphyrostachys* another species in the study area was reported by Droissart et al. (2006) as being endemic to Cameroon and Nigeria. These authors reported on *Polystachya letouzeyana* as being endemic to Mount Cameroon. The occurrence of several species of orchid in the study area was an indication that despite the anthropogenic activities taking place on Mount Cameroon, the site still remains rich in terms of orchid diversity.

The number of epiphytic species in this study was greater than the terrestrial species, a finding similar to that of Simo et al. (2009) in the Mbam Minkom hills of Cameroon.

Molecular systematics of orchids of the MCR

DNA was successfully extracted from all the orchid species, nonetheless, lots of challenges were observed with the amplification of DNA, especially from the *Bulbophyllum,* despite exposure to several annealing temperatures. Similar observations were noted by Li et al. (2016) in their study of *Bulbophyllum*. Marches were not found within the Genbank for most species of *Bulbophyllum* using FASTA for Mat K genes.

Givnish et al. (2015) observed no satisfactory distinction between the Malaxideae (comprising *Liparis*) and the Dendrobineae (comprising *Bulbophyllum*), but they seemed to be separate groups and probably are only very distantly related. They also observed that

Malaxideae were closely allied to Arethusieae. Molecular phylogenetic studies of Orchidaceae in the study reported in Givnish et al. (2015) strongly positioned Malaxideae as sister to Dendrobineae, and removed from either Collabineae or Arethusieae. Thus, naked pollinia do appear to serve as a shared, derived character between these 2 tribes (Cameron, 2011; Mytnik-Ejsmont, 2011).

The phylogenetic results in this study were found to be in synchrony with Russell et al. (2010) who found that, *P. caloglossa, P. laxiflora, Polystachya galeata, Polystachya supfiana* and *Polystachya fulvilabia* were part of a sub clade within the *Polystachya* genus*.*

Furthermore, similar to this work, they equally found that *Polystachya bifida* represented the earliest extant lineage to diverge from the said sub clade. Just like in this study, Russell et al. (2010) equally found that *P. affinis* represented the earliest extant lineage to diverge from the rest of the *Polystachya* genus. Moreover, they found that *P. henrici, P. odorata* and *P. concreta* are part of another sub monophyletic group in the *Polystachya* genus which equally agrees with this study. Hence, both studies demonstrate similarity in results despite the method that were employed in inferring phylogenetic relationship. In a similar vein, a study conducted by Carlsward et al. (2006), who worked on “Molecular Phylogenetics of Vandeae and the Evolution of Leaflessness and inferred phylogenetic relationship using the maximum parsimony method, had similar results with those of this study, in which they observed that *C. arcuata* and *C. chailluana* were part of a sub monophyletic group with good support (BP 100%).

Conclusion

The MCR is an orchid-rich ecosystem with occurrence of different orchids species based on altitude. There is no similarity of orchids found in the wild and those found in the cultivated gardens clearly stipulating that none of the wild species of orchid were used as ornamentals. Orchid diversity and distribution largely depended on climatic factors with higher diversities in areas with high relative humidity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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