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CELLULAR AND MOLECULAR BIOLOGY

Phylogeography of the neotropical epiphytic orchid, genus *Dracula*

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Abstract: The tropical Andes constitute a natural barrier between the Pacific Ocean and the Atlantic; in these mountains, are a great variety of Ecosystems, defined by factors such as orography, winds, humidity, temperature, among others. Some of these Ecosystems have different environmental conditions from tropical ones. In them, there is a great Biodiversity, in some cases endemic and associated with relatively small geographic areas. An example of this biodiversity is the orchids of the genus *Dracula*, about which discussions are currently generated due to the difficulty in classifying their members. The present work shows a study where DNA was isolated and sequenced from plant samples obtained from 52 species of orchids of the genus *Dracula*, which were analyzed using the MEGA7 software. Phylogenetic analysis of the DNA sequences showed a well-resolved topology that reflects a geographical pattern of several major clades of the Pacific and Atlantic watersheds. Geophysical conditions of the Andes have generated greater biodiversity of the genus *Dracula* on the side of the Pacific. Although the species *Dracula cordobae* and *alessandroi* reported on both sides of the study site belong to the same clade and show limited mobility through the drier area to the South of the mountain range.

Key words: Andean region, ITS, molecular biology, Neotropical, orchids, phylogeny.

INTRODUCTION

The tropical Andes top the list of worldwide hotspots about numbers endemics and endemic species/area ratio for plants and vertebrates (Myers et al. 2000).

This is the result of a variety of processes, mainly geological and climatic. The Andes mountain range divides South America from North to South into two large blocks: to the West the Pacific Ocean watershed and the East the Atlantic Ocean watershed. In Ecuador, the Andes constitute an impressive meridian mountain barrier whose width generally varies between 100 and 150 km from East to West, the central part being the narrowest; while, in the South near the Peruvian border, the Andean mountainous reliefs prolonged by the Amazonian sub-Andean mountain ranges are characterized by lower annual average rainfall (Maldonado & Solano 2013), a general drop in altitudes to constitute a mountain range, certainly less marked but measuring 180 to 200 km wide (Maldonado & Solano 2013, Winckell 1997).

The vegetation of the Amazon is much older than that of central Ecuador. The flora of the mountains was formed after the geological survey of the Andes (Patzelt 1996). Besides, climatic fluctuations during the Pleistocene influenced the distribution of plants (Prance 1982). The territory does not have a completely tropical climate, presenting a wide variety depending on the orographic or topographic location. The snowy peaks, steep slopes, deep canyons and isolated valleys of these mountains have resulted in a great diversity of microhabitats that favour speciation. Also, its location between the lowlands of the Amazon, the Chiquitanía and the Gran Chaco to the East and the Chocó, Tumbes-Guayaquil, and the arid systems of the Sechura desert to the West, determine complex dynamics of species exchange and isolation (Herzog & Jørgensen 2011).

Ecuadorian flora exceeds twenty thousand species distributed in a great diversity of habitats, more than two thousand are arboreal and about four thousand are orchids (FAO & MINISTERIO DEL AMBIENTE 2012, Patzelt 1996). In the Guayas river basin, with an approximate area of 34500 km^2, 12.57% of the territory of Ecuador presents, for instance, more than two hundred species of orchids that are not found in other parts of the country (Patzelt 1996). Orchids are one of the largest, most diverse and widely distributed plant families and can be found in all habitats except the polar regions (Cox 2013, Cribb et al. 2003). A high proportion of them are epiphytes, forming part of the canopy in humid forests where they play a relevant role in the processes of storage and circulation of water (Castellanos Castro & Torres Morales 2018, Pypker et al. 2006).

Therefore, they are related to a wide variety of organisms, such as fungi, insects, birds, and even mammals, participating, for example, in the cycling of nutrients (Castellanos Castro & Torres Morales 2018). Also, they support specialized pollinating organisms as well as the abundance and diversity of flowers that generate pollinator biodiversity (Lázaro et al. 2020, Storck-Tonon & Peres 2017).

The Orchidaceae form one of the largest families of angiosperms, as well as one of the most fascinating by reason of their diversity and specialization in floral. While many workers have described new genera and species of orchids, there has been very little monographic work, and we may safely say that the family has really been very little studied, considering its size and complexity (Dressler & Calaway 2009). A characteristic of orchids is their production of up to six million seeds/fruits in powder form that are dispersed in the wind over considerable distances (Trapnell et al. 2019), is surprisingly poorly known in a fossil state. The shortage of orchid fossil data is discussed mainly in the context of limitations in evolutionary and phylogenetic studies of this group of plants (Gołaszewska et al. 2019, Poinar & Rasmussen 2017). So a classification of orchids in tribes and subtribes is not yet available (Dressler & Calaway 2009).

The first species of *Dracula* to be described was *Masdevallia chimaera* by Professor Reichenbach in 1872. Following his example, every species of *Dracula* continued to be described in *Masdevallia* until 1978. Luer (1993) included in *Dracula* the species with carinated leaves, internally pubescent sepals, widened and bilaminated or bivalved petals apically; and, without the tooth that *Masdevallia* has at the label margin or the base, a generally sacciform labellum with radial veins and divided into a hypochile and epichile. Vegetative differences allow the *Dracula* genus to be easily distinguished from other genera of Pleurothallidinae, but generally, it does not allow the differentiation between *Dracula* species when plants are not blooming (Meyer & Cameron 2009). Some complexes of species or variable species, that cause much confusion and debate, are minimally distinguishable from other species due to the difficulty to differentiate them from other species of the same genus (group). Natural variations can be found within a species complex (Karremans 2016, Solano-Gómez et al. 2008). It is even suspected that some taxa collected in nature are natural hybrids, such as *D. hawleyi* and *D. radiosyndactyla* (Luer 1993). Currently, 120 species of the *Dracula* genus are recognized for the neotropics from Central America to South America; 55 species in the genus have been described in the territory of the Republic of Ecuador and recently several new species have been described (Baquero & Meyer 2014, Luer 1993, Peláez et al. 2009). They mainly grow in cloud forests, where they can be found at an altitude between 300 and 2800 meters (Baquero & Moncayo 2017, Luer 1993).

The advent of molecular techniques has dramatically advanced our understanding of the phylogenetic relationships in family Orchidaceae. The Internal Transcribed Spacer (ITS) region of nrDNA possesses moderate interspecific variation and has been the primary source of characters for phylogenetic analysis at lower taxonomic levels (Baldwin et al. 1995, Hu et al. 2016). Several papers on nucleotide sequences in the Orchidaceae family of the ITS nuclear genome region in molecular phylogenetics are presented in the literature (Batista et al. 2013, Whitten et al. 2012) Phylogeography examines the correspondence between genetic characteristics and geographic distribution of different species (Avise et al. 1987). The genetic structure of the population is as much a product of history as of current migration patterns and isolation of populations (Avise et al. 1987, Bermingham & Mortiz 1998). In recent decades molecular studies have established classical biogeographic patterns, associated with past geological and bioclimatic changes that led to genetic divergences in many groups of species. (Schmitt 2007). The species diversification process is often associated with niche changes in the newly emerged lineages. However, an opposing force known as niche conservatism means that related species tend to resemble each other in their niche requirements (Šmíd et al. 2019). One of those possible components is the species' climatic niche (Šmíd et al. 2019). Phylogenetic studies used cp DNA sequences as matK from the ITS region to test models of historical biogeography (Fritsch & Cruz 2012), showing a well-resolved topology that reflects a geographical pattern of several clades (Holderegger & Abbott 2003, Loera et al. 2012, Romaschenko et al. 2014).

Microclimatic variability in Tropical Andes plays a key role in shaping species distributions and their ability to cope with environmental change (Ayala-Izurieta et al. 2017, Montejo-Kovacevich et al. 2020). The present work shows a study where DNA was isolated and sequenced from plant samples obtained from 52 species of orchids of the genus *Dracula*, which were analyzed using the MEGA7 software. Phylogenetic analysis of the DNA sequences obtained show a geographical pattern of the main clades of the Pacific and Atlantic watersheds. As greater biodiversity on the Pacific Ocean watershed generated by the geological uplift of the Andes is seen, the trade winds, which in the Andean range of Ecuador always go from east to west, as well as the effect of low temperatures, gave rise to the development of different intraspecific lineages in the genus *Dracula*. In the south of the country, the mountain range presents lower rainfall and heights than the study area; the species *D. cordobae* and *D. alssandroi* have been reported on both the Pacific and Atlantic watersheds, suggesting the presence of a genetic exchange corridor for orchids of the *Dracula* genus. To fulfill this task, the ITS1 region of the rDNA was analyzed from 52 samples of species of the genus *Dracula* out of 55 described in Ecuador. For comparison, some taxa of the genus *Masdevallia* were included.

MATERIALS AND METHODS

Plants

The *Dracula* genus consists of 120 species including those recently described in Central and South America (Baquero & Meyer 2014, Peláez et al. 2009), they grow preferably in cloud forests where they can be found at an altitude between 300 and 2800 meters (Baquero & Moncayo 2017, Luer 1993). For the present study, samples of plant tissue were collected from private and public collections located in the province of Pichincha: "Orquideario de Sarina", "Jardín Botánico de Quito", " Alberto Guerrero's Collection", and in the province of Carchi canton Huaca: "Digna Chacón's Collection" and "Guadalupe Utreras' Collection", representing a total of 104 samples of leaf tissue from 52 of the 55 species of the genus *Dracula* described in Ecuador (Baquero & Meyer 2014, Peláez et al. 2009) which corresponds to 44% of the total number of known species of the genus *Dracula* in the Neotropics (Valencia & Pitman 2000). The fresh leaf samples of each of the specimens were transported in kraft paper envelopes and in hermetically sealed plastic bags with silica gel. In the laboratory they were kept at a temperature of -20˚C. The samples were identified by Luis Baquero, curator of the orchid collection of the Botanical Garden of Quito. The living samples are kept in the Botanical Garden "Orquídeas de Sarina", with the collection code from Marco Cerna's field book, Project "Identification molecular of the genus *Dracula* Orchidaceae 2018".

Geographical location of species and wind

The geographical location of the species under study was obtained from the Red Book of Endemic Plants of Ecuador, Dracula-species and Tropics (Figure 1). Ecuador's wind system is dominated by tropical trade winds with strong easterly winds throughout the year (Beck et al. 2008), effect that has the greatest intensity on the peaks of the Andes. However, local climatic conditions may differ due to the microclimatic influence caused by the topography and the presence of the Pacific Ocean to the west (Jantz et al. 2013).

Of the fifty-five species of *Dracula* reported for Ecuador (Baquero & Moncayo 2017), only the species *D. Felix, D. Trinympharum* and *D. Soennemarkii* were not included in this study, fifty-two species were evaluated, of which forty-one species have been reported for the Pacific Ocean watershed, twelve species have been found in the Amazon River basin, two species have been identified on both watersheds at the south of the study area, and one species have not yet been reported in the wild in the study area, see Table I. As a functional group, species of the genera *Masdevallia yungasensis, Masdevallia picturata, and Diodonopsis erinacea* were used, whose sequences were downloaded from GENBANK.

DNA extraction

Genomic DNA from leaves samples was extracted using the chemical protocol described by Doyle & Doyle (1987). A sample of approximately 30 mg was sprayed in liquid nitrogen and placed in a eppendorf tube, to which 600 µL of lysis buffer was added, 94 µL SDS at 10%, 118 µL Potassium Acetate (5M). The mixture was stirred and then the supernatant was taken and added to a new tube. Next, 600 µL of a phenol-chloroform mixture (1: 1 v / v) was added, stirred and centrifuged to induce the formation of two phases, 250 µL of the upper phase were taken and transferred to a new one microtube, adding 360 µL of cold isopropanol, mixing by inversion. The obtained mixture was stored at -20 °C overnight to obtain the precipitated DNA. After this time, 3 washes were performed, adding 400 µL of 70% ethanol and centrifuging each time a

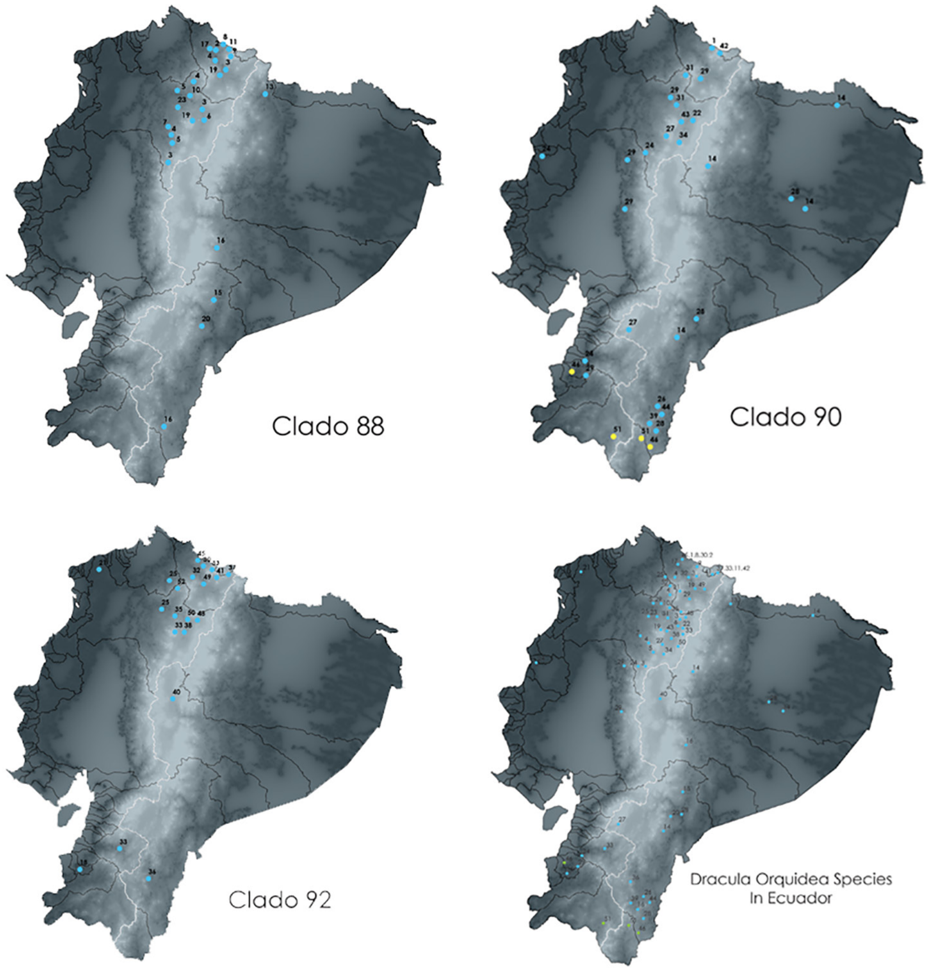

Figure 1. Geographical location of the species belonging to the clade and distribution of *Dracula* orchid species in Ecuador. It is observed that each species is located on one side of the mountain range (Pacific slope and Atlantic slope) between 300 and 3000 meters above sea level, with the exception of *Dracula alessandroi* and *Dracula cordobae* species highlighted with yellow circles, which they are found on both sides of the mountain range (The numbers identify the species according to the cladogram, the white line represents the highest part of the mountain range).

wash was performed. The DNA pellet obtained were resuspended in 30 µL of TE Buffer and stored at -20 °C until use.

Internal transcribed spacer region amplification

Using the Polymerase Chain Reaction technique (PCR) in a conventional thermal cycler type MultiGene™ OptiMax, the amplification of the ITS region was performed using the ITS-1 primer (F-TCCGTAGGTGAACCTGCGG). The PCR products were visualized by horizontal electrophoresis in 1% agarose gels at 90 V for 40 minutes and stained with SYBR Safe, to be visualized under UV light using a MicroBis DNR Bio-Imaging Systems. The PCR amplified product was sequenced (forward) using the Sanger method (Bernardos et al. 2006), by the company MACROGEN (Korea). In 1977, Frederick Sanger developed a new method for DNA sequencing based on the chain termination method, where nucleotides in a single-stranded DNA molecules are determined by complementary synthesis of polynucleotide chains, based on the selective incorporation of chain-terminating dideoxynucleotides driven by the DNA polymerase enzyme (Totomoch-Serra et al. 2017).

Table I. Altitude and watershed to which the species of the genus *Dracula* have been reported.

Id. Number	Scientific name	Altitude (masl)	Watershed	
			Pacific	Atlantic
1	*Dracula terborchii**	1200	North	
2	*Dracula lindstroemii**	1500–2000	North	
3	*Dracula vespertilio***		North	
4	*Dracula venosa**	2500–3000	North	
5	*Dracula vampira**	1500–2500	North	
6	*Dracula ubangina**	1500–2000	North	
7	*Dracula tubeana**	1500–2500	North	
8	*Dracula trigonopetala***	1800	North	
9	*Dracula smaug***	1900	North	
10	*Dracula sodiroi**	1500–2250	North	
11	*Dracula radiella**	1800–2500	North	
12	*Dracula sijmii****	1800-2400	North	
13	*Dracula sibundoyensis***	2300		North
14	*Dracula rezekiana**	800–1700		The whole watershed
15	*Dracula hirsuta**	1200-1600		South
16	*Dracula simia**	1500–2000		South
17	*Dracula psyche**	2000	North	
18	*Dracula pinasensis****	1200	South	
19	*Dracula polyphemus**	1000–2000	North	
20	*Dracula portillae**	2000		South
21	*Dracula papillosa**	300	North	
22	*Dracula nigritella**	> 2000	North	
23	*Dracula navarrorum***	2000-2200	North	
24	*Dracula mopsus**	400–1500	Central and South	
25	*Dracula marsupialis**	2000–2500	North	
26	*Dracula mendozae***	1500		South
27	*Dracula mantissa***	1800-2200 m	North	
28	*Dracula lotax**	800–1600		Central and South
29	*Dracula woolwardiae**	1000–2000	The whole watershed	
30	*Dracula levii***	2000-2600	North	
31	*Dracula lafleurii**	1200–1700	North	
32	*Dracula kareniae**	2000	North	
33	*Dracula iricolor****	2000-2600	North	

Table I. Continuation.

34	*Dracula hirtzii***	2000	North	
35	*Dracula radiosa***	2400	North	
36	*Dracula hawleyi**	2500	North	
37	*Dracula gigas****	1700 a 2500	North	
38	*Dracula gastrophora**	1500–2000	North	
39	*Dracula fafnir**	1400		South
40	*Dracula fuligifera**	1500–2000		Central
41	*Dracula fuliginosa****	1800 a 2500	North	
42	*Dracula erythrocodon****	2000	North	
43	*Dracula dodsonii**	1600–2160	North	
44	*Dracula deltoidea**	2800–3000		South
45	*Dracula dalstroemii**	2500	North	
46	*Dracula cordobae**	750–1000	South	South
47	*Dracula chistineana*	Have not yet been reported in the wild in the study area		
48	*Dracula chiroptera***	1800	North	
49	*Dracula andreettae****	1500-2200	North	
50	*Dracula alcithoe***		North	
51	*Dracula dalessandroi**	2000–2450	South	South
52	*Dracula morleyi**	1900	North	

Source: * Red Book (Valencia & Pitman 2000);Tropics (Missouri Botanical Garden n.d.); ***Dracula-species (Herman ter Borch n.d.).**

Data analysis

The sequences were individually verified using the free software Finch Tv DNA Sequencing for Windows Geospiza (2012). The GenBank BLAST tool was used to make a comparison of the amplified regions, determining that 24 species of those analyzed are already in the database in the ITS region. Nucleotide sequences were aligned using the MUSCLE software implemented in the Molecular Evolutionary Genetics Analysis (MEGA) version 7.0 program (Tamura et al. 2013). MEGA is computer software originally created at Pennsylvania State University, its latest version of MEGA7 software updated in January 2016 produces relative divergence times for nodes, which are useful in determining the order and spacing of divergence events in species and family trees (Kumar et al. 2016).

The evolutionary history was inferred using the Maximum Probability method based on the Tamura-Nei model. The model is based on rates of two types of transition substitutions between purines and pyrimidines and the rate of cross-substitutions are considered separately, taking into account the unequal frequencies of four nucleotides (Tao et al. 2020). As a result, the tree with the highest recording probability (-19876.71) was obtained (Figure 2). The initial tree for the heuristic search was obtained automatically by applying the Neighbor-joining and BioNJ algorithms to a distance matrix in nucleotide pairs estimated using the Maximum Composite Likelihood (MCL), and then selecting the topology with a higher recording probability value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 52 nucleotide sequences. All positions containing gaps and missing data were eliminated. The evolutionary analyses were performed in MEGA7.

RESULTS AND DISCUSSION

The present work shows a study where DNA was isolated and sequenced from plant samples obtained from 52 species of orchids of the genus *Dracula*. The species evaluated in this study that have sequences in the NCBI GenBank are 24, and the others 28 species are not registered yet (Table II).

Phylogenetic analyses of the genus *Dracula* showed, in general, a well-resolved topology reflecting a geographical pattern of several major clades of the Pacific and Atlantic watersheds. When analyzing the tree (Figure 2), with the location of the reports of wild species of the genus *Dracula* in the territory of the Republic of Ecuador. It is found that in clade 92 (Figure 1), the sixteen species have been reported to the Pacific slope, fifteen of the eighteen species have been reported to the North, only *D. pinasensis* has been found in the southern part of the Pacific watershed and the species *D. chistineana* distribution is unknown in wild form in the study area. Clade 76 the five species have been reported in the south, *D. fafnir, D. deltoridea*, *D. lotax* and *D. mendozae*, have been reported in the wild on the Atlantic watershed. Nevertheless, the species *D. mopsus* has been reported along the Pacific watershed (Figure 1).

In clade 88, fourteen species have been reported in the northern part of the Pacific slope. In the clade 70, three of the five species have been reported in the southern part Atlantic watersheds where the height of the mountain range decreases showing the presence of a natural corridor in the south of the Andean mountain range in the study area between the Pacific and Atlantic watersheds (Figure 1). The trade winds from the East throughout the year stimulate mobility from East to West. However, the influence of the trade winds decreases with the height of the mountain range. Therefore, it seems possible the diffusion of pollen and seeds from the West to the East through this biological corridor.

The Andes and the trade winds surround the hotspot to the Northwest of Ecuador limiting the possibility of the diffusion of the genus *Dracula* species towards the Amazon basin. The only two species reported in the wild on both the Pacific and Atlantic watersheds are *D. cordobae* and *D. dalessandroi* from the same main clade 90 (Figure 1). The species in the study area, which have been reported only to the north of the Pacific watershed, do not share the main clade in any case with species reported to the north of the Atlantic watershed where the Andean mountain range maintains high altitudes.

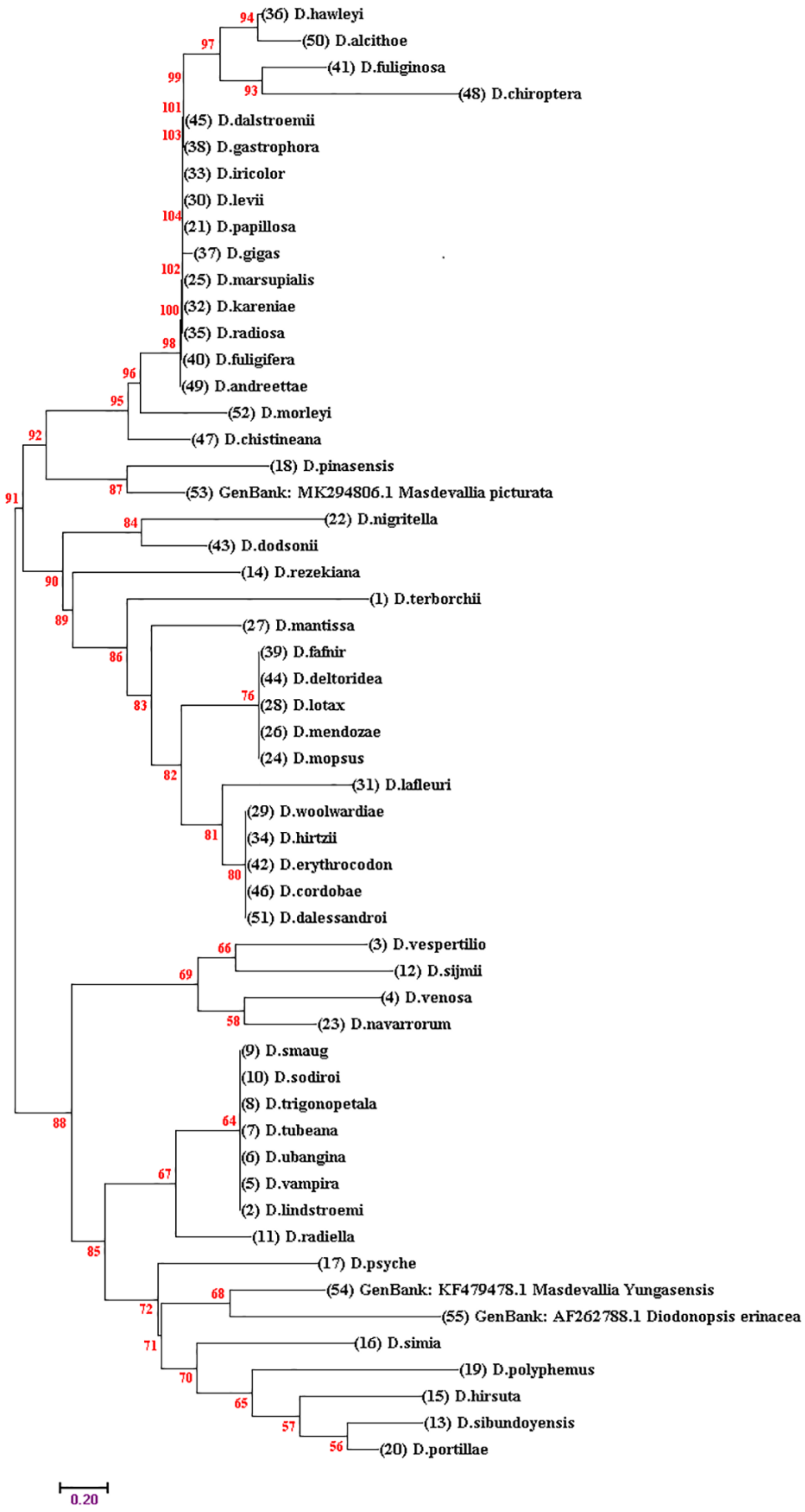

Figure 2. Phylogenetic tree of *Dracula* species based on the DNA sequencing data of the ITS region.

Table II. List of the species of orchids of the genus *Dracula* evaluated in this study that have sequences in the GenBank. It should be noted that except for *Dracula radiosa*, which has 86% similarity, all sequences have values greater than 95%.

SUBJECT				SCORE		IDENTIDAD			CODE
Scientific name	**Length**	**Start**	**End**	**Bit**	**Raw**	**Match**	**Total**	**Identify (%)**	**GENBANK**
Dracula sodiroi	767	120	767	1146	620	640	649	99	AF262764.1
Dracula dodsonii	771	121	769	1166	631	644	650	99	AF262759.1
Dracula tubeana	767	127	767	1173	635	640	642	99	EF372384.1
Dracula Simia	762	122	755	1064	576	624	645	97	EF372362.1
Dracula erythrochaete	771	123	769	1173	635	644	648	99	EF372364.1
Dracula vampire	762	120	762	1160	628	639	644	99	AF262761.1
Dracula cordobae	762	123	762	1166	631	638	641	99	EF372355.1
Dracula andreettae	767	125	767	1166	631	640	644	99	AF262765.1
Dracula Polyphemus	762	121	762	1155	625	639	645	99	EF372368.1
Dracula Gigas	767	125	767	1173	635	642	645	99	EF372366.1
Dracula rezekiana	762	122	756	1133	613	632	640	99	EF372376.1
Dracula navarrorum	664	41	662	1088	589	618	630	98	EF372379.1
Dracula Fafnir	764	121	762	1155	625	640	646	99	EF372388.1
Dracula fuligifera	666	34	661	1035	560	619	644	96	EF372372.1
Dracula vespertilio	762	124	762	1160	628	637	641	99	EF372387.1
Dracula radiosa	760	132	557	462	250	369	427	86	EF372373.1
Dracula iricolor	762	123	344	1166	631	638	641	99	EF372392.1
Dracula mopsus	666	42	642	1083	586	597	602	99	EF372389.1
Dracula alcithoe	664	41	662	1088	589	618	630	98	EF372353.1
Dracula Lotax	664	36	659	1107	599	620	629	99	EF372367.1
Dracula morleyi	664	37	535	839	454	485	499	97	EF372391.1
Dracula dalstroemii	668	51	660	957	518	581	612	95	EF372374.1
Dracula hitzii	762	121	762	1175	636	641	643	99	EF372382.1
Dracula dalessandroi	689	50	668	1011	503	638	611	98	EF372352.1

CONCLUSIONS

The DNA samples obtained of orchids of the genus *Dracula* show that about 54 % of species analyzed not registered in the NCBI GenBank. Phylogenetic analysis of the DNA sequences obtained showed a well-resolved topology that reflects a geographical pattern of several major clades of the Pacific and Atlantic watersheds. Geophysical conditions of the Andes have generated greater biodiversity of the genus *Dracula* on the side of the Pacific. Although the species *Dracula cordobae* and *alessandroi* reported on both sides of the study area belong to the same clade and show limited mobility through the drier site to the South of the mountain range. Microclimatic variability in the Tropical Andes, the trade winds, among other phenomena plays a crucial role in shaping species distributions created one of the most important hotspots in the world. Although, at the same time, they limit the mobility of species of the *Dracula* genus to the extensive Amazon River basin.

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