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Chemotaxonomy of the Amazonian *Unonopsis* Species Based on GC-MS and Chemometric Analysis of the Leaf Essential Oils

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Abstract: Twelve *Unonopsis* specimens, comprising five species commonly found at Amazonas state (Brazil) were collected in three different sites. The leaves of the specimens were extracted by hydrodistillation and analyzed by gas chromatography coupled to mass spectrometry (GC-MS). The data were treated by chemometric analysis with the objective of verify the potential of their chemical profiles for chemotaxonomic approaches. Despite the essential oils presented spathulenol and caryophyllene oxide as a main constituent in most samples, the multivariate analysis showed significant differences between the species and their collection sites. The obtained results suggest high chemical similarity between *U. floribunda* and *U. rufescens* species and proved that *U. guatterioide*s has a distinct chemistry when compared to the analyzed species. The chemical identification points to α-guaiene, α-calacorene and widdrol as possible chemical markers for *U. floribunda* and *U. rufescens* species.

Keywords: *Unonopsis;* chemotaxonomy; Essential oil; GC-MS; Annonaceae; Amazon region.

1. Introduction

The genus *Unonopsis* is encountered in Central America and tropical South American (neotropical) regions [1]. Its name is derived from the old genus *Unona* L.f. (= *Xylopia* L.) and 'opsis' (Old Greek 'face'), because of the superficial similarity. The first botanical description was performed by Robert E. Fries in 1900, being after some years incorporated into the informal "Unonopsis-Gruppe", along with *Bocageopsis* and *Onychopetalum* genera, also described by Fries (1931). A recent botanical review performed with these 3 genera changed substantially the structure of *Unonopsis*, rising from 27 to nearly 50 species, being the additional 23 species described as new.

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Within *Unonopsis* genus some species present problems regarding taxonomic classifications, where *U. guatterioides* plays a central role, once 13 species were recently incorporated as synonymins [1]. In Brazil, 15 species of *Unonopsis* are described, some of them unique to certain states, as the cases of *Unonopsis bahiensis* (Bahia), *U. bauxitae* (Minas Gerais), *U. heterotricha* (Pará), *U. renati* (Espírito Santo), *U. riedeliana* (Rio de Janeiro) and *U. sanctae-teresae* (Espírito Santo). At Amazonas state the species *U. duckei*, *U. floribunda*, *U. guatterioides*, *U. rufescens*, *U. stipitata*, *U. spectabilis* and *U. veneficiorum* are found [1]. Some species are described as used for medicinal purposes, as example *Unonopsis floribunda*, whose barks of the trunks are popularly employed in the treatment of arthritis, bronchitis, rheumatism and diarrhea, as well in malaria treatment by native populations of Peru [2,3]; *U. stipitata* leaves (powder) are added to the food of indigenous people that have speaking difficulties; *U. veneficiorum* leaves are also added to food, but for the treatment of elderly indigenous suffering from dementia [4].

Unonopsis is a genus well explored from the chemical and biological views [5,6,7,8,9]. Chemical studies with *Unonopsis* showed this genus as a promising source of aporphine alkaloids and their derivatives [5]. Regarding essential oil constitution, only three previous studies are reported for *U. stipitata* (flowers) [10], *U. guatterioides* (roots, barks and fruits) [11] and *U. costaricensis* (leaves) [12]. This paper describes the chemical analysis by gas chromatography coupled to mass spectrometry (GC-MS) of the essential oils from *U. duckei*, *U. floribunda*, *U. rufescens*, *U. stipitata* and *U. guatterioides* and the chemometric treatment of the results aiming a chemotaxonomic grouping of the selected species found at the Amazonas state of Brazil.

2. Materials and Methods

2.1. Plant material

Leafs from the cited species were collected from the three following sites at the Amazonas state (Figure 1): SUFRAMA Agricultural District (DAS), located in the rural zone of Manaus being an area of approximately 600.000 hectares, with 468 km of feeder roads and being cut in the North/South direction by the BR-174 highway and partly towards East/West, the AM-010 road (Manaus-Itacoatiara). Adolpho Ducke Forest Reserve (RFAD), located 25 km away from the city of Manaus, has 10.000 hectares of protected area. The green area of the Federal University of Amazonas (UFAM) campus, located in the city of Manaus, occupying approximately 700 hectares.

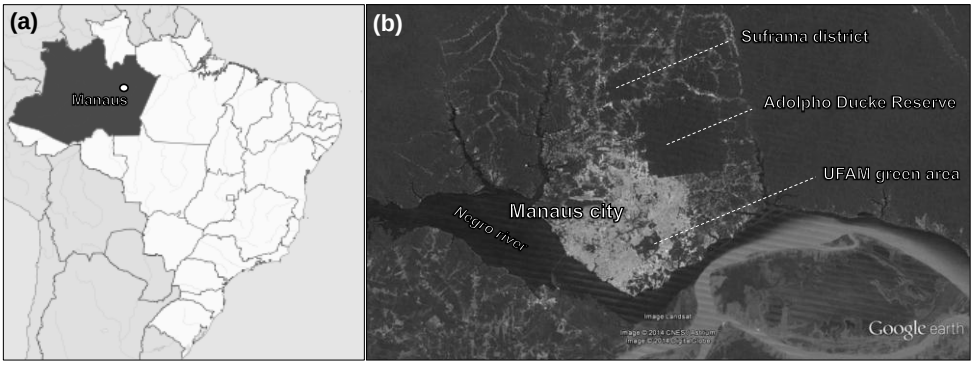

Figure 1. (a) Amazonas state (Brazil) and the capital Manaus. (b) Collection sites near the metropolitan area of Manaus.

From DAS, 4 specimens of *U. duckei*, 2 specimens of *U. floribunda*, and 1 specimen of *U. rufescens* were collected. At RFAD, 2 specimens of *U. duckei* were collected. From UFAM, 2 specimens of *U. stipitata* and 1 specimen of *U. guatterioides* were collected. All the species were sampled in September of 2012. For Adolpho Ducke Forest Reserve as well as in the agricultural district of SUFRAMA the specimens were previously identified. Specimens collected on the campus of UFAM were identified by Prof. Antonio Carlos Webber from the Department of Biology at the

Institute of Biological Sciences (ICB) of the Federal University of Amazonas. A voucher specimen of each individual is deposited according to Table 1.

Table 1. Collection of *Unonopsis* from Amazonas state, Brazil.

Plant	Code	Collection site	Voucher Nº	Mass of oil
U. duckei	DRA	RFAD	2627[a]	89 mg (0.089 %)
U. duckei	DRB	RFAD	3289[a]	56 mg (0.056 %)
U. duckei	DDA	DAS	3478[b]	63 mg (0.063 %)
U. duckei	DDB	DAS	3504[b]	65 mg (0.065 %)
U. floribunda	FDA	DAS	6701[b]	429 mg (0.429 %)
U. floribunda	FDB	DAS	7394[b]	380 mg (0.380 %)
U. rufescens	RD	DAS	3767[b]	527 mg (0.527 %)
U. duckei	DDC	DAS	80[b]	64 mg (0.064 %)
U. duckei	DDD	DAS	610[b]	88 mg (0.088 %)
U. stipitata	SUA	UFAM	8164[c]	150 mg (0.150 %)
U. stipitata	SUB	UFAM	8250[c]	137 mg (0.137 %)
U. guatterioides	GU	UFAM	8249[c]	105 mg (0.105 %)

[a]Herbarium of INPA (Instituto Nacional de Pesquisas da Amazônia); [b]Botany collection of PDBFF/INPA (Projeto Dinâmica Biológica de Fragmentos Florestais); [c]Herbarium of UFAM (Universidade Federal do Amazonas).

2.2. Essential oil analysis

After the collected, the leaves of each individual were dried at room temperature for a period of 20 days. For the extraction, 100 g of dried leaves were pulverized and subjected to extraction by hydrodistillation in a Clevenger-type apparatus for a period of four hours. The obtained oils were extracted with CH_2Cl_2, dried over anhydrous Na_2SO_4 and filtered. The extracted oils were placed in vials and stored at -15 °C until analysis. The analysis was performed in a GC-MS equipment, Model GC2010/QP2010 Plus (Shimadzu), using a selective detector and a capillary column Rtx-5 MS (30 m x 0.25 mm x 0.25). Helium gas was used as carrier gas with a flow of 1.02 ml/min. The injection solution was prepared by dissolving about 15 mg of oil in 1 ml of ethyl acetate, and 1 microliter of the solution injected using a split ratio of 1:50. The column temperature program was 60 to 280 °C with gradual increase of 3 °C/min. The temperatures of the injector and the ion source were 220 °C and 260 °C, respectively. To obtain the retention index were injected a homologous series of linear hydrocarbons (C7-C30) and the calculation was done according to the Van den Dool and Kratz equation [13].

The identification of the constituents was based on comparison of spectra with those stored in the Wiley 8th edition library and comparison of retention indexes with literature data [14].

2.3 Statistical analysis

The multivariate analysis was performed in the free software Chemoface, version 1.5 [15]. Principal components analysis (PCA) was calculated through the normalization of the 57 variables, corresponding to the 57 substances identified, being the hierarchical cluster analysis (HCA) calculated through the Euclidian distances and average linkage of the first tree principal components, whose cumulative variance represents 93.45%.

3. Results and Discussion

In the analysis of the essential oils was possible to identify 57 different constituents (Table 2), being observed identification coverages ranging from 76.02 to 95.72 %. Among the identified substances, a predominance of sesquiterpenes was observed. Trace amounts of monoterpenes were recorded only for *U. duckei*. The sesquiterpenes spathulenol and caryophyllene oxide were the main constituents of the essential oils for all specimens, with the exception of *U. guatterioides* and *U. stipitata*. As can be seen in Table 2, the highest amount of spathulenol was found in *U. duckei* specimens, reaching 40.20% of the total ion chromatogram (TIC) signal in DDB specimen, and the lowest was found in *U. guatterioides* (4.80%). The highest amount of caryophyllene oxide was observed in *U. rufescens* (15.95%) and the lowest in *U. guatterioides* (4.80). *U. stipitata* displayed relative high concentrations of (*E*)-caryophyllene (7.99-18.76%) and bicyclogermacrene (8.6-19.97 %) along with elemol (11.20%) for SUB, which was not observed in other specimens.

Table 2. Essential oil composition of different populations of Amazonian *Unonopsis* species.

RI[a]	Compounds	GU	SUA	SUB	FDA	FDB	RD	DRA	DDA	DDC	DDD	DRB	DDB
1095	linalool	-	-	-	-	-	-	0.29	-	-	0.25	-	-
1223	citronellol	-	-	-	-	-	-	-	-	0.40	-	-	-
1335	δ-elemene	2.51	0.99	-	0.27	-	-	0.48	0.46	0.70	1.29	2.07	-
1345	α-cubebene	1.19	-	-	0.83	0.61	0.24	0.34	-	0.17	0.21	0.36	0.18
1354	citronellyl acetate	-	-	-	-	-	-	-	-	0.60	-	-	-
1369	cyclosativene	0.71	0.11	0.27	0.27	0.38	-	0.40	-	0.33	-	-	0.17
1373	α-ylangene	0.34	-	-	0.17	0.60	0.31	-	-	0.26	-	-	0.22
1374	α-copaene	11.26	0.40	0.66	6.26	6.96	3.71	1.99	1.65	3.05	1.39	3.88	2.59
1387	β-bourbonene	0.90	-	0.45	1.24	1.48	1.44	0.46	0.30	0.31	0.37	0.30	0.18
1387	β-cubebene	0.84	-	5.64	0.84	0.74	0.26	0.49	0.36	0.55	0.43	1.00	0.63
1389	β-elemene	2.03	1.31	-	0.89	1.00	0.42	2.65	2.17	4.38	2.37	2.60	1.89
1409	α-gurjunene	-	-	-	0.19	0.16	-	1.26	0.49	-	0.11	-	-
1417	(*E*)-caryophyllene	3.91	18.76	7.99	4.06	4.04	3.97	1.22	0.70	0.61	0.58	1.32	0.46
1431	β-gurjunene	0.76	-	-	0.15	0.20	0.32	0.80	-	0.40	0.38	0.42	-
1434	γ-elemene	0.44	-	-	0.12	-	-	-	-	0.47	-	-	-
1437	α-guaiene	-	-	-	2.48	2.30	2.58	-	-	-	-	-	-
1439	aromadendrene	1.13	2.38	0.74	-	-	-	0.44	0.48	0.55	0.59	0.21	0.50
1449	sinularene	0.18	0.21	-	-	-	-	-	-	0.29	-	-	-
1452	α-humulene	1.74	5.18	1.13	0.94	1.06	0.84	0.96	-	0.75	0.56	0.92	0.79
1458	allo-aromadendrene	3.55	-	-	-	-	-	0.40	-	-	-	-	-
1478	γ-muurolene	2.79	0.45	-	4.18	3.90	6.37	1.11	0.58	0.52	0.54	1.11	0.45
1484	germacrene D	1.62	4.56	0.90	1.54	1.17	2.59	1.24	1.82	1.05	0.93	0.90	-
1489	β-selinene	0.31	0.30	0.64	0.80	1.17	0.50	0.54	-	-	0.50	0.57	-
1492	δ-selinene	-	0.22	-	0.53	0.24	-	-	-	-	-	-	-

1496	valencene	1.94	-	-	2.10	1.30	-	0.37	0.66	0.47	0.48	0.48	-
1500	bicyclogermacrene	-	19.97	8.86	1.55	0.70	3.67	-	-	-	-	-	-
1500	α-muurolene	1.26	0.23	0.25	1.93	2.13	2.38	0.57	-	0.32	0.34	-	0.18
1509	α-bulnesene	-	0.40	1.46	1.69	1.70	1.24	-	-	-	-	-	-
1513	γ-cadinene	1.91	0.26	0.67	4.96	3.54	4.26	0.92	0.84	2.82	0.65	1.01	0.56
1522	δ-cadinene	2.24	0.70	0.60	5.37	2.90	4.02	0.83	0.86	0.72	0.75	1.23	0.41
1533	(*E*)-cadina-1,4-diene	-	-	0.21	0.43	-	-	-	-	0.28	0.50	-	0.40
1537	α-cadinene	0.25	-	-	0.39	0.41	0.55	-	-	-	-	-	-
1544	α-calacorene	-	-	-	2.16	2.31	2.56	-	-	-	0.19	-	-
1548	elemol	1.60	1.29	11.20	1.30	1.70	0.68	7.86	1.84	1.48	1.70	1.17	4.73
1554	β-vetivenene	0.63	0.53	1.07	1.14	1.46	1.09	1.29	1.39	0.87	0.88	0.66	0.95
1559	germacrene B	1.84	0.56	-	0.52	0.50	0.29	-	1.38	3.63	-	0.25	0.28
1561	(*E*)-nerolidol	0.52	-	-	-	-	-	0.63	-	-	-	-	-
1565	(3*Z*)-hexenyl benzoate	-	-	-	-	-	-	0.53	-	0.75	1.06	0.74	-
1567	1,5-epoxysalvial-4(14)-ene	0.46	0.88	1.75	0.30	0.33	0.26	3.65	2.18	0.96	1.97	4.29	2.48
1577	spathulenol	4.80	20.46	17.9	15.66	13.96	17.13	19.10	37.49	30.6	28.77	20.7	40.2
1582	caryophyllene oxide	4.80	8.41	8.47	9.77	10.54	15.95	9.67	9.75	8.16	7.60	9.19	10.64
1590	β-Copaen-4α-ol	0.32	0.27	-	0.75	0.81	0.56	-	0.54	1.05	0.54	0.70	0.34
1592	viiridiflorol	1.61	1.60	0.87	0.99	1.30	0.84	-	-	1.05	1.22	-	-
1594	salvial-4(14)-en-1-one	-	0.47	0.26	-	-	-	0.25	1.04	-	-	1.71	0.50
1599	widdrol	-	-	-	1.14	1.36	0.63	-	-	-	-	-	-
1600	guaiol	5.14	0.85	5.06	1.43	1.60	1.57	6.41	2.78	1.57	1.52	0.49	3.16
1600	rosifoliol	-	0.80	-	-	-	-	-	-	-	-	-	-
1602	ledol	-	-	2.82	-	-	-	1.83	0.93	-	-	-	-
1608	humulene epoxy II	0.95	0.98	2.69	2.26	3.32	2.89	3.21	2.71	2.04	3.15	2.91	3.21
1631	isospathulenol	5.51	1.60	0.36	3.14	1.47	2.21	2.57	4.54	2.86	6.39	10.81	1.96
1644	α-muurolol	2.45	-	0.86	1.35	4.30	0.78	1.37	1.77	0.98	2.06	2.40	1.43
1649	β-eudesmol	1.13	-	0.97	1.10	1.51	1.16	1.49	1.85	1.01	1.52	1.24	1.16
1652	α-cadinol	3.64	0.59	1.70	1.62	1.85	2.09	3.05	2.90	2.07	2.9	3.58	2
1670	bulnesol	2.65	-	1.85	0.70	0.69	0.80	2.40	1.49	0.52	0.48	-	1.03
1675	cadalene	-	-	-	0.48	0.41	0.62	0.52	-	-	0.56	0.23	0.36
1845	(2*E*, 6*E*)-farnesyl acetate	-	-	-	-	-	-	0.24	-	-	0.29	-	-
1913	(5*E*, 9*E*)-farnesyl acetone	1.52	-	-	-	-	-	-	-	-	-	-	-
	Total identified (%)	83.38	95.72	88.30	89.99	88.11	91.78	83.83	85.95	79.60	76.02	79.45	84.04

[a]RI = Retention Index observed in literature[14]

U. floribunda and U. rufescens species presents as remarkable fact in their chemical composition, the exclusive presence of α-guaiene (2.30-2.58%), α-calacorene (2.16-2.56%) and widdrol (0.63-1.36%). The absence of aromadendrene is another important feature for *U. floribunda* and *U. rufescens*. The species *U. guatterioides* was the unique that presented α-copaene (11.26 %) as a major constituent. The predominance of sesquiterpenes in the leaf essential oils for all the species is in agreement with previous studies performed with *U. costaricensis* [12], where was reported the sesquiterpenes germacrene D (62.9%), viridiflorol (12.1%) and bicyclogermacrene (10.0%) as main constituents of the leaves essential oil. The study of the roots and fruits of *U. guatterioides* [11] displayed sesquiterpenes as the major compounds for the different tissues, being observed δ-cadinol (21.6%), terpinen-4-ol (15.7%) and caryophyllene oxide (15.3%) in the roots oil, and mainly (*E*)-caryophyllene (22.5%), α-pinene (11.7%) and caryophyllene oxide (10.4%) for the fruits. Although

monoterpenes were only detected as trace constituents for *U. duckei* and not observed for the other species, a previous work [10] reports this class in high concentrations at the flowers *U. stipitata*, suggesting that moneterpenes play an important role in the pollination processes.

The statistical analysis through principal component analysis (PCA) and hierarchical cluster analysis (HCA) of the GC-MS data from the essential oil allowed the unequivocal correlation between the studied species (Figures 2 and 3). Four main groups were observed for the hierarchical analysis, where *U. guatterioides* and *U.* stipitata were clearly separated (groups 1 and 2). The Group 3 was constituted by *U. rufescens* and *U. floribunda* where the chemical composition was slightly different, being observed as punctual differences the presence of cyclosativene, α-gurjenene, δ-selinene and valencene for *U. floribunda* specimens. The group 4 was constituted by the *U. duckei* specimens, where is observed the formation of two subgroups. The two subgroups represent the two collection sites for this species. Analyzing the chemical variability of these populations, it is observed that the individuals collected in the Adolpho Ducke Forest Reserve (RFAD) exhibit greater difference in the chemical composition when compared to individuals collected in agricultural district of SUFRAMA (DAS). When the chemical variability of group 4 is compared with the chemical variability of group 3, is observed how *U. floribunda* and *U. rufescens* are chemically closer than *U. duckei* species collected in the same environment (RFAD). Morphologically, the difference between *U. floribunda* and *U. rufescens* is the glaucous monocarps (vs brown or blackish in sicco) and the thicker monocarp wall (1–2.5 vs 0.2–0.3 mm) [1].

As described above, the presence of α-guaiene (2.30-2.58%), α-calacorene (2.16-2.56%) and widdrol (0.63-1.36%) may be an indicative of chemical markers for these species, once they does not appear in other studied individuals. In group 2 specimens of *U. stipitata* are observed, where a lower chemical similarity is recorded when compared to individuals of *U. duckei* collected from different sites (DAS and RFAD) (Figure 2) suggesting that *U. stipitata* presents a high chemical variability. Recently several species of Annonaceae were submitted to phylogenetic analysis based on rbcL and trnL-F plastid DNA sequences, being *U. sipitata*, *U. rufescens*, *U. pittieri*, *Bocageopsis pleiosperma*, *Bocageopsis multiflora* and *Onychopetalum piriquino* segregated by genus. Despite the segregation observed between the three genera, is observed for species of *Unonopsis* genus a minor genetic variability between *U. rufescens* and *U. pittieri*, being the variability observed to *U. stipitata* near to that observed between *Bocageposis* and *Onychopetalum* genus [16]. The group 1, constituted only by *U. guatterioides*, presented the largest chemical variability (Figures 2 and 3). This finding is not surprising since *U. guatterioides* is by far the most problematical and variable species in the genus from the morphological point of view [1].

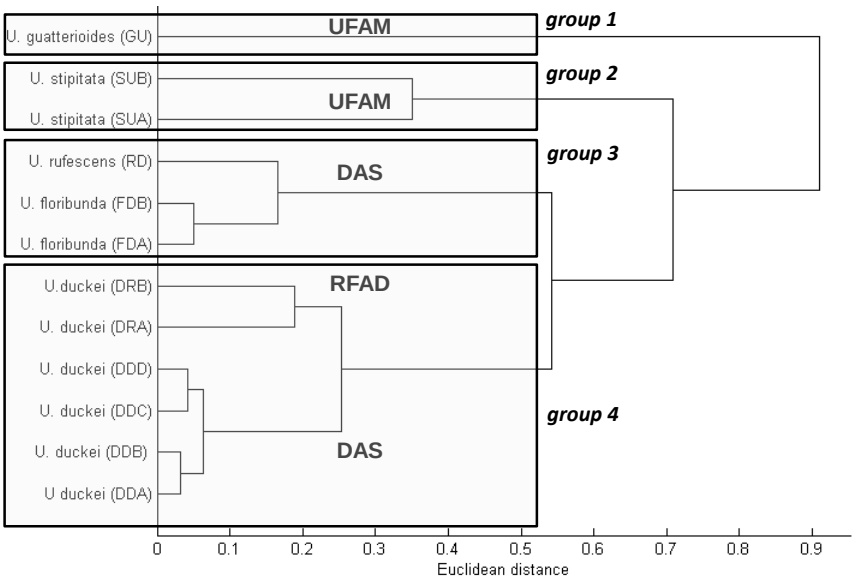

Figure 2. Cluster analysis of the different populations of Amazonian *Unonopsi*s species.

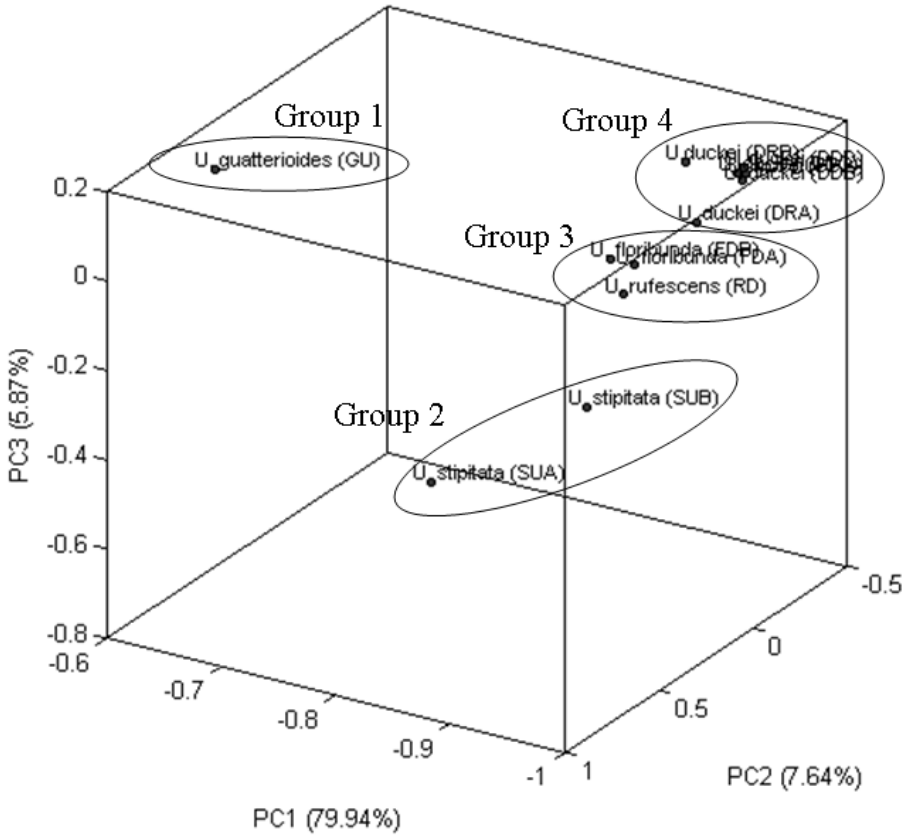

Figure 3. PCA analysis of the different populations of Amazonian *Unonopsis* species.

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