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Chemotaxonomy of Portuguese *Ulex*: Quinolizidine alkaloids as taxonomical markers

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Abstract

Six species of Portuguese *Ulex* L. in a total of nineteen populations were studied by GC–EIMS as to their content in quinolizidine alkaloids. Sparteine, β-isosparteine, jussiaeiine A, *N*-methylcytisine, cytisine, 5,6-dehydrolupanine, rhombifoline, lupanine, jussiaeiine B, *N*-formylcytisine, *N*-acetylcytisine, anagyrine, jussiaeiine C, jussiaeiine D, pohakuline, baptifoline, and epibaptifoline were detected. Analysis of the chromatograms showed that the chemical profile of all species was mainly composed of *N*-methylcytisine, cytisine, anagyrine, and jussiaeiines A, B, C and D. Therefore a quantification study of these alkaloids in all the populations studied was done by GC. These data were then submitted to cluster analysis and principal component analysis, which allowed the definition of five chemotypes and the recognition of hybrids. *N*-methylcytisine, cytisine, and jussiaeiines A, C and D are recognized as markers of this genus in Portugal.

Keywords: *Ulex airensis*; *Ulex australis*; *Ulex densus*; *Ulex europaeus*; *Ulex jussiaei*; *Ulex minor*; Fabaceae; Chemotaxonomy; Quinolizidine alkaloids

1. Introduction

The *Ulex* L. genus (Fabaceae) grows in Portugal as several different species. Some of these occur in restricted areas and do not present any problem in their botanical classification since they have distinct morphological characters. Some examples are *Ulex densus* Welw. ex Webb, *Ulex europaeus* L., and *Ulex minor* Roth. However, several species growing in the central zone of Portugal do not possess well defined morphological characters and have been reclassified by several authors (Sampaio, 1924; Coutinho, 1939; Rothmaler, 1941; Vicioso, 1962; Guinea and Webb, 1968; Franco, 1971). These species have been commonly known as *Ulex parviflorus* Pourr. *sensu lato*. More recently, Espirito-Santo et al. (1997), based on morphological and kariological characters, further reclassified these taxa and proposed for the central zone of Portugal four different species including: *Ulex airensis* M.D. Espirito-Santo, P. Cubas, M.F. Lousã, C. Pardo, & J.C. Costa, *Ulex australis* Welw. ex Webb, and *Ulex jussiaei* Webb. Considering the known botanical classification and with the intention to add chemical information to it, we present here a chemotaxonomical study based on the quinolizidine alkaloids (QA) content of six species of *Ulex* L.

QA occur mainly within the Fabaceae (Wink, 1993) and are characteristic of the Faboideae subfamily (Hegnauer, 1988). Previous work on their use as taxonomic markers can be found in the works of Kinghorn et al. (1980, 1982), Salatino and Gottlieb (1980, 1981, 1983), and Wink and Waterman (1999).

2. Results and discussion

With the purpose of establishing a chemical classification of Portuguese *Ulex* L. we investigated the composition of this genus in QA. A total of six species (nineteen populations) collected in Portugal during the years of 1994–1998

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were studied: *U. airensis* M.D. Espirito-Santo, P. Cubas, M.F. Lousã, C. Pardo, & J.C. Costa (2/98, 3/98), *U. australis* Welw. ex Webb (2/97), *U. densus* Welw. ex Webb (1/95, 3/95, 1/96, 2/96, 3/96, 1/97), *U. europaeus* L. (1.3/94), *U. jussiaei* Webb (1.1/94, 2.1/94, 3.1/94, 2/95, 4/95, 4/96, 5/96, 1/98) and *U. minor* Roth (1.2/94). The composition in QA was done by GC–EIMS and sparteine **1**, β-isosparteine **2**, jussiaeiine A **3**, *N*-methylcytisine **4**, cytisine **5**, 5,6-dehydrolupanine **6**, rhombifoline **7**, lupanine **8**, jussiaeiine B **9**, *N*-formylcytisine **10**, *N*-acetylcytisine **11**, anagyrine **12**, jussiaeiine C **13**, jussiaeiine D **14**, pohakuline **15**, baptifoline **16**, and epibaptifoline **17** were identified by their fragmentation pattern and Kovats retention indexes (Wink et al., 1995; Máximo and Lourenço, 2000). The distribution of these alkaloids in the various populations is in Table 1. These results show that Portuguese *Ulex* L. is rich in α-pyridone type alkaloids and jussiaeiines A, B, C and D. Although a clear distinction between species cannot be established by these data, GC analysis showed that all the specimens have as main constituents *N*-methylcytisine **4**, cytisine **5**, anagyrine **12**, and jussiaeiines A **3**, B **9**, C **13** and D **14** (Fig. 1). As such, a quantification study of these alkaloids in all the populations studied was done by GC, using caffeine as internal standard (Priddis, 1983). Five standard solutions of the seven alkaloids and 45 μg of caffeine were used (Table 2) and the calibration plots of area of alkaloid/area of caffeine versus mass of alkaloid/mass of caffeine were made. Although jussiaeiines C **13** and D **14** had to be quantified together due to poor resolution, all the results proved good linearity in the 20-fold range of concentrations used. Statistical treatment of the linear regressions allowed the calculation of the limit of detection (lod) for each compound. The obtained values

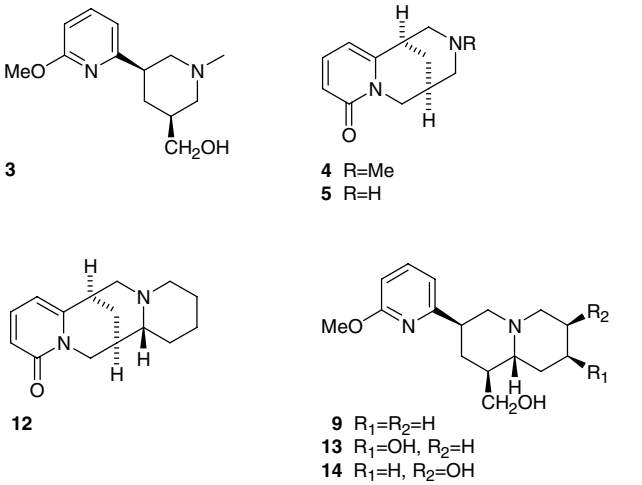

Fig. 1. Major constituents of Portuguese *Ulex* L. *N*-methylcytisine **4**, cytisine **5**, anagyrine **12** and jussiaeiines A **3**, B **9**, C **13** and D **14**.

Table 2
Standard solutions (1–5) used for the calibration plots

Alkaloid	R_I	Amount (μg)				
		1	2	3	4	5
Caffeine	1769	45.0	45.0	45.0	45.0	45.0
Jussiaeiine A **3**	1856	92.6	54.5	27.2	13.6	5.40
N-Methylcytisine **4**	1936	47.5	23.8	11.9	4.80	2.40
Cytisine **5**	1967	85.0	50.0	25.0	12.5	5.00
Jussiaeiine B **9**	2210	55.8	27.9	13.9	5.60	2.80
Anagyrine **12**	2358	52.0	26.0	13.0	5.20	2.60
Jussiaeiine C **13**	2426	47.5	23.8	11.9	4.80	2.40
Jussiaeiine D **14**	2436	50.0	25.0	12.5	5.00	2.50
Jussiaeiine C **13** + jussiaeiine D **14**		97.5	48.8	24.4	9.80	4.90

Table 1
Composition in quinolizidine alkaloids of the nineteen populations of *Ulex* L.

Species	Population	Alkaloid																
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
U. airensis	2/98	•		•	•	•	•	•	•	•	•	•	•	•	•		•	•
	3/98		•	•	•		•	•	•	•	•		•	•	•			
U. australis	2/97	•	•	•	•	•	•	•	•	•			•	•	•	•	•	•
U. densus	1/95	•		•	•	•	•	•	•	•	•		•	•	•	•	•	•
	3/95			•	•	•	•	•	•	•	•		•	•	•		•	•
	1/96			•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	2/96			•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	3/96	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	1/97			•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
U. europaeus	1.3/94				•	•	•	•	•		•	•	•				•	
U. jussiaei	1.1/94			•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
	2.1/94			•	•	•	•		•	•	•		•	•	•	•	•	
	3.1/94	•		•	•	•	•	•	•	•	•		•	•	•	•	•	
	2/95			•	•	•	•	•	•	•	•		•	•	•	•	•	•
	4/95		•	•	•	•	•	•	•	•	•		•	•	•	•	•	•
	4/96			•	•	•	•	•	•	•	•		•	•	•	•	•	•
	5/96	•	•	•	•	•	•	•	•	•	•		•	•	•	•	•	•
	1/98			•	•	•	•		•	•	•		•	•	•			
U. minor	1.2/94	•			•	•	•	•	•		•	•	•	•	•	•	•	•

Sparteine **1**, β-isosparteine **2**, jussiaeiine A **3**, *N*-methylcytisine **4**, cytisine **5**, 5,6-dehydrolupanine **6**, rhombifoline **7**, lupanine **8**, and jussiaeiine B **9**, *N*-formylcytisine **10**, *N*-acetylcytisine **11**, anagyrine **12**, jussiaeiine C **13**, jussiaeiine D **14**, pohakuline **15**, baptifoline **16**, and epibaptifoline **17**.

of area of alkaloid/area of caffeine, together with the correlation coefficients of the linear regressions, and the lod for each compound are presented in Table 3. Analysis of the plant extracts after proper dilution with a solution of caffeine allowed the determination of the amount of each alkaloid in all of the populations, together with the standard deviation. These results are presented in Table 4, expressed as ppm/dry wt. of plant. In the cases were the analysis by GC–EIMS allowed the identification of the compound but its quantity is inferior to lod its presence is identified as a trace amount.

These data were then submitted to cluster analysis using the Pearson correlation coefficient and the unweighted pair group average method. The dendogram obtained with a cofenetic correlation coefficient (r_{cs}) of 0.915 is depicted in Fig. 2 and the plot of linkage distance versus the number of clusters is illustrated in Fig. 3. This plot allows the validation of four clusters: one including *U. europaeus* 1.3/94 and *U. minor* 1.2/94, other including *U. australis* 2/97 and three populations classified as *U. jussiaei* (4/95, 4/96 and 5/96), other including two *U. densus* (3/95 and 2/96), and a final cluster with *U. airensis* 2/98 and 3/98, four *U. densus* (1/95, 1/96, 3/96 and 1/97), and five *U. jussiaei* (1.1/94, 2.1/94, 3.1/94, 2/95 and 1/98). This analysis clearly differentiates *U. europaeus* and *U. minor* from the remaining species,

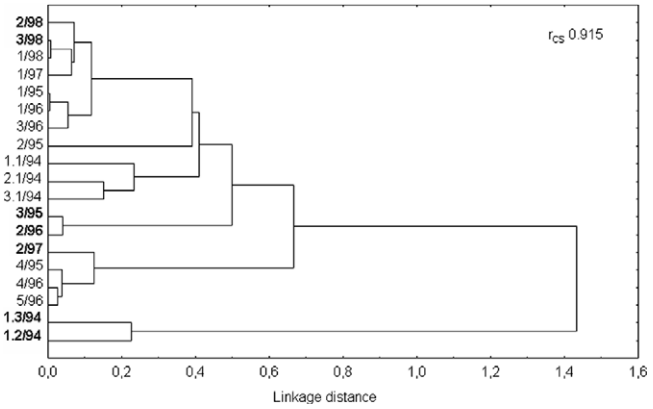

Fig. 2. Dendogram obtained by the Pearson correlation coefficient and the unweighted pair group average method.

Table 3
Values of area of alkaloid/area of caffeine used in the calibration plots

Alkaloid	Area of alkaloid/area of caffeine					*r*	lod (μg/μl)
	1	2	3	4	5		
Jussiaeiine A **3**	2.683	1.418	0.564	0.246	0.064	0.998	0.16
N-Methylcytisine **4**	0.808	0.345	0.151	0.039	0.023	0.998	0.09
Cytisine **5**	2.749	1.442	0.605	0.222	0.050	0.998	0.12
Jussiaeiine B **9**	1.753	0.801	0.394	0.125	0.056	0.999	0.05
Anagyrine **12**	2.001	0.948	0.472	0.148	0.071	1.00	0.03
Jussiaeiine C **13** + jussiaeiine D **14**	2.381	1.065	0.533	0.156	0.064	0.999	0.11

r, correlation coefficient; lod, limit of detection.

Table 4
Amounts of *N*-methylcytisine **4**, cytisine **5**, anagyrine **12**, and jussiaeiines A **3**, B **9** and C **13** + D **14** in the plant extracts in ppm/dry wt. of plant

Species	Pop.	ppm/dry wt. of plant (s.d.)					
		3	**4**	**5**	**9**	**12**	**13** + **14**
U. airensis	2/98	25.1 (4.6)	•	•	4.4 (1.5)	•	18.5 (3.2)
	3/98	64.9 (8.0)	•		•	•	25.9 (5.7)
U. australis	2/97	36.9 (3.7)	9.7 (2.1)	47.2 (2.9)	8.5 (1.2)	4.3 (0.7)	75.4 (2.6)
U. densus	1/95	45.2 (5.9)	16.9 (3.3)	•	•	•	15.6 (4.2)
	3/95	35.2 (7.1)	49.8 (4.0)	•	•	•	16.8 (5.0)
	1/96	36.0 (1.7)	10.3 (1.0)	•	•	•	10.9 (1.2)
	2/96	44.2 (6.5)	73.3 (4.2)	15.6 (5.3)	•	4.6 (1.2)	39.3 (4.5)
	3/96	47.4 (4.8)	15.2 (2.7)	12.8 (4.0)	•	•	10.9 (3.5)
	1/97	185.6 (13.9)	51.0 (7.8)	59.3 (11.4)	17.2 (4.7)	•	127.9 (9.7)
U. europaeus	1.3/94		•	83.8 (4.6)		32.6 (1.1)	
U. jussiaei	1.1/94	18.8 (6.8)	23.1 (3.6)	•	8.4 (2.2)	6.8 (1.3)	30.1 (4.7)
	2.1/94	3.4 (0.7)	1.4 (0.4)	•	1.7 (0.2)	•	2.4 (0.5)
	3.1/94	15.3 (1.4)	12.3 (0.8)	•	15.8 (0.4)	2.6 (0.3)	22.2 (1.0)
	2/95	9.7 (0.8)	7.3 (0.5)	2.0 (0.7)	6.4 (0.3)	6.1 (0.2)	5.5 (0.6)
	4/95	26.6 (6.7)	•	•	•	5.8 (1.3)	116.6 (4.5)
	4/96	114.9 (14.0)	74.9 (7.8)	106.2 (11.2)	51.6 (4.5)	38.4 (2.7)	317.4 (10.1)
	5/96	48.6 (6.9)	15.2 (3.8)	41.4 (5.5)	37.8 (2.2)	10.8 (1.3)	141.3 (4.8)
	1/98	33.0 (1.2)	2.6 (0.6)	5.8 (0.9)	2.9 (0.4)	1.7 (0.2)	13.7 (0.8)
U. minor	1.2/94		20.2 (1.1)	29.0 (1.1)		12.3 (0.3)	•

Pop., population; s.d., standard deviation; •, trace amount (<lod).

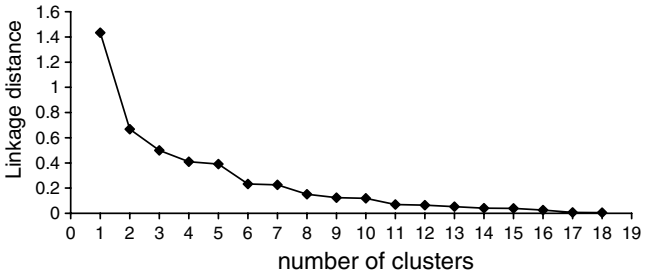

Fig. 3. Linkage distance versus number of clusters.

according to their well established taxonomical classification. It also separates *U. australis* from *U. airensis* and isolates two of the populations classified as *U. densus* (3/95 and 2/96). The fact that the remaining populations of *U. densus* (1/95, 1/96, 3/96 and 1/97) are grouped with *U. airensis* can be explaining if hybridization between these two species is taken into account: these populations of *U. densus* could have morphological characters closer to *U. densus* and a chemotype closer to *U. airensis*. Hybridization can also explain the apparent random distribution of *U. jussiaei*. These populations could in fact represent hybrids and as such do not form a well characterized species, in accordance with its controversial botanical classification.

Since hybridization was taken into account we performed principal components analysis (PCA) as it has been known to recognize this type of phenomena: hybrids present an intermediate position between the groups recognized for the two parents (Edmonds, 1978). This analysis was first performed on the nineteen populations studied. Three principal components were extracted (Fig. 4, 89.2% of variance explained). This analysis again clearly shows the distinction of *U. europaeus* 1.3/94 and *U. minor* 1.2/94 from the remaining species and this separation is accomplished by PC3. The analysis of the factor loadings of each compound, after varimax rotation (Table 5) allows its identification with cytisine **5**, pointing out the relevance of this compound in the distinction of the two species. Although these two species seem to form a unique group their chemotypes can be distinguished on the basis of the presence of *N*-methylcytisine **4** (Table 4). Having differentiated two of the species another PCA was performed on the remaining seventeen populations. Again three components were extracted (Fig. 5, 92.1% variance explained). This second analysis, in agreement with the cluster analysis, allows the clear separation of three major groups: one led by *U. airensis* 2/98 and 3/98, other led by *U. australis* 2/97, and other led by *U. densus* 3/95 and 2/96. Once again four *U. densus* (1/95, 1/96, 3/96 and 1/97) are closer to *U. airensis* although showing an intermediate position between the groups defined by *U. airensis* (2/98 and 3/98) and *U. densus* (3/95 and 2/96). They are thus recognized as hybrids of the two species. As such the chemotype of *U. densus* is thus recognized as the one represented by the populations 3/95 and 2/96. The fact that the populations classified as *U. jussiaei* are distributed throughout the three major groups clearly suggests they are in fact hybrids of *U. airensis* × *U. densus* or *U. australis* × *U. densus* and do not consist of an isolated

Table 5
Factor loadings for the PCA of nineteen populations of *Ulex* L.

Alkaloid	PC1	PC2	PC3
Jussiaeiine A **3**	1.921	−0.170	0.224
N-Methylcytisine **4**	−0.064	−0.928	0.764
Cytisine **5**	−0.054	0.179	−1.888
Jussiaeiine B **9**	−0.889	−0.337	0.611
Anagyrine **12**	−0.675	−0.632	−0.295
Jussiaeiine C **13** + jussiaeiine D **14**	−0.238	1.888	0.584

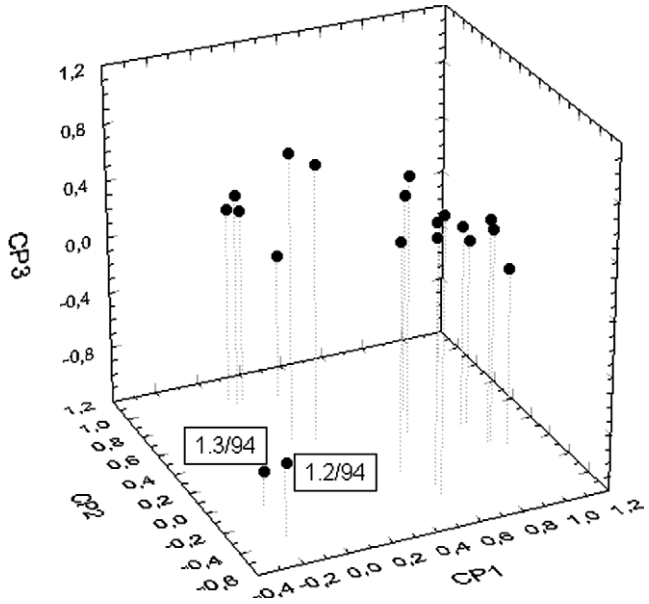

Fig. 4. PCA of nineteen populations of *Ulex* L.

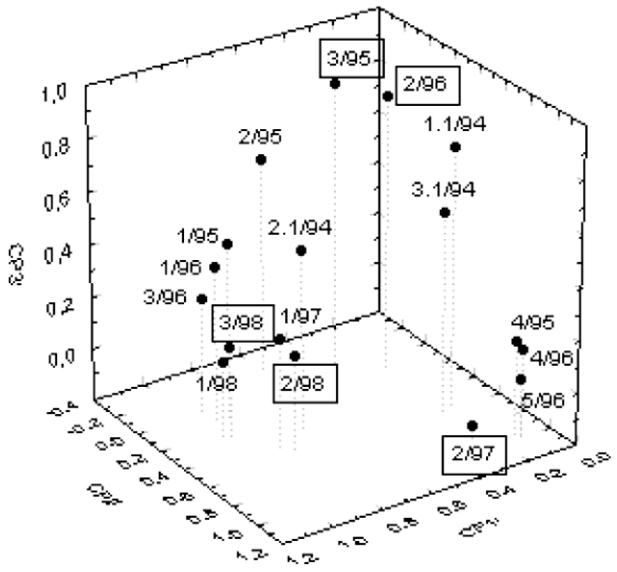

Fig. 5. PCA of seventeen populations of *Ulex* L.

species with a well-defined chemotype. Some have chemotypes similar to *U. airensis*, some are similar to *U. australis* and some are similar to *U. densus*.

Analysis of the factor loadings of each compound for this analysis (Table 6) allows the identification of PC′1 with jussiaeiine A **3**, of PC′2 with jussiaeiines C **13** + D **14**, and of PC′3 with *N*-methylcytisine **4**. Combining the results of the two PCA with the quantification study (Table 4) the following chemotypes can be established: *U. europaeus* and *U. minor* are mainly constituted by cytisine **5**, do not possess significant amounts of jussiaeiines A **3**, C **13** and D **14**, and can be differentiated from each other on the basis of the presence of *N*-methylcytisine **4**. *U. airensis* are mainly constituted by jussiaeiine A **3**, possess a significant amount of jussiaeiines C **13** and D **14**, and only traces of *N*-methylcytisine **4**, cytisine **5**, and anagyrine **12**. *U. australis* is mainly constituted by jussiaeiines C **13** and D **14**, and possesses a significant amount of all the remaining alkaloids. Finally, *U. densus* (3/95 and 2/96) is mainly constituted by *N*-methylcytisine **4**, and contains a significant amount of jussiaeiines A **3**, C **13** and D **14**. The compounds *N*-methylcytisine **4**, cytisine **5**, and jussiaeiines A **3**, C **13** and D **14** can thus be selected as markers of this genus in Portugal.

Further comments on the evolution of these species can be made considering the biosynthesis of QA. Tetracyclic α-pyridone alkaloids are known to be the precursors of tricyclic structures and the sequence cytisine **5** → *N*-methylcytisine **4** is well established (Ohmiya et al., 1995). Jussiaeiines A **3**, B **9**, C **13** and D **14** are postulated to derive from the corresponding α-pyridone alkaloids by rupture of ring B (Máximo and Lourenço, 2000) and as such are considered to be derived from *N*-methylcytisine **4**, anagyrine **12**, baptifoline **16** and 14-β-hydroxyanagyrine, respectively. The more evolved metabolites can thus be considered jussiaeiine A **3**, followed by jussiaeiines C **13** and D **14**, followed by *N*-methylcytisine **4** and cytisine **5**. Considering the chemotypes previously established for the Portuguese *Ulex* L. the following comments can be made: *U. europaeus* and *U. minor* can be considered the more primitive species since they accumulate mainly cytisine **5** and have only traces of jussiaeiines A **3**, C **13** and D **14**; the next species in the evolution scale should be *U. densus* since it accumulates *N*-methylcytisine **4** and has significant amounts of jussiaeiines A **3**, C **13** and D **14**; this species should be followed by *U. australis* (mainly constituted by jussiaeiines C **13** and D **14** and with significant amounts of the remaining alkaloids); finally we should have *U. airensis* (accumulates mainly jussiaeiine A **3**, has significant amounts of jussiaeiines C **13** and D **14**, and has only traces of the remaining alkaloids).

Table 6
Factor loadings for the PCA of seventeen populations of *Ulex* L.

Alkaloid	PC′1	PC′2	PC′3
Jussiaeiine A **3**	2.012	−0.273	0.186
N-Methylcytisine **4**	−0.618	−0.663	1.663
Cytisine **5**	−0.221	−0.073	−1.313
Jussiaeiine B **9**	−0.470	−0.291	−0.282
Anagyrine **12**	−0.518	−0.683	−0.554
Jussiaeiine C **13** + jussiaeiine D **14**	−0.185	1.982	0.301

3. Concluding remarks

Chemotypes can be defined for *U. airensis* M.D. Espírito-Santo, P. Cubas, M.F. Lousã, C. Pardo, & J.C. Costa, *U. australis* Welw. ex Webb, *U. europaeus* L., *U. minor* Roth, and *U. densus* Welw. ex Webb, although for the later species its botanical classification should be reinforced with chemical characterization since hybrids of *U. airensis* × *U. densus* have been detected. The species commonly known as *U. jussiaei* Webb groups a number of plants that are in fact hybrids of *U. australis* × *U. densus* and *U. airensis* × *U. densus* and in all cases its chemical composition should be taken into account in their classification. The compounds *N*-methylcytisine **4**, cytisine **5**, and jussiaeiines A **3**, C **13** and D **14** are identified as markers of this genus in Portugal.

4. Experimental

4.1. Plant material

Ulex L. specimens were collected in Portugal in the years 1994–1998 in the blooming season and voucher specimens were deposited at the Herbário, Museu, Jardim Botânico da Universidade de Lisboa, as follows: *U. airensis* M.D. Espírito-Santo, P. Cubas, M.F. Lousã, C. Pardo, & J.C. Costa – 2/98 [LISU 171664] collected at Porto de Mós, Serra de Aire, July 1998, 3/98 [LISU 171667] collected at Ramalhal, Torres Vedras, December 1998; *U. australis* Welw. ex Webb – 2/97 [CTG 164972] collected at Terras do Risco, Arrábida, April 1997; *U. densus* Welw. ex Webb – 1/95 [LISU 171659] collected at Cabo Espichel, Arrábida, April 1995, 3/95 [LISU 171661] collected at El Carmen, Arrábida, April 1995, 1/96 [TO 42612A] collected at Casais da Serra, Arrábida, April 1996, 2/96 [TO 42613a] collected at Vale do Rasca, Arrábida, April 1996, 3/96 [TO 42609A] collected at Aldeia da Serra, Arrábida, April 1996, 1/97 [CTG 164971] collected at Terras do Risco, Arrábida, April 1997; *U. europaeus* L. – 1.3/94 [ASCE 2593] collected at Peninha, Sintra, April 1994; *U. jussiaiei* Webb – 1.1/94 [ASCE 2595] collected at Peninha, Sintra, April 1994, 2.1/94 [ASCE 2594] collected at Azenhas do Mar, Sintra, April 1994, 3.1/94 [ASCE 2591] collected at Quinta da Capela, Sintra, April 1994, 2/95 [LISU 171660] collected at Califórnia, Arrábida, April 1995, 4/95 [LISU 171662] collected at Casalinho, Arrábida, April 1995, 4/96 [TO 42611A] collected at Zambujal, Arrábida, April 1996, 5/96 [TO 42516A] collected at Terras do Risco, Arrábida, April 1996, 1/98 [LISU 171663] collected at Cabo da Roca, Sintra, July 1998; *U. minor* Roth – 1.2/94 [ASCE 2592] collected at Peninha, Sintra, April 1994.

4.2. Alkaloid extraction

The dried and finely powdered aerial parts of each population (ca. 40 g) were extracted with 0.5 M HCl as previously described (Wink et al., 1995). The crude extracts were further dissolved in 0.1 M HCl, and purified by funnel extraction with *n*-hexane. The aqueous layers were made basic to pH 12 with NH_3 25% and applied in isolute® (International Sorbent Technology Ltd.) columns. The pure alkaloid extracts were eluted with CH_2Cl_2 and the solvent was evaporated under reduced pressure.

4.3. GC–EIMS analysis

The pure alkaloid extracts were chromatographed in a fused-silica capillary column (OV 1 or DB1), using He as carrier gas and the following conditions: split ratio 1:10; injector temperature 250 °C; ion source temperature 140 °C; temperature program 120 °C, hold for 3 min, increase 6 °C/min to 312 °C, hold for 10 min. The MS measurements were performed on a Finnigan MAT 4500, at 45 eV and using chemical ionization with NH_3 for confirmation of molecular ions. The Kovats retention indexes (R_I) were determined by co-injection with a mixture of C-14 to C-20, C-24 and C-28 alkanes.

4.4. GC analysis

The pure alkaloid extracts were chromatographed on a fused-silica capillary column (WCOT DB 1) using a Shimadzu GLC 14A equipped with a FID detector and using He as carrier gas (0.85 ml min^{-1}). The following conditions were used: split ratio 1:20; injector temperature 300 °C; detector temperature 300 °C; temperature program 120 °C, hold for 2 min, increase 6 °C/min to 260 °C, increase 20 °C/min to 300 °C.

4.5. Quantification

(−)-*N*-Methylcytisine **4**, (−)-cytisine **5**, (−)-anagyrine **12**, and (+)-jussiaeiines A **3**, B **9**, C **13** and D **14** were extracted and purified from *U. jussiaiei* Webb 5/96 (Máximo and Lourenço, 2000). Caffeine was extracted and purified from black tea leaves. The purity of each compound was confirmed by ^1H and ^{13}C NMR, and GC analysis. For the calibration plots 1 μl of each standard mixture (Table 2) was injected into the gas chromatograph, and the alkaloids were identified by their R_I, by co-injection with a mixture of C-14 to C-20, C-24 and C-28 alkanes. Linear regressions of the ratios area of alkaloid/area of caffeine versus mass of alkaloid/mass of caffeine were plotted. The lod was calculated as $a + 3S_{YX}$, where a is the Y intercept and S_{YX} is the standard deviation of the Y values. The content in QA of the analysed populations was established by the injection of 1 μl of the pure extracts after proper dilution with a solution of caffeine 0.9 mg/ml. The ratio of area of alkaloid/area of caffeine was measured and the amounts of each alkaloid were calculated by the linear regressions.

4.6. Statistic treatment

For the cluster analysis and PCA *STATISTICA for Windows* v. 5.1, 1997 was used. The Pearson correlation coefficient and unweighted pair group average method were used in cluster analysis and the data of the principal component analysis were rotated by the varimax method.

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