Biogeographical Patterns and Phenological Changes in *Lapiedra martinezii* Lag. Related to Its Alkaloid Diversity

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The aim of this work was to investigate the alkaloid patterns of *Lapiedra martinezii* and their relation to biogeography and phenology focused in a phylogenetic comparison. Plants from 14 populations of *L. martinezii*, covering almost its entire distribution area, were subjected to morphological, ecological, and phytochemical analysis. Experiments for different alkaloid-type content are proposed as a new tool for analysis of plant distribution. Several plants were transplanted for weekly observation of their phenological changes, and alkaloids from different plant organs were extracted, listed, and compared. The alkaloid pattern of *L. martinezii* comprises 49 compounds of homolycorine, lycorine, tazettine, haemantamine, and narciclasine types. The populations located in the north and south margins of the distribution area displayed alkaloid patterns different from those of the central area. Changes in these patterns during their phenological cycle may be related to a better defence for plant reproduction. *L. martinezii* is an old relict plant, and it has maintained some of the more primitive morphological features and alkaloid profiles of the Mediterranean Amaryllidaceae. The variations in alkaloid content observed could be interpreted in a phylogenetic sense, and those found in their phenological changes, in an adaptive one.

Introduction. – Amaryllidaceae is a cosmopolitan, mainly pantropical family that originated in western Gondwana [1], with different systematic treatments throughout botanical history. Using molecular data, *Fay* and *Chase* demonstrated that Amaryllidaceae form a monophyletic group related to Agapanthaceae and Alliaceae [2]. Nowadays, however, many authors maintain the independent status of the three families [1][3]. Amaryllidaceae is one of the few families of Asparagales with clear morphological characters, namely the combination of umbella cymes, inferior ovaries, and typical alkaloid content [3].

Since the genetic studies in Amaryllidaceae, what is known as the Eurasian Clade is considered as a monophyletic group [1][4]. This group presents a vicariant divergence in two branches, the Asian Lycorideae tribe and the rest (Narcisseae, Galantheae, and other genera). The second branch is centered within the Mediterranean region, mainly between the Iberian Peninsula and the north of Africa [4]. This area is the center of origin of the genera *Narcissus*, *Lapiedra, Hannonia*, and *Vagaria* [4][5]. The old genus *Lapiedra* Lag. is restricted to the Mediterranean coastal side of the Baetic mountain chain (from Málaga to Valencia), with a few populations in the nearby Spanish city of Melilla (North Africa).

Taxonomically, the location of *Lapiedra* in Amaryllidaceae is not definitely established. In a traditional sense, *Lapiedra* was closely related to Pancratieae [6] or Galantheae [5][7]. But despite the differences in the shape of the corolla, its relation with Narcisseae has been shown, because this endemic Iberian genus shares a common gene pool with two other endemic North African genera, *Hannonia* and *Vagaria* [1]. Others placed *Lapiedra* close to *Acis,* the western Mediterranean vicariant of *Leucojum* [8]. *Lapiedra* is a diploid, with $2n=22$, the ancestral chromosome number for the Amaryllidaceae, and it is one of the most primitive genera of the Galantheae [5]. *Hannonia* and *Vagaria* sorting took place after the separation of Galantheae and Narcisseae, but they still retain a mosaic of ancestral haplotypes [4][9]. Also, these two genera and *Lapiedra* share old morphological characters [4], such as an actinomorphic corolla and solid scapes. *Vagaria* and *Lapiedra* have lorate leaves with an adaxial whitish midrib stripe and a floral tube that is obsolete or very short. However, *Lapiedra* have complete sagittate anthers [5][10], a very rare character in Amaryllidaceae that is only shared with the Chilean *Traubia*, and is almost differentiated in *Vagaria* and *Acis* [11]. For all these reasons, these three genera may represent a relict taxa from the early differentiation of the Mediterranean Clade from the Galantheae [4].

Alkaloids of Amaryllidaceae have been considered to possess taxonomic significance [3]. Many of them have been identified and evaluated [12–15], but despite the early works on the alkaloids of *Lapiedra martinezii* [16][17], they have not been studied fully. Further than the alkaloid content, the relations between its phytochemistry and its biogeographical, ecological, and phenological aspects have never been investigated.

The present study may contribute, for the first time, to a better understanding of the actual distribution of this genus in a phytogeographic and phylogenetic scenario, together with its major alkaloid pattern throughout the distribution area. Changes in its alkaloid composition during its phenological stages reveals the adaptive strategies of *L. martinezii.*

Results and Discussion. – *Study Site and Plant Material. L. martinezii* has not shown any great variability in its morphology throughout the 14 populations studied, which covers most of its distribution area (*Fig. 1*).

L. martinezii is a small herbaceous bulbous plant usually of 30 cm (average) height, but we also found specimens between 15 and 70 cm in height. In populations as BE, PB, SG, and AL, we found the largest individuals, over 40 cm in height. The size of the bulb ranges from 1.3 to 7.0 cm (average 4 cm), but only AL and BE populations exceed 4 cm in diameter. The bulb is more or less rounded, covered with blackish scales, extended as a sheath up to the lower part of the leaves. Each bulb has (1)2–4(5) leaves that are narrowly linear with a rounded apex, *ca.* 1-cm-wide and 8–50-cm-long (average 20 cm), with BE, SG, and AL populations displaying the largest. The dark-green leaves have a glossy aspect and a typical central whitish band. In flowering, the plant develops an elliptic scape solid to lacunose-fistulose (reported solid and smooth in other works [10][18]) and clearly striate with two keels (exceptionally three). The apical inflorescence in pseudoumbella, has (4)6–13(14) flowers 2–3 cm long with scariose margin into two bracts (exceptionally three). Flowers are white, actinomorphic, pedicelate (pedicel 0.7–3.0 cm long), with oblanceolate tepals showing a typical green

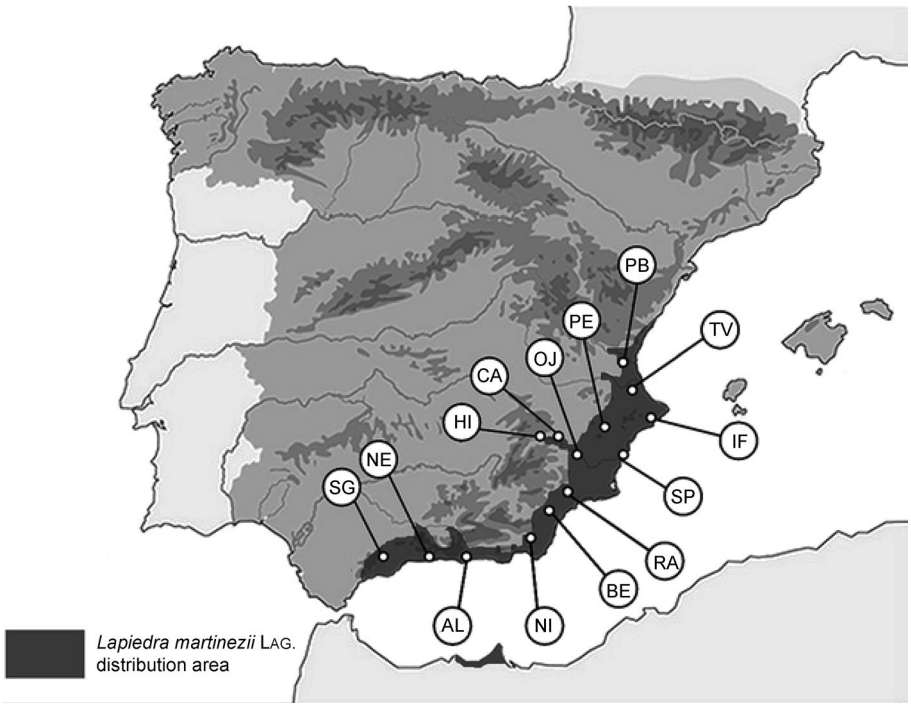


Fig. 1. *Geographical locations of the 14* Lapiedra martinezii *populations studied throughout their complete distribution area* (darkest zone) *in the Iberian Peninsula*. For location codes, see *Table 1*.

midrib within (with variable intensity in the studied populations). Tepals are persistent after complete ripening. Flowers have six stamens 0.4–0.7 long, with sagittate anthers. Fruits are rounded capsules of 0.7–2 cm (1.1 cm average) diameter with three carpels (exceptionally 4–5) and 8–22 seeds inside (12 average). Seeds are ovate and black, with a large strophiole discolorous at first, but finally also blackish. The size of dried seed is 0.2–0.3 mm, and for fresh seed, it is 0.4–0.5 mm.

Vegetative propagations frequently take place by subequal division in 2–3 or more, so that the new bulbs appeared grouped in tightened colonies of up to a dozen bulbs. We did not find any bulbet formations in field or in cultivated conditions. Plants that originated from seeds were only found in populations with no overgrazing.

Observations in cultivated and wild populations of *L. martinezii* showed non-contemporaneous development of leaves and flowers. Notably, there were only three populations, OJ, SP, and IF, where individuals were found that had leaves and flowers at stage III, or that had leaves, flowers, and fruits (stages III–IV) simultaneously. Flowering scapes emerge after a dormancy period, with a different bud origin (*Fig. 2*). New leaves appeared during the complete fruit-ripening stage in most populations, although some exceptions were observed. The capsules are dehiscent, but the seed-dispersion mechanism is not explosive. After the opening of fruit, many seeds remained joined to the placenta, and were collected and dispersed by ants, whilst the rest of the seeds fell gradually.

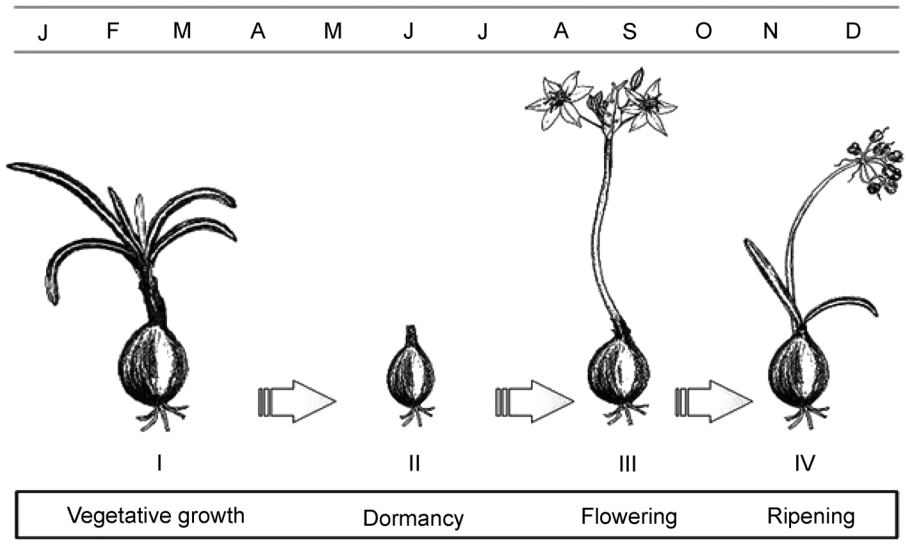


Fig. 2. *Phenological stages of the life cycle in* Lapiedra martinezii *throughout the year* (per month, from January (J) to December (D))

Local names found are *cebolla marranera*, *hierba de la estrella*, and *narciset valencià* for Andalucia, Murcia, and Valencia, respectively.

Biogeography, Ecology, and Major Plant Communities. The populations of *L. martinezii* occur from sea level up to 650 m altitude in the whole distribution area. *Lapiedra* lives in several termophilous plant communities dominated or co-dominated by the dwarf palm *Chamaerops humilis*, *Osyris lanceolata*, and *Maytenus senegalensis*, as well as in several types of Mediterranean macchias and woodland with *Pistacia lentiscus* and *Quercus coccifera* (*Table 1*). Nevertheless, they have usually been found in less developed plant communities, which act as a refuge from overgrazing, under some thorny plants, or on vertical rocky slopes or in crevices. Although the plant grows in several soil types, it is more frequent in limestone (80% of samples), but is also found in volcanic, metamorphic (schists, gneiss), and igneous rock (quarcite, sandstone) soils. Only the BE population displays a different alkaloid pattern that is probably correlated with soil conditions (quarcite rocks and acid soils).

More than 35 species living alongside *L. martinezii* were identified in the 14 populations studied (*Table 1*). *Asparagus albus*, *Chamaerops humilis*, and *Osyris lanceolata* are the most reliable bioindicators for predicting potential *Lapiedra* habitat, and maintaining them in good conditions of conservation.

In this study, *L. martinezii* has been found in five different major scrub plant communities, of a total of 22 habitats of European Community Interest (ECI; 50% corresponding to habitats for small areas of rocky plant communities) according to D 92/43/EEC on Habitats conservation [19].

Alkaloid Diversity. So far, five alkaloids have been reported for *L. martinezii*, namely 8,9-methylenedioxyphenantridine (trisphaeridine), *N*-methyl-8,9-(methylenedioxy)phenantridin-6-one, *N*-methyl-(8,9-methylenedioxy)-phenantridinium chloride,

Table 1. *Localities of* Lapiedra martinezii *Studied, in Order from Higher to Lower Altitude, and Compared with Respect to Some Geological, Biogeographical, Climatological, and Ecological Parameters*

Code	Localities	Altitude [m]	Temp. [°]	*P* [mm]	Chorological provinces	Geology type	Grazing conditions	Plant communities[a])
HI	Bridge of Híjar, Letur, Albacete	650	15	400	Murcian-Almerian	Limestone	Moderate	Less thermophilous
CA	Road Calasparra-Socovos, Albacete	550	15	420	Murcian-Almerian	Limestone	Moderate	Less thermophilous
BE	Bédar, Almería	466	17	380	Murcian-Almerian	Quarcite	Severe	Thermophilous
PE	Sierra del Caballo, Petrer, Alicante	450	15	370	Murcian-Almerian	Limestone	Severe	Thermophilous
NI	Níjar, Almería	429	18	250	Murcian-Almerian	Volcanic	Severe	Thermophilous
AL	Albuñol, Granada	301	17	410	Baetic	Limestone	Severe	Thermophilous
OJ	Sierra del Cajal, Ojós, Murcia	276	17	300	Murcian-Almerian	Limestone	Moderate	Thermophilous
RA	El Ramonete, Lorca, Murcia	204	16	280	Murcian-Almerian	Limestone	Severe	Thermophilous
SG	Sierra Grosa, Coín, Málaga	188	18	640	Baetic	Metamorphic	Moderate	Thermophilous
NE	Nerja, Málaga	173	19	480	Baetic	Limestone	Severe	Thermophilous
PB	Pedralba, Valencia	174	16	480	Catalan-Valencian	Limestone	Moderate	Thermophilous
IF	Near Peñón de Ifach, Calpe, Alicante	85	18	300	Murcian-Almerian	Limestone	Moderate	Thermophilous
SP	Near Santa Pola Cape, Alicante	50	18	330	Murcian-Almerian	Limestone	Ungrazed	Thermophilous
TV	Tavernes de Valldigna, Valencia	10	16	720	Catalan-Valencian	Limestone	Moderate	Thermophilous

[a]) Thermophilous: dominated by the dwarf palm (*Chamaerops humilis*) and other similar species; Less thermophylous: plant communities dominated by *Quercus coccifera* and others.

6-*O*-methyllycorenine *N*-oxide and homolycorine *N*-oxide [16][17]. GC/MS has been verified as a powerful tool for both rapid identification of compounds and for searching for new natural products in complex alkaloid mixtures from Amaryllidaceae plants [15][20–22].

Alkaloid profiles of different parts of *L. martinezii* plants in vegetative, flowering and ripening stages were studied for the first time. More than 65 alkaloids were detected in the extracts and 49 of them, whose contribution to the alkaloid mixture is more than 0.1% (of TIC (=total ion current)), are compiled in *Table 2*. Twenty-two were left unidentified due to a lack of reference spectra. For many of them, however, it was possible to assign structural types based on the characteristic mass-spectral pattern for each type of Amaryllidaceae alkaloids. Thus, the homolycorine-type alkaloids have a barely detectable molecular-ion peak and a base peak at *m*/*z* 109 for unsubstituted, or at *m*/*z* 125, for 2-hydroxy-substituted homolycorine derivatives [23]. Lycorine-type compounds show intensive molecular M^+ and $[M-H]^+$ peaks [24]. These unknown compounds may be new and perhaps bioactive molecules, but most of them are in low abundance, generally less than 5% of TIC, and, therefore, their isolation would be difficult.

The results revealed that alkaloid synthesis in the 14 populations of *L. martinezii* studied, covering the entire distribution area of this species, was dominated by compounds arising from *ortho-para'* phenol oxidative coupling of *O*-methylnorbelladine (*Fig. 3* and *Table 2*). Thus, lycorine and/or homolycorine were the dominant structural types in all the populations, in both bulbs and leaves, during the vegetative growth (stage I, *Fig. 2*). Tazettine-type compounds, arising from *para-para'* phenol oxidative coupling, were found in nine of the populations, ranging from 1.2–10.4% of the alkaloid mixtures, showing a tendency for higher accumulation in the underground parts of the plants that increase in the flowering period, *i.e.*, stage III (*cf. Figs. 2* and *4*). Exceptionally, haemanthamine-type compounds, also formed by *para-para'* phenol oxidative coupling, were detected in only one population (NE). Narciclasine-type compounds were found in the bulbs of five populations in amounts lower than 5% of TIC.

All the populations of *L. martinezii* exhibited a high level of variability in the percentage contribution of both main structural types and individual alkaloids in alkaloid mixtures. During vegetative growth, only one population (BE), as a singular case, displays a clear tendency towards accumulation of higher amounts of lycorine-type compounds in the bulbs (83%) and leaves (*ca.* 100%). In SP and SG populations, compounds of this type were found to range from 2 to 62% of TIC in leaves, and from 33–72% in the bulbs.

The content of homolycorine-type compounds ranged from traces to 91% of TIC in the leaves of BE and RA populations, respectively, and in the range of 7–62% in the bulbs of BE and SP populations. Deoxylycorenine (**5**) in the bulbs and 6-*O*-methyllycorenine (**6**) in the leaves were dominant alkaloids in most populations at the vegetative growth stage. Exceptions were found in populations such as PB and SG growing along the north and south borderlines of the distribution area, as well as in the BE population (the only one that grew in siliceous soil). Lycorine (**20**) was the main alkaloid in the leaves of populations BE, PB, and SG, and the main compound in the bulbs of populations PB and OJ. Another lycorine-type compound, 11,12-didehy-

Table 2. *Alkaloid Composition of Leaves and Bulbs from 14 Localities of* L. martinezii

Alkaloid	t_R	M^+	IF[a])		OJ		AL		BE		SP		PE	
			l[b])	b[c])	l	b	l	b	l	b	l	b	l	b
Tyramine (**1**)[d])	7.40	137					1.0							
Ismine (**2**)[d])	19.22	257						2.3		4.7				1.6
Trisphaeridine (**3**)[d])	19.53	223												
A-1 (HLY)[e])	20.06	–	0.3											
5,6-Dihydrobicolorine (**4**)[f])	20.19	239								1.6				1.2
Deoxylycorenine (**5**)[g])	21.55	301	16.3	19.7	24.8	19.1	2.5	16.0		1.5	11.8	25.8	14.8	16.9
A-2	22.15	299		0.5										
A-3 (HLT)	22.36	–	2.4	0.5	1.8		4.5				8.0	3.3	5.4	
6-*O*-Methyllycorenine (**6**)[d])	22.68	–	38	7.7	25.0	6.9	59.0	8.9			62.9	21.3	47.7	5.1
A-4	22.79	281						1.0		2.1				
Anhydrolycorine (**7**)[h])	22.94	251	0.1	1.3		1.9		1.5	14.0	8.7		2.3		5.3
1-*O*-Acetylnorpluviine (**8**)[i])	23.08	315								5.2				1.3
6-Deoxypretazettine (**9**)[h])	23.40	315		0.1				1.4		2.8				2.2
Lycosenine (**10**)[d])	23.34	297	0.8	0.9	1.6						1.6	2.3	1.1	
Norpluviine (**11**)[h])	23.60	273				1.2								1.0
Kirkine (**12**)[d])	23.66	273												1.1
Assoanine (**13**)[d])	24.03	267	0.2	0.7	1.9	5.4	9.1	3.3		1.0		2.3		2.5
A-5	24.28	329												
11,12-Didehydroan-hydrolycorine (**14**)[i])	24.46	249	1.2	8.7		3.7		6.2	3.3	23.1		2.7	1.0	12.8
Lycorenine (**15**)[d])	24.47	317	0.2		16.6	1.0								
Tazettine (**16**)[d])	24.98	331	0.9	4.7				9.0		4.0		1.4	1.2	2.3
Hippamine (**17**)[d])	25.39	301	0.2			1.0				2.4				1.9
A-6 (LY)	25.43	265	0.7	2.6				6.9	2.9	6.9		2.1		1.9
A-7	25.72	373						1.3						
A-8	25.86	389						1.3						
Sternbergine (**18**)[d])	25.89	331		0.2						2.3				
11-Hydroxyvittatine (**19**)[d])	25.92	287												
A-9 (LY)	26.36	299	0.4	2.4				1.8		1.7		1.7		1.0
Lycorine (**20**)[d])	26.50	287	13.6	12.3		21.3	2.6	12.0	65.2	8.0		7.2	4.5	13.0
Homolycorine (**21**)[d])	26.57	315	6.0	14.2	7.4	7.2	4.1	2.9				9.5	12.8	5.5
A-10 (LY)	26.88	315	0.3	0.5			1.1	3.4	1.6	1		1.3		0.4
A-11 (LY)	27.18	359												
A-12 (LY)	27.20	339						1.2						
8-*O*-Demethyl-homolycorine (**22**)[d])	27.28	301			9.9						3.8			
A-13 (LY)	27.80	266				1.4								
A-14 (LY)	28.11	343			4.9		1.1						2.7	
A-15 (LY)	28.39	279	4.4	11.2		3.8		6.7		12.1		6.5		11.7
Hippeastrine (**23**)[d])	28.35	315	0.5	0.9	1.1	1.3								2.0
A-16 (LY)	28.40	417			1.0								1.1	
Ungiminorine acetate (**24**)[j])	28.90	359	4.5							1.0				1.4
Ungiminorine (**25**)[k])	28.64	317				12								
A-17	28.80	281	0.5		1.4		2.7						1.1	
Narcissidine (**26**)[l])[g])	28.84	333				1.4								
A-18 (LY)	28.94	265				2.5				1.8				1.3
Narcissidine acetate (**27**)[m])	28.97	375	1.1	4.1		2.1		1.5	3.7	1.3		2.5		
A-19 (LY)	29.10	295	2.7	4.4			3.3	5.1	3.0	3.9	1.6	3.9	1.6	1.8

HI		RA		TV		PB		NE		NI		CA		SG	
l	b	l	b	l	b	l	b	l	b	l	b	l	b	l	b
							3.7								1.0
							1.0							1.2	3.4
13.9	31.9	62.6	26.4	21.1	17.2	16.1	3.2	33.7	18.2	4.5	13.5	20.2	24.1	19.3	9.1
															1.3
7.1		1.6		3.8			2.7	4.2		3.3		6.4		1.0	
47.1		5.7	5.8	25.9	1.5	10.9		18.0	4.7	32.4		44.7	3.7	7.3	
							1.7								2.2
	5.0		3.6	1.6	4.0	4.4	2.0		3.4		3.8		2.8	3.0	6.3
					1.9		3.0		1.1						1.7
							1.2				1.4			1.2	7.7
1.3	2.9	6.2	1.0	1.3	1.7			1.4	0.9			2.3	1.4		
			1.9				1.5								
			1.4												
	1.7		2.0	6.3	1.5	1.7	1.4	2.9	4.3	1.8	1.1	1.0	1.3	1.3	
						2.9									
	11.8		14.1	3.6	8.3	2.5	11.3		8.9		16.5		14.5	1.5	24.5
			1.5		3.7	2.4	7.1							2.2	2.1
					2.0		8.2						1.4		
	7.9					1.7	2.4	3.1	7.8	6.6	14	1.0	5.9	2.4	9.2
					2.6	1.0									
					1.8										
									3.6						
			2.2	1.0	3.2		2.3		1.1		0.5		1.2		
	4.0		7.7		2.6	37.7	13.0		5.0		4.6		7.2	39.3	6.0
11.7		10.9	5.5	6.6	8.7	3.4	4.7	19	4.7	7.7	3.6	9.6	9.2	4.7	1.3
									2.6	3.6	1.4				
				6.8											
2.1		2.0		2.1		1.8				1.1		2.0			
5.8		2.5						2.5		1.4		2.1		1.4	
	11.7		15.8	14.3	11.4	2.9	9.3		7.5		10.5	1.3	14.7		11.1
							1.0			1.1		1.1	1.5		
2.2												1.8		1.5	
			3.3	1.8	19.0				5.1		3.9				1.0
							5.7								
1.9		5.2						3.5		6.4		1.4		1.3	
	1.0		2.4	1.6	2.8		3.4								1.4
1.3	1.0					1.9	1.3	2.7	8.2	6.3	8.8		1.3	3.4	
2.7	8.6	1.1				2.6	1.7	6.3	6.7	19.4	9.8	4.0	5.1	5.2	6.6
	1.2								2.2	1.0	2.5				1.1

Table 2 (cont.)

Alkaloid	t_R	M^+	IF[a])		OJ		AL		BE		SP		PE	
			l[b])	b[c])	l	b	l	b	l	b	l	b	l	b
A-20 (LY)	29.44	281				0.8		1.5		1.2				
A-21	36.05	432					2.1							
A-22	37.47	418					3.3							

[a]) Population codes according to *Table 1*. [b]) l, Leaves. [c]) b, Bulbs. [d]) Identification by co-chromatography with isolated standard. [e]) Compounds A1 – A23 are unidentified alkaloids of which the type (HLY: homolycorine and LY: lycorine), when determined, is indicated in brackets. [f]) Identification by comparison of with the data in [25]. [g]) Identification by comparison with the data in [23]. [h]) NIST08 Database. [i]) Comparison with other compounds with known MS spectra. [j]) Identification by comparison with the data in [26]. [k]) Identification by comparison with the data in [16]. [l]) Identification by comparison with the data in [24]. [m]) Identification by comparison with the data in [27].

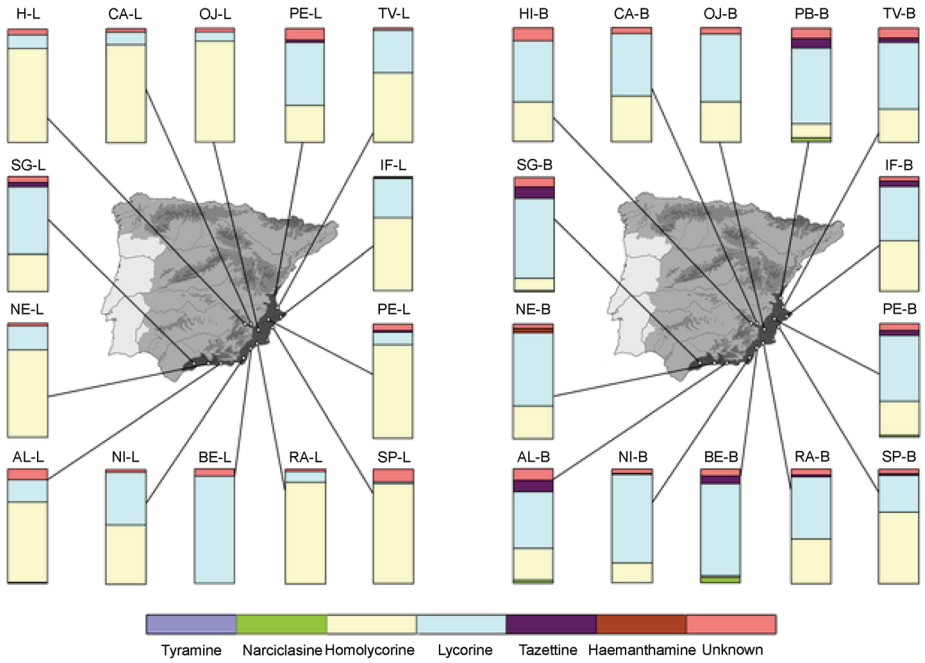


Fig. 3. *Biogeographical patterns of alkaloid-type profiles from the 14 populations of* Lapiedra martinezii *studied*. Population codes according to *Table 1*. L: Leaves, B: Bulbs.

droanhydrolycorine (**14**), was found as the dominant alkaloid in the bulbs of populations BE, NI, and SG. Compound A-19 (lycorine type) was one of the main alkaloids in the leaves of population NI.

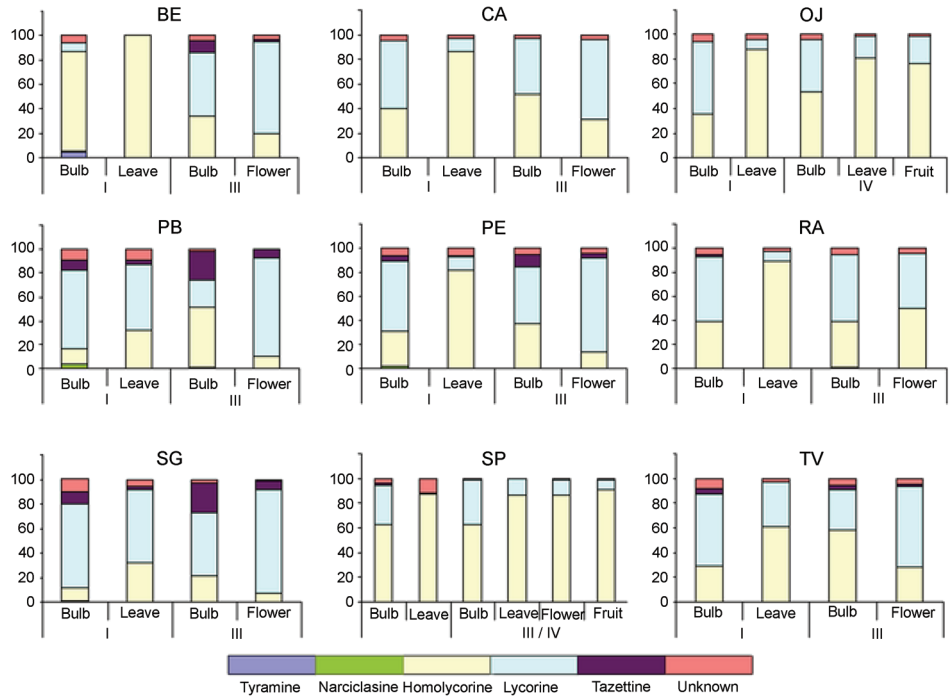


Fig. 4. *Phenological stages in nine populations of* L. martinezii *related to changes in alkaloid composition*. Population codes according to *Table 1,* and phenological stages I–IV according to *Fig. 3*.

The chemical study conducted, along their phenological cycles, revealed slight changes in the alkaloid patterns in the plants at the flowering stage (III), when most populations had no leaves (*Fig. 2*), compared to those found during the vegetative growth stage (I). A trend of decreasing lycorine-type and increasing homolycorine-type alkaloids was observed in the bulbs during this stage. Furthermore, the amount of tazettine-type alkaloids in the bulbs was higher, reaching up to 24% in SG population (*Fig. 4*). This type of alkaloid also appeared in all of the plants in BE population during stage III.

In comparison with stage I, a tendency for accumulation of higher amounts of lycorine-type compounds in the aerial parts (flowers or fruits) was observed in most populations (stage III–IV). These alternations in alkaloid synthesis during the flowering stage changed the major-alkaloid patterns in some populations. For instance, 6-*O*-methyllycorenine (**6**) was the major alkaloid in the bulbs of PB population instead of lycorine (**20**). Interestingly, ungiminorine acetate (**24**) in TV and PE populations, and narcissidine acetate (**27**) in BE population were found as major compounds in their flowers. These compounds were rarely detected in the populations during the vegetative growth stage. Lycorine (**20**) or 6-*O*-methyllycorenine (**6**) were the major alkaloids in flowers in the rest of studied populations.

In summary, we can argue that 70% of the populations of *L. martinezii* exhibited a general biogeographic lycorine-type pattern according to PCA analysis (*Fig. 5*), with

homolycorine-type alkaloids as a second component. The first-component lycorine-type indicated deviations of BE, and also SG and PB (jointed to tazettine) populations. The AL population showed a different deviation, with can be explained partially by the second component and the presence of many unknown alkaloids.

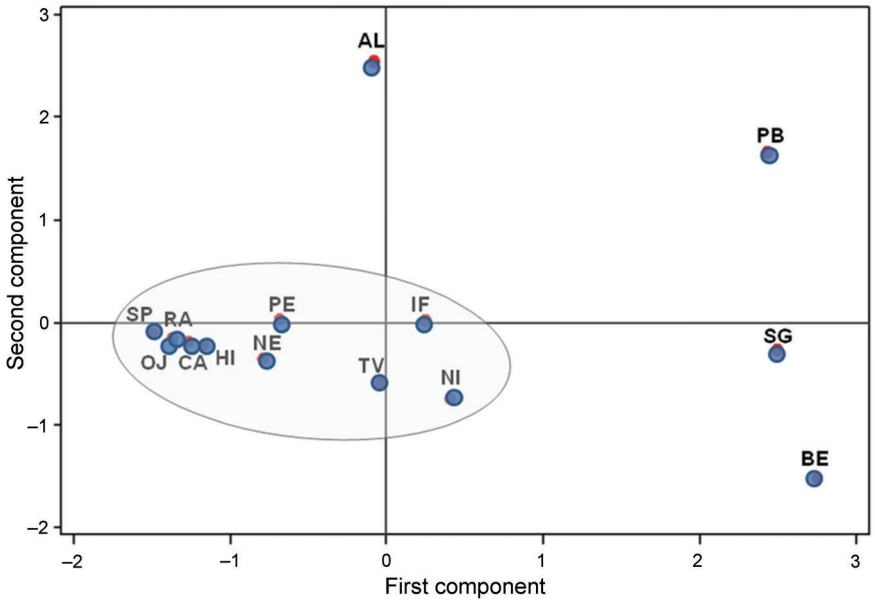


Fig. 5. *PCA of alkaloid-type content in the 14 populations of* L. martinezii *at stage I* (bulbs and leaves). First component: lycorine type; second component: homolycorine type. Population codes according to *Table 1*.

Alkaloids are considered as defence metabolites in plants [28]. The chemo-ecological role of the alkaloids identified in *L. martinezii* has been poorly studied, however, their pharmacological effects have been assayed [12][13]. In our case, one of the major alkaloids, deoxylycorenine (**5**), has not been studied for any pharmacological or biological activity. 6-*O*-Methyllycorenine (**6**) has shown cytotoxic activity [12][13]. Compound **6** may arise from lycorenine (**15**) as an artefact of the isolation procedure with MeOH, as in other alkaloids with a OH group at C(6) [22]. Lycorenine (**15**) is known to affect blood pressure and heart rate in dogs and rats [29][30]. The other relatively abundant homolycorine-type alkaloids homolycorine (**21**), 8-*O*-demethyl-homolycorine (**22**), and hippeastrine (**23**) have been shown to exibit hypotensive effects in rats and citotoxic activity. In addition, **23** has weak insect antifeedant and antifungal activities [12][13]. Lycorine (**20**), one of the most frequently occurring alkaloids in Amaryllidaceae plants, possesses a vast array of biological properties. It has been reported as a potent inhibitor of ascorbic acid synthesis, and cell growth, division, and organogenesis in higher plants, algae, and yeasts, inhibiting the cell cycle during the interphase [12][13]. Additionally, **20** exhibits antiviral, antifungal, antiprotozoan [31], and insect-antifeedant activities [32]. Its administration to dogs invokes nausea and emesis in dose-dependent manner [33]. It is able to decrease tumor cell growth and

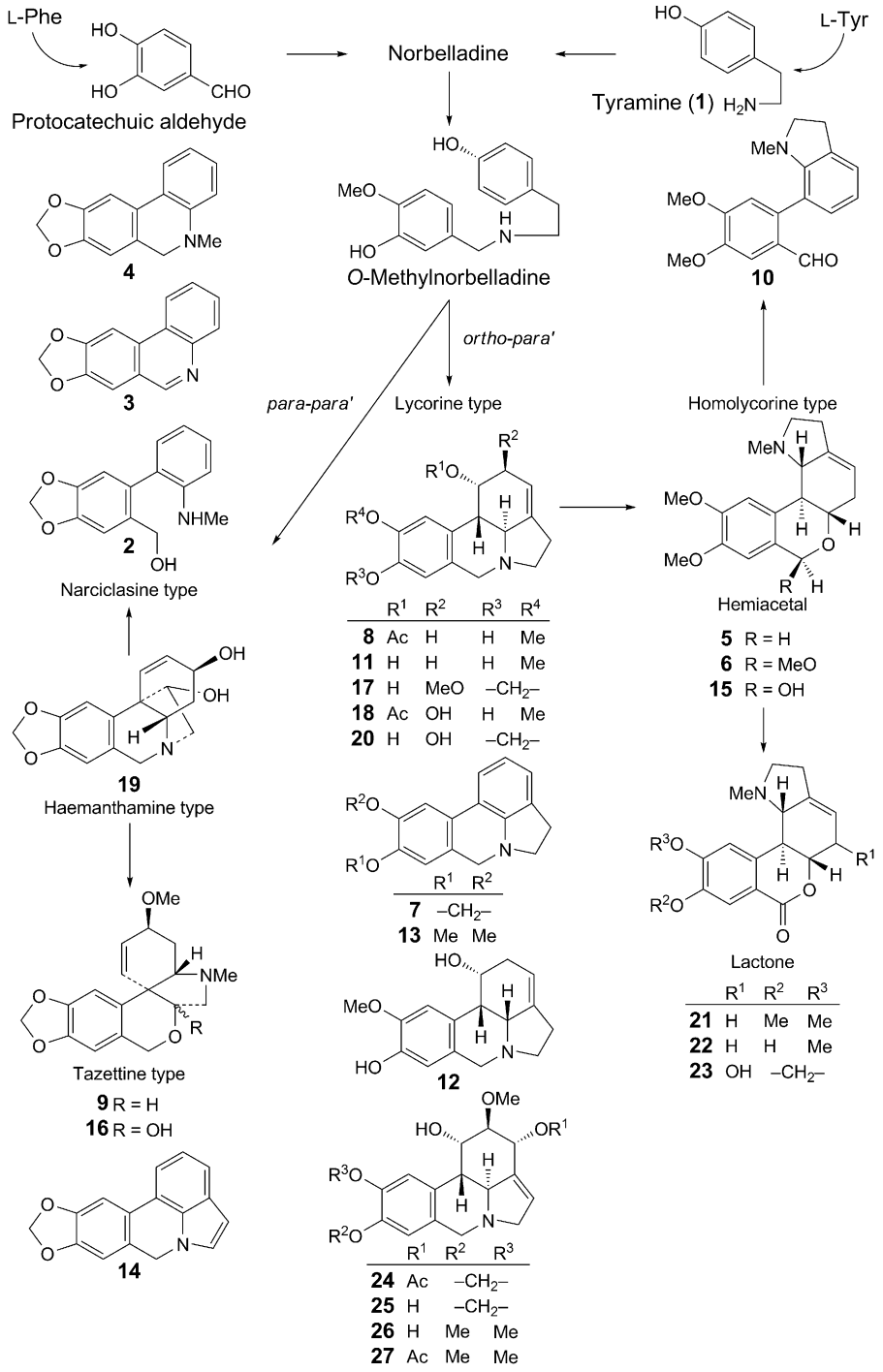
increase survival rates with no observable adverse effects in treated animals [34], thus being a good candidate for a therapeutic agent against leukaemia [35]. 11,12-Didehydroanhydrolycorine (**14**) has been found in bulbs of *Pancracium biflorum* that had been attacked by a parasite [36]. Its derivative, anhydrolycorine (**7**), was found as a degradation product of lycorine (**20**) in a medium with *Pseudomonas* ssp. [32]. The Quaternary chloride of compound **7** has been reported as a potent cytotoxic agent [37]. Tazettine (**16**), an isolation artefact of pretazettine, has shown cytotoxic activity, whereas pretazettine, which in fact is synthesised by plants, possesses potent cytotoxic and antiviral activities [12][13].

The pharmacological activities of the major alkaloids found in *L. martinezii* indicate that the plant has an effective chemical defence mechanism against fungal and animal attacks. It has developed a rich spectrum of bioactive compounds, mainly from lycorine, homolycorine, and, in some populations, from tazettine types. Despite the fact that the distribution area of the species is relatively restricted, all the populations studied showed a diversity in their alkaloid patterns. This is evident for the marginal populations SG, PB and TV, as well as for the BE population (the only one growing in quarcite acid soils). On the other hand, spatially adjacent populations such as HI, CA, and OJ show an identical alkaloid pattern, suggesting that genetic factors may determine the alkaloid patterns.

A vast biochemical diversity was observed in the alkaloid synthesis of other amaryllidaceous plants, *Galanthus nivalis* and *G. elwesii* [23] with homolycorine, lycorine, galanthamine, haemanthamine, tazettine, tyramine chemotypes found. In contrast, only lycorine and homolycorine chemotypes have been found in *L. martinezii*. These results imply that the lycorine chemotype, one of the first structural types of Amaryllidaceae alkaloids, is closely related to the origin, phylogeny, and distribution of *Lapiedra*. Lycorine (**20**), in fact, is one of the most distributed alkaloids in the family, due to its unique and multitarget biological activities. Homolycorine-type alkaloids arise from the lycorine type (*Scheme*). The biosynthesis of lycorenine (**15**) proceeds *via* *O*-methylnorbelladine and norpluviine (**11**). Norpluviine is converted in Narcissus '*King Alfred*', primarily to alkaloids of the homolycorine type. Hemiacetal formation and methylation could provide lycorenine and, on subsequent oxidation, provide the lactone homolycorine (**21**) [12][13]. It is worth mentioning that *L. martinezii* accumulates mainly hemiacetal homolycorine-type alkaloids but not the biosynthetically later lactone derivatives (compounds **21**, **22**, and **23**), as found for more recent species such as *G. elwesii* and *Leucojum aestivum* [15][21]. The appearance of low amounts of tazettine-type compounds (products of *para-para'* phenolic oxidative coupling) in some populations indicates that these compounds may appear later in the evolution. Interestingly, neither galanthamine-type nor montanine-type compounds (*Scheme*) have been found in *L. martinezii*, as expected from the results obtained in other phylogeneticaly related genus like *Pancracium* [14][38].

Conservation. According to the data from the check list of Habitats in D92/43/EEC, the endemic *Lapiedra martinezii* lives in seven EEC Priority Habitats and also 15 Habitats with EEC Interest in the main part of its distribution area (south to east of Spain). Most of these habitats are characterized by *Lapiedra martinezii*, which is considered to be a good bioindicator. Moreover, *L. martinezii* occur in 22 natural Spanish protected areas (four Andalusian, eight Murcian, eight Valencian, and two

Scheme 1. *Structures and Biosynthetic Relationship of the Alkaloids of* L. martinezii



from Castilla-La Mancha) distributed along the Mediterranean coast where they are very well preserved.

The knowledge about the North African presence of this plant is very scarce. Only one report [39] mentions its occurrence in a narrow coastal belt from Melilla to Peñón de Alhucemas where actually a preserved area exists, the Morrocan Al-Hoceima Natural Park. Moreover, *L. martinezii* has never been catalogued as a threatened species in Spanish Red Books [40], probably due to the high number of populations present in the entire area. However, we observed an unexpected sensitivity to overgrazing by goats in six of the total populations studied (*Table 1*). All of them are far away from the legally protected Spanish areas and, therefore, available for free grazing. These overgrazed populations are now under their limit of survival possibilities, with a reduced population (less than 20 plants) located on inaccessible rocky slopes.

Biogeographic Origin of L. martinezii. Nevertheless, why does *L. martinezii* not show a greater distribution area like other related plants, *e.g.*, *Pancratium*, or a major taxonomic diversification like *Narcissus*, in the western Mediterranean? The current distribution of *L. martinezii* extends to the external Baetic mountains, nearby areas, and perhaps a few populations occurring in North Africa, around the Spanish city of Melilla [10][11][18][40].

According to a recent biogeographycal and phylogenetic work [8], the ancestor of the genus *Narcissus* appeared in the Iberian plate, the emerged land of the actual Iberian Peninsula, joined to the Pyrenean-Alpine plate. This is where the separation into the main greater sections began, between the late Oligocene and the early Miocene (*ca.* 23.6 Ma). Later, a few members of only three sections crossed to the north of Africa. The same authors confirmed that the ancestor of *Lapiedra-Acis*, could be differentiated from a *Narcissus-Sternbergia* ancestor at 23.6 Ma and from its *Pancratium* ancestor at 22 Ma. Later, *Lapiedra* probably separated from *Acis* during the middle Miocene (between 12.5 to 8 Ma), before the great species diversification of *Narcissus.*

During this period (*Fig. 6*), the actual Baetic mountain chain was an archipelago formed by many islands (where the *Lapiedra* ancestor could have been present), allowing communication between the Atlantic Ocean and the old Thetys Sea through the Guadalquivir marine grave [41–43]. These isolated areas suffered some successive climatic changes, first becoming warm and arid in the Mesiniense period, later warm and humid in the late Pliocene, and finally cold and humid in the late Pliocene-Pleistocene [42][44]. These drew the current reduced area of *Lapiedra*, which lives not very far from the seashores, but in areas that exclude the sandy beaches that are colonized by other related genera like *Pancratium*.

These events, with scarce morphological variation and poor adaptability to ecological conditions, could explain why *L. martinezii* does not exceed the Baetic mountain boarder towards the inner part of the Iberian Peninsula (as occurs in North Africa). This limitation to warm and never freezing conditions is the reason why all the localities studied in its distribution area are no higher than 700 m. It occurs in several Mediterranean macchias and woodland plant communities, mainly dominated or co-dominated by the dwarf palm *Chamaerops humilis*, *Osyris lanceolata*, *Maytenus senegalensis*, and *Pistacia lentiscus* (*Table 1*).

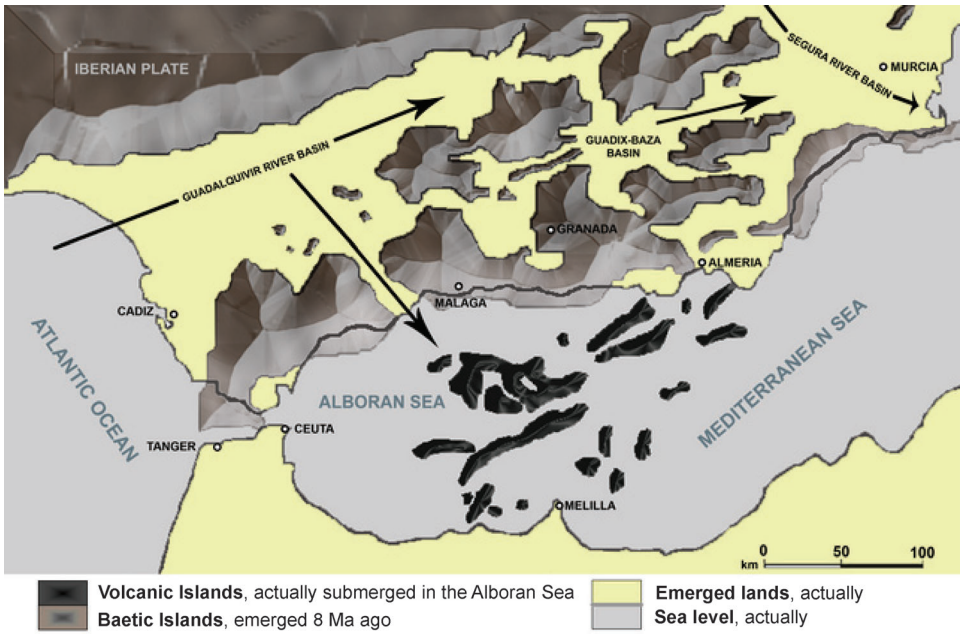


Fig. 6. *Composition of theoretically emerged lands 9–8 Ma ago.* In light brown-grey, several isolated lands are represented, surrounded by marine channels, which correspond to Alpine orogene-Baetic Cordillera. In blackish grey, the volcanic submarine islands are represented; these are now under the Alboran Sea (modified from [41][43]).

Therefore, to understand the biogeographic origin of *Lapiedra* in line with the chemical and historical arguments above, we need several assumptions. The first is to accept the origin of *Lapiedra* genus from an island of the Baetic-Archipelago, *ca.* 12–8 Ma ago. The second is to accept the lycorine-type biosynthesis pathway as one of the oldest alkaloid strategies in the Amaryllidaceae. Then, according to our results and the alkaloid pattern found (*Table 2*, *Figs. 2* and *3*), compared using PCA analysis (*Fig. 5*), we could speculate on three different theories about its biogeographic origin:

1. *L. martinezii* could have originated on a large occidental island (between Málaga and Almería), colonizing later towards the southeastern Spanish Mediterranean coast up to the north of the actual area (Valencia-Castellón). This theory is supported by the major lycorine-type populations now located in the occidental Andalusian littoral and the homolycorine-type populations centered around the Segura river basin. In this case, we consider that individuals from BE population (with an almost pure lycorine-type pattern) which grow in an old Quarcitic soil, could be close to the ancestral form of *Lapiedra*.

2. *L. martinezii* could have originated on a small island (between Almería, Murcia, or Alicante) later colonizing the emerged earth from the north of its actual distribution area to the southwest Andalusian coast. This theory is supported by the great homogeneity in homolycorine/lycorine profiles (*Scheme*) grouped in the central part of its area, coinciding with the major altitudinal range and density of populations (70% of

herbaria records). The heterogeneity is relegated to the NE and SW borderline populations (with mixed tazettine, narciclasine, or other alkaloid types, and the exceptional BE population with pure lycorine type).

3. *L. martinezii* could have originated on a central volcanic Alboran island emerged between the Miocene to Pliocene periods, which submerged later under the sea (*Fig. 6*). The colonization of the southeastern Spanish Mediterranean coast and the North African narrow belt between Melilla-Alhucemas could have occurred later. Nowadays, unfortunately, *Lapiedra* is no longer present on the tiny arid Alboran Island located between Almeria and Melilla.

However, in any of the three scenarios mentioned above, the center of origin has to have been close to the southern Iberian Peninsula and North Africa, which was colonized later. The scarce presence now in this area, and the relations between the Melilla cape and the submarine volcanic Alboran archipelago suggest a bridge during the low marine-level situations during the Mesiniense period. The later rise of the Mediterranean climate and the successive Pleistocene glaciations finalized drawing its current narrow area.

Conclusions. – The relict Mediterranean bulbous *L. martinezii* grows in most warm and arid lands of Europe, and displays a great richness, in alkaloids with clearly dominant homolycorine/lycorine-type biosynthesis pathways, closely related to the most ancestral genus of Amaryllidaceae. A biogeographical alkaloid pattern can be observed (homogeneous in the central part of the distribution area, *i.e.*, Segura river basin and nearby zones, and highly diversified on the SW and NE borders) that could be used to interpret its ancient phylogenetic origin. In addition, the chemical changes found at different phenological stages, and increasing levels of lycorine and other minor alkaloid types in aerial parts during flowering and fruiting may be related to defence against pests, diseases, and overgrazing, an important threat for this species.

Chemical data of endemic plants can be a useful tool to promote the conservation of the major genetic biodiversity for them, mainly in their borderline areas. Further research about unknown alkaloids of the *L. martinezii* may also provide new bioactive compounds of pharmacological interest.

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Experimental Part

Study Site and Plant Material. For this study, a large number of localities from south and south-east Spain (from the coastal areas of Málaga to the Valencia-Castellón boundary) were preselected using botanical literature, some previous works, and the Iberian flora database ANTHOS (http://www.programanthos.org). Finally, only 14 of these were selected for collection (deliberately excluding all protected areas) according to their accessibility and equal distribution in the entire area.

The plant material of *L. martinezii* was obtained from February to March 2009, by random collection of 13–15 individuals, from each sampling location, at the vegetative stage. Only one sample (near the Peñón de Ifach, IF) was collected later, due to its exclusive phenology, with leaves, flowers, and fruits at the same time (unlike other populations). The bulbs collected were preserved and cultivated at the Torretes Field Station-Botanical Garden, Biodiversity Institute CIBIO (University of Alicante), due to

the difficulty in observing phenological changes in the field for all populations simultaneously. Also, voucher specimens, seeds and dry samples were deposited with the Amaryllidaceae collection of Torretes Field Station-Botanical Garden.

At collection time, the size and diameter of bulbs, as well as the presence/absence of bulblets, number, length, and width of leaves, were measured in all plants. Other morphological characters studied during the flowering stage of *Lapiedra* were the number of scapes per bulb, length of scape, shape and length to the floral bracts, number of flowers per scape, and the size of all the floral pieces. Finally, capsule size and number of seeds were also measured in successful fructifying populations.

For chemical analysis, fresh plant organs (*ca.* 2–3 g) from each individual were macerated separately in glass vials with 40 ml of MeOH for 3 days. The alkaloid profiles obtained belong to three of the four phenological stages of *L. martinezii:* I (vegetative growth), III (flowering), and IV (ripening). During the dormancy period (stage II), no bulbs were analyzed to avoid losses at the following stages.

Biogeography, Ecology, and Major Plant Communities. The bioclimatic units (Bioclimatic Belt) and the biogeographical data (Corological Provinces) of this study, have been considered according to [45–47]. The coastal belt of distribution of this species includes Málaga, Granada, Almería, Albacete, Murcia, Alicante, and Valencia provinces. In addition, in order to select a good representation from a wide ecological range and the major diversity of plant communities [10][18][19][48], all the plants present in the studied populations, as well as geographical coordinates, altitude, geology, ecology, and grazing conditions were inventoried. Taxonomic data are according to the published volumes of *Flora Iberica*, and other regional flora for the rest.

Biogeography and Alkaloid Diversity. A major biogeographical gradient was studied to look for differences in alkaloid patterns, as in other related genera [21].

Alkaloid Extraction and Instruments. Aliquots (40 ml) of the MeOH extracts were evaporated under vacuum, and the residues were dissolved in 3 ml of 2% H_2SO_4 and defatted with Et_2O (5×3 ml). Next, the aq. layers were basified with 25% NH_3 to pH 9–10, and the alkaloids were extracted with AcOEt (3×5 ml). After evaporation of the org. solvent, the dried alkaloid fractions were dissolved in 250 µl of MeOH for further analysis.

Gas Chromatography/Mass Spectrometry (GC/MS). The GC/MS analysis was performed on a *Hewlett Packard 6890$^+$/MSD 5975* instrument (*Hewlett Packard*, Palo Alto, CA, USA) operating in EI mode at 70 eV. An *HP-5* MS column ($30\ m \times 0.25\ mm \times 0.25\ \mu m$) was used. The temp. program was: 100–180° at $15° \times min^{-1}$, 180–300° at $5° \times min^{-1}$, and 10 min hold at 300°. The injector temp. was 250°. The flow rate of carrier gas (He) was $0{\cdot}8\ ml \times min^{-1}$. The split ratio was 1:20. One µl of the soln. was injected.

The alkaloids were identified by comparing their masspectra and *RI* (*Kovats* retention index) values with those of previously isolated standards identified by other spectroscopic methods (NMR, UV, CD); the mass-spectral fragmentation of the compounds were compared with those from standard reference spectra from the *NIST* 05 database. The spectra obtained were compared with those reported in the literature, or with those of closely related standard compounds. The percentage contribution of the compounds in alkaloid mixtures are expressed as a percentage of total ion current (TIC). The *RI* values of the compounds were recorded with standard calibration *n*-hydrocarbon mixture (C_9–C_{36}) using AMDIS 2.64 software (NIST).

Phenology and Seasonal Changes. Lapiedra is an uncommon Amaryllidaceae genus, in which leaves appear at a different time than the flowers and fruits. For this reason, the phenological stages were estimated using data from the Iberian flora database (ANTHOS), from different North African and Spanish flora [10][18][39][48], and from other botanical works [5][6][11]. In addition, once the material had been collected, samples were placed under cultivation conditions for comparative observations to obtain accurate phenological data for all populations. The following year, only 9 of the 14 populations completed the blossoming and ripening stages under cultivation (May–September 2010). The closer populations of Alicante and Murcia were field-observed during the different phenological stages studied, as a control for the cultivated ones.

Conservation. We compiled all the available information on protection rules about *L. martinezii* and its habitats for the European Economic Community (EEC) and Spain, however, during the collection period we also observed the particular degree of threat for each population.

Statistical Analysis. The main values of the alkaloids from all plants analyzed per population were used for Principal Component Analysis (PCA), which was performed by Minitab 16 software (*Minitab Inc.*).

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