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Population-Specific Chemotypes in *Pseudomisopates rivas-martinezii* (Sanchez Mata) Güemes From La Serrota and Gredos Massifs (Iberian Central Range)

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ABSTRACT

Pseudomisopates rivas-martinezii (Sánchez Mata) Güemes (Plantaginaceae) is a critically endangered endemic species from the Iberian Central Range. This study characterizes population-specific chemotypes in aerial and underground tissues from La Serrota and Gredos populations using elemental analysis, Fourier–transform infrared (FTIR) spectroscopy, and gas chromatography–mass spectrometry (GC–MS). Elemental analysis revealed interpopulation differences in aerial nitrogen content (1.0% vs. 1.9%). FTIR showed interpopulation and tissue-specific variations. GC–MS characterization revealed convergent composition of underground parts dominated by histidine-derived metabolites, but significant divergence in aerial profiles reflecting differential aromatic amino acid metabolism from the shikimic acid pathway. La Serrota aerial tissues were characterized by phenolic derivatives (phenylalanine/tyrosine products), while Gredos specimens exhibited predominance of nitrogenous heterocyclic compounds (tryptophan catabolism products). These findings suggest differential selective pressures acting on organs and populations, with implications for conservation strategies and taxonomic understanding of this monospecific genus.

1 | Introduction

Pseudomisopates rivas-martinezii (Sánchez Mata) Güemes is the sole species within the monospecific genus *Pseudomisopates* Güemes, one of the 16 genera endemic to the Iberian Peninsula [1, 2] and one of four monospecific genera that are critically endangered [3]. This perennial herb, which has underground stolons that can extend over a meter between ramets [4], inhabits the Iberian Central Range—a biodiversity hotspot and glacial refugium within the Mediterranean Basin [5]. The species is ecologically linked to the *Cytisus oromediterraneus* Rivas Mart. et al. scrubland belt formations, extending from 1400 to 1990

m.a.s.l. across the Central Range [6, 7]. After decades of intensive field surveys, only eight populations occurring on two mountain massifs (Gredos and La Serrota, separated by the Alberche and Tormes river valleys) were initially reported [8], although additional records have raised the total to 13 populations [6]. *Pseudomisopates* is a pyrophyte in which germination is strongly stimulated by fire [6, 9], and it has been shown to be self-incompatible with pollen-limited reproduction [7]. Recent phylogeographical studies using molecular markers have revealed moderate to high levels of genetic diversity within populations, with late Pleistocene divergence between the two mountain massifs despite their geographical proximity [10].

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TABLE 1 | Elemental analysis of composite samples of underground and aerial parts of *Pseudomisopates rivas-martinezii* from La Serrota and Gredos populations, and estimated calorific values. Values are means $\pm$ standard deviations of analytical triplicates of a single homogenized composite sample per population ($n = 3$ analytical replicates). These error terms reflect instrumental precision, not biological variance within populations.

Sample	Origin	Nitrogen (%)	Carbon (%)	Hydrogen (%)	Sulfur (%)	Oxygen (%, by difference)	HHV (kJ/g)
Underground part	La Serrota	0.74 ± 0.01	44.48 ± 0.06	6.00 ± 0.00	0.00 ± 0.00	48.77	20.23
	Gredos	0.68 ± 0.07	46.07 ± 0.40	6.14 ± 0.08	0.00 ± 0.00	47.11	21.27
Aerial part	La Serrota	1.00 ± 0.02	45.29 ± 0.15	6.09 ± 0.04	0.00 ± 0.00	47.61	20.79
	Gredos	1.89 ± 0.22	44.80 ± 0.55	6.23 ± 0.13	0.00 ± 0.00	47.09	20.73

To generate baseline chemical data for conservation planning and to evaluate chemotaxonomic variation within this monospecific genus, we performed physicochemical analysis of aerial and underground tissues from La Serrota and Gredos populations.

2 | Results and Discussion

2.1 | Characterization by Elemental Analysis

The results of elemental analysis of the composite samples of *P. rivas-martinezii* from La Serrota and Gredos populations are presented in Table 1.

The elemental analysis revealed relatively homogeneous composition between the two populations studied, with carbon values ranging from $44.48 \pm 0.06\%$ to $46.07 \pm 0.40\%$ and hydrogen values between $6.00 \pm 0.00\%$ and $6.23 \pm 0.13\%$. These values are within the typical range reported for Mediterranean herbaceous species [11]. The Gredos population showed slightly higher carbon values in the underground part (46.07% vs. 44.48%) compared to La Serrota.

Notably, no sulfur was detected in any of the analyzed samples, suggesting an absence of sulfur-containing amino acids or secondary metabolites in significant quantities. This characteristic has been observed in other species from Mediterranean environments with nutrient limitations [12].

The nitrogen content showed marked differences between organs, being consistently higher in the aerial part ($1.00 \pm 0.02\%$ in La Serrota and $1.89 \pm 0.22\%$ in Gredos) compared to the underground part ($0.74 \pm 0.01\%$ in La Serrota and $0.68 \pm 0.07\%$ in Gredos). This organ-specific nitrogen distribution likely reflects the higher concentration of structural and enzymatic proteins in photosynthetic tissues, and/or the presence of alkaloids and other nitrogenous metabolites in aerial tissues [13, 14].

Further, the interpopulation variability in aerial nitrogen content of the Gredos composite sample (1.89%) was markedly higher than that of the La Serrota composite sample (1.00%). Because these values derive from a single pooled sample per population, this difference cannot be attributed to statistical significance and may reflect differences in individual plant composition, microsite nutrient availability, or both. This elevated nitrogen content in Gredos aerial tissues is consistent with the GC-MS identification of nitrogen-rich heterocyclic compounds (pyrrole, acridine, and

polyaminated pyridine derivatives) in this population, whereas La Serrota's lower nitrogen content aligns with its phenolic-dominated chemical profile, as discussed below.

Given that *P. rivas-martinezii* is a pyrophyte whose germination is fire-stimulated [6, 9], we calculated the higher heating value (HHV) to provide baseline combustion data relevant to the species' fire ecology. HHV values ranged between 20.23 and 21.27 kJ/g, within the typical range for herbaceous Mediterranean biomass ($18\text{--}22$ kJ/g) [15] and comparable to co-occurring species in the *C. oromediterraneus* scrubland belt. The underground part of the Gredos population exhibited the highest HHV (21.27 kJ/g), which is consistent with its higher carbon content (46.07%). These data may inform future fire-management modeling for habitat conservation, as tissue calorific values influence flame characteristics and soil heat penetration relevant to seed bank stimulation.

2.2 | Vibrational Characterization

FTIR spectroscopy revealed distinct spectral signatures between aerial and underground tissues, as well as interpopulation differences (Table 2). All four sample types (aerial and underground tissues from both populations) displayed complex FTIR spectra consistent with lignocellulosic plant material containing diverse secondary metabolites. The main functional groups identified across all samples included hydroxyl groups ($3295\text{--}3335$ cm^{-1}), methylene groups (2918 cm^{-1}), carbonyl groups ($1726\text{--}1730$ cm^{-1}), and various aromatic structures ($1511\text{--}1691$ cm^{-1}).

The C—H stretching band at 2850 cm^{-1} , present in leaves of both chemotypes, was absent in underground samples. The marked shift in the band at ~ 1650 cm^{-1} suggests different types of aromatic compounds or proteins between populations.

2.3 | Extract Characterization by GC-MS

GC-MS analysis allowed the identification of complex chemical profiles in the derivatized extracts from composite samples of the aerial and underground parts of *P. rivas-martinezii* from the La Serrota and Gredos populations. The identified chemical species (Tables S1–S4) revealed specific distribution patterns both at the organ and population levels. Derivatization by-products (*N*-methyltrifluoroacetamide and 2,2,2-trifluoro-*N*-methylacetamide), siloxane contaminants from chromatographic

TABLE 2 | FTIR band assignments for *P. rivis-martinezii* tissues.

Serrota leaves	Gredos leaves	Serrota underground	Gredos underground	Assignment
3295	3304	3329	3335	O—H stretching (hydroxyl groups of phenols, alcohols, and adsorbed water)
2918	2918	2918	2918	Asymmetric C—H stretching of methylene groups (—CH ₂ —)
2850	2850	—	—	Symmetric C—H stretching of methylene groups (—CH ₂ —)
1727	1728	1726	1730	C=O stretching (carbonyl groups, possibly esters or acids)
1642	1650	1691,1657	1641	Aromatic C=C stretching and/or amide I (C=O of proteins)
1548,1515	1548,1515	1512	1511	C=C and C=N vibrations of heterocyclic rings
1409	1414	1414	1416	In-plane C—H and O—H bending
1367	1369	1370	1368	C—H bending of methyl groups
1309	1314	1323	1319	C—O stretching (secondary alcohols, phenols)
1229	1230	1231	1229	C—O stretching of phenols
1015	1015	1019	1016	C—O stretching of primary alcohols
897	897	—	896	Out-of-plane C—H bending (aromatics)
832	832	—	831	Out-of-plane C—H bending (disubstituted aromatics)
522	522	530	523	Molecular skeleton deformations
430	430	425	424	Low-frequency skeleton deformations

consumables, and identified inorganic contaminants were excluded from the total chromatogram area before normalization. The relative renormalized area (%) values reported in this study, therefore, represent normalization strictly against the pool of plant-derived metabolites. The original (raw) area (%) values are retained in each supplementary table for full transparency.

2.3.1 | Chemical Composition of the Underground Part

The extracts of the underground part from both populations showed notable similarity in their chemical composition (Table 3, Figure 1a), with the consistent presence of four compounds: 1-methyl-4-nitro-1*H*-imidazole, *N*-methylformamide, 3-methoxy-1*H*-pyrazol-5-ylacetamide, and 3-quinuclidinol.

This phytochemical convergence between geographically separated populations suggests an important functional role for these metabolites in the underground system, possibly related to defense against soil-borne pathogens or root growth regulation [16]. It should be noted that these identifications are based on mass spectral library matching alone and must be considered tentative (see Experimental Section). In particular, 1-methyl-4-nitro-1*H*-imidazole, while detected with a quality score of 82 in both populations, is a rare structural motif in plant tissues and could represent a degradation product or an extraction artifact. Its consistent detection in both underground samples with matching retention times lends some confidence to the assignment, but confirmation with an authentic standard is necessary.

2.3.2 | Chemical Composition of the Aerial Part

In contrast to the convergence observed in underground parts, the chemical profiles of the aerial part differed markedly between populations. Both populations shared α ,2,2,6-tetramethyl-cyclohexanepropanol (tetrahydroionol, a fragrance ingredient with a woody, sandalwood-like scent, which is used in perfumery) and mandelic acid. Beyond these shared constituents, La Serrota extracts were dominated by oxygenated phenolic derivatives, including 3,5-dihydroxybenzamide (Figure 1b). As discussed in the Methodological Considerations section, this compound is almost certainly an extraction artifact formed by amidation of a native phenolic acid (most likely 3,5-dihydroxybenzoic acid or a conjugated ester) in the aqueous ammonia medium. Gredos extracts, by contrast, were characterized by nitrogenous heterocyclic compounds (3-methyl-1*H*-pyrrole and pyridine-2,3,5,6-tetraamine; Figure 1b), the latter of which may likewise be an artifact of Chichibabin-type amination of a simpler pyridine precursor. Crucially, because both populations were extracted under identical conditions, the qualitatively distinct artifact suites indicate genuinely divergent pools of precursor metabolites—phenolic acids/esters in La Serrota versus nitrogen–heterocyclic intermediates in Gredos.

2.3.3 | Correlations Between Ftir and Gc-Ms

In underground tissues, GC-MS results show that the composition is dominated by histidine-derived metabolites (nitrogenous heterocyclic compounds: 1*H*-imidazole-1-methyl-4-nitro,

TABLE 3 | Comparison of the most abundant chemical species identified by GC–MS in derivatized extracts of composite samples of underground and aerial parts of *Pseudomisopates rivas-martinezii* from La Serrota and Gredos populations. Complete compound lists with retention times and quality scores are provided in Tables S1–S4.

La Serrota population	
<i>Underground part</i>	<i>Aerial part</i>
1H-Imidazole, 1-methyl-4-nitro- (15.45%)	3,5-Dihydroxybenzamide (38.67%)
Formamide, N-methyl- (8.47%)	Cyclohexanepropanol, α ,2,2,6-tetramethyl- (12.71%)
3-Methoxy-1H-pyrazol-5-ylacetamide (7.89%)	Benzo[h]quinoline, 2,4-dimethyl- (7.06%)
Catechol (5.14%)	1,4-Dihydroxybenzene (Hydroquinone) (4.84%)
3-Quinuclidinol (5.02%)	Benzenecarboxylic acid, α -hydroxy- (mandelic acid) (2.62%)
Butanoic acid (4.14%)	Benzenethiol, 4-methoxy- (2.25%)
Gredos population	
1H-Imidazole, 1-methyl-4-nitro- (12.88%)	Cyclohexanepropanol, α ,2,2,6-tetramethyl- (24.89%)
Formamide, N-methyl- (12.03%)	1H-Pyrrole, 3-methyl- (8.81%)
3-Methoxy-1H-pyrazol-5-ylacetamide (11.20%)	N-Acridin-9-yl-N'-phenyl-hydrazine (5.97%)
3-Methyl-1-pentanol (3.96%)	Pyridine-2,3,5,6-tetraamine (5.96%)
3-Quinuclidinol (3.40%)	Dianhydromannitol (5.59%)
Cyclohexene,3-(2-propenyl)- (2.54%)	Benzeneacetic acid, α -hydroxy- (mandelic acid) (3.23%)
3-Formyl-4,5-dimethyl-pyrrole (2.30%)	

Note: While underground tissues' extracts show convergent composition overall, minor quantitative differences exist in low-abundance compounds not shown in this summary table.

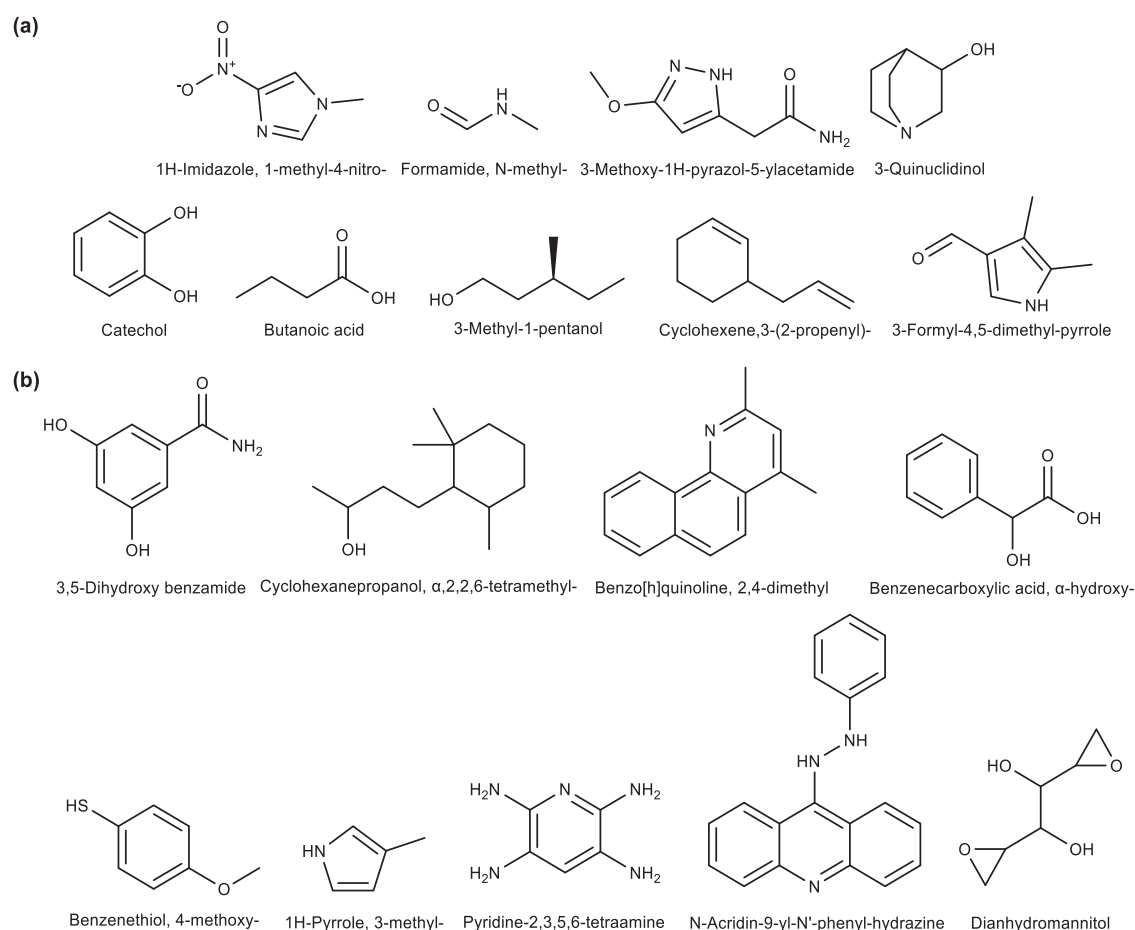


FIGURE 1 | Chemical structures of the most abundant species identified by GC–MS in: (a) underground part extracts of *Pseudomisopates rivas-martinezii*; (b) aerial part extracts of *P. rivas-martinezii* from La Serrota and Gredos.

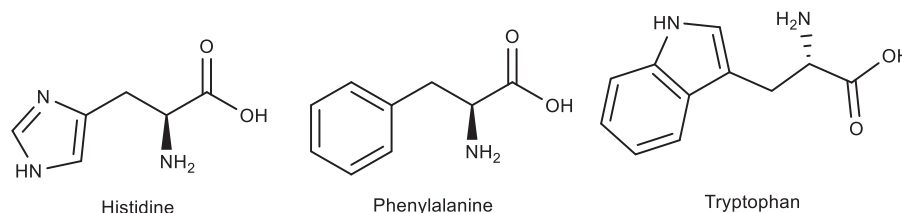


FIGURE 2 | Chemical structures of potential precursors of some of the chemical species identified in the *P. rivas-martinezii* extracts.

N-methylformamide, 3-methoxy-1*H*-pyrazol-5-ylacetamide). This correlates with the absence in FTIR spectra of bands at 2850 cm^{-1} (symmetric C—H stretching), indicative of reduced presence of complex aliphatic structures, and the consistent presence of aromatic bands (1511–1512 cm^{-1}) corresponding to C=C and C=N vibrations of the heterocyclic rings identified by GC–MS.

In aerial parts, the chemical divergence between populations detected by GC–MS is reflected in FTIR spectral variations. The La Serrota aerial sample, characterized by phenolic derivatives (3,5-dihydroxybenzamide, 4-methoxybenzenethiol, benzo[*h*]quinoline-2,4-dimethyl), shows intense bands at 1309 cm^{-1} and 1229 cm^{-1} (C—O stretching of phenols) and bands at 832 and 897 cm^{-1} (out-of-plane C—H bending of disubstituted aromatics). The ~1645 cm^{-1} band, which encompasses Amide I protein vibrations, bound water deformation, and aromatic C=C stretching, showed a shift from 1642 cm^{-1} (La Serrota) to 1650 cm^{-1} (Gredos) in the aerial samples. In a complex plant powder matrix, it is not possible to attribute such a small shift to a single class of secondary metabolites, as proteins, structural polysaccharides, and moisture all contribute to absorption in this region. Nonetheless, this spectral variation is consistent with broad-level compositional differences between the two populations and aligns directionally with the higher bulk nitrogen content measured in Gredos aerial tissues (1.89% vs. 1.00%), which would increase the relative contribution of nitrogenous functional groups (Amide I, C=N) to this spectral region. The FTIR data should therefore be interpreted as providing corroborative, but not independently diagnostic, evidence of the population-level chemical differences characterized more precisely by GC–MS.

2.3.4 | Biosynthetic Pathways

Analysis of the chemical structures identified in extracts from underground tissues suggest that several of the main compounds could derive from L-histidine, phenylalanine, or tryptophan through different degradation pathways (Figure 2).

2.3.5 | Histidine-Derived Metabolites

The presence of 1*H*-imidazole-1-methyl-4-nitro can be explained by decarboxylation of histidine, followed by methylation (of nitrogen) and nitration (at position 4) of the imidazole ring through additional reactions. This type of transformation has been documented in other plant species that produce imidazole alkaloids [17, 18]. *N*-Methylformamide could originate through oxidative cleavage of the histidine side chain, a process analogous

to that observed in amino acid degradation under oxidative stress conditions [19]. Concerning 3-formyl-4,5-dimethyl-pyrrole, while not a direct histidine metabolite, pyrrole derivatives can form through secondary reactions: histidine metabolism generates formyl groups (via formyl-glutamate), and formylated compounds could potentially undergo condensation reactions to form pyrrole structures. However, the specific pathway producing this compound in *P. rivas-martinezii* underground parts remains to be elucidated.

The preferential accumulation of these metabolites in the underground system may be related to the function of histidine in heavy-metal chelation and tolerance to abiotic stress in granitic soils, characteristic of the *P. rivas-martinezii* habitat [20].

Regarding catechol, which was detected exclusively in the underground tissues' extracts of the La Serrota population, this compound is typically derived from the shikimate pathway [21, 22] via hydroxylation of phenylalanine or tyrosine. Its population-specific presence suggests that the shikimate pathway is active in the underground parts of the La Serrota population, highlighting a subtle but important layer of metabolic divergence even in below-ground tissues. As will be elaborated upon later, this finding provides an interesting contrast to the otherwise convergent, histidine-dominated profile of the underground part.

2.3.6 | Aromatic Amino Acid-Derived Metabolites

The compounds identified in the aerial part show biosynthetic differentiation between populations in the downstream modifications of aromatic amino acids produced by the shikimic acid pathway [21]. The shikimic acid pathway is the common biosynthetic route for all three aromatic amino acids (phenylalanine, tyrosine, and tryptophan) through the key intermediate chorismate [23]. Both populations, therefore, utilize the same primary biosynthetic pathway, with chemotypic differences reflecting divergent secondary transformations of these aromatic amino acid intermediates.

In La Serrota, the chemical profile suggests preferential metabolism of phenylalanine and tyrosine alongside some tryptophan derivatives. 3,5-Dihydroxybenzamide could be formed through hydroxylation of the aromatic ring of phenylalanine or tyrosine, followed by decarboxylation and amidation. Benzo[*h*]quinoline, 2,4-dimethyl- represents a modified tryptophan-derived quinoline derivative; quinoline structures can arise from tryptophan through indole ring rearrangements and cyclization processes. Mandelic acid, present in both populations, is produced from phenylalanine

through enzymatic pathways (phenylalanine → phenylpyruvate → benzoylformate → mandelic acid) [24, 25].

In contrast, specimens from Gredos showed a chemical profile suggesting greater activity of tryptophan-specific downstream pathways following its production via the shikimic acid route. The presence of 3-methyl-1*H*-pyrrole and acridine derivatives (such as *N*-acridin-9-yl-*N'*-phenyl-hydrazine) points toward indole ring degradation processes characteristic of tryptophan catabolism [26], related to the kynurenine pathway. The presence of pyridine-2,3,5,6-tetraamine could be linked to the kynurenine pathway, which produces quinolinate (a pyridine-containing intermediate) during NAD biosynthesis. However, it is plausible that the four amino substituents are an artifact of the aqueous ammonia extraction. Through a Chichibabin-type reaction, a simpler precursor like pyridine could have been aminated during sample preparation. Therefore, the native compound might be pyridine itself, a hypothesis that requires further investigation with alternative extraction methods.

2.3.7 | Methodological Considerations

The aqueous ammonia extraction method employed in this study was chosen because lignified underground stolons require aggressive solvent conditions to achieve adequate extraction yields [27, 28], and the same medium was applied to aerial parts to enable direct organ-to-organ comparison. However, this alkaline extraction medium is reactive and generates artifacts through chemical reactions with native plant constituents. Two specific cases merit emphasis. First, 3,5-dihydroxybenzamide, the most abundant species detected in La Serrota aerial extracts, is almost certainly an extraction artifact produced by ammonolysis of a native carboxylic acid or ester (3,5-dihydroxybenzoic acid). Second, pyridine-2,3,5,6-tetraamine in Gredos aerial extracts likely arises from Chichibabin-type amination of a simpler pyridine precursor, possibly an intermediate of the NAD biosynthesis pathway. While the exact native structures therefore remain undetermined, population-level divergence in chemical profiles is robust: both populations were extracted identically, and the differential formation of phenolic amides versus aminated heterocycles reflects genuinely different metabolite pools in the starting tissues. This interpretation is further supported by the independent elemental analysis data, which show substantially higher nitrogen content in Gredos aerial tissues—consistent with a metabolome enriched in nitrogenous heterocycles. Validation by extraction with a neutral, non-reactive solvent system (e.g., 80% methanol–water, or sequential ethyl acetate/methanol extraction) is the essential next step and constitutes a primary objective for our future investigations.

2.4 | Ecological and Evolutionary Implications

The chemical divergence observed between La Serrota and Gredos populations in the aerial parts, contrasted with convergence in the underground parts, is consistent with the hypothesis that different selective pressures may be acting above and below ground. The relatively homogeneous granitic soil substrate shared by both populations could have favored the conservation of a common chemical profile for underground

parts, while microclimatic differences between the two massifs (i.e., temperature, precipitation regime, UV radiation, and abiotic interactions, etc.) might have promoted divergence in aerial secondary metabolites [29]. However, the observed differences could equally arise from neutral genetic drift following the late Pleistocene divergence documented by Jiménez-Mejías et al. [10] or from phenotypic plasticity in response to site-specific environmental conditions. Discriminating among these alternatives will require the integration of environmental metadata, transcriptomic profiling, and common-garden or reciprocal-transplant experiments.

The presence of imidazole alkaloids in the underground stolons of both populations deserves special attention. As noted by Rosenthal and Berenbaum [30], these compounds are typically associated with defense against soil-borne fungal pathogens, particularly relevant in plants that colonize extreme and limiting substrates where microbial depletion and competition are intense.

2.5 | Chemotaxonomic Considerations

The phytochemical profile of *P. rivas-martinezii* shows distinctive characteristics within the genus *Pseudomisopates* and, more broadly, within the Plantaginaceae family. The presence of imidazole alkaloids has not been documented in this family, where alkaloids are limited to simple compounds like indicain and plantagonin in *Plantago major* L. [31], with imidazole alkaloids being primarily documented in Rutaceae and occasionally reported in Cactaceae [16]. This chemical singularity could reflect adaptive evolution to a specialized habitat or be a chemical relic from an ancestor with a different metabolic profile.

The interpopulation variability observed in aerial part metabolites raises questions about the degree of genetic isolation between La Serrota and Gredos populations. Future studies should address whether the observed chemical differences correlate with neutral genetic divergence or represent local adaptations mediated by natural selection. Critically, the present study cannot determine whether the observed chemotypic variation is genetically encoded (i.e., heritable) or environmentally induced (i.e., phenotypic plasticity). Common garden experiments, in which individuals from both populations are cultivated under identical conditions, represent the most direct test of heritability. Reciprocal transplant experiments would further clarify the relative contributions of genotype and environment. Until such data are available, we refer to the observed chemical differences as ‘chemotypes’ in a descriptive, rather than strictly genetic, sense.

Our hypotheses regarding differential selective pressures remain to be tested through direct environmental characterization. Future work should include: (1) comparative soil analysis (pH, heavy metal content, nutrient availability, and metataxonomic profiling) between sites to test both the metal-chelation hypothesis for histidine metabolites, and the existence of soil microbial depletion; (2) microclimate monitoring (temperature, precipitation, and growing season length) to identify abiotic factors that might favor different secondary metabolite profiles; and (3) herbivore surveys to determine whether phenolic compounds in La Serrota provide defense against site-specific herbivore

assemblages. Such environmental data would be crucial for predicting how populations might respond to climate change and informing habitat management strategies.

2.6 | Conservation Management Implications

From a conservation perspective, the chemical divergence documented here has several practical implications. First, the distinct chemotypes between La Serrota and Gredos populations suggest that *ex situ* conservation programs should maintain separate breeding lines for each population to preserve potentially adaptive metabolic profiles and their associated genotypes [10]. Second, any future reintroduction efforts should prioritize source-matched planting (i.e., La Serrota genotypes returned to La Serrota sites) to maintain population-specific chemical adaptations that may confer local fitness advantages [29]. Third, the elevated histidine-derived metabolites in underground parts of both populations suggest potential metal-chelation adaptations to granitic soils [20]; habitat restoration efforts should therefore prioritize maintaining similar soil geochemistry to support these specialized metabolic requirements. Finally, the chemical divergence observed here reinforces the genetic distinctiveness previously documented through molecular markers [10], supporting the designation of these populations as separate management units for conservation planning. Given the species' self-incompatibility, pollen limitation [7], and fire-dependent germination [9], maintaining both populations with their distinct metabolic profiles is critical for long-term adaptive potential under changing environmental conditions.

3 | Conclusions

This study provides the first comprehensive phytochemical characterization of *Pseudomisopates rivas-martinezii* and reveals population-specific chemotypes with clear conservation and taxonomic implications. The elemental analysis demonstrated relatively homogeneous composition between populations, though notable differences in nitrogen content suggest differential nutrient availability or utilization strategies between sites. FTIR spectroscopy revealed tissue-specific differences, with the C–H stretching band at 2850 cm⁻¹ present in leaves but absent in underground parts of both populations, suggesting different chemical compositions between above- and below-ground tissues. The GC–MS analysis revealed organ-specific and population-specific chemical signatures, with underground tissues showing convergent histidine-derived metabolite profiles across populations, likely reflecting shared adaptive responses to granitic soil conditions.

The most striking finding was the divergent aerial chemotypes between populations. La Serrota specimens were characterized by phenolic compounds derived primarily from the phenylalanine/tyrosine branches of the shikimic acid pathway (3,5-dihydroxybenzamide, 4-methoxybenzenethiol, mandelic acid), while Gredos specimens showed predominant nitrogenous heterocycles derived from the tryptophan branch (3-methyl-1H-pyrrole, *N*-acridin-9-yl-*N'*-phenyl-hydrazine, pyridine-2,3,5,6-tetraamine). All identified aromatic compounds originate from the shikimic acid pathway, which produces the precursor aro-

matic amino acids phenylalanine, tyrosine, and tryptophan through the common intermediate chorismate; the observed chemotypic differences reflect population-specific downstream modifications and secondary transformations of these pathway intermediates rather than fundamental differences in primary biosynthetic routes. This chemical divergence, contrasted with underground chemotype convergence, suggests differential selective pressures acting on above- and below-ground tissues, potentially driven by microclimatic variations or other site-specific factors. These findings should be interpreted in light of the study's narrow sampling scope, which was deliberately constrained to minimize demographic impact on the species. The chemical patterns reported here, therefore, require confirmation through replicate sampling across seasons and years, and through individual-level profiling when population sizes permit.

The presence of rare imidazole alkaloids in both populations highlights the unique metabolic profile of this critically endangered species within the Plantaginaceae family. These findings underscore the importance of preserving both known populations, as they represent distinct evolutionary units with unique chemical adaptations. The chemical divergence observed between populations may reflect incipient speciation processes or local adaptations that could be crucial for the species' long-term survival under changing environmental conditions.

4 | Experimental Section

4.1 | Samples Collection

Samples of *P. rivas-martinezii* were collected in August 2024 from the Sierra de Gredos (ca. 1750 m a.s.l.) and the Sierra de La Serrota (ca. 1850 m a.s.l.), Ávila, Spain. Precise GPS coordinates are not disclosed in the published record to protect this critically endangered species from unauthorized collection; they are available upon reasonable request to the corresponding author. Collections were conducted under authorization from the General Directorate of Natural Heritage and Forest Policy of the Junta de Castilla y León regional government. Specimens were identified and authenticated by Dr. María Elena Amate-León (Real Jardín Botánico, CSIC, Madrid, Spain). Voucher specimens are deposited at the herbarium of the Real Jardín Botánico de Madrid (MA) under registration numbers MA-01-00644711 (Gredos population) and MA-01-00890773 (La Serrota population).

To minimize clonal sampling, tissue samples (aerial and underground parts) were collected from ramets separated by several meters, without compromising their viability. Composite samples of aerial parts ($n = 20$ per location) and underground parts ($n = 20$ per location) were prepared, which were subsequently shade-dried, ground to a fine powder, homogenized, and sieved (1 mm mesh) for elemental analysis and extract preparation. A limitation inherent to working with this critically endangered species is the necessarily narrow sampling scope. Composite samples from 20 ramets per population were the minimum required to yield sufficient biomass for the full analytical pipeline (elemental analysis, FTIR, GC–MS), while avoiding lethal damage to individual plants. As a consequence, intrapopulation chemical variance was not captured by the present design. Future studies should aim for

individual-level sampling, contingent upon population recovery and regulatory approval.

4.2 | Extract Preparation

The ultrasonic extraction procedures for underground and aerial parts followed protocols previously described in Sánchez-Hernández et al. [32]. An aqueous ammonia solution digestion, suitable for materials with tough or lignified cell walls, was chosen for the underground stolon of *P. rivas-martinezii* to maximize the extraction yield of relevant chemical constituents. While other extraction media (e.g., a conventional hydromethanolic medium) would have been suitable for the less recalcitrant aerial part tissues, the same aqueous ammonia medium was chosen to allow for direct comparison between the chemical profiles of underground and aerial parts and ensure that any observed differences in chemical profiles between organs or populations are likely due to genuine biological variation, not solvent-induced artifacts.

Briefly, the underground stolon powder sample was digested in an aqueous ammonia solution (200 mL H₂O + 50 mL NH₃) for 2 h, and then subjected to pulsed sonication for 10 min (with a 2-min pause every 2.5 min), using an UP400St ultrasonicator (Hielscher Ultrasonics, Teltow, Germany). The solution was left to stand for 24 h, neutralized to pH 7 with acetic acid, centrifuged at 9000 rpm for 15 min, and the supernatant was filtered through Whatman No. 1 paper. For the aerial part, the same extraction procedure was followed. After extraction, aliquots of the underground stolon and aerial part extracts were freeze-dried.

The freeze-dried extracts were redissolved in methanol (HPLC grade) and underwent a derivatization step before GC–MS analysis using *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA, for GC derivatization, LiChropur) according to the procedure described in [33]. The obtained solutions, with a concentration of 5 mg·mL^{−1}, were filtered before injection.

4.3 | Physicochemical Characterization

Elemental analyses of the dried ground samples (3 mg/sample) were performed prior to extraction using a CHNS-932 analyzer (LECO, St. Joseph, MI, USA). Calorific values were calculated from the elemental analysis data according to Talwalkar [34], using the following equation:

$$\text{HHV} = (0.341 \times \%C) + (1.322 \times \%H) - 0.12 (\%O + \%N)$$

where the higher heating value (HHV) is the calorific value for dry material, expressed in kJ/g, and %C, %H, %O, and %N are the mass fractions, expressed as % by weight of dry material.

The infrared vibrational spectra of both the aerial and underground parts (pre-extraction) and the freeze-dried extracts were recorded using a Nicolet iS50 Fourier-transform infrared (FTIR) spectrometer (Thermo Scientific, Waltham, MA, USA) equipped with a diamond attenuated total reflection (ATR) system. Interferograms were generated by co-adding 64 scans across the 400–4000 cm^{−1} spectral range with a 1 cm^{−1} resolution.

The extracts were analyzed by GC–MS at the Technical Research Services (SSTTI) of the University of Alicante (Alicante, Spain), using an Agilent Technologies model 7890A gas chromatograph coupled to a model 5975C quadrupole mass spectrometer. The chromatographic conditions were as follows: injection volume = 1 µL; injector temperature = 280°C, in split-less mode; initial oven temperature = 60°C, held for 2 min, followed by a 10°C·min^{−1} ramp to a final temperature of 300°C, held for 15 min. The chromatographic column used for compound separation was an Agilent Technologies HP-5MS UI column with a length of 30 m, a diameter of 0.250 mm, and a film thickness of 0.25 µm. The mass spectrometer conditions were as follows: mass spectrometer electron impact source temperature = 230°C; quadrupole temperature = 150°C; ionization energy = 70 eV. Component identification was based on comparison of their mass spectra and retention times with the National Institute of Standards and Technology (NIST11) and Wiley databases. Compounds with mass spectral quality scores ≥70 were considered reliably identified. Compounds with scores between 50 and 69 were designated as tentative identifications, and those with scores <50 were excluded from biological interpretation or reported with appropriate caveats. A limitation of the present study is that compound identification relies exclusively on mass spectral library matching (NIST11 and Wiley) and constitutes tentative identification only. No authentic reference standards or calculated retention indices (e.g., Kovats indices) were employed for confirmation. Structural assignments involving rare motifs –particularly nitrated heterocycles such as 1-methyl-4-nitro-1*H*-imidazole– should be regarded as provisional and will require targeted validation by co-injection of commercial standards, high-resolution MS (HRMS), and/or NMR spectroscopy of isolated fractions.

Supporting Information

Supporting information for this article is available on the WWW under <https://doi.org/10.1002/cbdv.71377>.

Author Contributions

Eva Sánchez-Hernández: conceptualization, investigation, resources, writing – original draft, writing – review and editing, project administration, funding acquisition. **María Elena Amat:** validation, investigation, writing – original draft. **Mario Cano-Pérez:** investigation, writing – original draft. **Vicente González-García:** validation, investigation, writing – original draft. **Jesús Martín-Gil:** formal analysis, investigation, writing – original draft. **Pablo Martín-Ramos:** conceptualization, methodology, formal analysis, investigation, writing – original draft, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available in the supporting information of this article.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File 1: cbdv71377-sup-0001-SuppMat.docx.