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IONOMICS SUGGESTS NICHE DIFFERENCES BETWEEN SYMPATRIC HEATHERS (ERICACEAE)

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ABSTRACT

Background and Aims The co-existence of large number of competing plant species with the same basic needs is a major question in ecology, particularly when this involves closely related species.

Methods We investigated the ecology of six heather species (*Calluna vulgaris* & *Erica* spp., Ericaceae) able to cohabit in the same heathlands. We characterised the ionome, i.e. the mineral composition of the plants using a sampling strategy specifically designed to control for soil effects. Nine plant communities in Limousin, France, were investigated, representing eight combinations of different species, on serpentine and non-serpentine substrates.

Results Ionome was influenced by both taxonomic and environmental factors with significant interactions between them. We found that species growing in sympatry had distinct ionomic profiles, i.e. they differ in their leaf mineral content, suggesting different nutritional strategies. Different mycorrhizal associations may be hypothesised to explain these different chemical signatures.

Conclusions Differential use of the soil nutrients could explain the co-existence of closely related species. It may also explain the diversity of certain shrubby ecosystems or large shrubby genera such as *Erica*. Ionomics is therefore a promising tool for ecological studies in non-model organisms.

Keywords: heathland, ionome, ericoid mycorrhiza, serpentine, ultramafic

INTRODUCTION

The coexistence of a large number of competing plant species with the same basic needs in a single community is a major question in ecology (Silvertown 2004). Contrasting views exist as to whether plant species occupy different niches or if the richness of local communities can be explained by a balance between migration, extinction and speciation ("neutral" theory, Hubbell 2001). It is particularly difficult to explain how several closely related species (e.g. from the same genus) can share the same space when they have so many inherited morphological, physiological, and other characters in common, and competition between them would thus be expected to be severe (Darwin 1859 p. 79; Richards 1969; Weber and Strauss 2016). This question is particularly enigmatic in the case of tropical rainforests which are considered as the most diverse terrestrial ecosystems, but shrublands can also be extremely diverse, for example, the fynbos of South Africa or the kwongan of south-west Australia, which were recently qualified as old climatically buffered infertile landscapes (OCBILs, Hopper 2009; Hopper et al. 2016).

The diverse shrublands in south-west Australian are characterised by a range of different nutrient acquisition strategies (Teste et al. 2017), e.g. arbuscular mycorrhizal, ectomycorrhizal, ericoid mycorrhizal, N-fixing and non-mycorrhizal cluster-rooted, but these are relatively conserved traits (Maherali et al. 2016). In addition, nutritional specialisation on soils is one expectation predicted by the OCBIL theory (Hopper 2009). Indeed the occurrence of nickel hyperaccumulating and aluminium hyperaccumulating species was recently discovered in a recently evolved genus of trees from New Caledonia (Pillon et al. 2014), suggesting that nutritional diversity may be found within a young taxonomic group.

The ionome can be defined as "all the mineral nutrient and trace elements found in an organism—extending the metallome to include metals, metalloids and non-metals" (Lahner et al. 2003). Ionomics, the study of the ionome, has been mostly applied to functional analysis of genes in model organisms (e.g. Arabidopsis, Salt et al. 2008), but also offers perspectives for the study of environmental adaptions (Baxter and Dilkes 2012; Huang and Salt 2016). However the approach has rarely been applied to wild non-model plants (but see Garten 1978; Pillon et al. 2014; Verboom et al. 2017).

Shrublands are less frequently studied and are more difficult to investigate and understand than rainforests. For example, in a forest, the number of trunks in a plot is roughly the same as the number of individual trees, whereas shrubs are often multi-stemmed and the notion of 'individual' is complex in the case of vegetative reproduction. We chose to investigate European heathlands as they are relatively simple shrublands dominated by a few

species of heather (*Erica* spp.). It has been assumed that differences in pollinator explain the survival of different heather species in sympatry but this implies that pollinators are a limiting resources for seed set and that recruitment is seed-limited (Heystek and Pauw 2014). However this hypothesis has received limited experimental support (Heystek and Pauw 2014; Bouman et al. 2017). The aim of this study was thus to investigate whether ionomics could reveal differences in nutrient and trace element uses between congeneric heather species growing side by side, thereby revealing putative niche differences that could explain their sympatry. We investigated a diverse range of heather communities in contrasting environments, including on serpentine substrates. Pedogenesis processes on serpentine and ultramafic rocks generally differ most from those on other type of rocks (Echevarria 2018).The resulting soils offer major challenges to plant growth: low availability of some macronutrients, a high Ca/Mg ratio, high concentrations of potentially toxic metal elements (Kazakou et al. 2008). They can be considered as extreme environments and therefore a good model to test the applicability of ionomics.

MATERIAL AND METHODS

Study group—We investigated the six heather species which occur naturally in the Limousin area (France): *Calluna vulgaris* (L.) Hull, *Erica ciliaris* L., *E. cinerea* L., *E. scoparia* L., *E. tetralix* L. and *E. vagans* L. The species *Calluna vulgaris* was originally described as *Erica vulgaris* by Linnaeus and is the only member of the genus *Calluna*, that is, the sister group to the genus *Erica* (Pirie et al. 2011). These species are of similar size (generally < 50 cm in height), except *E. scoparia* which can reach a few metres. *E. scoparia* is also anemophilous while the other species are entomophilous and may attract different insect species (Hagerup and Hagerup 1953; Bannister 1965, 1966; Rose et al. 1996). *Erica cinerea*, *E. scoparia* and *E. tetralix* flower in early summer while *C. vulgaris*, *E. ciliaris* and *E. vagans* flower in late summer in our study area. The species are well-defined and easily distinguished and only *E. ciliaris* and *E. tetralix* are known to hybridise (*E.* × *watsonii* Benth.).

Study sites—We selected nine sites in the Limousin area and one in the nearby Charente (France) to sample a diversity of heather communities/combinations (table 1, figure 1). The sampled locations were either on serpentine substrates: three distinct plots/communities at Cluzeau, and one at Pierres du Mas, and five on other substrates: Judie, Maîtres Jacques, Massaloux, Puy Chabrol and Rochechouart. Soil electrical conductivity (EC) and pH were

determined in a 1:5 suspension of soil to double deionized water. Organic matter content was determined by the loss ignition method. Cation exchange capacity (CEC) and exchangeable Ca and Mg were determined according to French norms NF X 31-130. According to the World Reference Base (IUSS Working Group WRB 2015), the soils developed on serpentine are Hypereutric Magnesic Cambisols, while most of the other soils are Eutric Cambisols. The physical-chemical properties of the soil samples are given in supplementary data.
The nine sites investigated represented six different combinations of three species (two sites, Pierres du Mas and Massaloux, had the same combination but on different substrates) and two sites (Judie, Puy Chabrol) with two different combinations of two species. All the samples were collected between the 10th and the 28th of June 2016, except for the site of Puy Chabrol, which was sampled on the 12th of September 2016.

Sampling strategy—The main sampling strategy was to avoid microsite effects and to sample the different heather species in the same soil conditions. At each site, we selected a homogenous zone where the three target species were well mixed. A bamboo cane was planted each time we found the association of the three species within a radius of ca. 1 metre and this was repeated 15 times. Each cane was planted at least 2 metres apart. Next we selected the 10 most closely located bamboo canes to minimize the size of the study plot and the environmental gradient. We then collected a branch of each target species within a distance of 1 metre from each bamboo cane (Figure S1), giving a total of 10 samples per species per site. One soil sample was collected in the centre of each plot at each site. None of the plots exceeded 400 m^2 in size.

Sample treatments—The heather branches were taken back to the lab, where they were cut into 10-20 cm fragments. Because heathers have very small leaves, large samples (i.e. branches) were needed to obtain 1 g of dried leaves. Due to their size, samples of each individual were rinsed separately in a c. 5 L bucket of tap water to remove any adhering soil particles rather than a more thorough procedure such as using demineralized water with Alconox (Faucon et al. 2007). Samples were then wiped briefly with paper towels, placed in a paper envelope and dried in an oven at 45 °C for 48 h or more. More than 1 g of leaves was then ground to powder in a mortar with a pestle and liquid nitrogen. The plant samples were sent to Bureau Veritas Minerals (Vancouver, Canada) where they were digested in HNO_3 and then Aqua Regia before analysis by ICP-MS (*Analyse* “VG101”). The soil samples were dried at ca. 45 °C for > 1 week, sieved (2 mm mesh) and more than 15 g were sent for analysis to

Bureau Veritas Minerals where the samples were further sieved (180 μm), digested (Aqua Regia) and analysed with ICP-MS (*Analyse* "AQ251"). The 37 elements measured in both plant and soil samples were Ag, Al, As, Au, B, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, Ga, Hg, K, La, Mg, Mn, Mo, Na, Ni, P, Pb, S, Sb, Sc, Se, Sr, Te, Th, Ti, Tl, U, V, W, and Zn.

Data analysis— Elemental concentrations below the detection threshold were replaced by zero values. Two-way ANOVA was conducted in R (Casgrain and Legendre 1999) and principal component analyses using correlation matrices in PAST (Hammer et al. 2001).

RESULTS

Leaf elemental concentrations for the 37 elements and 225 individuals (9 individual per species per site) are given in supplementary data. The concentrations of two elements, W and Ga, were below the detection threshold in all accessions and were excluded from subsequent analyses. No anomalously high concentration of elements such as Al, Cr, Ti, Fe that could indicate soil contamination (Reeves and Baker 2000) were found in any of the leaf samples, suggesting that our washing procedure was sufficient for these microphyllous, non-hyperaccumulating and glabrous to sparsely hairy species. A principal component analysis of the 37 element concentrations, pH, CEC, and EC of the soil samples (supplementary data) at the nine locations clearly separated plots on serpentine substrates from plots on other substrates (Figure S2).

The results of principal component analysis of the leaf elemental concentration at the nine sites (representing eight combinations of different species) are shown in figure 2. In most communities, the species were separated according to their ionomic profile, with the exception of some minor overlaps between the profiles of *C. vulgaris* and *E. tetralix* at Pierre du Mas, and *E. vagans* and *E. scoparia* at Maîtres Jacques. In most cases, species were better separated by the principal components 1 and 2, whereas for the sites Cluzeau 2 and Rochechouart, better separation was obtained with the principal components 1 and 3. Principal component analysis also showed that populations of the same species on different substrates had different elemental profiles (Figure S3).

Leaf elemental concentrations differed significantly between sites and species, and in most cases, the interaction between these two parameters was also significant (ANOVA, Table S1). The species effect was not significant for only two of the essential elements tested: K and Mo. The taxonomic effect was particularly strong in some elements (figure 3) such as

Mn, concentrations of which were consistently high in *C. vulgaris* and low in *E. vagans*, irrespective of the substrate. Tl (Thallium) was generally detected in leaves of *C. vulgaris*, whereas Tl was below the detection threshold in most accessions of the other species. *Calluna vulgaris* also had the highest average leaf P content of all species in all the communities sampled. The substrate effect was more prominent in some other elements (figure 4). Higher concentrations of Ni were found in the leaves of all species on serpentine substrates, where soil Ni concentrations ranged from 119.3 to 851.2 mg/kg (vs. 3.2-14.1 mg/kg on non-serpentine soils). Similar results were found for Cr and V (data not shown). By contrast, concentrations of Mo were consistently lower in leaves of all species growing at serpentine sites (0.64-1.27 mg/kg in soils) compared to species growing at non-serpentine sites (0.35-4.37 mg/kg) where the two lowest concentrations of Mo in soils were recorded (Massaloux and Puy Chabrol).

DISCUSSION

Ionomic profiles showed that sympatric species of heathers differed in the elements taken up from the soil and translocated to their aerial photosynthetic organs. This confirms our previous observations (Pillon et al. 2014) that closely related species may exploit soil nutrients differently, but the sampling strategy used in the present study allowed us to rule out environmental effects. A large portion of our knowledge on plant physiology (including ionomics) is derived from Brassicaceae (*Arabidopsis* and relatives) which are unusual among Angiosperms in being non-mycorrhizal. In contrast, heathers are known to be involved in a particular type of root symbiosis, ericoid mycorrhiza, in association with fungi that are mostly ascomycetes (Read 1996). These mycorrhiza have been shown to reduce uptake and hence the toxicity of certain metals (Zn, Cu) in *C. vulgaris* (Bradley et al. 1981). Thus, there are several possible explanations for the differences observed in leaf elemental concentrations among heather species. The species may be associated with different fungi with different nutritional strategies (Waterman et al. 2011), absorb elements from the soil / fungal hypha differently, and / or differ in the translocation of the element from the roots to the leaves. Differences have indeed been found between root fungal communities of Ericaceae species growing on the same mountain (Toju et al. 2016), and Osborne et al. (2018) recently found evidence that arbuscular mycorrhizal fungi play a role in the co-existence and speciation of the palms on Lord Howe Island. Further investigations are required to confirm that the “microbial mediation” hypothesis (*sensu* Silvertown 2004) can explain plant coexistence.

Considerable variations can be observed in leaf elemental concentrations, for example a factor of 10 for Mn between sympatric *C. vulgaris* and *E. vagans* or a factor of 10 for Ni in *C. vulgaris* growing on serpentine vs. non-serpentine soils. It is consequently difficult to investigate large scale phylogenetic variations in leaf elemental concentrations without controlling for soil effects (Broadley et al. 2001; Watanabe et al. 2007; Neugebauer et al. 2018). Future studies on phylogenetic variation of leaf mineral content should be carried out on data collected on species growing in the same soil conditions. Similarly, different species respond differently to soil conditions, reflecting previously observed differences between populations of *Arabidopsis thaliana* (Buescher et al. 2010). Elements are clearly not independent variables, and although several correlations are known (Baxter 2009), their characterisation is rendered difficult by the significant interactions between genetic (taxonomic) and environmental (soil) effects, as observed in our study. The limited number of individuals we collected per population (species × sites) made this type of correlation difficult to investigate with sufficient statistical power, and, what is more, the correlation between elemental concentrations in plants and soils is not necessarily straightforward. Cultivating individuals from each population in controlled conditions would provide the insights needed to unravel environmental effects and putative local adaptations. Such experiments have already been conducted on *Arabidopsis* species (Arnold et al. 2016; Stein et al. 2017), and revealed the existence of ecotypes adapted to metalliferous soils with distinct ionomic signatures. This approach has not yet been used for slower growing shrubs such as *Erica* species but would be useful to test for the existence of character displacement.

As could be expected, we observed higher Ni concentration in the leaves of all heather species on serpentine soils, where this element is much more abundant; although the reverse has been observed in *Arabidopsis arenosa* (Arnold et al. 2016). In contrast, low levels of Mo have consistently been observed in plants growing on serpentine substrates although the concentrations of this element in this substrate were not the lowest of the elements, suggesting that the availability of Mo rather than its concentration is the limiting factor on serpentine (Walker 1948, 2001). High leaf Mn contents were previously reported in *C. vulgaris* in Poland (Wojtuń et al. 2017), our values are even higher (average > 1,000 mg/kg). The fact that this species also had on average the highest leaf P content in each community, reinforces the hypothesis that Mn content could be used as a surrogate to detect efficient strategies to acquire P from soil (Lambers et al. 2015).

Thus, there seems to be some niche partitioning between congeneric species which could explain their cohabitation at the same site. As mentioned above, the role of mycorrhizal

symbioses in this ecological separation remains to be determined. Based on the evidence presented here, it is not yet possible to infer a general role for ionome differentiation in the speciation of *Erica*, globally recognized as a "big" plant genus (Frodin 2004). However, it is possible that the differences in soil resource use implied by such differentiation, facilitate coexistence and may partly explain the high concentration of *Erica* species in the Cape Floristic Province of South Africa (Goldblatt and Manning 2002). There, *Erica* is represented by more than 600 endemic species that descend from a single recent radiation (Pirie et al. 2011, 2016). The factors that promoted this remarkable diversification remain to be clearly identified, the role of pollinators has not been convincingly demonstrated (Heystek and Pauw 2014; Pirie et al. 2016; Bouman et al. 2017). Plant-soil interactions are likely an important driver of speciation in the Cape flora (Schnitzler et al. 2011), and nutritional specialisation would be expected in old-climatically buffered infertile landscapes (Hopper 2009). Ionomics is therefore a promising tool to understand the origin and functioning of species-rich ecosystems.

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Table 1. List of study sites with coordinates, substrates, and sampled species.

Site number	Site name	Latitude	Longitude	Elevation	Substrate	Soil type (WRB)	Species
1	Cluzeau 1	45°34’58.9’’N	01’24’’02.7’’E	438 m	serpentinite	hypereutric magnesic cambisol	*cinerea*, *tetralix*, *vagans*
2	Cluzeau 2	45°34’58.3’’N	01°24’01.5’’E	434 m	serpentinite	hypereutric magnesic cambisol	*tetralix*, *vagans*, *vulgaris*
3	Cluzeau 3	45°34’50.6’’N	01°24’00.4’’E	423 m	serpentinite	hypereutric magnesic cambisol	*cinerea*, *vagans*, *vulgaris*
4	Pierres du Mas	45°34’13.1’’N	01°31’46.3’’E	438 m	serpentinite	hypereutric magnesic cambisol	*cinerea*, *tetralix*, *vulgaris*
5	Judie	45°41’46.9’’N	00°56’42.3’’E	370 m	gneiss / schist	eutric cambisol	*ciliaris*, *tetralix*
6	Maîtres Jacques	45°24’40.5’’N	00°03’03.4’’W	86 m	marlstone	eutric cambisol	*scoparia*, *vagans*, *vulgaris*
7	Massaloux	45°44’04.3’’N	01°00’19.4’’E	317 m	Leptinic gneiss	hypereutric magnesic cambisol	*cinerea*, *tetralix*, *vulgaris*

8	Puy Chabrol	45°36’16.4’’N	01°02’14.1’’E	446 m	granite	eutric cambisol	*ciliaris*, *vulgaris*
9	Rochechouart	45°48’55.0’’N	00’52’35.8’’E	279 m	weathered gneiss		*scoparia*, *tetralix*, *vulgaris*

1

Figure 1. Locations of the study sites. Triangles and squares indicate heather populations on serpentine and non-serpentine sites, respectively.

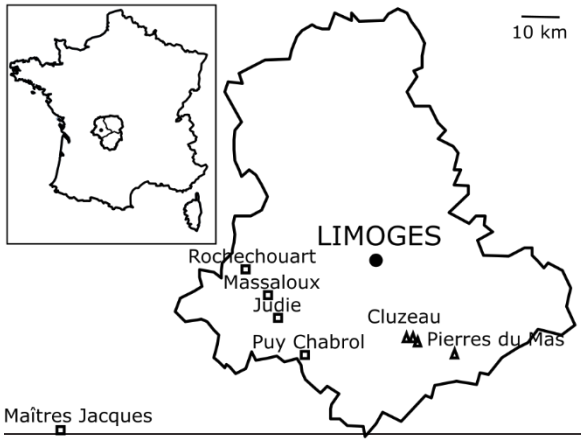

Figure 2. Principal component analysis on leaf concentration of 35 elements at nine sites.

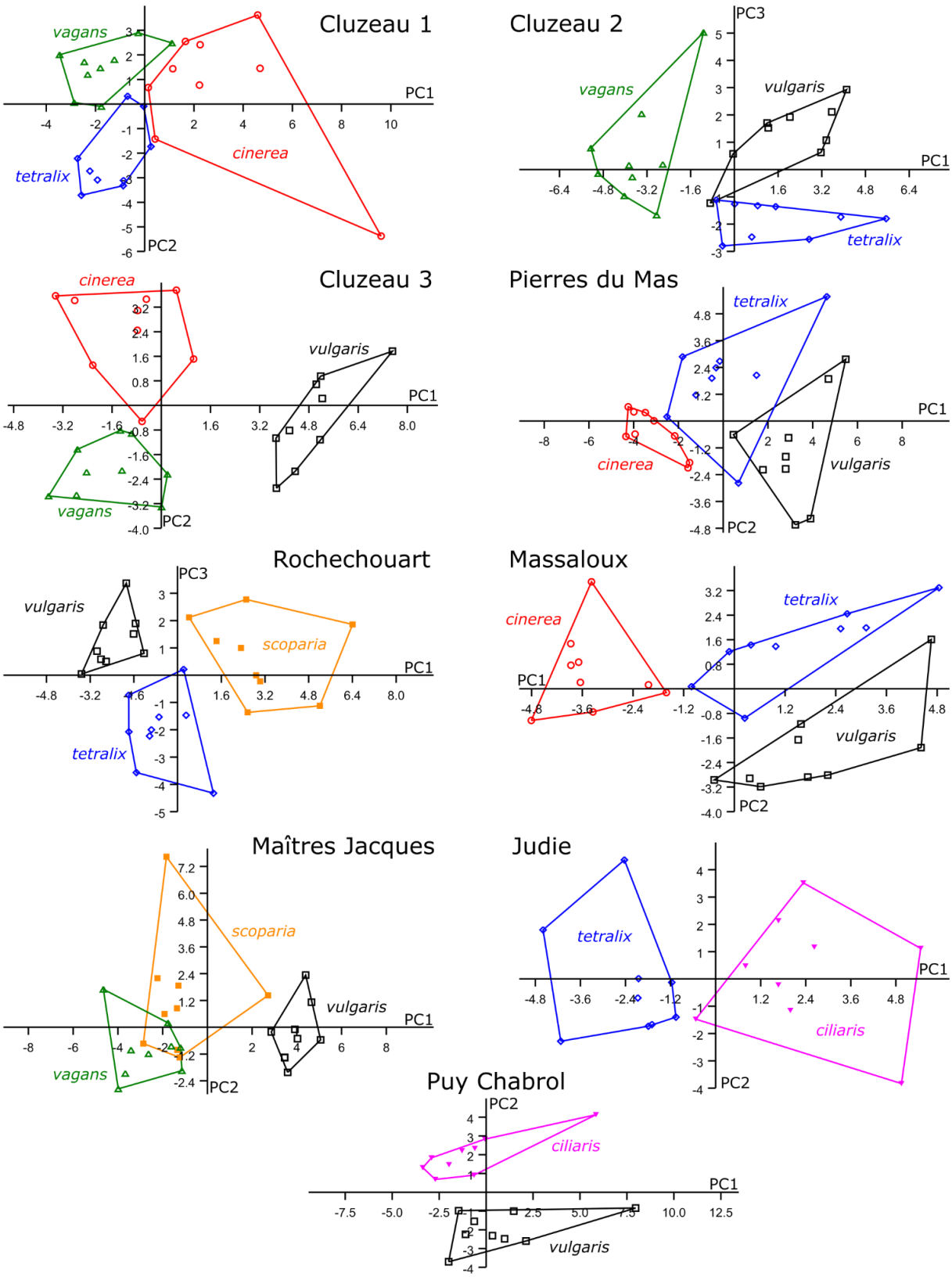

Figure 3. Variation across populations in the leaf concentrations (mg/kg) of manganese (Mn) and thallium (Tl), two elements with a strong taxonomic signature. vul: *Calluna vulgaris*; cil: *Erica ciliaris*; cin: *E. cinerea*; sco: *E. scoparia*; tet: *E. tetralix*; vag: *E. vagans*. Site numbers are the same as in table 1. Red: serpentine sites; Yellow: non-serpentine sites.

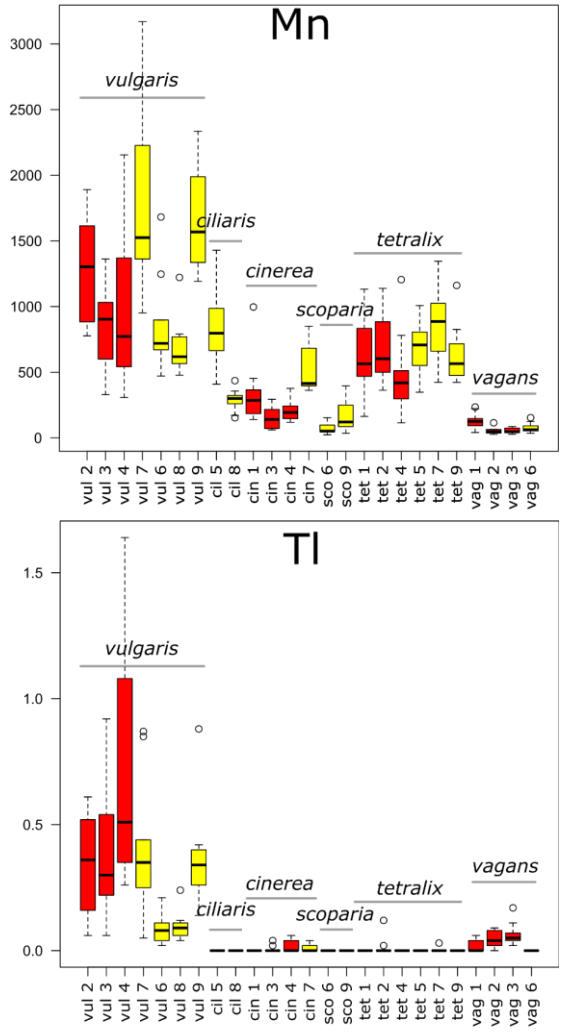
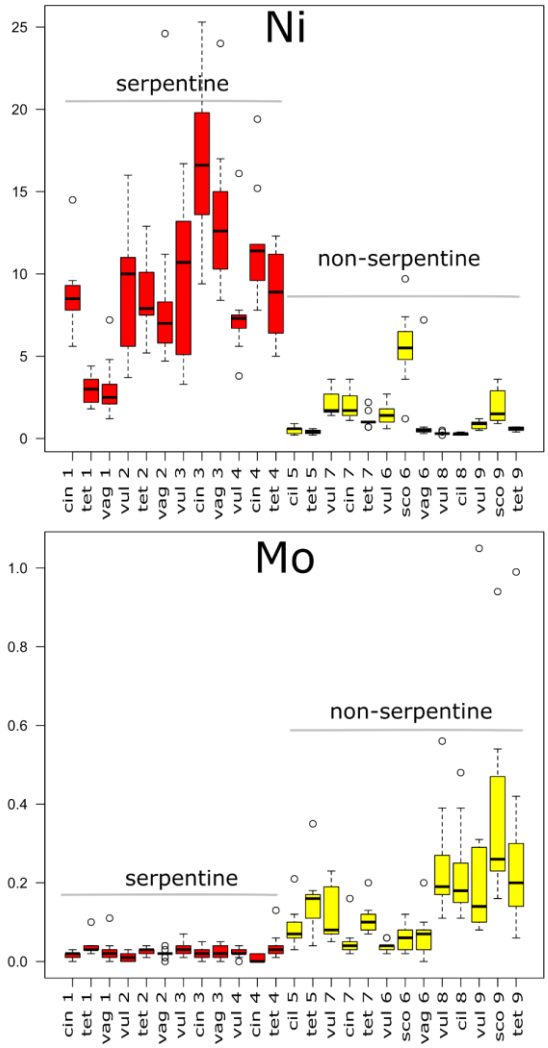
Figure 4. Variation across populations in the leaf concentrations (mg/kg) of nickel (Ni) and molybdenum (Mo), two elements with a strong environmental effect. vul: *Calluna vulgaris*; cil: *Erica ciliaris*; cin: *E. cinerea*; sco: *E. scoparia*; tet: *E. tetralix*; vag: *E. vagans*. Site numbers are the same as in table 1. Red: serpentine sites; Yellow: non-serpentine sites.