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1 **The X-ray fluorescence screening of multiple elements in herbarium**
2 **specimens from the Neotropical region reveals new records of metal**
3 **accumulation in plants**

4
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5 Running head: Metal Accumulation in the Neotropical Flora

6

7 **KEYWORDS**

8 X-Ray Fluorescence, Metal, Hyperaccumulator, Cuba, Ionomics, Agromining

9

10

1 **ABSTRACT**

2 Plants have developed a diversity of strategies to take up and store essential metals in order to
3 colonize various types of soils including mineralized soils. Yet, our knowledge of the capacity
4 of plant species to accumulate metals is still fragmentary across the plant kingdom. In this study,
5 we have used the X-Ray Fluorescence technology to analyze metal content in a wide diversity
6 of species of the Neotropical flora that was not extensively investigated so far. In total, we
7 screened more than 11 000 specimens representing about 5000 species from herbaria in Paris
8 and Cuba. Our study provides a large overview of the accumulation of metals such as
9 manganese, zinc and nickel in the Neotropical flora. We report 30 new nickel
10 hyperaccumulating species from Cuba, including the first records in the families Connaraceae,
11 Melastomataceae, Polygonaceae, Santalaceae and Urticaceae. We also identified the first
12 species from this region of the world that can be considered as manganese hyperaccumulators
13 in the genera *Lomatia* (Proteaceae), *Calycogonium* (Melastomataceae), *Ilex* (Aquifoliaceae),
14 *Morella* (Myricaceae) and *Pimenta* (Myrtaceae). Finally, we report the first zinc
15 hyperaccumulator, *Rinorea multivenosa* (Violaceae), from the Amazonas region. The
16 identification of species able to accumulate high amounts of metals will become instrumental
17 to support the development of phytotechnologies in order to limit the impact of soil metal
18 pollution in this region of the world.

19

1 **Introduction**

2 The ability of plants to colonize most of the terrestrial ecosystems reside in their capacity to
3 take up and store nutrients and metals from the soil. Metals such as iron (Fe), copper (Cu),
4 manganese (Mn), zinc (Zn), and nickel (Ni) are cofactors of numerous enzymes playing
5 essential biological functions including respiration and photosynthesis. However, when present
6 in excess these metals play adverse effects, triggering oxidative and genotoxic stresses.¹⁻³
7 Therefore, every plant has to regulate the homeostasis of metals according to the availability of
8 metals and their needs during life cycle. The regulation of metal homeostasis is particularly
9 challenging for plant species growing on soils depleted in available metals or on metalliferous
10 soils enriched in metals such as ultramafic (e.g. serpentine) and calamine soils.^{4,5} A limited
11 number of species have acquired the capacity to tolerate and accumulate high concentration of
12 metals in their above-ground tissues. These species are called metal hyperaccumulators when
13 the concentration of metal in above-ground tissues collected on plants growing in their natural
14 environment reaches a threshold presently fixed at 1000 ppm (or 1000 ug/g dry weight) for Ni,
15 3000 ppm for Zn and 10 000 ppm for Mn, corresponding to 2-3 orders of magnitude above the
16 levels recorded in plant leaves growing on common soils.^{6,7} Today, about 700 metal
17 hyperaccumulator species have been identified, with the large majority (c. 530 species)
18 hyperaccumulating Ni.⁸ Metal hyperaccumulators are widely distributed across the tree of life
19 in more than 70 families but are found more frequently in some specific eudicotyledon families,
20 *ie* Phyllanthaceae, Brassicaceae, Asteraceae, Cunoniaceae, Euphorbiaceae and Salicaceae. The
21 capacity of plants to accumulate metals is a rare trait even in metalliferous flora suggesting an
22 important evolutionary cost. However, metal accumulation is also proposed to provide selective
23 advantage through the protection from insects and pathogens, allelopathy or adaptation to
24 drought.^{9,10} Species that are able to accumulate metals thus represent good models to identify
25 the mechanisms involved in the regulation of metal homeostasis in plants and to study the

1 evolution of this complex adaptive trait.^{11–13} Hyperaccumulator species are also foreseen as
2 crops to extract and recycle metals from contaminated or naturally metal-rich soils.^{14,15}
3 The number of plant species known to accumulate metals has considerably increased in the past
4 few years thanks to the use of handheld X-ray fluorescence (XRF) analyzer to screen herbarium
5 collections from Malaysia, Papua New Guinea and New Caledonia. These regions contain very
6 diverse floras developing on ultramafic soil.^{16–18} Handheld XRF analyzers are particularly well
7 suited to screen large herbarium collections because they allow to measure non-destructively
8 virtually all elements from magnesium to uranium, very rapidly and with a good sensitivity on
9 dry leaf material.^{19,20} However, the XRF spectra analysis software used in these analyzers are
10 usually not developed for the quantification of elements from carbon-based matrix. Therefore,
11 the precise quantification of metals from dry leaves still requires a calibration and a
12 confirmation of XRF data using quantitative methods including inductively coupled plasma
13 (ICP) or microwave plasma (MP)-atomic emission spectrometers (AES).
14 Despite the fact that the Neotropical region is the richest region for plant species on Earth,²¹
15 handheld XRF was not used yet to perform large scale screens of herbarium specimens from
16 this area. The dimethylglyoxime assay was previously used to screen herbaria from Cuba and
17 Brazil, both countries containing large ultramafic outcrops, and allowed the identification of
18 130 and 20 Ni hyperaccumulators respectively.^{22–25} In addition, Ni hyperaccumulators of the
19 *Psychotria* genus (Rubiaceae) were identified in several countries of the Neotropical region.^{26,27}
20 However, to our knowledge, no Zn or Mn hyperaccumulators had yet been reported from the
21 Neotropical region.
22 In this work, we used handheld XRF to screen large collections of plant specimens in the
23 herbarium of the French National Museum of Natural History in Paris (P) and in herbaria in
24 Cuba (HAJB and HAC) to identify plant species able to accumulate metals from the Neotropical
25 region. This analysis provides a large picture of metal accumulation in plant species from this

1 region. In particular, we have identified new accumulating species for Ni, Mn and Zn. Some of
2 these species belong to plant families or genera that were not known so far to contain metal
3 accumulating species. We believe that these original data will be instrumental to broaden our
4 knowledge of the mechanisms involved in metal accumulation in plants and to develop
5 sustainable phytotechnologies to extract metals from soil in the neotropical region.

6

1 **Materials and methods**

2 **Handheld XRF screening of herbarium specimens**

3 We used a Niton XL3T 950 GOLDD+ handheld XRF analyzer (Thermo Fisher Scientific,
4 Waltham, Massachusetts, USA) to screen herbarium specimens essentially as previously
5 described¹⁸. Each specimen was placed on a 2 mm titanium foil (#269489, Sigma-Aldrich,
6 Saint Louis, Missouri, USA) and the X-ray fluorescent spectra were recorded in the soil mode
7 for 30 sec. using the main filter. The analysis of the spectra by the proprietary XRF
8 quantification software (ver. HH-XRF_8.4G) provided an estimated concentration in part per
9 million (ppm) for the following elements: As, Au, Co, Cu, Fe, Hg, Mn, Mo, Pb, Rb, Sr, Th, U,
10 W, Zn, Zr. The handheld XRF analyzer was associated with a barcode reader (Motorola
11 CS3070) to record the specimen identification number when available.

12 **Strategy of herbarium screening**

13 We screened the Herbarium of the French National Museum of Natural History in Paris (P)
14 containing c. 6 million specimens of vascular plants organized according to the APGIII
15 classification.²⁸ We restricted the screen to the America sector (AME) as defined in P,
16 excluding specimens from USA and Canada. The screen of P was mostly targeted at species
17 belonging to clades known to contain metal hyperaccumulator species including families of the
18 COM clade (eg Cunoniaceae, Euphorbiaceae, Phyllanthaceae, Violaceae, Salicaceae),
19 Proteaceae and Rubiaceae (entire genus *Psychotria*). When possible, we screened 3 to 5
20 specimens collected from distinct geographical areas per species. The metadata linked to each
21 specimen were recovered from the P herbarium database Sonnerat using the unique barcode
22 number, or manually collected from the label of the specimen with the help of the citizen
23 science program “Les herbonautes” (<http://lesherbonautes.mnhn.fr/missions/13907009>).

1 In addition, species of the Cuban flora were screened at the Johannes Bisse Herbarium (HAJB)
2 of the Jardín Botánico Nacional in Havana. In this case, we screened every dicotyledonous
3 species from the reference collection that contains one specimen of all species present in Cuba.
4 For species and genera with noticeable concentration of metals, we screened additional
5 specimens at HAJB and at the Onaney Muñíz Gutiérrez Herbarium (HAC) of the Institute of
6 Ecology and Systematics in Havana. For the Cuban flora, we used the most recent nomenclature
7 of the species available.²⁹ Following preliminary results, additional specimens of *Rinorea*
8 *multivenosa* and *R. longistipulata* were kindly provided as a loan by the Missouri Botanical
9 Garden Herbarium (MO).

10

11 **Quantitative elemental analysis and calibration of the XRF data**

12 Dry leaf samples (c. 50 mg) were weighted and mineralized in polypropylene tubes (#352070,
13 Falcon brand-Corning, Tewksbury, USA) at 120°C in a heating block for 4 h in 2 mL of 65 %
14 HNO₃ (#30709-M, Sigma-Aldrich, Saint Louis, USA) and additionally for 4 h after the addition
15 1 mL of 30% H₂O₂ (#7722-84-1, Sigma-Aldrich, Saint Louis, Missouri, USA). Mineralized
16 samples were analyzed on an Agilent 4200 MP-AES (Agilent Technologies, Santa Clara, USA).
17 The concentration of metals in the samples were calculated using metal standard calibration
18 curves. For the calibration of the XRF analyses, we gathered 124 samples of dry leaves from
19 plant species accumulating various amounts of Ni and Mn originating from herbarium
20 specimens and samples collected in the field. The samples were analyzed in parallel by XRF
21 and MP-AES as described above. A linear regression modelling the relationship between the
22 elemental concentration measured by XRF and MP-AES was obtained for Ni ($[\text{Ni}]_{\text{MP-AES}} =$
23 $0.6762 [\text{Ni}]_{\text{XRF}}; r^2=0.94$) and Mn ($[\text{Mn}]_{\text{MP-AES}} = 0.2793 [\text{Mn}]_{\text{XRF}}; r^2=0.95$) (Fig. S1).

24

1 **Data representation and statistical analyses**

2 The cartographies were generated using the QGIS software (version 2.18.28, QGIS Geographic
3 Information System, Open Source Geospatial Foundation Project, <http://qgis.osgeo.org>) with
4 the GRASS v. 7.6.0 extension. Publicly available base maps originate from Natural Earth
5 (Admin 0 - Countries, <https://www.naturalearthdata.com>).

6 For metal concentration analysis, unless indicated, we used the elemental concentration
7 provided by the handheld XRF analyzer. Metal concentrations below to the limit of detection
8 (LOD) were replaced by the lowest concentration measured in this study: Zn (6 ppm), Cu (8
9 ppm), Ni (29 ppm) and Mn (39 ppm).

10 Statistical analyses and data representation were performed using R v.3.6.3³⁰ and R studio
11 v.1.3.109. The visualization of data was performed with R packages ggplot2 v.3.3.2³¹ and dplyr
12 v.1.0.2.³² The analysis of the relationship between metal concentration and plant families was
13 analyzed using a linear mixed effect model (LMM) with the R packages lme4 v.1.1.23,
14 lmerTest v.3.1.2 and car v3.0-10.³³⁻³⁵ Metal concentration data were Log10 transformed. The
15 models were constructed using the plant family as a fixed effect and both genus and species as
16 random effects. p-values were obtained using the Satterthwaite's approximation for degrees of
17 freedom. The R script used for this analysis is available upon request to the authors.

18

1 **Results and discussion**

2 **Extent of the X-Ray Fluorescent screen of the Neotropical flora**

3 Combining the X-Ray Fluorescent (XRF) screen of the herbaria in Paris (P) and in Cuba (HAJB
4 and HAC), we analyzed a total of 11 321 herbarium specimens originating from 39 countries
5 and territories of the Neotropical region (Fig. 1, Table S1). We emphasized the investigation of
6 the Cuban flora (4282 specimens) because Cuba is known to contain a large diversity of Ni
7 accumulating species. However, this flora was not investigated so far to identify species able
8 to accumulate other metals than Ni.^{22,23} The important representation of specimens from Brazil
9 (2155 specimens) and French Guiana (1181 specimens) reflects the richness of the P herbarium
10 for these flora.²⁸ The screened specimens correspond to 5053 plant species, mostly
11 dicotyledonous, distributed in 933 genus and representing 161 families (Fig. S2). The screen of
12 the P herbarium was targeted to species from families and genera known to contain metal
13 hyperaccumulators. As a consequence, the most represented families in our study are Rubiaceae
14 (2325 specimens), Phyllanthaceae (832 specimens), Salicaceae (777 specimens), Celastraceae
15 (741 specimens), Chrysobalanaceae (644 specimens) and Violaceae (539 specimens).
16 Overall, our screen covers approximately 5% of the diversity of the Neotropical flora that is
17 estimated to contain from 90 000 to 100 000 seed plant species.²¹

18 **Distribution of metal concentration in herbarium specimens**

19 The concentration of several elements was measured in parallel by handheld XRF in leaves of
20 the 11 321 herbarium specimens (Table S1). The distributions of the concentrations of the
21 essential metals Cu, Mn, Zn and Ni measured in these specimens are presented in Fig. 2. The
22 concentration of Fe measured in herbarium specimens was not considered in our study because

1 a fraction of specimens is contaminated with Fe-rich soil particles, randomly affecting the
2 quantification of the concentration of this element.

3 The distribution of Cu concentrations measured in the herbarium specimens is relatively
4 restricted around the median value of 79 ppm (mean=91 ppm), with only few outliers or
5 samples below the limit of detection (LOD) of 8 ppm. In contrast, the concentrations of Mn and
6 Zn measured in the specimens showed a wider distribution around the median values of 218
7 ppm (mean=650 ppm) for Mn and 50 ppm (mean=93 ppm) for Zn. Only a limited number of
8 specimens displayed a concentration below the LOD for these metals that was estimated at 39
9 ppm for Mn and 6 ppm for Zn. For both metals, we also observed high outliers with metal
10 concentrations up to 48 811 ppm for Mn and 24 267 ppm for Zn, which likely indicates the
11 presence of hyperaccumulator specimens. These results indicated that these two essential
12 metals are available to plants in most of the soils where the specimens have been collected and
13 that plant species can accommodate with wide concentrations of Mn and Zn. The Ni
14 concentrations measured in herbarium specimens showed a very different distribution. A large
15 fraction (85%) of the specimens contains Ni below the LOD of 29 ppm. This result indicates
16 that the sensitivity of handheld XRF is not sufficient to detect Ni in the majority of specimens
17 originating from standard soils. We were however able to detect and measure Ni concentration
18 in 1742 specimens, likely originating from Ni enriched soils such as the ultramafic outcrops
19 from Cuba. In these specimens, Ni concentrations showed a right skewed distribution with a
20 median value of 124 ppm (mean=4306 ppm) and ranging from 29 ppm to 71 901 ppm Ni. This
21 very large distribution of Ni concentrations can be explained by the presence in this dataset of
22 Ni concentrations measured from either excluder or hyperaccumulator species.³⁶

23

1 **Analysis of manganese accumulation at the family level**

2 Because the concentration of Mn can be measured in most of the specimens and displayed a
3 wide distribution, it was possible to test for differences in Mn accumulation at the plant family
4 level. The analysis of the distribution of Mn concentrations in 10 families selected from visual
5 inspection of the data and containing more than 30 specimens revealed large variations of the
6 mean Mn concentration from 443 ppm in Rubiaceae to 4030 ppm in Aquifoliaceae (Fig. 3;
7 Table 1). Using a linear mixed effect model (Fig. 3, Table S2), we did not reveal significant
8 difference ($p>0.01$) in the accumulation of Mn between the Proteaceae family, known for its
9 propensity to accumulate high concentrations of Mn,³⁷ and the Ochnaceae, Clusiaceae,
10 Melastomataceae, Theaceae and Aquifoliaceae families. In contrast, we observed significant
11 differences ($p<0.01$) in the mean concentration of Mn between Proteaceae and the Violaceae,
12 Salicaceae, Phyllanthaceae and Rubiaceae families.

13 Our data thus reveal that several plant families tend to accumulate higher concentration of Mn
14 suggesting that the species from these families have developed specific nutrition strategies
15 leading to an increased uptake, tolerance and accumulation of Mn.³⁸ The high capacity of
16 Proteaceae species to take up and accumulate Mn is proposed to be the consequence of their
17 phosphorus nutrition strategy based on the exudation of large amounts of organic acid in the
18 rhizosphere. The acidification of the rhizosphere leads to the solubilization of phosphate and
19 other elements including Mn.³⁷ It was also showed that Proteaceae species are able to
20 accumulate high amount of Aluminum (Al) and that there is a trade-off between Al and Mn
21 accumulation in these species.^{39,40} These observations suggest that the uptake and accumulation
22 of both Mn and Al in Proteaceae depends on the acidification of the rhizosphere. Interestingly,
23 species of the Melastomataceae and Theaceae families are known to accumulate high amounts
24 of Al.^{41,42} Here, we observed that species of the Melastomataceae and Theaceae families also
25 have a tendency to accumulate high amounts of Mn. It will be therefore interesting to test if

1 species of these families as well as species of the Aquifoliaceae and Ochnaceae families use a
2 nutrition strategy based on the important acidification of the rhizosphere, leading to the
3 solubilization and accumulation of Mn or Al depending on the availability of these metals in
4 the soil.

5

6 **Identification of species accumulating manganese**

7 The XRF screen of the herbaria revealed 69 specimens containing above 10 000 ppm Mn in
8 leaves (Table S1). These specimens belong to 30 species from 18 genera (14 families) including
9 *Roupala* and *Lomatia* (Proteaceae), *Calycogonium* and *Miconia* (Melastomataceae), *Morella*
10 (Myricaceae), *Pimenta* (Myrtaceae), and *Ilex* (Aquifoliaceae) (Table 2; Fig. 4). The analysis of
11 several specimens from these species revealed a large variability of Mn content suggesting a
12 high intraspecific genetic variability and/or a strong influence of edaphic conditions on Mn
13 accumulation. To obtain a better quantification of Mn accumulation in these specimens, we
14 calibrated the XRF measurements with MP-AES on a set of plant samples accumulating various
15 amounts of metals. This calibration indicated that the XRF analysis overestimates the
16 concentration of Mn in dry leaves material by a factor of 3.58 in our experimental set-up (Fig.
17 S1). According to this calibration, only three species, *Lomatia dentata*, *Pimenta oligantha* and
18 *Morella shaferei*, are predicted to accumulate more than 10 000 ppm Mn currently used as a
19 threshold to define Mn hyperaccumulators (Table 2). The direct measurement of Mn
20 concentration in herbarium specimens by MP-AES confirmed that *P. oligantha* and *M. shaferei*
21 are able to accumulate more than 10 000 ppm Mn in leaves and further revealed that *Ilex*
22 *obcordata* and *Calycogonium bissei* can also be considered as Mn hyperaccumulators. The
23 confirmation that the other species accumulating high concentration of Mn can be considered
24 as hyperaccumulators will require further sampling and quantitative elemental analyses.

1 The species accumulating above 10 000 ppm Mn in their leaves described in this study
2 represent, to our knowledge, the first Mn hyperaccumulator species described in the
3 Neotropical flora including Cuba. It was recently shown that several Proteaceae species
4 belonging to the *Roupala*, *Embothrium* and *Lomatia* genera growing in Chile accumulate high
5 concentrations of Mn but so far none of the analyzed sample had ever been reported to reach
6 the hyperaccumulation threshold.³⁹ Here, we have identified a sample of *Lomatia dentata*
7 collected in the Valdivia region in Chile that is predicted to accumulate more than 10 000 ppm
8 Mn. This result also suggests that additional sampling of Proteaceae species from South
9 America may reveal additional Mn hyperaccumulators. More interestingly, we identified new
10 Mn hyperaccumulators such as *Ilex obcordata* (Aquifoliaceae), *Morella shaferi* (Myricaceae)
11 and *Calycogonium bissei* (Melastomataceae) belonging to families that were not known to
12 contain Mn hyperaccumulators. Recently, *Ilex paraguariensis*, also known as “yerba mate”,
13 was shown to be able to accumulate more than 10 000 ppm Mn when grown *ex-situ* on an Mn
14 enriched soil.⁴³ Together with the identification of *I. obcordata*, these results suggest that Mn
15 hyperaccumulation could be revealed in other *Ilex* species. More generally, our results support
16 the idea that, in contrast to Ni hyperaccumulation, the hyperaccumulation of Mn does not define
17 a specific biological adaptation strategy but rather describe the capacity of some species to
18 accumulate and tolerate very high concentration of Mn when this metal is highly available in
19 soils.³⁶ The frequency distribution of Mn concentrations at the level of the Neotropical flora
20 appears to be unimodal (Fig. 2). Furthermore, the species in which we have recorded Mn
21 concentrations above 10 000 ppm belong to plant families that have a propensity to accumulate
22 elevated concentration of Mn (Fig. 3). Finally, even in these Mn hyperaccumulator species we
23 observed a very dispersed distribution of Mn concentration between specimens (Fig. 4). These
24 results suggest that the threshold of 10 000 ppm Mn used to qualify Mn hyperaccumulators
25 defines a limit in the upper tail of the distribution of Mn concentrations measured in plants

1 rather than separates two distinctive modes (*ie* non-hyperaccumulator and hyperaccumulator)
2 in this distribution as previously described for Ni.³⁶

3

4 **Analysis of the accumulation of zinc**

5 We identified 43 specimens containing more than 1000 ppm Zn in leaves as measured by XRF
6 (Table S1). However, we suspected that several specimens from Cuban herbaria collections
7 were contaminated by a Zn-rich glue used for the mounting of the specimens. Such
8 contamination with a glue was previously observed in specimens from the herbarium of Sabah.
9 ¹⁸ Nevertheless, we identified in the P herbarium four specimens of *Rinorea multivenosa*
10 (Violaceae) collected in Brazil, containing from 9280 to 17 830 ppm Zn (Fig. 5). The
11 concentration of Zn measured in *R. multivenosa* specimens is significantly higher than the Zn
12 concentration recorded in the other species of the *Rinorea* genus or more generally in the
13 Violaceae family. To confirm the high accumulation of Zn in leaves of *R. multivenosa*, we
14 analyzed six additional specimens collected in Brazil and Colombia obtained from the Missouri
15 Botanical Garden herbarium (MO). The analysis of the samples from MO confirmed the
16 capacity of *R. multivenosa* to accumulate high concentration of Zn (8517 ± 4936 ppm, n=10)
17 in leaves (Table 3). As a comparison, we measured low concentrations of Zn (56 ± 26 ppm,
18 n=6) in *R. paniculata*. Interestingly, the analysis of 3 specimens of *R. longistipulata* from MO
19 revealed that this species, closely related to *R. multivenosa* and originating from the same
20 geographical region,⁴⁴ is also able to accumulate noticeable amount of Zn (1055 ± 314 ppm,
21 n=3).

22 The highest concentrations of Zn measured by XRF in leaves of *R. multivenosa* are very likely
23 to exceed the 3000 ppm threshold value currently used to define Zn hyperaccumulators. *R.*
24 *multivenosa*, a 2-3 m tall tree growing in the Amazonia region,⁴⁴ would thus correspond to the
25 first Zn hyperaccumulator species identified in the Neotropical region. The labels of the *R.*

1 *multivenosa* specimens that have been analyzed do not provide precise information of the nature
2 of the soil on which they have been collected. However, the accumulation of high Zn in leaves
3 of one cultivated specimen collected in 1874 in the Quinta da Boa Vista park (P02141358), that
4 later became the zoological garden of Rio de Janeiro, suggest that *R. multivenosa* is able to
5 accumulate Zn from soils that are not particularly enriched in Zn. This hypothesis still needs to
6 be supported by quantitative measurement of Zn content in freshly-collected *R. multivenosa*
7 leaves and corresponding soil samples. Interestingly, the closely related species *R.*
8 *longistipulata* is also able to accumulate noticeable amounts of Zn, suggesting that the Zn
9 accumulation trait was present in the common ancestor of these 2 species. Previously,
10 specimens of *Rinorea longiracemosa* from Sabah (Malaysia, Borneo Island) were shown to
11 accumulate high concentrations of Zn in leaves.⁸ The *Rinorea* genus is also known to host
12 several noticeable Ni hyperaccumulators from the south east Asian region, eg *R. bengalensis*
13 ⁴⁵⁻⁴⁷ It should be noted that the limits of the genus *Rinorea* require re-appraisal as suggested by
14 its polyphyly observed in molecular phylogenetic studies.⁴⁸ These results thus indicate that both
15 Zn and Ni hyperaccumulation appeared repeatedly in the *Rinorea* genus as this was previously
16 documented for the *Noccaea* (Brassicaceae) and *Dichapetalum* (Dichapetalaceae)
17 genera.^{8,47,49,50}

18

19 **Analysis of the accumulation of nickel**

20 We screened the P herbarium for species belonging to families and genera known to contain Ni
21 hyperaccumulators including *Phyllanthus* (Phyllanthaceae), *Psychotria* (Rubiaceae) and
22 Violaceae. As expected, our XRF screen identified specimens with foliar Ni concentration
23 largely exceeding 1000 ppm in the *Psychotria* species *P. grandis*, *P. costivenia* and *P.*
24 *papantlensis* that are known Ni hyperaccumulators from the Neotropical region.^{22,27} We also
25 confirmed the capacity of *Phyllanthus nummularoides* from the Dominican Republic to

1 hyperaccumulate Ni.⁵¹ More interestingly, we measured high concentration of Ni in leaves of
2 *Blepharidium guatemalense* (\equiv *B. mexicanum*, Rubiaceae) and in four *Orthion* (Violaceae)
3 species *O. caudatum*, *O. malpighiifolium*, *O. oblanceolatum* and *O. subsessile*, as well as in the
4 closely related monospecific genus *Mayanea* (*M. caudata*) from Guatemala, that were recently
5 identified as new Ni hyperaccumulators from Guatemala and Mexico.^{52,53}

6 The systematic screen of the HAJB herbarium and the screen of the HAC herbarium in Cuba
7 confirmed the accumulation of Ni in most of the hyperaccumulator species previously identified
8 in the Cuban flora (Table S3).^{22,23} Our work provides an updated nomenclature of the Cuban
9 species and references to herbarium specimens. We further observed the accumulation of Ni
10 above 1000 ppm in new species belonging to genera that had previously been investigated in
11 the Cuban flora, including *Buxus braimbridgeorum*, *Mosiera acunae*, *Sapium parvifolium*,
12 *Euphorbia podocarpifolia* and *Phyllanthus excisus* (Table 4). In contrast, we did not confirm
13 the accumulation of high Ni concentration in *Anaestrophia crassifolia* (\equiv *Gochnatia crassifolia*,
14 Asteraceae), *Garcinia ruscifolia* (Clusiaceae), *Bonania erythrosperma* (\equiv *Sapium*
15 *erythrospermum*, Euphorbiaceae), *Ouratea nitida* (Ochnaceae) and *Psychotria osseana*
16 (Rubiaceae). In few cases (eg *Leucocroton* genus), specimens corresponding to species
17 previously identified as Ni hyperaccumulators were not available at the time of our study in
18 HAJB, precluding the confirmation of previous observations. The differences observed with
19 previous studies could be explained by changes in the identification of species or because we
20 analyzed different specimens collected on non-ultramafic soils. More interestingly, we
21 identified species able to accumulate high concentrations of Ni in families and genera that were
22 not known to contain Ni accumulators (Fig. 6, Table 4). We measured above 1000 ppm Ni by
23 XRF in specimens from the Aristolochiaceae, Urticaceae, Connaraceae, Melastomataceae,
24 Santalaceae and Polygonaceae families. In particular, 3 species of the *Pilea* genus (Urticaceae),
25 *P. fruticulosa*, *P. mayarensis* and *P. microphylla* accumulates more than 10 000 ppm Ni in

1 leaves. We also measured above 1000 ppm Ni in specimens from new species from families
2 and genera previously known to contain Ni hyperaccumulators as in *Pimenta* and *Calyptantes*
3 (Myrtaceae), *Daphnopsis* (Thymelaeaceae), *Aristolochia* (Aristolochiaceae)⁵⁴ and *Allophylus*
4 (Sapindaceae) genera. The identification of 2 specimens (Ekman10003, Alain7754) of *Pimenta*
5 *oligantha* accumulating more than 1000 ppm Ni is intriguing because several specimens of this
6 species accumulate high amounts of Mn but not Ni (Table 2).

7 The calibration of XRF data with MP-AES quantification indicated that our XRF analysis
8 overestimates the Ni concentration by a factor of 1.48 (Fig. S1). To confirm the quantification
9 of Ni, we directly measured metal concentration by MP-AES on some herbarium specimens
10 corresponding to new Ni accumulating species (Table 4). These results confirmed that *Pilea*
11 *fruticulosa* is able to accumulate up to 25 700 ppm Ni indicating that this species, and very
12 likely two other *Pilea* species, *P. mayarensis* and *P. microphylla*, are novel Ni
13 hyperaccumulators from the Urticaceae family. This analysis also confirmed that
14 *Crossopetalum rhacoma* (Celastraceae), *Rourea glabra* (Connaraceae), *Casearia crassinervis*
15 (Salicaceae), *Allophylus reticulatus* (Sapindaceae), *Daphnopsis angustifolia* (Thymelaeaceae),
16 *Dendrophthora tetrastachya* (Santalaceae) and *Coccoloba oligantha* (Polygonaceae), are able
17 to accumulate more than 1000 ppm Ni in leaves and can therefore be considered as new Ni
18 hyperaccumulators from Cuba. However, for other species including *Aristolochia lindeniana*
19 (Aristolochiaceae), *Miconia costata* (Melastomataceae) and species of the *Calyptanthes* genus
20 (Myrtaceae), Ni quantification on additional leaf samples will be necessary to confirm their Ni
21 hyperaccumulator status.

22 Our analysis revealed new Ni hyperaccumulator species in the Neotropical flora, thus
23 expanding our knowledge of the diversity of Ni hyperaccumulators in this region of the world.
24 However, except for the Cuban flora, our screen was targeted to selected plant families and
25 genera known to contain Ni hyperaccumulator species. Other plant groups known to contain Ni

1 hyperaccumulators in the Tropics were not specifically targeted.^{24,25} We therefore think that
2 further efforts to screen tropical floras will reveal additional Ni hyperaccumulator species. We
3 show here that the systematic screen of the Cuban flora allowed us to identify new Ni
4 hyperaccumulators, such as *Pilea fruticulosa* (Urticaceae), that belong to families and genera
5 that were not known to contain hyperaccumulators before. These new findings will likely
6 motivate the targeted investigation of related species in future screens to identify new Ni
7 hyperaccumulator species in flora of the pantropical region and in other regions of the world.

8

9 **New combinations of functional traits**

10 Our screening has revealed several combinations of functional traits that had never been
11 reported before in angiosperms, including metal (hyper)accumulation associated with particular
12 root symbioses. *Morella shaferi* is a new Mn hyperaccumulator from Cuba (Table 2) and, as a
13 member of Myricaceae, a likely actinorrhizal plant forming root nodule with nitrogen fixing
14 *Frankia*.⁵⁵ Ni hyperaccumulation has been revealed in the Polygonaceae species *Coccoloba*
15 *baracoensis* and possibly in *C. oligantha* (Table 4), which both belong to a genus that is
16 considered as ectomycorrhizal.⁵⁶ The only similar case known so far was *Shorea tenuiramulosa*
17 from Borneo, accumulating up to 1787 ppm Ni and belonging to Dipterocarpaceae, a family
18 widely considered as entirely ectomycorrhizal.⁵⁷⁻⁵⁹ Although the symbiotic status of *Morella*
19 and *Coccoloba* remains to be validated, the combination of hyperaccumulation with root
20 symbiosis makes those plants ideal candidates as nurse plants for ecological restoration.

21 In addition, we report novel cases of metal accumulation in parasitic plants. We
22 observed high Ni content in *Dendrophthora tetrastachya* (Santalaceae), an aerial ligneous
23 parasite, which unidentified host was likely a nickel accumulator (Table 4). We also observed
24 high Mn content in *Schoepfia cubensis* (Table 2), that belongs to a woody genus considered as
25 hemi-parasitic.⁶⁰ Therefore, these two members of the order Santalales, which partially or

1 entirely depend on other plants for their mineral nutrition, can withstand high metal
2 concentrations in their leaf tissue. Previous reports of parasitism on nickel hyperaccumulating
3 plants include the leafless vine *Cuscuta* (Convolvulaceae) on herbaceous *Streptanthus*
4 *polygaloides* (Brassicaceae) in California, and the non-photosynthetic herbaceous root-parasite
5 *Phelipanche* (Orobanchaceae) on another herbaceous Brassicaceae, *Odontarrhena* (*Alyssum*)
6 in Albania and Lesbos Island.^{61–63} In these cases, the Ni content of the parasite was much lower
7 than that of the hosts, but sometimes exceeding 1000 ppm. Recently, a Ni concentration of 1350
8 ppm was reported in *Amyema scandens* (Loranthaceae), a root semi-parasitic woody vine from
9 New Caledonia.¹⁷ Thus, even if metal hyperaccumulation is proposed to provide elemental
10 allelopathy and a protection against parasitic plants,¹⁰ our results further illustrate that several
11 unrelated lineages of plants with different parasitic strategies evolved metal tolerance and
12 accumulation mechanisms to circumvented the toxicity of their hyperaccumulator host.

13

14 **Conclusions**

15 In this work, we have used XRF to analyze the elemental composition of more than 11 000
16 herbarium specimens representing more than 5000 plant species and 161 families from the
17 Neotropical region. The quantification of metals in leaves of a wide diversity of plant species
18 can help to identify the phylogenetic effects on plant ionomes, reflecting both nutrition and ion
19 homeostasis strategies.^{64–68} The screen of the Neotropical flora with handheld XRF led us to
20 the identification of the first hyperaccumulators of Zn and Mn in this region of the world. In
21 addition, the systematic screen of the Cuban flora allowed us to identify new Ni
22 hyperaccumulators belonging to families and genera in which Ni hyperaccumulation was not
23 described before. Our results suggest that further efforts to screen the flora of yet
24 underinvestigated regions such as tropical Africa and Madagascar will undoubtedly yield new
25 metal hyperaccumulator species. Our results also indicate that the systematic screen of flora is

1 essential to identify hyperaccumulator species in new plant groups. However, because of the
2 scarcity of digitalized information about the nature of the soil linked to the majority of
3 herbarium specimens, systematic screens are difficult to develop in large herbaria and are
4 probably better adapted to herbaria dedicated to specific geographic regions containing
5 metalliferous outcrops.²⁴

6 Our study also reinforces the position of Cuba as a major hot spot for the diversity of metal
7 hyperaccumulator species. While previous studies reported 130 Ni hyperaccumulators on this
8 island,^{22,23} we identified 30 new species predicted to hyperaccumulate Ni (Table S3) and the
9 first 4 species hyperaccumulating Mn (Table 2). New Caledonia, which flora contains from 70
10 to 100 Ni hyperaccumulators and from 10 to 70 Mn hyperaccumulators, is another very rich
11 territory for metal hyperaccumulators.^{8,17} Even if the recent XRF analyses of these flora are
12 difficult to compare because of differences in calibration and because results still need to be
13 confirmed by quantitative analyses,¹⁷ Cuba and New Caledonia have a comparable number and
14 diversity of Ni hyperaccumulators, but Cuba has a more limited number of Mn
15 hyperaccumulators (Table S4). The Cuban and New Caledonian metallophyte flora have
16 interesting similarities, but also differences associated with their distinct biogeographical
17 situation.⁶⁹ On both islands, Ni hyperaccumulation is observed in species of the Celastraceae
18 and Euphorbiaceae families, and Mn hyperaccumulation in Myrtaceae. In addition, Ni
19 hyperaccumulation has been reported in endemic species of the genera *Casearia* (Salicaceae),
20 *Phyllanthus* (Phyllanthaceae), and *Psychotria* (Rubiaceae), that is likely the outcome of
21 convergence. Other important families for Ni hyperaccumulation in Cuba are Asteraceae,
22 Buxaceae, and Ochnaceae, that are poorly represented or absent in New Caledonia.⁷⁰ On the
23 other hand, in New Caledonia Ni and Mn accumulation are common in the locally diverse
24 families Cunoniaceae and Proteaceae, that are virtually absent in Cuba.^{29,71}

1 A better knowledge of the metal hyperaccumulator species at the physiological and molecular
2 levels will become instrumental for the development of phytotechnologies to limit the impact
3 of metal pollution in different region of the world.⁷² For example the Mn hyperaccumulator and
4 putative nitrogen fixer *Morella shaferi*, represents a good candidate species for the restoration
5 of degraded soils in Cuba and Mn accumulated in its biomass could be recycled to produce Mn
6 for green chemistry.⁷³ The identification metal hyperaccumulators, as well as the identification
7 of related non-accumulator species in a large diversity of plant families is a prerequisite to
8 identify the genes involved in metal accumulation in plants using comparative studies ⁷⁴⁻⁷⁶.
9

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13

1 **Figure legends**

2

3 **Fig. 1.** Extent of the XRF analysis in the Neotropical region. The size of the circles is
4 proportional to the number of screened specimens (indicated within the circle). Countries with
5 the highest number of screened specimens are highlighted.

6

7 **Fig. 2.** Distribution of metal concentrations measured by XRF in the specimens of the
8 Neotropical region. The distributions of Cu, Mn, Zn and Ni concentrations measured in leaves
9 in all specimens (n=11 321) by XRF are represented using violin plots with a Log10 scale. To
10 better visualize the distribution of Ni, a violin plot containing only the specimens with a
11 concentration above the LOD is represented. In the box plots, the median is represented as a
12 bold line with both the first (Q1) and the third quartile (Q3). Whiskers extend from the
13 minimum to the maximum [$\pm 1.5 (Q3-Q1)$]. The outliers, located outside the whiskers, are
14 represented by brown dots.

15

16 **Fig. 3.** Distribution of Mn accumulations in 10 selected families. The distributions of Mn
17 concentrations are represented using violin plots with a Log10 scale. Families are organized
18 according to the APG IV classification. n represents the number of specimens per family.
19 Significant differences between the mean concentration of Mn in Proteaceae and other families
20 are shown ($p < 0.01$, linear mixed effect model, [Table S2](#)).

21

22 **Fig. 4.** Distribution of Mn accumulations in various genera. The distributions of Mn
23 concentrations are represented using violin plots with a Log10 scale. Black dots represent
24 individual XRF measurements. Because of the high number of specimens in *Psychotria* and
25 *Phyllanthus*, only the outliers are represented (brown dots). Genera are organized according to

1 the APG IV classification of the corresponding families. n represents the number of specimens
2 per genus.

3

4 **Fig. 5.** Zn accumulation in the Violaceae family. The distributions of Zn concentration in the
5 Violaceae family, the *Rinorea* genus, and in the *R. longistipulata*, *R. multivenosa* and *R.*
6 *paniculata* species are represented using box plots. Dots represent individual XRF
7 measurements. n represents the number of individual XRF scans.

8

9 **Fig. 6.** Distribution of Ni accumulations in genera from the Cuban flora containing
10 accumulating species. The distributions of Ni accumulations are represented using box plots
11 with a Log10 scale. Dots represent individual XRF measurements. n represents the number of
12 specimens. Genera are classified according to the APG IV classification of the corresponding
13 families.

14

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1 **Supplementary data legend**

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3 **Fig. S1.** MP-AES calibration of the XRF data. The concentration of Mn (A) and Ni (B) was
4 measured by XRF and MP-AES in a collection of 124 dry leaves samples from species
5 accumulating various amounts of metals. Only samples with a metal concentration above the
6 LOD for XRF were used for the calibration. The linear regression modelling the relationship
7 between XRF and MP-AES measurements is represented on the graphs. The coefficient of
8 determination (r^2) is used to measure the goodness-of-fit of the model.

9

10 **Fig. S2.** Extent of the XRF analysis in the Neotropical region. The number of families (A),
11 genera (B) and species (C) analyzed per country is represented by the size of colored circles.
12 The countries represented by the most important number of specimens are highlighted.

13

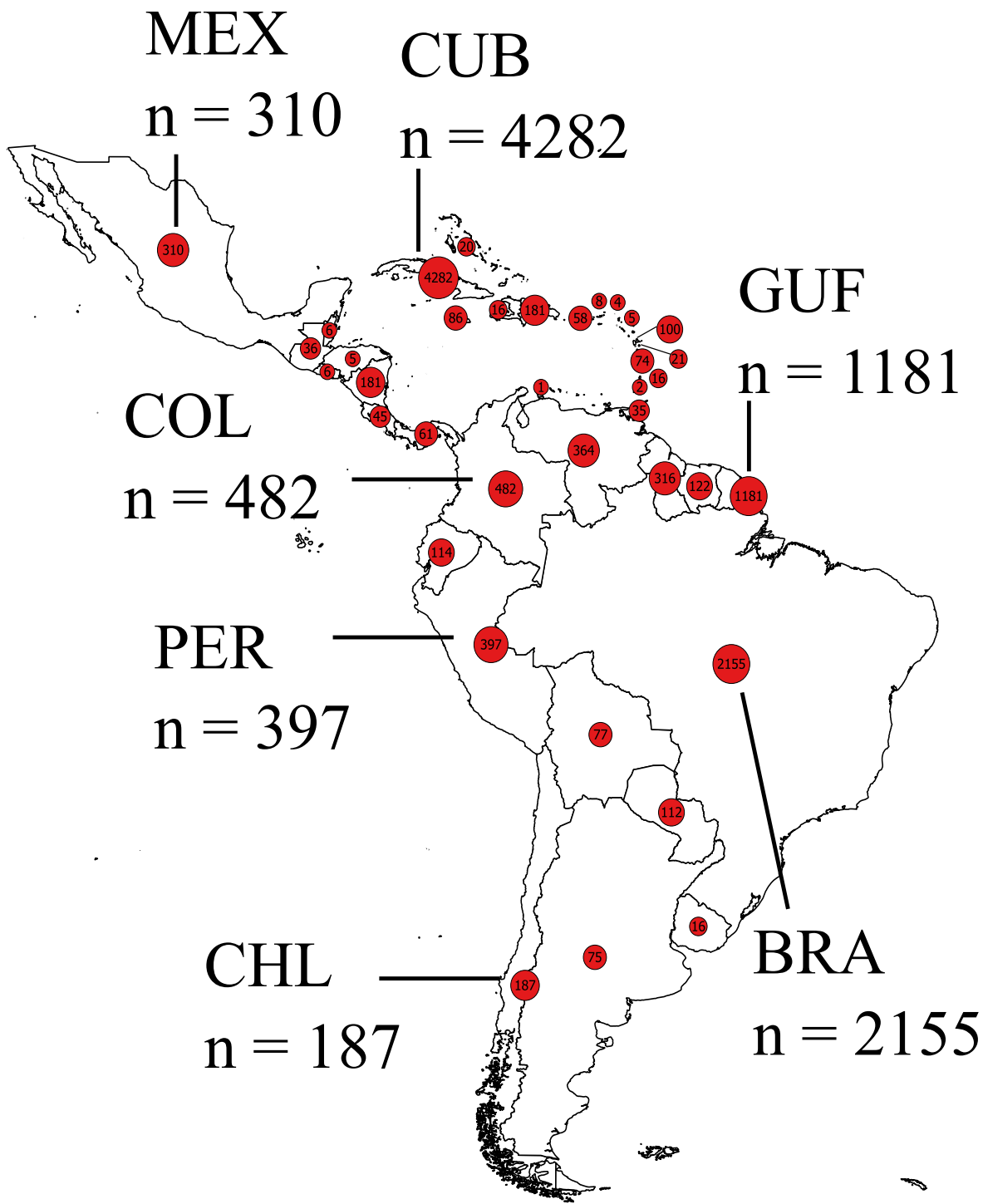


Fig. 1

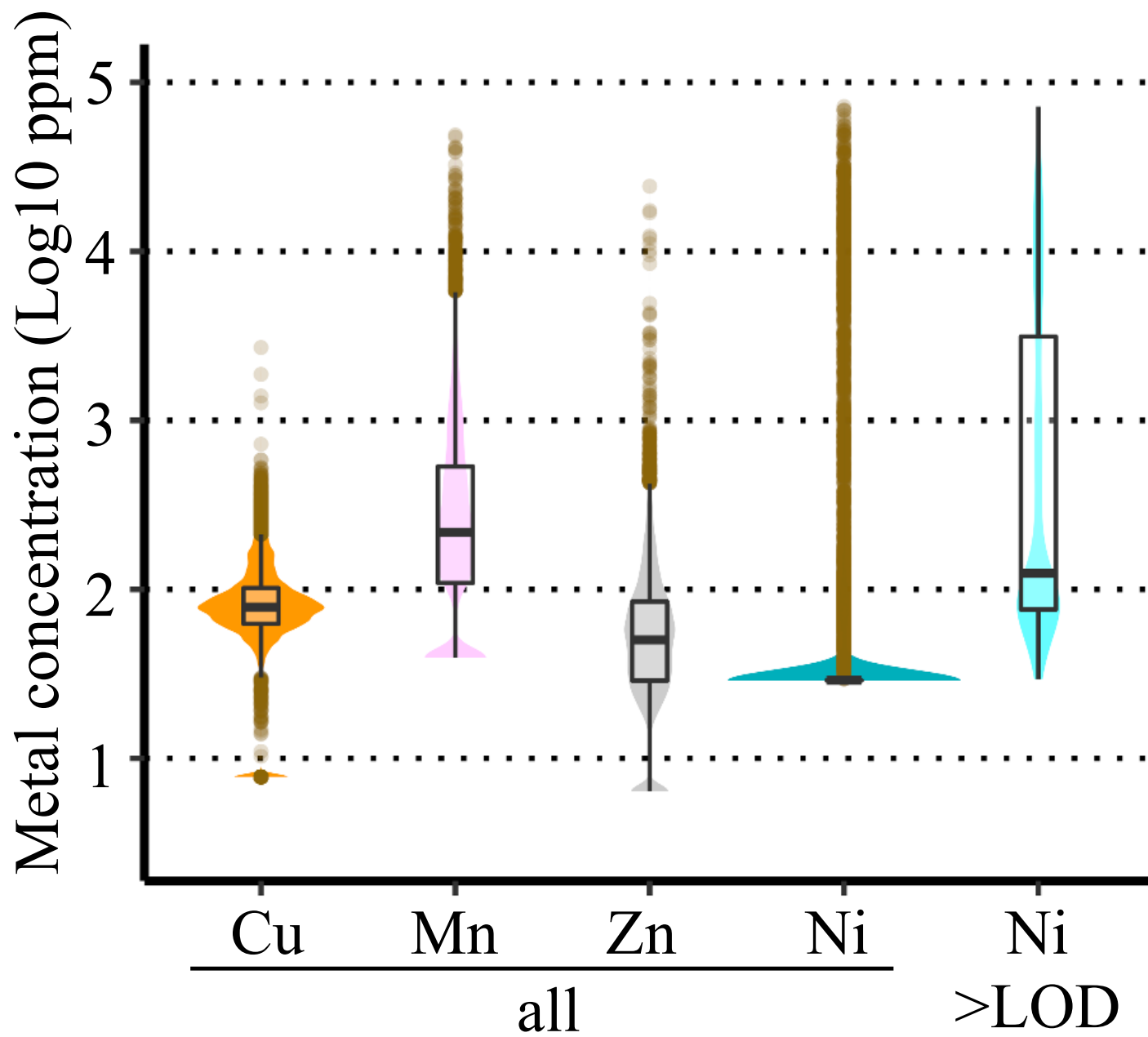


Fig. 2

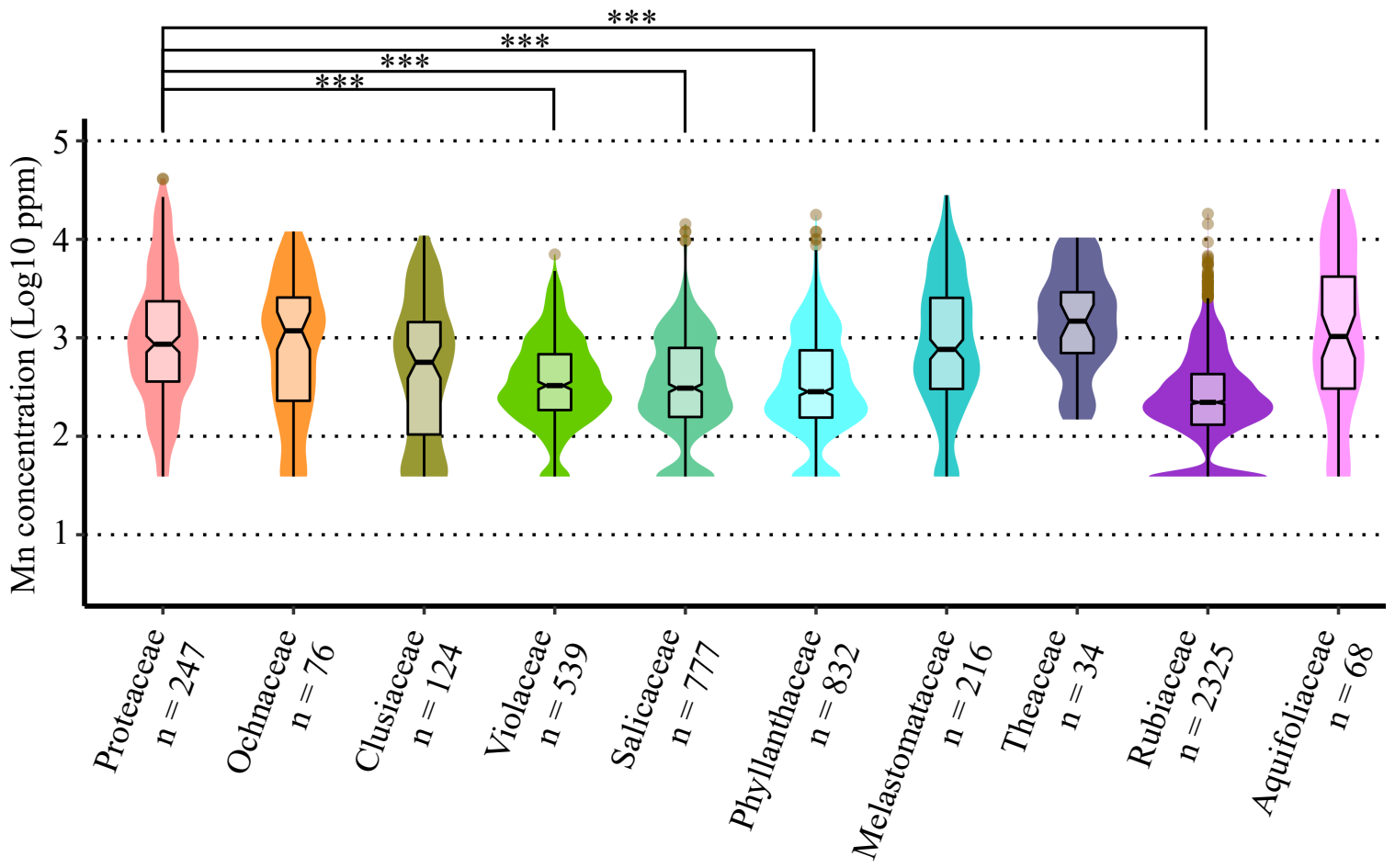


Fig. 3

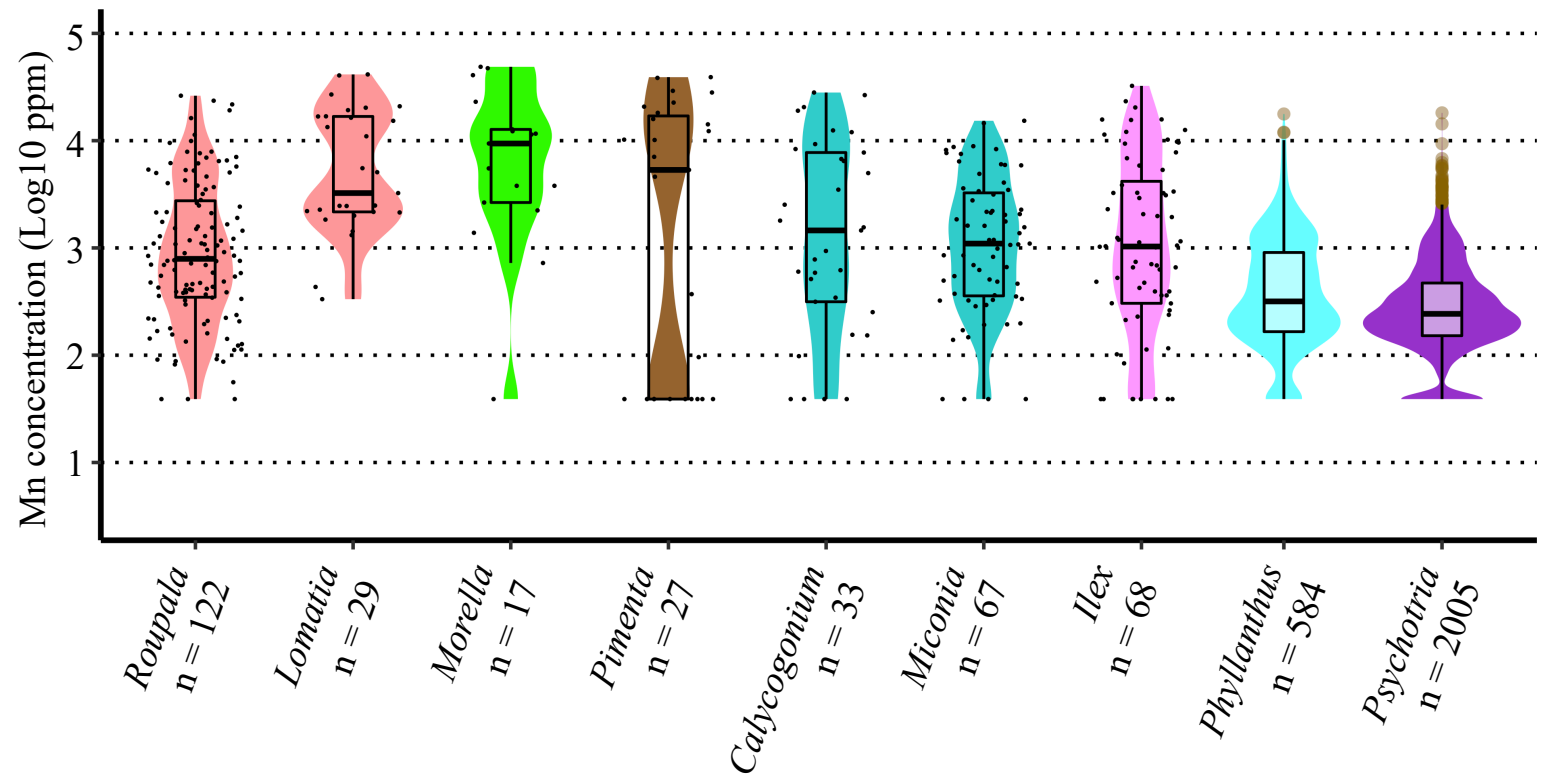


Fig. 4

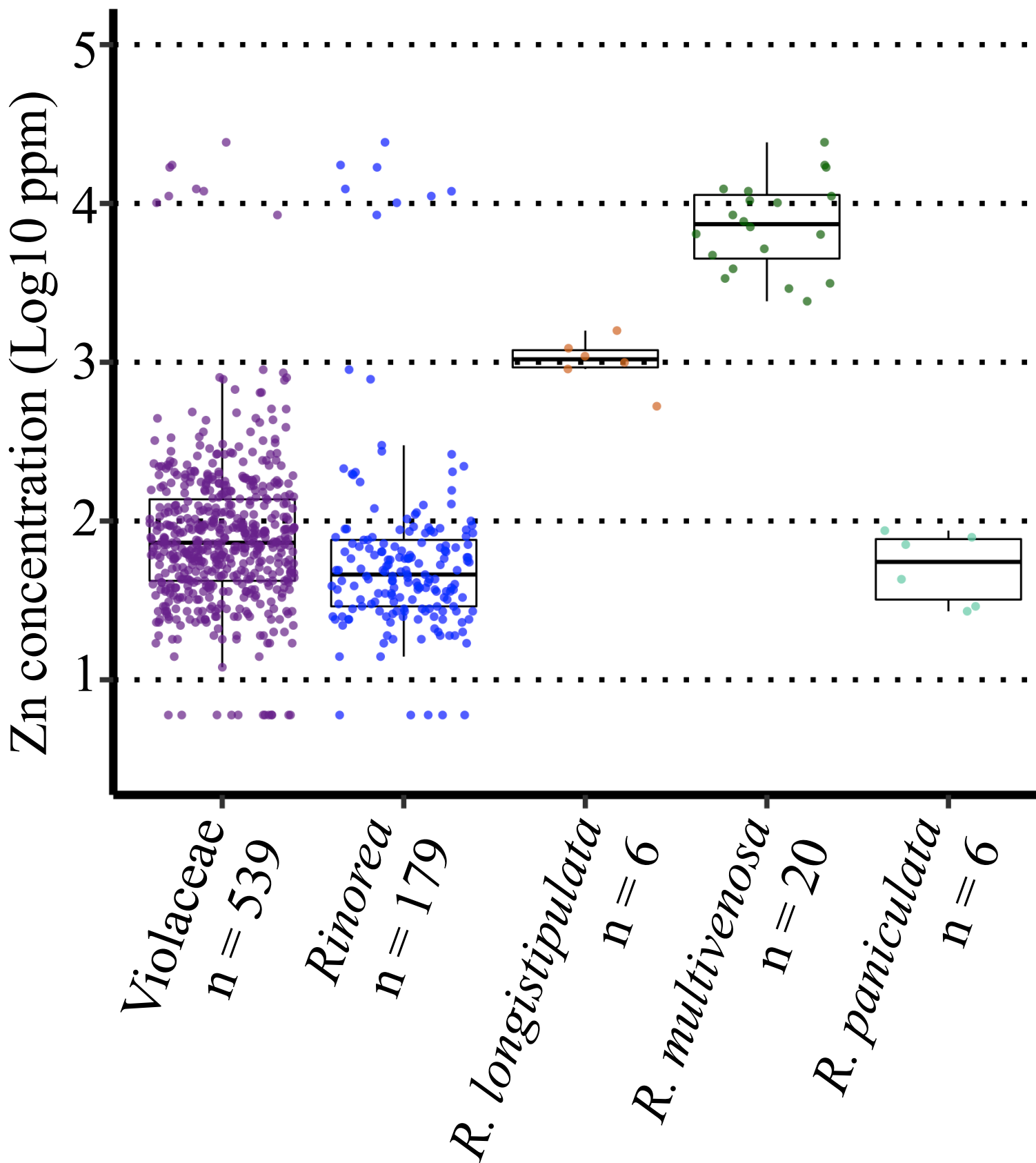


Fig. 5

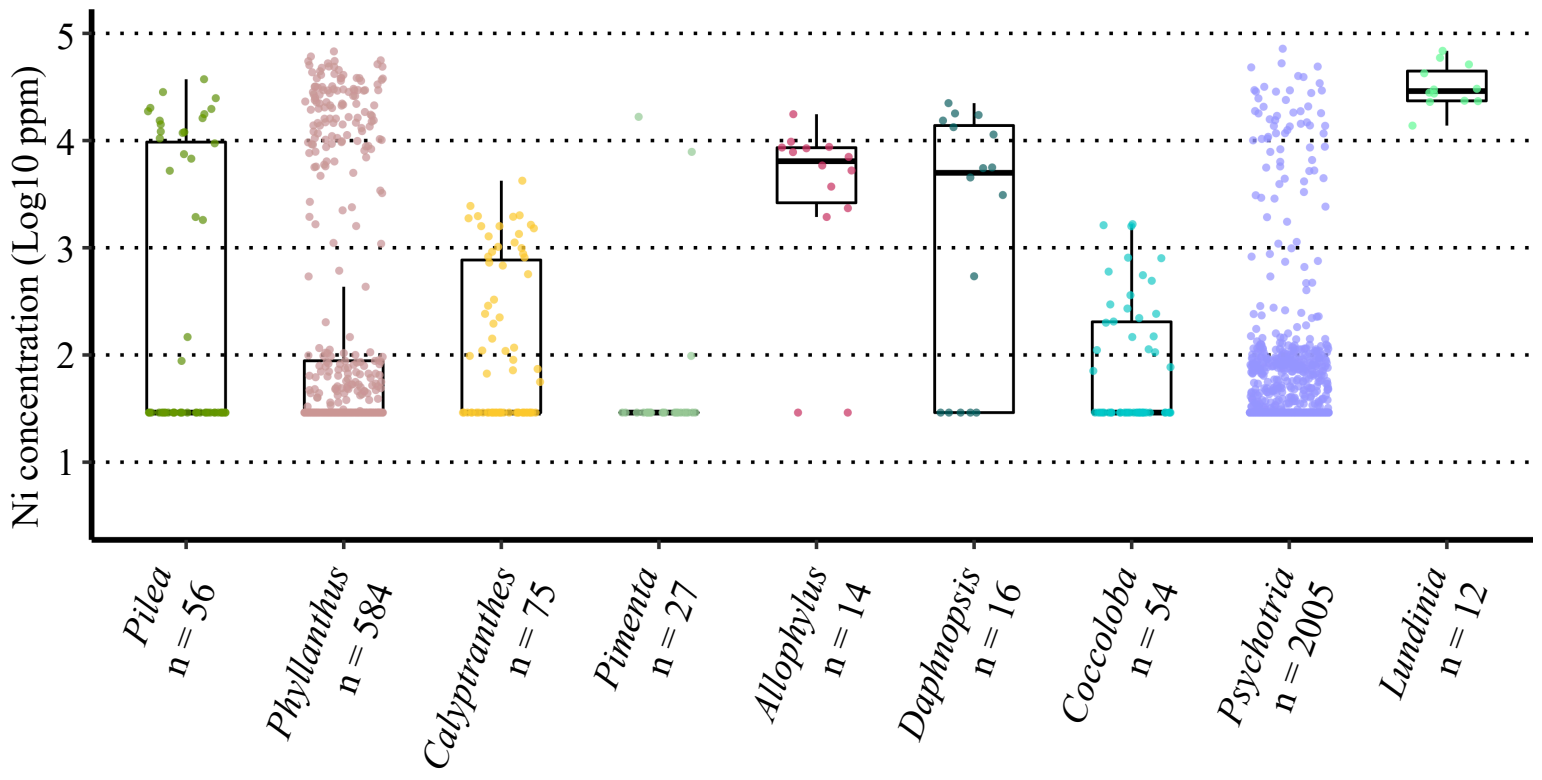


Fig. 6

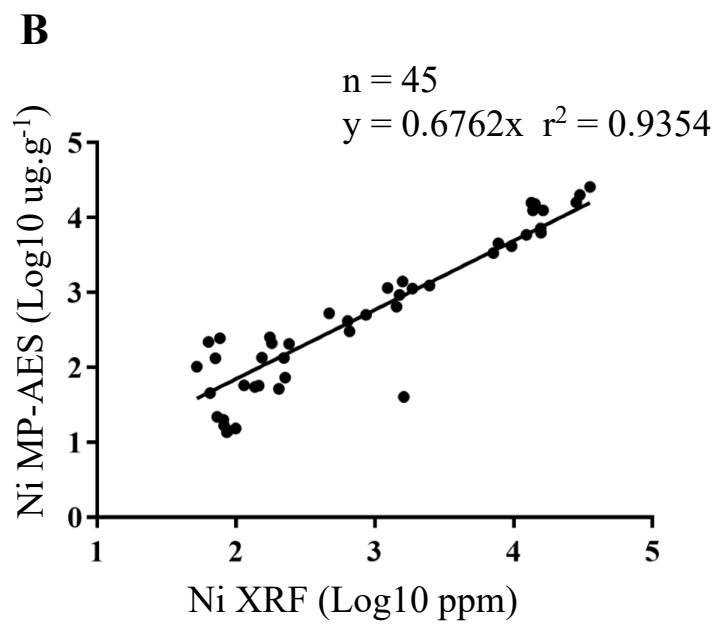
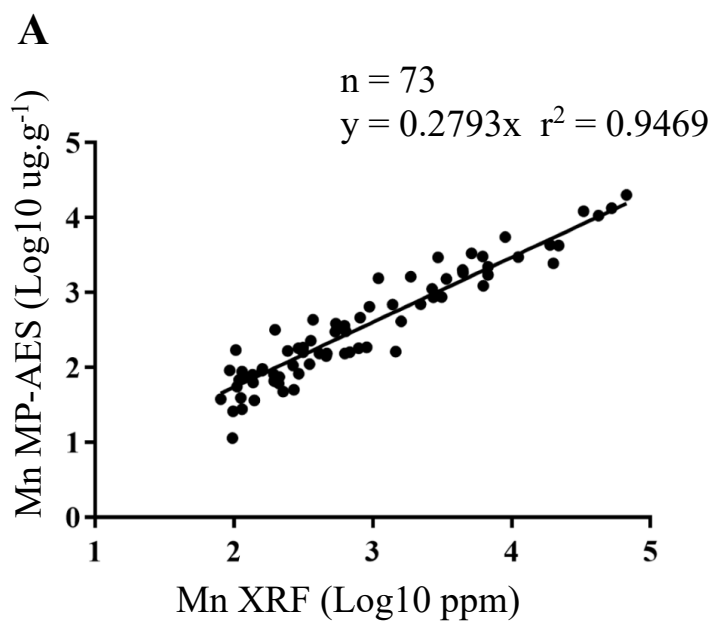


Fig. S1

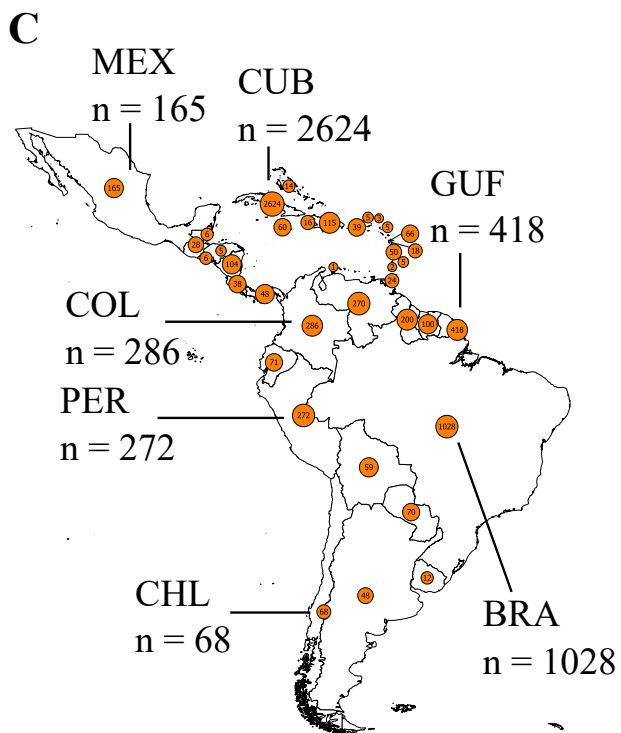
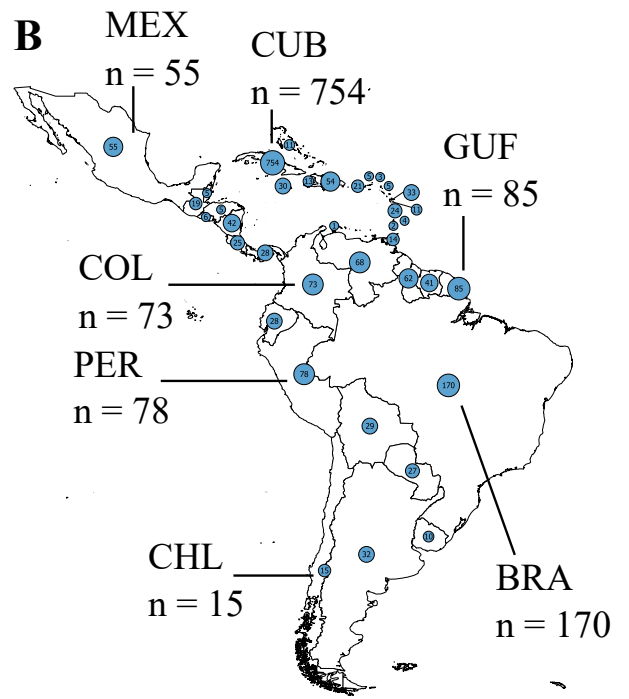
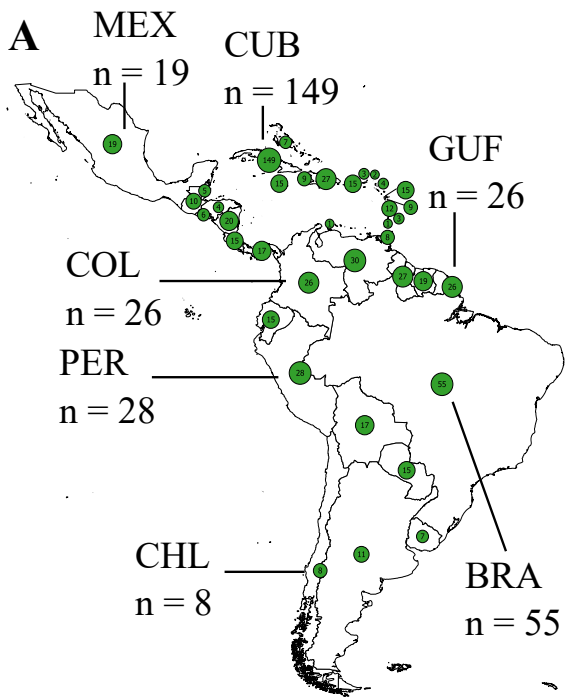


Fig. S2