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Herbarium X-ray fluorescence screening for nickel, cobalt and manganese hyperaccumulator plants in the flora of Sabah (Malaysia, Borneo Island)

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ABSTRACT

Sabah (Malaysia) on the Island of Borneo has high plant diversity (>8000 species) occurring on a wide range of soils, including ultramafic soils which are known to host hyperaccumulator plants. In this study a new approach ("Herbarium X-ray Fluorescence Ionomics") was used to obtain elemental data from herbarium specimens using non-destructive X-ray Fluorescence spectroscopy analysis. In total ~7300 specimens were thus analysed for nickel, cobalt, and manganese concentrations at the Herbarium of the Forest Research Centre (FRC) in Sepilok, Sabah. The measurements led to recording a total of 759 specimens (originating from 17 families in 30 genera and 74 species) as trace element hyperaccumulators, including 28 nickel hyperaccumulator species (in 10 families, 17 genera), 12 cobalt hyperaccumulator species (in 3 families, 7 genera), and 51 manganese hyperaccumulator species (in 12 families, 24 genera). The outcomes of this research demonstrate that handheld XRF is highly useful approach for hyperaccumulator plant discovery in herbarium collections that has the potential to add vast numbers of hyperaccumulating taxa to the global inventory.

1. Introduction

Some rare plants restricted to ultramafic soils have evolved ecophysiological mechanisms to accumulate trace elements in extreme concentrations and are known as hyperaccumulator plants (Jaffré et al., 1976; Reeves, 2003; van der Ent et al., 2013a). Nominal threshold values for 'hyperaccumulation status' have been established for various elements, including 1000 $\mu g\, g^{-1}$ for Ni, 300 $\mu g\, g^{-1}$ for Co, and 10,000 $\mu g\, g^{-1}$ for Mn (Reeves, 2003; van der Ent et al., 2013a). Hyperaccumulators can be either 'facultative' or 'obligate' hyperaccumulator plants (Pollard et al., 2014). Obligate hyperaccumulating taxa are exclusively found on ultramafic soils where they consistently hyperaccumulate, whereas facultative hyperaccumulator taxa can also grow on non-ultramafic soils where they do not hyperaccumulate (Pollard et al., 2014; van der Ent et al., 2013a). To date >700 hyperaccumulator species have been reported globally of which ~70% are Ni hyperaccumulators (Reeves et al., 2018). Hyperaccumulators with >1 w% (*i.e.* 10,000 $\mu g\, g^{-1}$ Ni) are also called 'hypernickelophores' and approximately 50 such taxa are presently known (Jaffré and Schmid, 1974; Reeves et al., 2018). Global hotspots for hyperaccumulator plants include Brazil, Cuba, and New Caledonia (Reeves et al., 1999, 2007; Isnard et al., 2016).

North Borneo (principally the Malaysian state of Sabah) is one of the top five biodiversity centres in the world (Barthlott et al., 2007) and the influence of ultramafic soils on biodiversity in this region have been studied extensively in recent years (van der Ent et al., 2015b, van der Ent et al., 2018a, 2018b). Plant adaptation to ultramafic soils represents a valuable experiment system for evolutionary ecologists and geneticists (Brady et al., 2005). Ultramafic soils contain toxic concentrations of some elements (*e.g.* Mg and Ni) and a lack of mineral nutrients (*e.g.* P and K), resulting in edaphic stresses for plants (Proctor, 2003; Galey et al., 2017). Ultramafic outcrops are relatively widespread and cover 4.6% (3500 km^2) of the total land area of Sabah (Repin, 1998; van der Ent et al., 2013b, 2015b). In Sabah, 33 plant species are currently known as Ni hyperaccumulators, seven species as

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Co hyperaccumulators, and 14 species as Mn hyperaccumulators (van der Ent et al., 2015a, 2015b, 2016a, 2018d; Nkrumah et al., 2018).

Globally the discovery of hyperaccumulator plants has been hindered by the lack of systematic screening of plant species and is highly biased towards particular regions (Reeves, 2003; van der Ent et al., 2013a). The greatest numbers of Ni hyperaccumulator plants are from Malpighiales order (Jaffré et al., 2013; Reeves et al., 2018). The Malpighiales is one the most diverse groups of flowering plants, comprising about 8% of all eudicots and 6% of all angiosperms and has approximately 40 families containing over 16,000 species (Davis et al., 2005; Korotkova et al., 2009; Wurdack and Davis, 2009). The Malpighiales has its greatest diversity in the Neo- and Old-World Tropics (Berry et al., 2016), and contains the highest proportion of known of Ni hyperaccumulator species in Cuba and New Caledonia (Reeves, 2003; Jaffré et al., 2013).

The value of herbaria as references for taxonomic and biogeographical information on floras is widely established, but herbaria may also supply information on the elemental profile in stored specimens (van der Ent et al., 2016a, 2016b, 2016c; Nkrumah et al., 2018a). This is not a new concept, and the use of herbarium specimens for discovering hyperaccumulator plants goes back to the 1970s, and tropical hyperaccumulators plants were studied first by Brooks and co-workers from the mid-1970s (Brooks et al., 1977; Jaffré et al., 1979; Kersten et al., 1979). Until the 1980's, testing for hyperaccumulation used mainly atomic absorption spectrophotometry (AAS), which can only analyse one element at a time, which limited discovery of the range of possible hyperaccumulated elements (*i.e.* frequently only Ni was analysed). Nowadays, trace elements in plant samples can be analysed using techniques that capture quantitative data from a suite of different elements simultaneously, such as inductively coupled plasma atomic emission spectrometry (ICP-AES) and inductively-coupled plasma mass spectrometry (ICP-MS). For example, fragments of herbarium specimens have been analysed with ICP-AES for suites of elements in the flora of the DR Congo (Yang et al., 1985), selected families of plants in New Caledonia, and more recently in search of Mn hyperaccumulators in selected lineages in Australia (Fernando et al., 2009). However, as small fragments (typically 20–30 mg) of herbarium material needs to be excised for the analysis, this is a destructive method of analysis, which is ultimately not compatible with preserving herbarium specimens for prosperity. Moreover, plant material acid digestion (or ashing) followed by ICP-AES analysis is relatively expensive and time-consuming. In contrast, X-ray fluorescence spectrometry (XRF) is a non-destructive technique to quantitatively determine multi-elemental concentrations within a sample. Handheld XRF devices have in-built X-ray source (miniature tube) that produces an incident beam that causes a sample to emit a spectrum of fluorescent X-rays which can be interrogated to determine the concentrations of elements present. In order to obtain reliable quantitative data, the XRF instrument has to be appropriately calibrated (Markowicz, 2008), which is particularly important for non-standard matrix samples such as herbarium material (Gei et al., 2018). The non-destructive nature and short duration of the measurements (*e.g.* 30 s), makes it possible to perform elemental analysis on a vast number of stored herbarium specimens (Gei et al., 2018). This approach has been termed "Herbarium X-ray Fluorescence Ionomics" and enables to obtain elemental information from entire phylogenetic lineages, and as such is expected to be transformative for the discovery of hyperaccumulator plants (van der Ent et al., 2019).

The flora of Sabah was already known to be rich in hyperaccumulator plant species (van der Ent et al., 2015a, 2015b, 2016a, 2016b, 2016c, 2018d), but these discoveries originated from field-collected material during ecological investigations. It was envisaged that the flora of Sabah would harbour numerous more hyperaccumulator species hitherto unknown, that could be discovered through analysis of herbarium specimens. Therefore, in this study ~7300 herbarium specimens were measured with handheld XRF for Ni, Co and Mn concentrations at the Forest Research Centre (FRC) Herbarium at Sepilok in Sabah, Malaysia. The aim was to prospect for novel hyperaccumulator plants to contribute to a broader understanding of the phylogenetic incidence of hyperaccumulation, and to allow for the identification of species potentially suitable for utilization in phyto-technologies, such as agromining (*e.g.* the use of hyperaccumulator plants to obtain Ni from ultramafic soils).

2. Materials and methods

2.1. Selection of herbarium specimens for the XRF scanning

Given that the FRC Herbarium contains ~350,000 specimens (~8000 species or 10,000 taxa) from the state of Sabah, a selection had to be made. All specimens in the Malpighiales families Phyllanthaceae, Salicaceae and Violaceae (totalling 5592 specimens) were selected. The selection further contained the genus *Walsura* (Meliaceae – 98 specimens), the genus *Mischocarpus* (Sapindaceae – 81 specimens), and the family Sapotaceae (all genera – 383 specimens). This selection of specimens was based on previous studies on hyperaccumulator plants in Sabah (van der Ent et al., 2015a, 2015b, 2016a, 2016b, 2016c, 2018d). Furthermore, single specimens from all species (1183 specimens) known to occur on ultramafic soils in Sabah were selected to provide a wide phylogenetic assay of the incidence of hyperaccumulation. In total, this resulted in 7337 specimens that were measured.

2.2. XRF calibration for measurement of herbarium specimens

The Thermo Fisher Scientific Niton XL3t 950 GOLDD + analyser uses a miniaturised X-ray tube (Ag anode, 6–50 kV, 0–200 μA max) as its excitation source. The X-ray tube irradiates the sample with high-energy X-rays which excite (characteristic) fluorescent X-rays in the sample. These fluorescent X-rays are detected and quantified with a large 20 mm^2 Silicon Drift Detector (SDD, 185 eV, up to 60,000 counts per second). It can detect elements from Mg to U within 15–60 s with absolute detection limits of 50–100 $\mu g\, g^{-1}$ (but typically $> 300\, \mu g\, g^{-1}$ depending on the element in real-life samples). The instrument incorporates Compton Normalisation; appropriate for the relatively low elemental concentrations found in plant material in a low-density (*i.e.* cellulose) matrix.

A total of 590 dried plant samples were used from earlier ecological studies in Sabah (van der Ent et al., 2015b; van der Ent et al., 2016b). These plant samples covered concentration ranges ranging from 'normal' to abnormal (*i.e.* hyperaccumulation) and includes known Ni, Co and Mn hyperaccumulator samples. From each sample, 6 mm diameter leaf discs were extracted using a paper punch. The XRF analysis was undertaken on a sheet of 'herbarium cardboard' on a pure titanium plate (~99.995%, 2 mm thick × 10 × 10 cm) to provide a uniform background and block transmitted X-rays. The XRF analysis used the 'Soils Mode' in the 'Main filter' configuration for 60s duration. After scanning, the leaf samples were weighed and pre-digested in 1 mL 70% HNO_3. The samples were subsequently digested at 125 °C for 2 h and diluted to 30 mL with ultra-pure water before analysis with ICP-AES (Varian Visa Pro II), which included measurements of the following elements: Ni, Co, Cr, Cu, Zn, Mn, Fe, Mg, Ca. The quality control included the NIST 1515 ('Apple Leaves') standard and blanks.

2.3. Herbarium specimen and sample contamination

Each herbarium specimen was measured for 30 s in 'Soil Mode' with the 'Main filter' and the raw XRF data was corrected using the regression formulas obtained from the empirical, calibration. The pure titanium plate was located under the herbarium specimens to provide a uniform background and to block penetrating X-rays. Consistently, old/mature leaves (*i.e.* the most basal leaves on a specimen) were measured, and care was taken to fully cover the 6 mm measurement area of the XRF.

The potential for contamination of leaves with soil particulates presents a problem for the accurate determination of elemental concentrations in plant material (Gei et al., 2017). This risk is highest for ground-herbs, and lesser for tall trees, but cannot be entirely avoided. Concomitant apparent high foliar concentrations of Cr (> 60 μg g^{-1}) and Fe (> 2500 μg g^{-1}) are useful indicators for soil contamination, as these elements are major constituents of ultramafic soils. Any suspect samples with anomalously high Cr and Fe were excluded from the dataset. Another possible problem is the practise of some tropical collectors to use methylated spirit for temporarily preserving sample collections to limit decomposition of the specimens during transport, which can potentially 'wash out' soluble elements from the plant material. These specimens are typically readily identified by their characteristic de-colouration ('yellowing'), and were treated with caution in the dataset. Note that affected specimens would have lower apparent Ni, Co, Mn concentrations, and hence would not lead to 'false-positives' for detecting hyperaccumulator plants.

Even though the handheld XRF instrument can accurately measure Zn concentrations, and Zn has been measured previously in herbarium specimens (Gei et al., 2017; Nkrumah et al., 2018a, 2018b), Zn data has been excluded from this dataset because some specimens at the FRC Herbarium have been glued to the cardboard sheets with a glue that has high concentrations of Zn (> 1 wt%). The type of glue that is Zn-rich was used mainly on older specimens (pre-1980s), but the identity of specimens that were affected could not reliably be established.

2.4. Statistical analyses

The apparent limits of detection (LOD) for Ni, Co and were estimated by visual inspection of the log-transformed linear regression models of the XRF data against corresponding ICP-AES measurements and set at: < 190 μg g^{-1} for Ni, < 140 μg g^{-1} for Co and < 336 μg g^{-1} for Mn. The residuals *vs.* fitted values were inspected for each linear regression analysis, and outliers (± 3 SD of the residual) were identified and removed. Secondary linear regression models were then derived after the samples with XRF values < LOD were removed. Herbarium specimens with XRF values < LOD were excluded from the dataset, and of the total 7337 specimens that were measured, 6775 specimens remained in the final dataset. The XRF-ICP-AES correction factors were then applied to all of the raw XRF measurements obtained from the herbarium specimens.

The data were analysed using Xlstat (Microsoft professional 2016) and comprised of computing range and means, and graphing plots. The phylogenetic data was analysed using the packages "ape" and "phylo4d" in R and the software PHYLOGENETIC (Phylomatic tree R20120829 Plants).

3. Results

3.1. General statistics and phylogenetic patterns

Overall, 6775 specimens (representing 119 different families) originating from ultramafic and non-ultramafic soils were analysed by XRF for Ni, Co and Mn and this yielded 759 specimens (in 17 families, 30 genera and 74 species) as trace element hyperaccumulators. This includes 28 Ni hyperaccumulator species (in 10 families, 17 genera), 12 Co hyperaccumulator species (in 3 families, 7 genera), and 51 Mn hyperaccumulator species (in 12 families, 24 genera). Some specimens hyperaccumulated a combination of Ni, and/or Co and/or Mn simultaneously. All specimens hyperaccumulating Ni and Co originated from ultramafic areas, whereas Mn hyperaccumulators are widespread and very few specimens originated from known ultramafic areas. Hyperaccumulators of Ni, Co, and Mn in the Malpighiales (Table 1) and for other clades (Table 2), and hyperaccumulation records are broken down into families and genera. The Phyllanthaceae had the greatest number of hyperaccumulators among the families tested, with nine genera containing hyperaccumulators and four genera containing hyperaccumulators of all three elements. The second highest number of hyperaccumulating genera was the Salicaceae, with four genera, but only Ni and Mn were hyperaccumulated in this family. This study examined the accumulation patterns of Ni, Co and Mn in 24 families, 78 genera and 351 species from the Malpighiales, and a phylogenetic tree was constructed based on these results (Fig. 1).

3.2. Nickel hyperaccumulation

In total, 271 specimens had Ni concentrations greater than the LOD (< 190 μg g^{-1}), of those 163 specimens exceeded hyperaccumulation threshold (> 1000 μg g^{-1}), with the highest value recorded in *Dichapetalum gelonioides* with 45,600 μg g^{-1} Ni. As such, only 4% of the specimens, had detectable Ni. The incidence of Ni hyperaccumulation is shown in Fig. 3. A total of 10 families with 17 genera and 28 species were identified as Ni hyperaccumulators. Nickel hyperaccumulation was recorded in unrelated families, such as the Achariaceae (*Hydnocarpus* – 2 spp.), Annonaceae (*Marsypopetalum* – 1 sp.), Dipterocarpaceae (*Shorea* – 1 sp.), Fabaceae (*Dalbergia* – 1 sp.), Loganiaceae (*Strychnos* – 1 sp.) and Meliaceae (*Walsura* – 2 spp.), but is most frequent in the Phyllanthaceae (genera: *Actephila*, *Antidesma*, *Aporusa*, *Ashtonia*, *Baccaurea*, *Cleistanthus Glochidion*, *Phyllanthus*). Particularly interesting new records include an unidentified species of *Hydnocarpus* (Achariaceae) with up to 3200 μg g^{-1} Ni, *Marsypopetalum pallidum* (Annonaceae) with 6000 μg g^{-1} Ni. The rare dipterocarp *Shorea tenuiramulosa* first reported by Proctor et al. (1989) from Mt. Silam on Sabah's east coast as a Ni hyperaccumulator was not detected in this survey, but it is a weak Ni hyperaccumulator with up to 1790 μg g^{-1} Ni (van der Ent and Mulligan, 2015). Values for *Dichapetalum gelonioides* (Dichapetalaceae) are extremely wide-ranging (340–45,600 μg g^{-1} Ni), which may be explained by the omission of infraspecific ranks in this study. The various *Dichapetalum* subspecies differ greatly in their hyperaccumulation characteristics, for example subsp. *sumatranum* and *pilosum* do not accumulate Ni, but Zn (Nkrumah et al., 2018b).

Some known Ni hyperaccumulator plants species, such as *Kibara coriacea* (Monimiaceae) with up to 5840 μg g^{-1} Ni (van der Ent and Mulligan, 2015) and *Actephila alanbakeri* (Phyllanthaceae) with up to 14,700 μg g^{-1} Ni (van der Ent et al., 2016a, 2016b, 2016c) were not detected because the FRC Herbarium does not hold any specimens of these taxa. This survey added another species of *Actephila*, *A. excelsa*, as a strong Ni hyperaccumulator with 5200–14,600 μg g^{-1} Ni. Another such instance is the known Ni hyperaccumulator *Walsura pinnata* (Meliaceae) which can accumulate up to 8000 μg g^{-1} Ni, with the new recording *W. pachycaulon* with 690–8600 μg g^{-1} Ni.

Antidesma montis-silam with 4600–32,700 μg g^{-1} Ni is a rare taxon principally known from Mt. Silam on Sabah's east coast, which has been the subject of a recent field-based study (Nkrumah et al. 2017). Other Ni hyperaccumulating species in this genus include *A. neurocarpum* (up to 21,500 μg g^{-1} Ni), *A. riparium* (up to 2900 μg g^{-1} Ni) and *A. puncticulatum* (up to 3500 μg g^{-1} Ni). The genus *Aporosa* also yielded several Ni hyperaccumulators, including *A. lucida* with up to 10,400 μg g^{-1} Ni (and up to 980 μg g^{-1} Co), all expect *A. chalarocarpa* were unknown previously. Numerous Ni hyperaccumulating species of *Glochidion* all of which were previously recorded, were also detected (see Suppl. Info for details). Note that several Glochidion taxa from Mount Kinabalu Park (G. *cf. mindorense*, *G. cf. sericeum*, *G.* "*panatanran*", *G.* "*bambangan*", *G.* "*Nalumad*"). There are at the Sabah Parks

Table 1
Co, Mn and Ni hyperaccumulator genera from the Malpighiales. The number of specimens, ranges and means above the hyperaccumulator threshold are shown. "–" is below the element-specific hyperaccumulation threshold (> 300 µg g^{-1} Co, > 1000 µg g^{-1} Ni, > 10,000 µg g^{-1} Mn).

Order	Family	Genera	Number of specimens/number of taxa[a]	Range of foliar concentrations (µg g^{-1})			Mean of foliar concentrations (µg g^{-1})[b]			Total number of hyperaccumulator species[c]		
				Co	Mn	Ni	Co	Mn	Ni	Co	Mn	Ni
Malpighiales	Achariaceae	*Hydnocarpus*	18/10	–	–	3000–3200	–	–	3100 (3)	–	–	[3]
	Dichapetalaceae	*Dichapetalum*	98/5 [2]	–	–	1000–45,600	–	–	15,900 (20)	–	–	1
	Phyllanthaceae	*Actephila*	23/2 [5]	–	–	2600–14,600	–	12,800 (1)	7500 (3)	–	[1]	1
		Antidesma	842/23 [98]	–	10,100–46,500	1000–32,700	560 (1)	14,600 (25)	9500 (27) (26)	1	8 [2]	5 [2]
		Aporosa	855/34 [74]	500–980	10,300–26,700	4000–10,400	740 (2)	14,300 (24)	6500 (3)	1 [1]	6 [1]	2 [1]
		Ashtonia	11/1	–	–	–	1500 (1)	–	8100 (1)	1	–	1
		Baccaurea	804/29 [44]	320–960	10,200–21,700	1000–8000	690 (4)	14,000 (7)	4400 (7)	3	5	3
		Cleistanthus	376/31 [1]	–	10,400–11,300	–	–	10,700 (5)	–	–	3	–
		Glochidion	644/30 [126]	590–780	10,200–12,600	1900–11,600	690 (2)	11,200 (3)	7100 (13)	1 [1]	3	1 [12]
	Salicaceae	*Casearia*	226/12 [47]	–	10,100–38,200	–	–	14,200 (8)	–	–	5 [3]	–
		Homalium	59/3 [17]	–	–	1400–5700	–	–	2700 (4)	–	–	[4]
		Scolopia	67/3 [13]	–	14,800–16,600	1200–9200	–	15,700 (2)	4700 (8)	–	[2]	[8]
		Xylosma	45/3 [13]	–	13,600–15,400	1300–23,000	–	14,500 (2)	7800 (16)	–	[2]	[16]
	Violaceae	*Rinorea*	188/12 [9]	300–620	–	1100–32,200	470 (4)	13,400 (1)	8500 (40)	1	1	1 [1]

a Number of specimens from unidentified species from this study between brackets.
b Number of hyperaccumulator specimens from this study between parentheses.
c number of hyperaccumulator specimens from unidentified species from this study between brackets.

Herbarium (SNP) and could not be matched with specimen sheets the FRC Herbarium, likely because there are yet undescribed taxa endemic to Mount Kinabalu. The genus *Glochidion*, which consists of > 300 species mainly distributed in the Indo-Pacific region, is in desperate need of a modern revision for Borneo, with regional herbaria typically housing thousands of unidentified *Glochidion* specimens. The FRC Herbarium only has specimens of six *Phyllanthus* taxa: *P. lamprophyllus*, *P. balgooyi* (synonym *P. hellwigii*), *P. kinabaluicus*, *P. rufuschaneyi* (formerly *P. cf. securinegioides* and identified as *P. gracilipes*), *P. urinaria* and *P. amarus*. The latter two a pan-tropical weeds, whereas *P. lamprophyllus* is a common species along rivers in Sabah. The other two taxa are hypernickelophores, whereas *P. kinabaluicus* is a hyper-endemic to Mount Kinabalu (and a Ni accumulator with 700 µg g^{-1} Ni, unpublished data).

In the large genus *Psychotria* (Rubiaceae) three taxa (*P. densifolia*, *P. gracilis*, *P. sarmentosa*) hyperaccumulate Ni, up to 24,500 µg g^{-1}, but it is uncertain whether these taxa are actually distinct given the taxonomical uncertainties in this genus. The large family Sapindaceae, with the well-known fruits *Nephelium lappaceum* (Rambutan) and *Dimocarpus longan* (Longan), only yielded one Ni hyperaccumulator in *Mischocarpus sundaicus*, which was previously known and can accumulate up to 4420 µg g^{-1} Ni (van der Ent et al. 2015f), although lower values up to 2400 µg g^{-1} Ni were recorded with XRF analysis. The Violaceae (genus: *Rinorea*) has two Ni hyperaccumulating taxa (*R. bengalensis*, *R. javanica*) which were previously known and again detected in this study. They can accumulate up to 22,200 µg g^{-1} and 9680 µg g^{-1} Ni respectively (van der Ent et al., 2017a). The Salicaceae has four genera (*Flacourtia*, *Homalium*, *Scolopia*, *Xylosma*) with Ni hyperaccumulators. Of these two unidentified *Scolopia* spp. (likely *S. luzonensis*) accumulates up to 9200 µg g^{-1} Ni, whereas *S. spinosa* accumulates 1240 µg g^{-1} Ni.

The majority of Ni hyperaccumulators are 'obligate' (*e.g.* hyperaccumulate Ni consistently for all specimens), for example *Phyllanthus rufuschaneyi* with 25 samples ranging from 2196 to 23,300 µg g^{-1} Ni (van der Ent et al. 2015f) or high but variable (but with some specimens below the Ni hyperaccumulation threshold *i.e.* > 1000 µg g^{-1} Ni) such as *Rinorea bengalensis* with 45 specimens ranging from 250 to 32,200 (with a mean of 7400 µg g^{-1} Ni), or *Walsura pinnata* with 22 specimens 290–8000 ranging from 290 to 8000 (with a mean of 2300 µg g^{-1} Ni). Only a few Ni hyperaccumulators are probably truly 'facultative', such as *Antidesma neurocarpum* with 196 specimens ranging from 370 to 21,500 µg g^{-1} Ni (with a mean of 2900 µg g^{-1} Ni). However, for most other Ni hyperaccumulating species not enough specimens (most species were represented by < 5 specimens) were available to draw any definitive conclusions about obligate or facultative hyperaccumulation status.

The frequency distribution of Ni concentrations in the herbarium specimens (Fig. 2) is distinct from Co and Mn (see further below). There are two separate modes on ultramafic and non-ultramafic soils (confirmed by statistical tests shown in Table 3). On non-ultramafic soils the distribution of Ni follows a pseudo 'log-normal' distribution with a geometric mean of ~320 µg g^{-1}. On ultramafic soils, two groups exist, the first follows a log-normal distribution with a geometric mean of ~500 µg g^{-1} (*e.g.* below the Ni hyperaccumulation threshold), whereas the second group also follows a log-normal distribution and has a geometric mean of ~10,000 µg g^{-1} (*e.g.* 'hypernickelophores'). The two groups are clearly separated at ~1000 µg g^{-1} which is the

Table 2
Co, Mn and Ni hyperaccumulator genera from Orders (excluding Malpighiales). With the number of specimens and foliar ranges above the hyperaccumulator threshold. "–" is below the element-specific hyperaccumulation threshold (> 300 μg g^{-1} Co, > 1000 μg g^{-1} Ni, > 10,000 μg g^{-1} Mn).

Clades	Order	Family	Genera	Number of specimens/number of taxa	Range foliar concentrations (μg g^{-1})			Mean foliar concentrations (μg g^{-1})[a]			Number of hyperaccumulator species[b]		
					Co	Mn	Ni	Co	Mn	Ni	Co	Mn	Ni
Asterids	Ericales	Ebenaceae	*Diospyros*	27/26	–	–	–	–	19,700 (1)	–	–	1	–
	Gentianales	Rubiaceae	*Psychotria*	7/7	–	–	–	–	–	20,700 (1)	–	–	1
Rosids	Fagales	Fagaceae	*Castanopsis*	3/3	–	–	–	–	18,200 (1)	–	–	1	–
	Magnoliales	Annonaceae	*Marsypopetalum*	1/1	–	–	–	–	–	6000 (1)	–	–	1
	Myrtales	Myrtaceae	*Syzygium*	33/33	–	–	–	–	28,800 (1)	–	–	1	–
	Sapindales	Meliaceae	*Walsura*	101/4	300–720	10,200–45,200	1300–8600	400 (9)	17,100 (23)	4500 (12)	1 [5]	1 [9]	1 [1]
		Sapindaceae	*Mischocarpus*	76/3	–	10,100–19,400	1200–2400	–	13,800 (6)	1900 (3)	–	1	1

[a] Number of hyperaccumulator specimens from this study between parentheses.
[b] Number of hyperaccumulator specimens from unidentified species from this study between brackets.

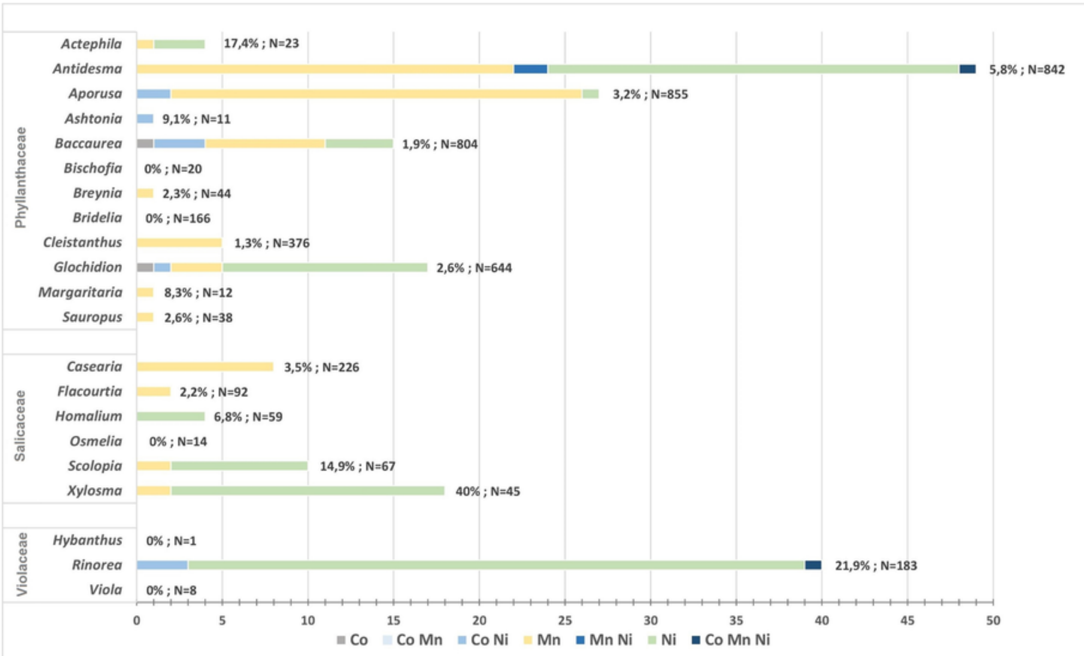


Fig. 1. Bar graphs showing a breakdown of combinations of Ni, Co and Mn hyperaccumulation in the Phyllanthaceae, Salicaceae and Violaceae genera. Shown are the number of specimens and percentages and non-hyperaccumulator number of specimens measured by XRF.

recognised threshold to define Ni hyperaccumulation. Statistical tests reveal that Ni concentrations in specimens from ultramafic and non-ultramafic soils are significantly different (Table 3). Only the genus *Baccaurea* (Phyllanthaceae) and the family Salicaceae show no significant difference between non-ultramafic and ultramafic soils.

3.3. Cobalt hyperaccumulation

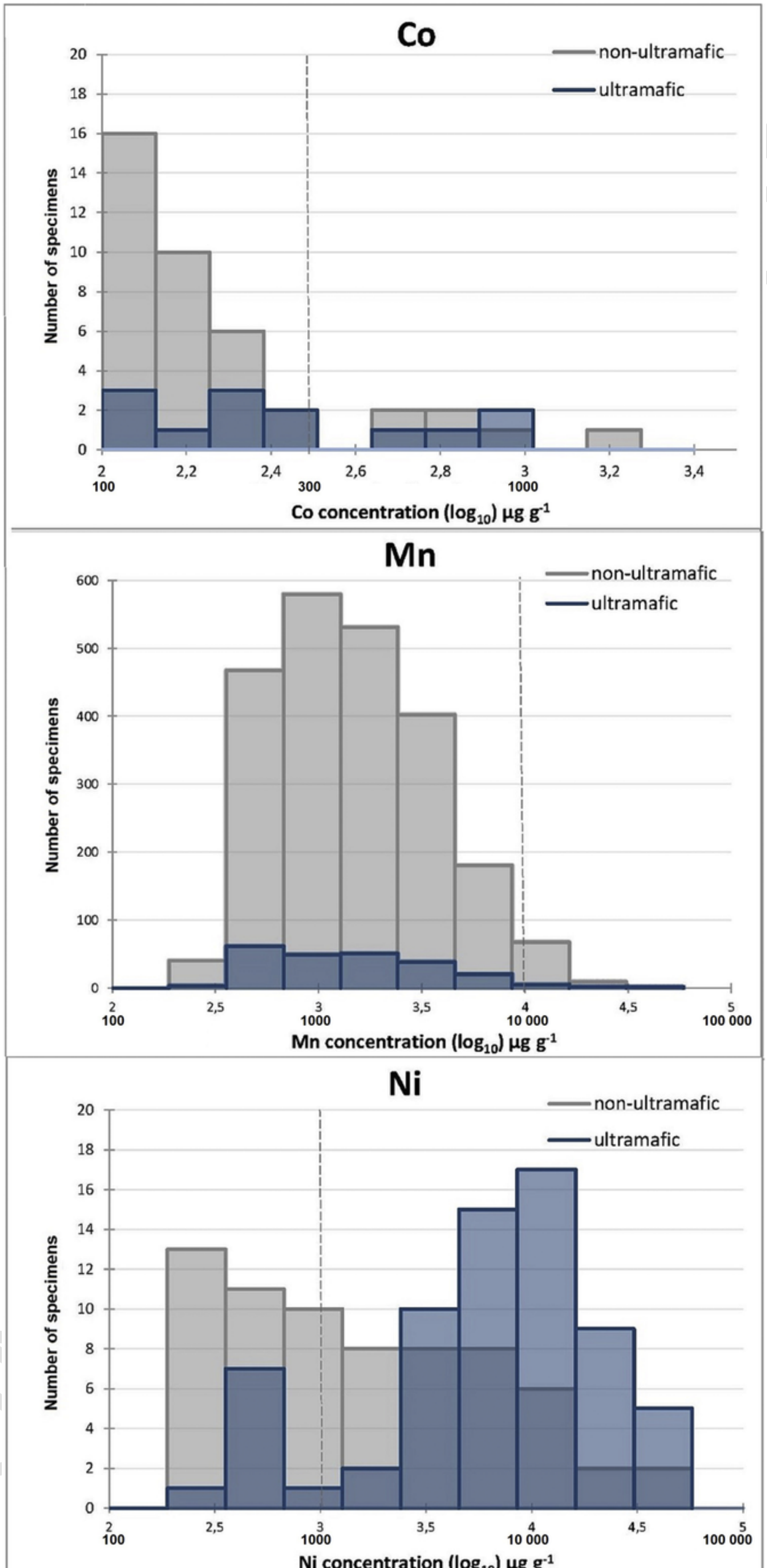
In total, 107 specimens had Co concentrations greater than the LOD (< 140 μg g^{-1}), of those 23 specimens exceeded the hyperaccumulation threshold (> 300 μg g^{-1}), with the highest value recorded in *Ashtonia excelsa* with 1500 μg g^{-1} Co. The frequency distribution shows that only a very limited number of specimens are clearly separated based on their concentrations (Fig. 2). There were only three families, nine genera and nine species which had Co concentrations > 300 μg g^{-1} (Tables 1 and 2). The Phyllanthaceae family emerged as the main Co (hyper)accumulator group. The known Co hyperaccumulator *Glochidion cf. sericeum* was not detected in this study, but that can be explained because the FRC Herbarium does not hold any specimens of this taxon, since they are deposited at the Sabah Parks Herbarium (van der Ent et al., 2018c). In the family Phyllanthaceae, samples originating from ultramafic soils tended to have higher foliar Co, notably in the genus *Baccaurea* (Table 3).

3.4. Manganese hyperaccumulation

In total 4218 specimens had Mn concentrations greater than the LOD (< 336 μg g^{-1}), of those 123 specimens exceeded the hyperaccumulation threshold (> 10,000 μg g^{-1}), with the highest value recorded in *Antidesma puncticulatum* with 46,480 μg g^{-1} Mn. As such, about one third of the specimens measured, originating from 46 families, had detectable Mn. Although hyperaccumulation of Mn is relatively rare, concentrations exceeding 550 μg g^{-1} are frequent in the flora of Sabah. A total of 12 families, 24 genera and 51 species were Mn hyperaccumulators (Tables 1 & 2). The Phyllanthaceae had the greatest numbers of Mn hyperaccumulators with six genera and 27 species. Manganese hyperaccumulation is clearly phylogenetically scattered, and in most instances just a single or a few specimens of a species were found with inordinately high Mn values. For example, *Diospyros singapurensis* (Ebenaceae) with 19,700 μg g^{-1} Mn, *Chrozophora oblongifolia* (Euphorbiaceae) with 11,600 μg g^{-1} Mn, *Gymnacranthera* sp. (Myristicaceae) with 10,200 μg g^{-1} Mn, and *Syzygium fastigiatum* (Myrtaceae) with up to 28,800 μg g^{-1} Mn. Such instances may be explained by local variations in soil Mn availability in which the particular specimen grew. In other cases, Mn hyperaccumulation is a more consistent trait with numerous specimens of the same species having extremely high values. Some of these are especially strong Mn hyperaccumulators, such as *Casearia tuberculata* with up to 38,200 μg g^{-1} Mn, *Baccaurea sumatrana* with up to 21,700 μg g^{-1} Mn, *Neoscortechinia philippinensis* (Euphorbiaceae) with three specimens with 3600–11,800 μg g^{-1} Mn, and *Aporosa illustris* with up to 26,600 μg g^{-1} Mn. The genus *Antidesma* is noteworthy for the numbers of Mn hyperaccumulating specimens, including *A. neurocarpum* with 192 specimens ranging from 370 to 13,900 μg g^{-1} Mn (mean of 2830 μg g^{-1} Mn), and *A. tomentosum* with 112 specimens ranging from 390 to 17,400 μg g^{-1} Mn (mean of 3190 μg g^{-1} Mn). The genus *Walsura* in the Meliaceae family is of similar interest because three taxa (*W. decipiens*, *W. pachycaulon*, *W. pinnata*) are strong Mn hyperaccumulators with up to 21,000 μg g^{-1}, Mn 45,200 μg g^{-1} Mn and 30,700 μg g^{-1} Mn respectively.

In all species, however, the mean Mn value does not exceed the Mn hyperaccumulation threshold (*i.e.* > 10,000 μg g^{-1} Mn). Nickel and Mn hyperaccumulation in *Antidesma*, focussing on *A. montis-silam*, has been subject of a dedicated study (Nkrumah et al. 2017). Yet other taxa have very wide-ranging Mn values, for example *Mischocarpus pentapetalus* (Sapindaceae) with 51 specimens ranging from 650 to 19,400 μg g^{-1} Mn. Interesting, the related *M. pinnata* is a strong Ni hyperaccumulator. Another example is *Cleistanthus sumatranus* (Phyllanthaceae) with 54 specimens ranging from 350 to 11,300 μg g^{-1} Mn.

The frequency distribution of Mn concentrations in the herbarium specimens (Fig. 2) shows that almost all the specimens fall in a log-normal distribution with a geometric mean of 1000 μg g^{-1}. There does not seem to be an effect of ultramafic soils in the pattern of foliar Mn concentrations. However, statistical differences between specimens from the Violaceae family from ultramafic and non-ultramafic soils were


Co
non-ultramafic
ultramafic
Number of specimens
Co concentration (log10) µg g-1
Mn
non-ultramafic
ultramafic
Number of specimens
Mn concentration (log10) µg g-1
Ni
non-ultramafic
ultramafic
Number of specimens
Ni concentration (log10) µg g-1

◀**Fig. 2.** Frequency distribution of Ni, Co and Mn elemental concentrations in herbarium specimens originating from ultramafic (shaded blue) and non-ultramafic soils (shaded grey). Concentration scales (X-axis) $\log_{10}$ transformed and actual values in μg g^{-1}. Numbers of specimens on Y-axis. Dotted line denotes the element-specific hyperaccumulation threshold (>300 μg g^{-1} Co, >1000 μg g^{-1} Ni, >10,000 μg g^{-1} Mn). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

found ($p < .05$) (Table 3). The Violaceae originating from non-ultramafic soils had Mn concentrations higher than the Violaceae originating from ultramafic soils ($p < .01$). Also, the Phyllanthaceae had more Mn hyperaccumulator species growing on non-ultramafic soils than on ultramafic soils.

3.5. Simultaneous hyperaccumulation of two or more elements

The genus *Baccaurea*, and especially *B. odoratissima* with up to 8000 μg g^{-1} Ni, is of interest because of its ability to also accumulate Co up to 960 μg g^{-1}. *Ashtonia excelsa*, with only one taxon in Borneo, also stands out because it accumulates up to 8100 μg g^{-1} Ni and 1500 μg g^{-1} Co. *Walsura pinnata* (Meliaceae) is another taxon that can hyperaccumulate Ni (up to 8000 μg g$^{-1)}$, Co (up to 4580 μg g^{-1} in van der Ent et al., 2015b) and Mn (up to 30,700 μg g^{-1}). *Rinorea bengalensis* and *R. javanica* incidentally also have high Co values, with up to 630 μg g^{-1}and 670 μg g^{-1} respectively (Brooks et al., 1977).

Hyperaccumulation of Co is relatively rare, mostly because soils with high available Co are infrequent (Lange et al., 2017; van der Ent et al., 2018c, 2018d). In this study, only a few specimens from the same genera in the Phyllanthaceae hyperaccumulated Co alone. Other species identified in this study as Co hyperaccumulators also hyperaccumulated Ni and Mn simultaneously. Furthermore, in most ultramafic soils worldwide, Co correlates with Ni (van der Ent and Mulligan, 2015; Tashakor et al., 2018; van der Ent et al., 2018c, 2018d).

3.6. New hyperaccumulator plant discoveries

The herbarium XRF scanning in this study led to the recording of a total of 28 Ni hyperaccumulator species from 10 families and 17 genera (Suppl. Table 2). Among the Ni hyperaccumulators were 10 species that can be classified as 'hypernickelophores' of which eight were already known prior to this study (Baker et al., 1992; van der Ent et al., 2015b, 2016c; Bouman et al., 2018; Nkrumah et al., 2018a). The 'hypernickelophores' were from the Dichapetalaceae, Phyllanthaceae, Violaceae and Rubiaceae, and the two newly reported hypernickelophores were from the genus *Xylosma* (Salicaceae); *Xylosma luzoniensis* and *X. sumatranum*. Moreover, the recently reported hypernickelophore *Antidesma montis-silam* was discovered through herbarium XRF scanning and plant material was subsequently collected in the field and analysed, confirming the findings from the XRF survey (Nkrumah et al., 2018a). The present study found that the highest Ni concentrations in herbarium specimens in *Dichapetalum gelonioides* subsp. *tuberculatum* with 4.56 Wt% Ni. This taxon was already the species with the highest Ni concentration previously reported with 2.7 wt% (Nkrumah et al., 2018b). *Dichapetalum gelonioides* subsp. *sumatranum* and *D. gelonioides* subsp. *pilosum* are strong Zn hyperaccumulators on normal soils, reaching up to 15,700 μg g^{-1} and 26,400 μg g^{-1} foliar Zn (Baker et al., 1992; Nkrumah et al., 2018b), and although extremely high Zn were detected in this study in *Dichapetalum* spp. (and Zn-rich glue could be eliminated in these cases after careful inspection of the specimens), we omitted Zn values from the overall dataset for consistency (as the presence or absence of the problematic glue could not be established in all cases).

This study also recorded a total of 51 Mn hyperaccumulator species from 13 families and 24 genera (Suppl. Table 3), of which 33 were previously known to be hyperaccumulators (van der Ent et al., 2018d). The present record for Mn hyperaccumulation in Sabah is *Antidesma puncticulatum* with 4.6 wt% Mn (Nkrumah et al. 2017), followed by *Walsura pachycaulon* with 4.5 wt% Mn, and *Casearia tuberculata* with 3.8 Wt% Mn. Additionally, 14 Co hyperaccumulator species from three families and eight genera (Suppl. Table 4) were found, of which seven were previously known to be hyperaccumulators (van der Ent et al., 2018d). The highest Co concentration in herbarium specimens was recorded in *Ashtonia excelsa* with 1500 μg g^{-1} foliar Co.

4. Discussion

Hyperaccumulator plants have been studied from many different angles for their unusual biochemistry and ecophysiology (Baker and Brooks, 1989; van der Ent et al., 2017a, 2017b), as well as for their potential utility in agromining applications (van der Ent et al., 2013b, 2015a). The identification of new hyperaccumulators can provide information about the evolution of hyperaccumulation in the Plant Kingdom. At the global scale Ni hyperaccumulator plants are known primarily to occur in the Brassicales (in the northern hemisphere temperate and Mediterranean climates) and Malpighiales (in tropical climates of the Old and New World) (Reeves, 1992, Reeves, 2003; Jaffré et al., 2013). Although Ni hyperaccumulation has been most intensively studied at global scale (Reeves et al., 2018), as well as in the Sabah (van der Ent et al., 2018a, 2018b, 2018c, 2018d), this study led to the discovery of numerous hyperaccumulators for Mn and several for Co. This places Sabah among the top regions in the world for the diversity of Ni hyperaccumulators after Cuba with 120 species (Reeves et al., 2007) and New Caledonia with 65 species (Jaffré et al., 2013). This study also confirmed that Ni hyperaccumulation is edaphically restricted to ultramafic soils and a phylogenetic proponency for the Malpighiales. In these respects, Ni hyperaccumulation is therefore a distinct phenomenon from Mn hyperaccumulation.

This study led to the recording of 51 hyperaccumulator species and hence added significantly to the global inventory of Mn hyperaccumulator plants (only 14 Mn hyperaccumulator species were previously known from Sabah). These Mn hyperaccumulator species are phylogenetically scattered and occur, among others, in the Celastraceae, Clusiaceae, Euphorbiaceae, Meliaceae, Phyllanthaceae, Salicaceae and Violaceae. Manganese is not specifically accumulated in plants occurring on ultramafic soils, in stark contrast to Ni (and Co), although ultramafic soils in Sabah are significantly higher in Mn than other 'normal' soils (van der Ent et al., 2017a). Given that Mn hyperaccumulation is phylogenetically widely distributed, and not associated with ultramafic soils, it is likely it is far more common than previously thought (Reeves, 2006). There are at least two mechanisms response for Mn hyperaccumulation: (i) rhizosphere acidification from the release of carboxylic acids (*i.e.* citrate) by roots for P uptake which can induce Mn accumulation (Lambers et al., 2015), as shown for *Lupinus albus* and *Phytolacca americana* (Jiang et al., 2015; DeGroote et al., 2018), and (ii) resulting from the mineralogical changes of Mn oxides in soils during oxidation/reduction cycles. Soil reduction results in dissolution of Mn oxides and increased availability of Mn under the Mn^{2+} ion. This mechanism has been identified as a cause for Co hyperaccumulation in Stagnosols by facultative Co hyperaccumulators (Co is extremely 'manganesophilic' in soils) (Lange et al., 2017).

This study also led to the recording of 12 Co hyperaccumulator species from three families and seven genera. Seven Co hyperaccumulator species were already known previously (Reeves, 2006; van der Ent et al., 2015b, 2018c, 2018d), and those recorded in this study occur in the Meliaceae (genus *Walsura*), Phyllanthaceae, and Violaceae (genus *Rinorea*). The highest Co concentration was found in *Ashtonia excelsa* with 1500 μg g^{-1}. The results of this study confirmed that Co hyperaccumulation is relatively rare, and that some taxa in the Phyl-

Table 3
Families and selected genera found on ultramafic and non-ultramafic substrates showing the corrected XRF values of their ranges, mean values, the total number of specimens and *t*-test (*p*-value at 0.05). "–" is below the element-specific LOD ($<190\ \mu g\ g^{-1}$ Ni, $<140\ \mu g\ g^{-1}$, $<336\ \mu g\ g^{-1}$ Mn). *values from all the genera in the family.

Order	Families	Genera	Co $\mu g\ g^{-1}$					Mn $\mu g\ g^{-1}$					Ni $\mu g\ g^{-1}$				
			Ultramafic		Non-ultramafic			Ultramafic		Non-ultramafic			Ultramafic		Non-ultramafic		
			Mean (n)	Range	Mean (n)	Range	*p*-Value	Mean (n)	Range	Mean (n)	Range	*p*-Value	Mean (n)	Range	Mean (n)	Range	*p*-Value
Malpighiales	Dichapetalaceae	*Dichapetalum*	–	–	–	–	–	1100 (2)	400–1900	1300 (39)	300–4300	0.83	23 9 (8)	8000–45,600	10,600 (12)	1000–37,100	0.02
	Phyllanthaceae	*	450 (9)	100–980	240 (30)	100–1500	0.11	2500 (193)	400–46,500	2300 (2136)	300–21,700	0.97	7800 (36)	300–32,800	2500 (36)	300–32,600	0.01
		Antidesma	340(2)	100–560	150 (9)	100–300	0.73	3100 (67)	400–46,500	2700 (490)	300–17,400	0.97	9700 (21)	400–32,800	3600 (14)	300–32,600	0.01
		Aporosa	560 (2)	100–0.98	170 (5)	100–200	0.57	3400 (43)	400–26,600	2700 (647)	300–19,800	0.11	10,400 (1)	–	500 (3)	300–600	–
		Baccaurea	810 (2)	700–1000	240 (8)	100–800	0.22	1800 (20)	400–11,600	1800 (279)	300–21,700	0.89	4000 (6)	400–8000	1000 (11)	300–5900	0.08
	Salicaceae	*	–	–	–	–	–	2800 (51)	400–38,200	2400 (273)	300–16,600	0.02	4000 (29)	200–2300	3400 (6)	400–10,400	0.98
	Violaceae	*Rinorea*	200 (4)	130–300	260 (10)	100–600	0.95	1700 (33)	400–6200	2100 (100)	300–13,400	0.01	10,700 (23)	500–32,200	3900 (20)	200–14,300	0.01
Sapindales	Meliaceae	*Walsura*	210 (3)	110–330	230 (34)	100–700	0.81	5400 (14)	400–15,300	7600 (81)	400–45,200	0.25	3900 (10)	300–8000	1300 (17)	300–8600	0.01
	Sapindaceae	*Mischocarpus*	–	–	–	–	–	4700 (7)	1700–8500	4200 (67)	400–19,400	0.28	23,900 (8)	8000–45,600	10,600 (12)	1000–37,100	0.02

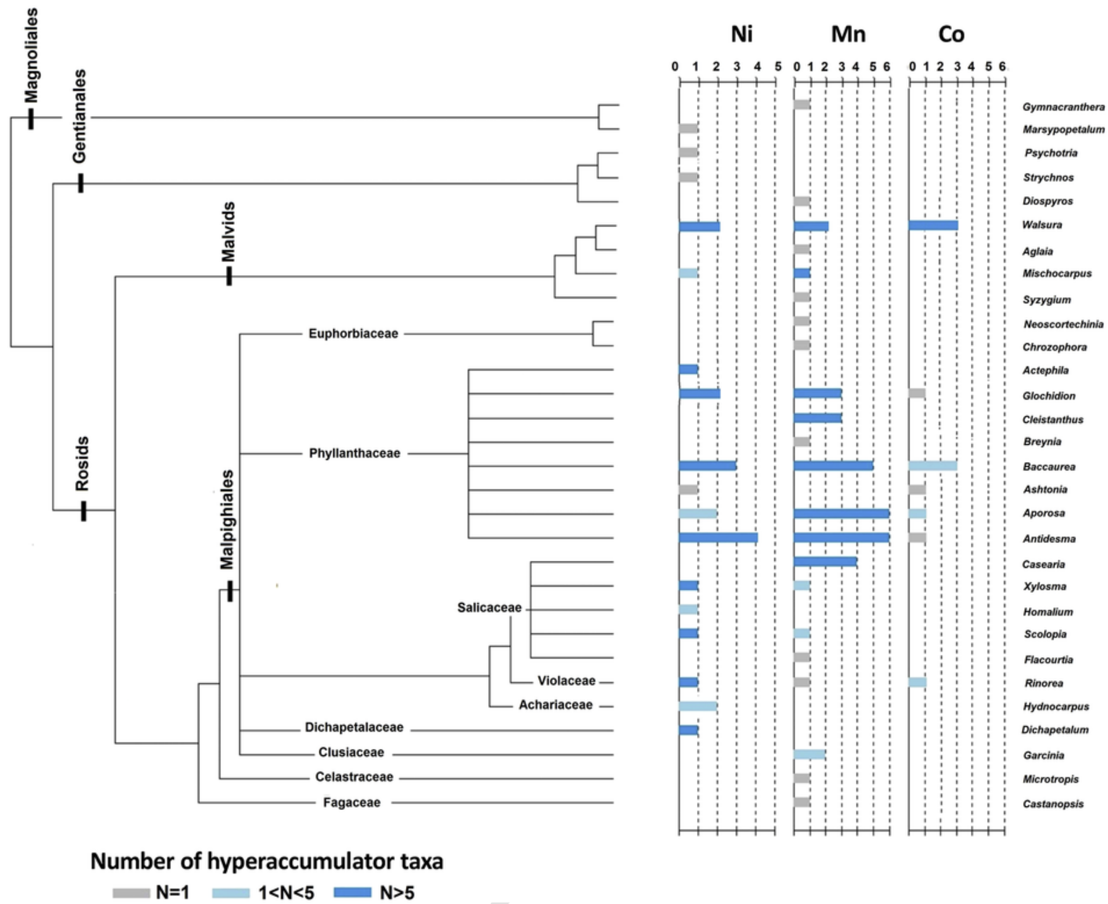


Fig. 3. Phylogenetic tree of Ni, Co and Mn hyperaccumulator taxa in the flora of Sabah. The tree was constructed using PHYLOGENETIC (phylomatic tree R20120829 Plants) and shows orders, clades. Families and genera with hyperaccumulators.

lanthaceae (*e.g. Baccaurea membranacea*) can accumulate both Co and Ni at concomitantly high concentrations.

Finally, the outcomes of this research demonstrate that handheld XRF is highly useful approach for hyperaccumulator plant discovery in herbarium collections that has the potential to add vast numbers of hyperaccumulating taxa to the global inventory. This research represents the largest single contribution to the global knowledgebase on hyperaccumulator plants, and includes a substantial expansion in phylogenetic records, many of the recorded genera being previously unknown to contain hyperaccumulator plants. This reiterates the importance of systematic screening across different families, as undertaken in this study, to avoid a bias. The current main limitation is the lack of sensitivity for transition elements present at concentrations $< 300\,\mu g\,g^{-1}$ and for light elements (elements with a $Z < 20$ such as Al). However, technological advances, including monochromatically excited XRF, will enable higher sensitivity for most elements of the Periodic Table. Newly discovered hyperaccumulators may eventually be used for genetic and ecophysiological experiments under controlled conditions to advance our fundamental understanding of trace element hyperaccumulation (Gei et al., 2018; Nkrumah et al., 2018a). The discovery of hypernickelophores (>1 wt% Ni) is promising for future advances in the development of Ni agromining. The two species of *Xylosma* discovered in this study are fast growing trees and might prove as useful as other species that are currently being used in agromining in Sabah, such as *Phyllanthus rufuschaneyi* (Bouman et al., 2018).

Uncited reference

Brooks et al., 1986

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gexplo.2019.03.013.

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