

An integrated approach to the study of *Hypericum* occurring in Sicily

Antonio GIOVINO¹, Alessandra CARRUBBA^{2*}, Silvia LAZZARA¹, Edoardo NAPOLI³, Giannantonio DOMINA²

¹Council for Agricultural Research and Economics (CREA)-Research Centre for Plant Protection and Certification (CREA-DC), Bagheria (PA), Italy

²Department of Agricultural, Food and Forest Sciences, University of Palermo, Palermo, Italy

³CNR- ICB Istituto di Chimica Biomolecolare, Via Paolo Gaifami, Catania

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Abstract: An integrated approach to the study of taxa of the genus *Hypericum* occurring in Sicily is proposed. The results of morphological, biochemical, and molecular analyses are combined to better assess the relationships between the species investigated and test the suitability of DNA barcoding technique in the discrimination of these taxa. For the name *Hypericum aegypticum* subsp. *webbii* (Spach) N. Robson a lectotype is designated. For *Hypericum triquetrifolium* Turra a lectotype and a supporting epitype are designated. The presence of *Hypericum perforatum* L. subsp. *perforatum* is excluded from Sicily and the previous reports have to be referred to *H. perforatum* subsp. *veronense* (Schrank) Ces. *Hypericum perfoliatum* L. and *H. pubescens* Boiss. are close morphologically and chemically, as well as based on the results from *rcbL* marker, although belonging to different sections. Biochemical analyses confirmed the relevant amounts in bioactive metabolites of the studied taxa. *Hypericum perfoliatum* L. is proposed as a valid alternative to *H. perforatum* L. for cultivation with phytotherapeutic purposes.

Key words: Taxonomy, morphometry, biochemistry, DNA barcoding, nomenclature

1. Introduction

Hypericum L. (*Hypericaceae*), with about 470 species, is widespread in temperate zones all over the world (Crockett and Robson, 2011). In Italy, 32 taxa are currently known, 30 species and 2 subspecies; 10 taxa occur to Sicily (Castellano and Spadaro, 2010; Bartolucci et al., 2018; Galasso et al., 2018).

Hypericum chemical constituents are well recognized for many pharmacological activities: antidepressant, antiphlogistic, improving blood circulation, against traumas, in wounds and burns recovering (Bombardelli and Morazzoni, 1995; Lazzara et al., 2015; Napoli et al., 2018). Which components of *Hypericum* plants are actually responsible for the demonstrated biological activities is still matter of debate. Notwithstanding, the most widely studied active compounds are phloroglucinols, such as hyperforin and adhyperforin, naphthodianthrones, such as hypericin and pseudohypericin, and polyphenols, including hyperoside, quercetin, rutin, quercitrin, and others (Castellano and Spadaro, 2010; Napoli et al., 2018). A previous work (Lazzara et al., 2020) allowed to assess a high variability in hyperforin and hypericins (hypericin + pseudohypericin) content in 6 *Hypericum* species from the Sicilian flora (*Hypericum perforatum* L., *H. perfoliatum* L.,

H. pubescens Boiss., *H. hircinum* L., *H. calycinum* L., and *H. tetrapterum* Fr.). Hyperforin content was on average much higher in *H. perforatum* and *H. perfoliatum* than in the other species, being absent at all from *H. hircinum* and *H. calycinum*. Yet, in *Hypericum*, a high biochemical variability showed also up within *H. perforatum* and *H. perfoliatum*, and some compounds such as hyperforin showed to be allocated in high-yielding and low-yielding genotypes from the same taxon.

Hence, in the perspective of their specialized cultivations, the awareness of which *Hypericum* taxa are most suitable for any given purpose has become a crucial issue. The availability of reliable plant material is of utmost importance both for its propagation and specialized cultivation, and to characterize market *Hypericum*-based products (Fascella et al., 2017).

In this scenario, the medical relevance of *Hypericum*, and the related commercial interest, push for improving the taxonomic identification method. Tools for fast and accurate identification of plant species are required to support morphological characterization.

With this purpose, in this study an integrated approach to all taxa of *Hypericum* currently recognized in Sicily is presented. The morphological analyses are compared

* Correspondence: alessandra.carrubba@unipa.it

with phytochemical and genetic discrimination. In particular, the suitability of DNA barcoding technique was investigated in discriminating the *Hypericum* taxa. As already successfully assessed in other species such as *Allium* spp. (İpek et al., 2014), this technique can contribute in developing an easy authentication assay, helpful in solving taxonomic doubts or in commercial trade traceability of whole plants, portions or derived products. This study also aimed to clarify the presence in Sicily of the subspecies of *H. perforatum*. In fact, Robson (2002) and Ciccarelli and Garbari (2004) reported *H. perforatum* subsp. *perforatum* occurring in Italy only in the northern part of the Peninsula and attribute the Sicilian populations to *H. perforatum* subsp. *veronense* (Schrank) Ces. Oppositely, Bartolucci et al. (2018) reported both subspecies in the whole peninsula, Sardinia, and Sicily.

2. Materials and Methods

2.1. Plant material

The 10 taxa of *Hypericum*, 9 species and 1 subspecies, were collected from natural populations in Sicily during the flowering period (from May to June) in 2013 and 2014 and were studied from the morphological, biochemical and genetic points of view. Voucher specimens were deposited in the Herbarium SAF (Table 1). The selection of sampled populations has followed extensive surveys of the whole regional territory.

The plant specimens were collected in bioclimatic belts between Lower Mesomediterranean to Lower Oromediterranean ones (Bazan et al., 2015) and in the subunits: Lampedusa Is., Northern Sicilian coast, Western Sicilian plain, Upper Madonie Mts, Lower and Upper Nebrodi Mts, Peloritani Mts, Lower Etna Mt., Iblei Mts (Domina et al., 2018). Field identification was based on morphological characters in the mature stage in comparison with the original descriptions, relevant literature (Robson and Adams, 1968; Robson, 1985, 1993, 2010), and with the original materials.

2.2. Morphological analyses

For each population, selected morphological traits were measured on 10 individuals, with 10 replicate measurements from each individual. Measurements were taken using an electronic calliper. The 11 quantitative characters considered were: #1 plant height (cm), #2 leaf length (mm), #3 leaf width (mm), #4 sepal length (mm), #5 sepal width (mm), #6 petal length (mm), #7 petal width (mm), #8 stamen length (mm), #9 stylus length (mm), #10 capsule length (mm), #11 capsule width (mm). The mean values of these measurements are presented in Supplementary Information 1. The range of each continuous numerical character was represented using box-and-whisker plots (Figure 1).

2.3. Chemical analyses

For chemical determinations, flowering tops (15–20 cm) of at least 10 individuals per population were collected in full flowering during the central hours of the day. The collected material was carried in paper bags and dried at 20–25 °C in the dark. The analyses were performed according to Napoli et al. (2018); briefly, 5 g of dry material were chopped up, homogenized and subjected to extraction in 50 mL of ethanol at room temperature for 72 h, in the dark and under constant agitation. The extract was filtered with filter paper and the filter was washed 3 times with 10 mL of ethanol. The obtained mixture was brought to dryness with a rotary evaporator. The chemical determinations were conducted by means of high performance liquid chromatography equipment with a diode array detector (HPLC-DAD), injecting 20 µL of a 10 mg/mL solution in methanol “HPLC grade VWR” for each extract. Each analysis was carried out in triplicate. Since the amount of *H. triquetrifolium* was too small for chemical determination, comparison data were obtained from literature (Hosni et al., 2011). Mean values of the 20 chemical determinations were used for multivariate analyses, and their totals, are reported in the Supplementary Information 2, whereas the box plots of these values, averaged by species, are reported in Figure 2.

2.4. DNA barcoding

The barcoding approach was adopted in support of the morphological and phytochemical investigation. Multiple individuals for each taxon were used for molecular analysis. Plant material for DNA extraction consisted of young lyophilized leaves. Genomic DNA extraction was based on CTAB protocol for plant tissue (Doyle and Doyle, 1987).

The 3 plastid barcoding regions *rbcl*, *matK*, *trnH-psbA*, were assessed by adopting polymerase chain reaction primers and conditions suggested by the Consortium for the Barcode of Life (CBOL) (Dunning and Savolainen, 2010; Fazekas et al., 2012) (Table 3).

When making the choice of markers we considered the relevance of the compromise between the discrimination level supported by a marker and amplification and sequencing success (Chase et al., 2005; Hollingsworth et al., 2009). The choice of *trnH-psbA*, as an additional marker, appeared logical to discriminate morphologically close samples.

Polymerase chain reaction amplifications were performed with the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). Products were purified and bidirectionally sequenced (Amersham Biosciences DYEnamic ET Terminator Cycle Sequencing Kits), according to the Sanger protocol for AB3730XL DNA Analyzer (Applied Biosystems). The resulting electropherograms were screened for errors and

Table 1. Synoptic table of the taxa studied reporting the field identification, site of collection, habitat characteristics, and voucher details.

Taxon	Locality	Coordinates	m a.s.l.	Bioclimatic belt (Bazan et al. 2015)	Subunit (Domina et al. 2018)	Voucher
<i>H. aegypticum</i> subsp. <i>webbii</i>	Cala Madonna, Lampedusa (AG)	35°30'08" N, 12°35'21" E	15	Lower Mesomediterranean	Lampedusa Is.	SAF100037
<i>H. androsaemum</i>	Vallone Canna, Madonie (PA)	37°51'00" N, 14°04'08" E	1395	Lower Oromediterranean	Upper Madonie Mts	SAF100038
<i>H. calycinum</i>	C.da Pardo, Ucria (ME)	38°03'05" N, 14°54'59" E	720	Upper Mesomediterranean	Lower Nebrodi Mts	SAF100036
<i>H. hircinum</i> subsp. <i>majus</i>	Monforte S. Giorgio (ME)	38°09'35" N, 15°22'47" E	250	Lower Mesomediterranean	Peloritani Mts	SAF100039
<i>H. perfoliatum</i>	Monte Catalfano (PA)	38°06'51" N, 13°30'55" E	75	Upper Thermomediterranean	Northern Sicilian coast	SAF100028
<i>H. perforatum</i> cfr. subsp. <i>perforatum</i>	Madonie, Piano Marcato (PA)	37°54'30" N, 14°04'78" E	1050	Lower Supramediterranean	Upper Madonie Mts	SAF100007
<i>H. perforatum</i> cfr. subsp. <i>perforatum</i>	Monte Etna (CT)	37°38'03" N, 15°01'25" E	900	Upper Mesomediterranean	Lower Etna Mt.	SAF100009
<i>H. perforatum</i> subsp. <i>veronense</i>	Madonie, Vicaretto (PA)	37°53'35" N, 14°05'48" E	800	Upper Mesomediterranean	Lower Madonie Mts	SAF100010
<i>H. perforatum</i> subsp. <i>veronense</i>	Capo Gallo (PA)	38°12'43" N, 13°17'39" E	25	Upper Thermomediterranean	Northern Sicilian coast	SAF100012
<i>H. pubescens</i>	Campobello di Mazara (TP)	37°39'09" N, 12°46'21" E	150	Lower Thermomediterranean	Western Sicilian plain	SAF100040
<i>H. tetrapterum</i>	Portella dello Zoppo, Floresta (ME)	37°59'34" N, 14°54'12" E	1350	Upper Supramediterranean	Upper Nebrodi Mts,	SAF100034
<i>H. triquetrifolium</i>	Valle dell'Anapo, Sortino (SR)	37°08'10" N, 14°59'31" E	450	Lower Mesomediterranean	Iblei Mts	SAF100041

assembled into contigs using Sequencer software 4.10 (Gene Codes Corporation, Ann Arbor, MI, USA). The sequence alignments were carried out by MUSCLE and phylogenetic Neighbour-Joining. A tree was generated for molecular identification, based on a Kimura 2 parameter model, using Mega 6 software (Kimura, 1980; Saitou and Nei, 1987; Tamura et al., 2013; Giovino et al., 2016). The comparison included all new sequences generated, a subset of the most closely related sequences, and the significant BLAST results, downloaded from GenBank database (Table 3).

2.5. Statistical treatment of data

According to Giovino et al. (2015), Domina et al. (2017), and Domina (2018), each morphological character was subjected to a preliminary univariate variance analysis (data not shown) according to the specific data structure, setting each morphological character as independent variable (X) and the taxon as dependent variable (Y), using PAST version 3.26b (Hammer et al., 2001; Hammer,

2019). Pearson correlation coefficients (r) among the 11 measured characters were calculated, as presented in the Supplementary Information 3. Multivariate analyses, including discriminant analysis (DA - Figure 3) and principal component analysis (PCA - Figure 4) were performed. A cluster analysis with paired group (UPGMA) algorithm and Euclidean similarity index was carried out for morphological observation (Figure 5), as well as for chemical components (Figure 6), and molecular markers (*rbcL*, Figure 7; *matK*, Figure 8; *trnH-psbA*, Figure 9).

Sampling, morphological and molecular data generated in this investigation were submitted to the BOLD database under the dedicated project code FMED (Ratnasingham and Hebert, 2007).

3. Results

3.1. Nomenclature

The nomenclatural types of the names *Hypericum androsaemum*, *H. calycinum*, *H. hircinum* subsp. *majus*,

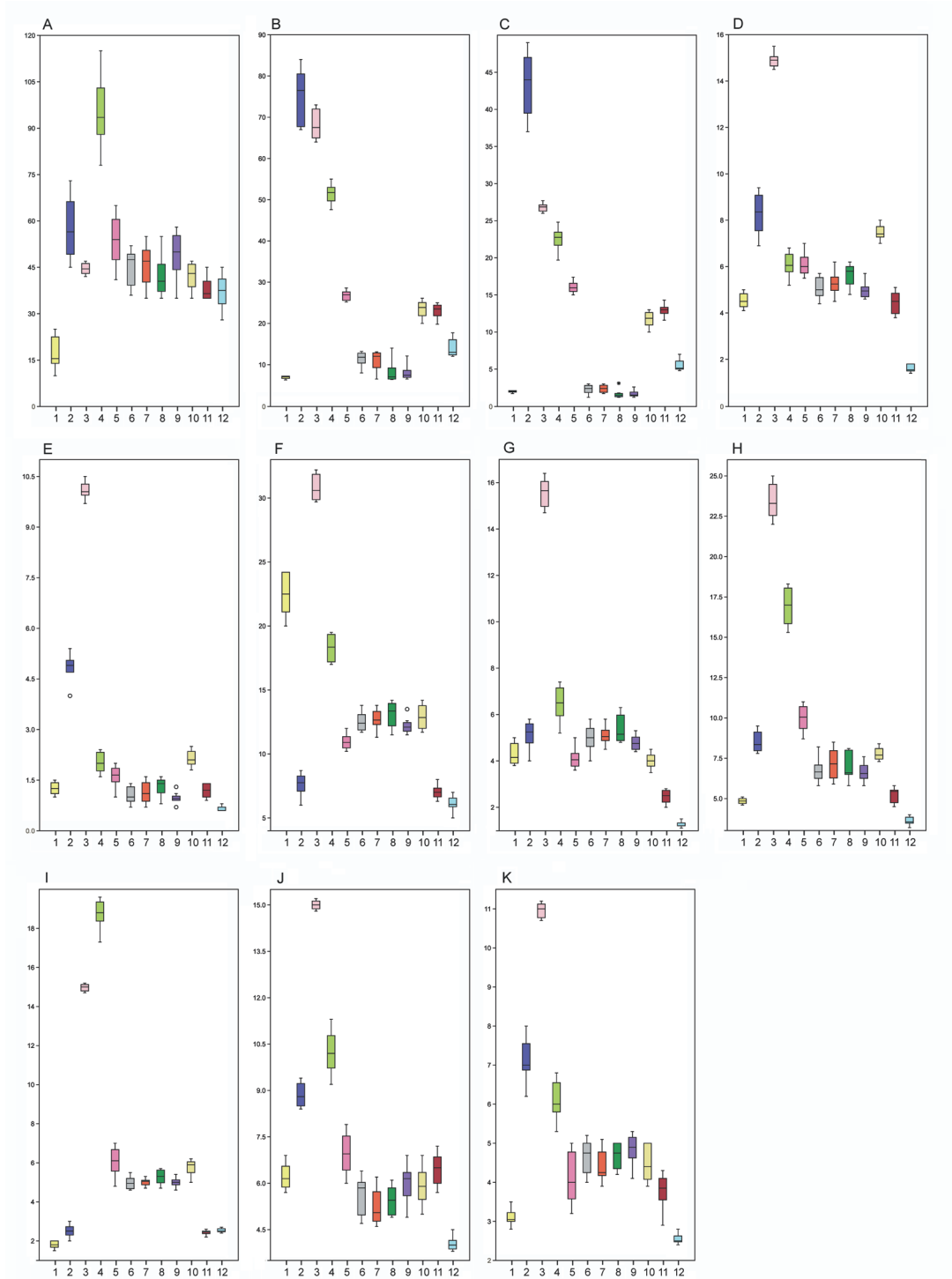


Figure 1. Box-plots of the 11 considered morphological characters (A: plant height, cm; B: leaf length, mm; C: leaf width, mm; D: sepal length, mm; E: sepal width, mm; F: petal length, mm; G: petal width, mm; H: stamen length, mm; I: stylus length, mm; J: capsule length, mm; K: capsule width, mm). For each sample, the 25–75% quartiles are drawn using a box. The median is shown with a horizontal line inside the box. The whiskers are drawn from the top of the box up to the largest data point less than 1.5 times the box height from the box, and similarly below the box. Outliers are shown as stars. 1(Yellow): *H. aegypticum* subsp. *webbii*; 2(Blue): *H. androsaemum*; 3(Pink): *H. calycinum*; 4(Light green): *H. hircinum* subsp. *majus*; 5(Hot pink): *H. perfoliatum*; 6(Grey): *H. perforatum* cfr. subsp. *perforatum*, Mt. Etna; 7(Red): *H. perforatum* cfr. subsp. *perforatum*, Madonie Piano Marcato; 8(Dark green): *H. perforatum* subsp. *veronense*, Madonie Vicaretto; 9(Violet): *H. perforatum* subsp. *veronense*, Capo Gallo; 10(Khaki): *H. pubescens*; 11(Brown): *H. tetrapterum*; 12(Light blue): *H. triquetrifolium*.

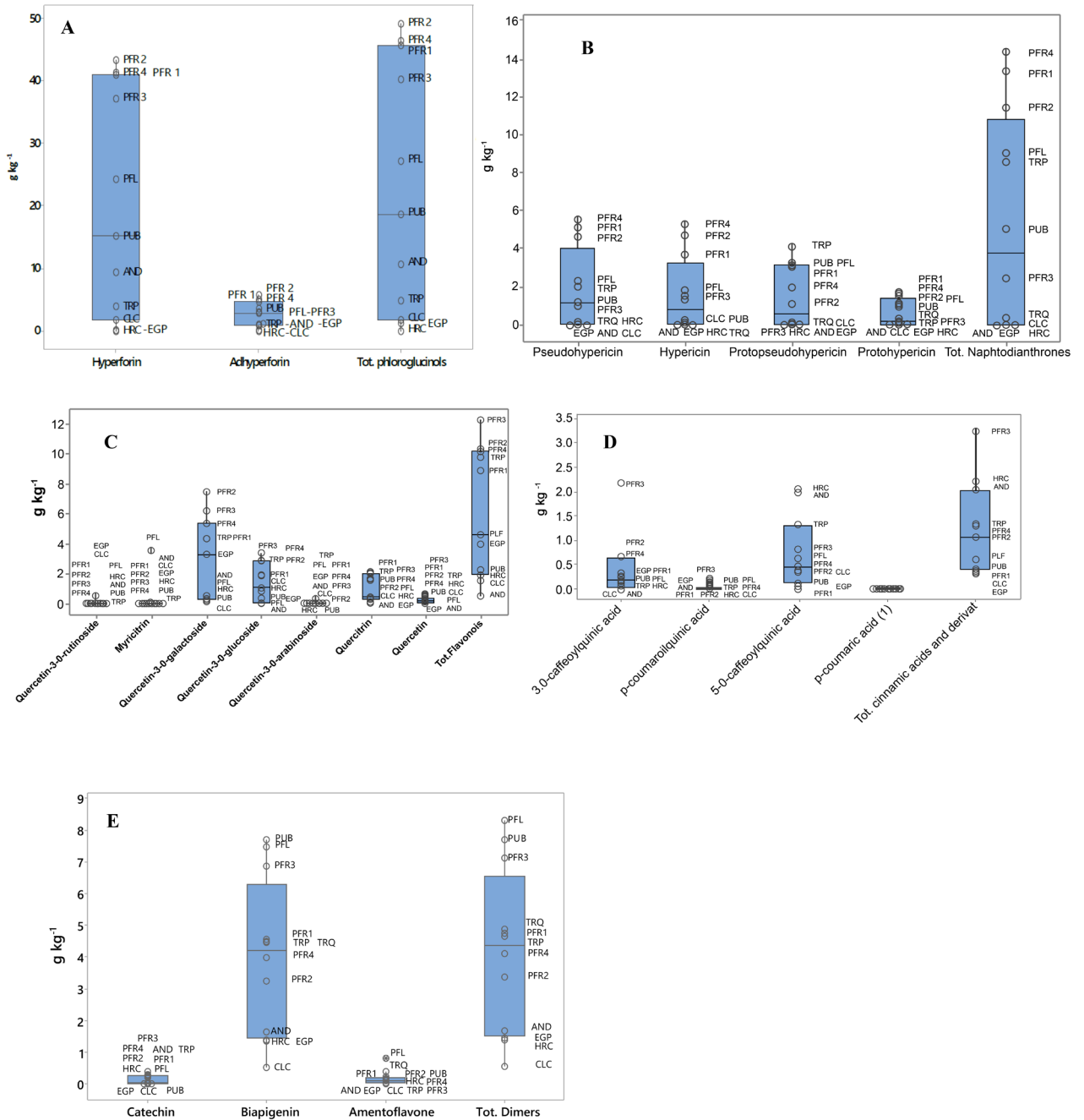


Figure 2. Box-plots of the major detected bioactive compounds, averaged by population. A: hyperforin, adhyperforin, and total identified phloroglucinols; B: pseudohypericin, hypericin, protopseudohypericin, protohypericin, and total identified naphthodianthrones; C: quercetin-3-O-rutinoside, myricitrin, quercetin-3-O-galactoside, quercetin-3-O-arabinsoside, quercitrin, quercetin, and total identified flavonols; D: 3-O-caffeoylquinic acid, *p*-coumaroilquinic acid, 5-O-caffeoylquinic acid, *p*-coumaric acid, and total identified cinnamic acids and derivatives; E: catechin, biapigenin, amentoflavone, total identified dimers. For each compound, the box represents quartiles Q1 to Q3, including 25% to 75% of data. The median is shown with an inner horizontal line. Outliers, i.e. values overpassing Q3 + 1.5 or Q1 - 1.5, are indicated by stars. EGP: *H. aegypticum* subsp. *webbii*; AND: *H. androsaemum*; CLC: *H. calycinum*; HRC: *H. hircinum* subsp. *majus*; PFL: *H. perfoliatum*; PFR1: *H. perforatum* cfr. subsp. *perforatum*, Mt. Etna; PFR2: *H. perforatum* cfr. subsp. *perforatum*, Madonie Piano Marcato; PFR3: *H. perforatum* subsp. *veronense*, Madonie Vicaretto; PFR4: *H. perforatum* subsp. *veronense*, Capo Gallo; PUB: *H. pubescens*; TRP: *H. tetrapterum*. (1): overlapping population names were removed.

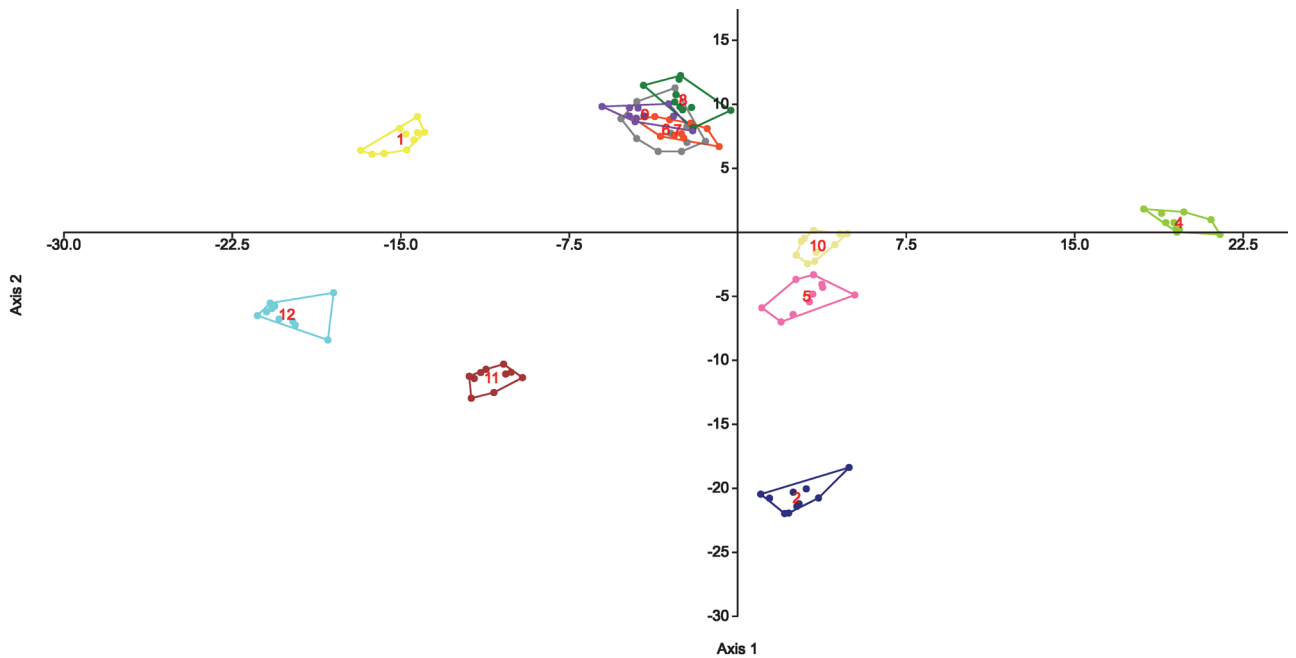


Figure 3. Discriminant analysis based on the 11 considered morphological characters, with groups corresponding to the 12 studied populations. Axis 1: Eigenvalue 176.24, % variance 48.51; Axis 2 Eigenvalue 88.654, % variance 24.4. 1(Yellow): *H. aegypticum* subsp. *webbii*; 2(Blue): *H. androsaemum*; 3(Pink): *H. calycinum*; 4(Light green): *H. hircinum* subsp. *majus*; 5(Hot pink): *H. perfoliatum*; 6(Grey): *H. perforatum* cfr. subsp. *perforatum*, Mt. Etna; 7(Red): *H. perforatum* cfr. subsp. *perforatum*, Madonie Piano Marcato; 8(Dark green): *H. perforatum* subsp. *veronense*, Madonie Vicaretto; 9(Violet): *H. perforatum* subsp. *veronense*, Capo Gallo; 10(Khaki): *H. pubescens*; 11(Brown): *H. tetrapterum*; 12(Light blue): *H. triquetrifolium*.

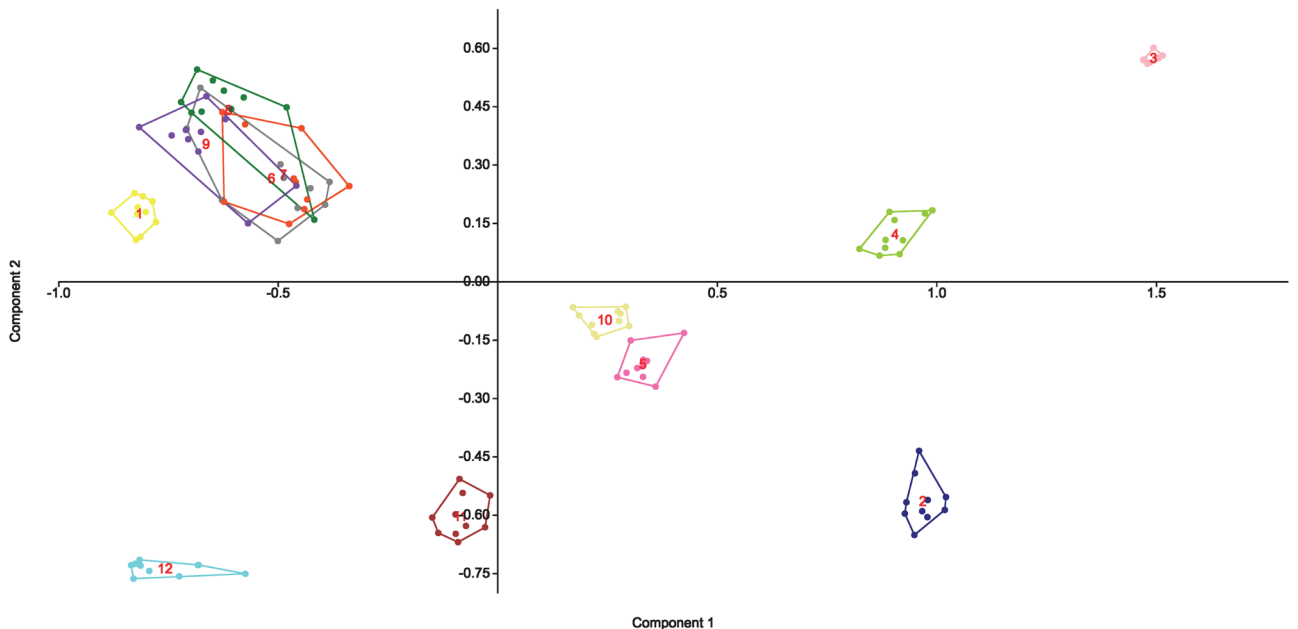


Figure 4. Principal component analysis based on the 11 considered morphological characters, with groups corresponding to the 12 studied populations. Axis 1: Eigenvalue 0.569655, % variance 66.107; Axis 2 Eigenvalue 0.182426, % variance 21.17. 1(Yellow): *H. aegypticum* subsp. *webbii*; 2(Blue): *H. androsaemum*; 3(Pink): *H. calycinum*; 4(Light green): *H. hircinum* subsp. *majus*; 5(Hot pink): *H. perfoliatum*; 6(Grey): *H. perforatum* cfr. subsp. *perforatum*, Mt. Etna; 7(Red): *H. perforatum* cfr. subsp. *perforatum*, Madonie Piano Marcato; 8(Dark green): *H. perforatum* subsp. *veronense*, Madonie Vicaretto; 9(Violet): *H. perforatum* subsp. *veronense*, Capo Gallo; 10(Khaki): *H. pubescens*; 11(Brown): *H. tetrapterum*; 12(Light blue): *H. triquetrifolium*.

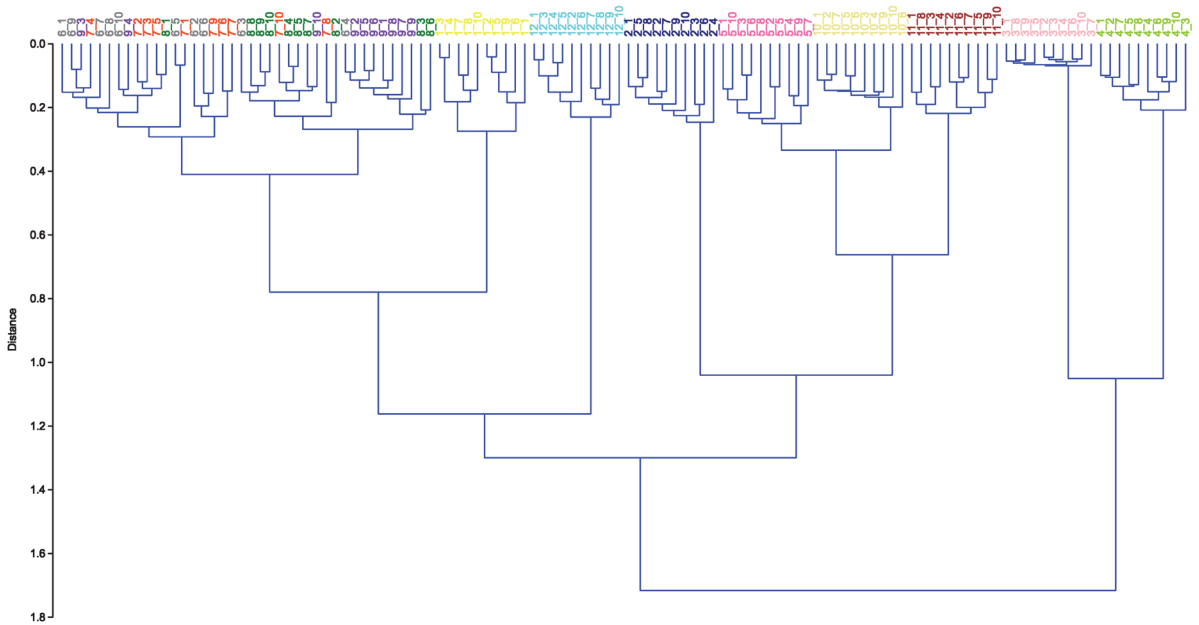


Figure 5. Cluster analysis based on the eleven considered morphological characters. 1(Yellow): *H. aegypticum* subsp. *webbii*; 2(Blue): *H. androsaemum*; 3(Pink): *H. calycinum*; 4(light green): *H. hircinum* subsp. *majus*; 5(Hot pink): *H. perfoliatum*; 6(Grey): *H. perforatum* cfr. subsp. *perforatum*, Mt. Etna; 7(red): *H. perforatum* cfr. subsp. *perforatum*, Madonie Piano Marcato; 8(dark green): *H. perforatum* subsp. *veronense*, Madonie Vicaretto; 9(Violet): *H. perforatum* subsp. *veronense*, Capo Gallo; 10(Khaki): *H. pubescens*; 11(Brown): *H. tetrapterum*; 12(light blue): *H. triquetrifolium*.

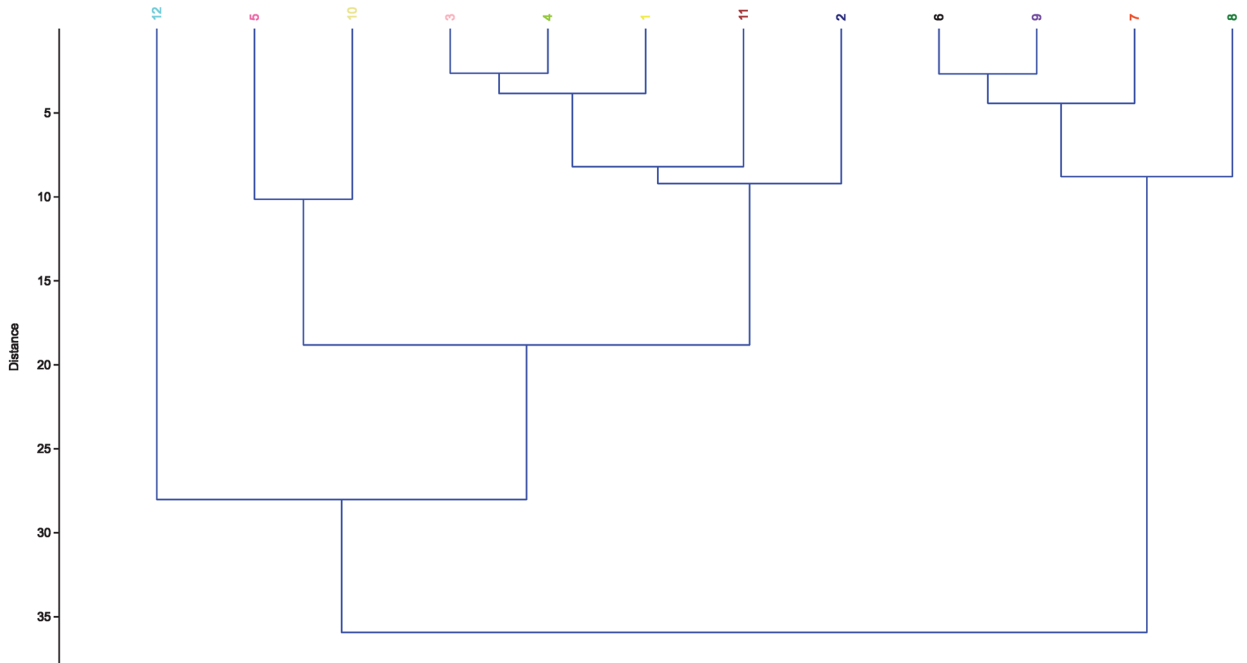


Figure 6. Cluster analysis based on the 20 considered detected bioactive compounds. 1(Yellow): *H. aegypticum* subsp. *webbii*; 2(Blue): *H. androsaemum*; 3(Pink): *H. calycinum*; 4(Light green): *H. hircinum* subsp. *majus*; 5(Hot pink): *H. perfoliatum*; 6(Grey): *H. perforatum* cfr. subsp. *perforatum*, Mt. Etna; 7(Red): *H. perforatum* cfr. subsp. *perforatum*, Madonie Piano Marcato; 8(Dark green): *H. perforatum* subsp. *veronense*, Madonie Vicaretto; 9(Violet): *H. perforatum* subsp. *veronense*, Capo Gallo; 10(Khaki): *H. pubescens*; 11(Brown): *H. tetrapterum*; 12(Light blue): *H. triquetrifolium*, Tunisia.

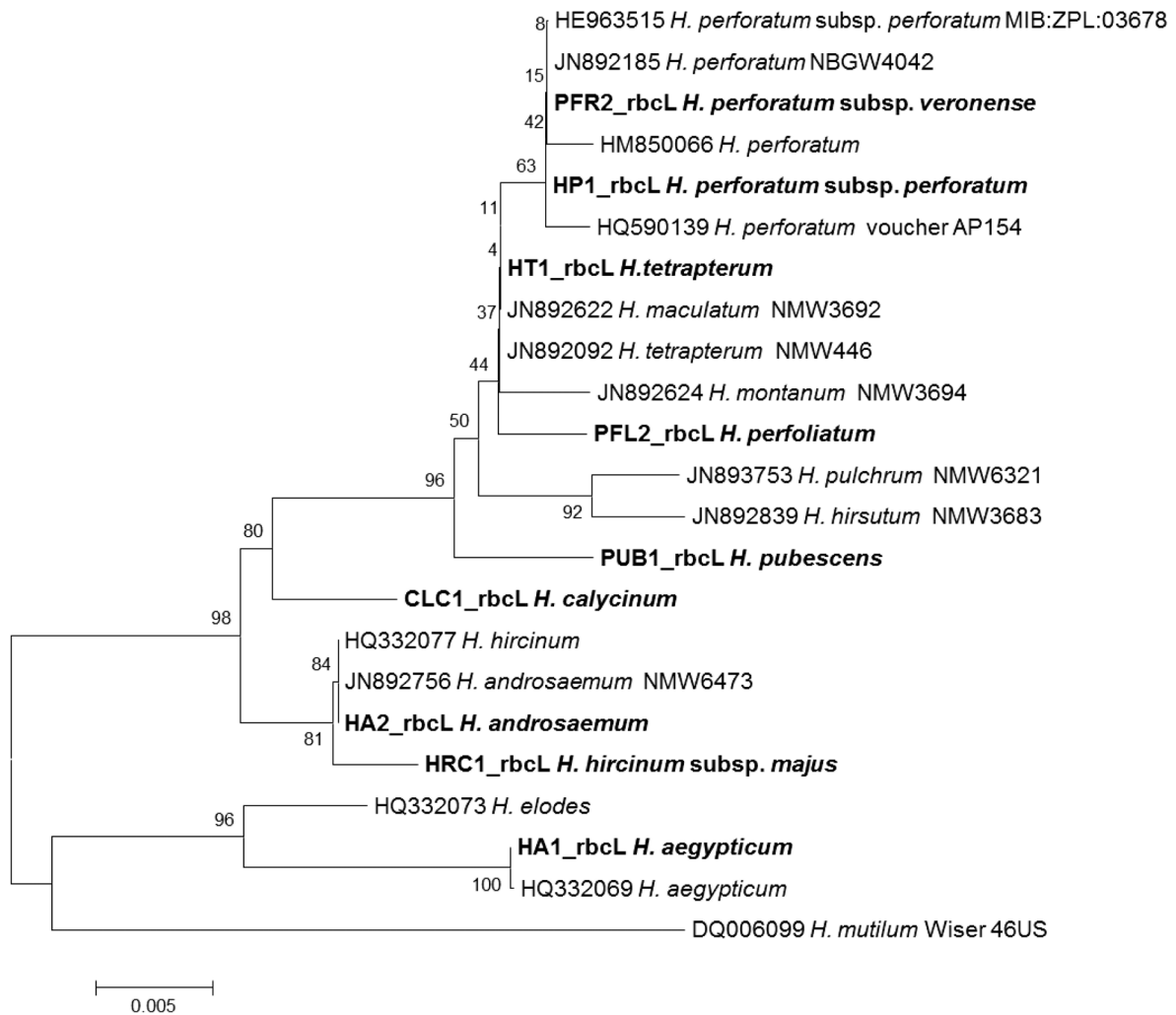


Figure 7. *rbcL* Neighbour-Joining phylogenetic tree of *Hypericum* sp. pl.

H. perfoliatum, *H. perforatum* subsp. *perforatum*, *H. perforatum* subsp. *veronense*, *H. pubescens*, and *H. tetrapterum* have been correctly designated and discussed in Burtt and Davis (1949), Burdet et al. (1984), Robson (1968, 1985, 2002, 2010). For *H. aegypticum* subsp. *webbii* and *H. triquetrifolium* lectotype designations are here proposed being, to the best of our knowledge, not yet typified (cfr. Peruzzi et al., 2019).

Hypericum aegypticum* subsp. *webbii (Spach) N. Robson, Bull. Brit. Mus. (Nat. Hist.), Bot. 23: 68. 1993 ≡ *Triadenia webbii* Spach in Annals Sci. Nat. (Bot.) II, 5: 174, t. 5A (1836)

Ind. Loc.: Malta, In rupestribus insulae Melitae legit cl. Webb.

Type (Lectotype designated here): Malta, In rupestribus insulae Melitae, *Webb s.n.* (FI!)

Notes: Robson (2010: 150) indicates the specimen collected by Webb, housed in FI, as the holotype of this

name. However, in the protologue different syntypes are reported, thus a lectotype designation was needed (Art. 9.3 of the ICN). The lectotype here designated is the specimen studied by Robson. It agrees with the protologue and the current usage of the name.

Hypericum triquetrifolium Turra, Farsetia: 12. 1765. [September 1765]

Ind. Loc.: Habitat in Grecia, Sicilia, Calabria.

Type (Lectotype designated here): Boccone (1697 2: pl. 12) (Epitype designated here): Sicily, Palermo a Camastra, Junio [second half of XIX Century], A. Todaro 1240 (PAL79039!); iso: (PAL79044!; PAL79045!; BM001201777 photo!; P05068965 photo!; P05118515 Photo!).

Notes: The protologue of this species contains reference to the plate from Boccone (1697 2: pl. 12) that can be considered original material. Turra's herbarium was hosted in the Museo civico in Vicenza (Italy) that was destroyed during the second World War (Stafleu and Cowan,

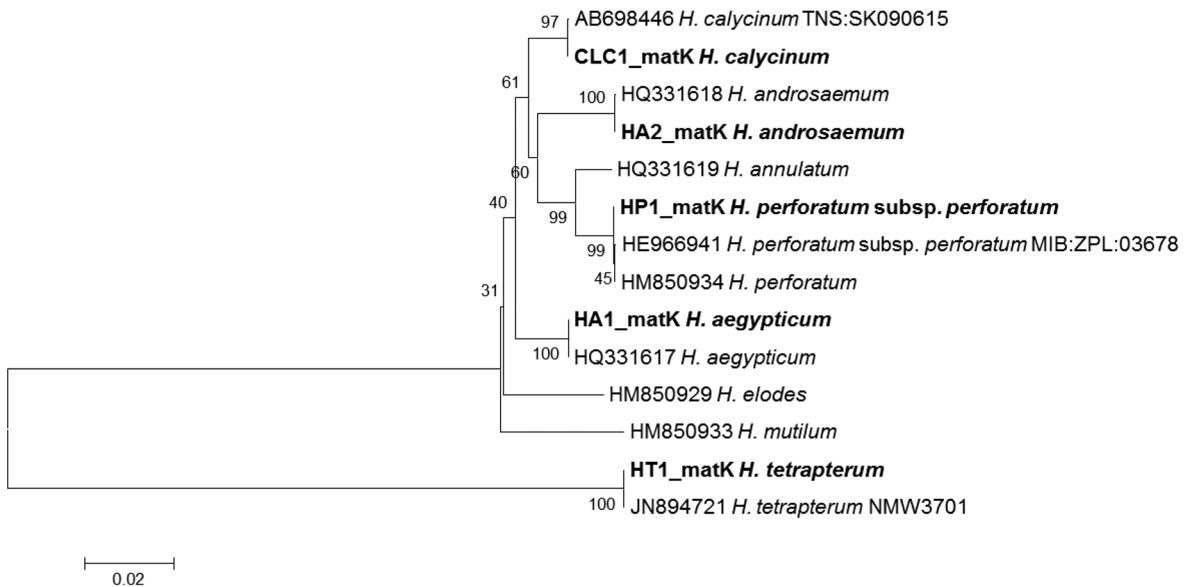


Figure 8. *matK* Neighbour-Joining phylogenetic tree of *Hypericum* sp. pl.

1986; Robson 2002). No relevant specimens suitable as original material have been found in the herbaria that could host duplicates of Turra's collections (FI, PI, RO, etc.). Therefore, we designate here the illustration by Boccone as lectotype (<https://www.biodiversitylibrary.org/item/14647#page/218>). This lectotype corresponds with the current concept of *H. triquetrum*. In support of this lectotype, we are designating an epitype using a specimen collected from Sicily with several duplicates in European herbaria.

3.2. Morphology

Pearson correlation coefficients (Supplementary Information 3) showed a high association level between the 2 measurements of leaf dimension (Leaf L and Leaf W, $r = 0.965$) and capsule dimension (caps L and Caps W, $r = 0.876$). Other highly correlated measurements were Petal W and Sepal L ($r = 0.880$) and Stylus L and Stamen L ($r = 0.864$). Otherwise, not significant negative correlations showed up about Petal L and Plant height ($r = -0.08$), and Petal L and Leaf W ($r = -0.05$).

The examined taxa were well discriminated using PCA (Fig. 4), with the only exception of the 4 populations identified as *H. perforatum* subsp. *perforatum* and *H. perforatum* subsp. *veronense*, which showed great variability and overlap. According to the DA (Figure 3, Table 2) the characters that showed a greater ability to discriminate were the Stylus length, the Sepal length, and the Stamen length. In addition, from the box-plots analysis (Figure 1) these characters had extreme values that allowed the discrimination of the largest part of taxa and partially overlapped in the populations of the *H. perforatum* group. The reduced morphological variability of the population of

H. calycinum observable in the box-plots analysis (Figure 1) could be explained by rather recent introduction of this taxon, reasonably originated from a reduced number of cultivated individuals.

More than 90% (94.17%) cases resulted were correctly classified by DA according to the *a priori* group assignment, and the only case that was not correctly classified belonged to the subspecies of *H. perforatum*. The cluster analysis (Figure 5) showed a branch with *H. calycinum* and *H. hircinum* subsp. *majus*, separated from the other taxa. It was highlighted the admixture of the specimens belonging to *H. perforatum* subsp. *perforatum* and *H. perforatum* subsp. *veronense*.

3.3. Chemical analysis

As main part of the complex bioactive secondary metabolism of *Hypericum*, in this study the attention was focused on polyphenols, naphthodianthrones and phloroglucinols contents of the ethanolic extract. Results are reported in Table S2.

A high intraspecific variability showed up, with different amounts of hypericins (hypericin + pseudohypericin) and hyperforin according to the genotype. From the biochemical aspect, the taxa were well distinguished, above all based on their content in hyperforin and, to a lesser extent, quercetin-3-*O*-rutinoside (rutin) and quercetin-3-*O*-galactoside (hyperoside).

The high discriminatory power found for the hyperforin content confirmed previous findings (Napoli et al., 2018). Hyperforin was detected in quite high amounts in *H. perforatum* (37–43 g kg⁻¹), followed by *H. perforatum* (24 g kg⁻¹) and *H. pubescens* (15 g kg⁻¹). Relevant quantities of this metabolite were also recorded

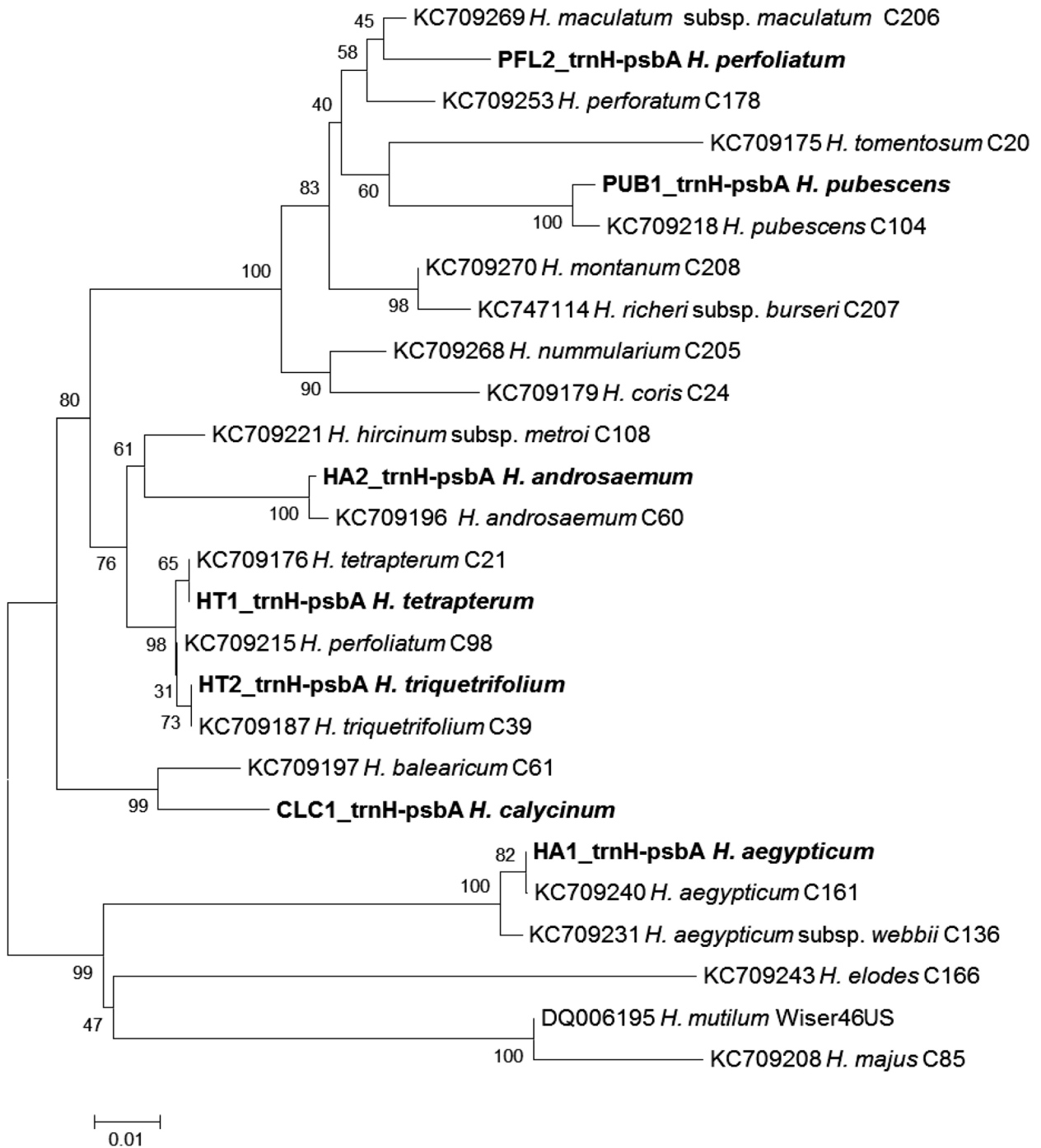


Figure 9. *trnH-psbA* intergenic spacer Neighbour-Joining phylogenetic tree of *Hypericum* sp. pl.

in *H. androsaemum* (9 g kg⁻¹). Noticeably, hypericins (given as the sum of hypericin and its 3 biosynthetic precursors, namely protohypericin, pseudohypericin, and protopseudohypericin) were found in larger amounts in *H. perforatum* and in rather similar quantities in *H. perfoliatum* and *H. tetrapterum*, whereas they were almost absent in *H. androsaemum*, *H. calycinum*, *H. hircinum*,

and *H. triquetrifolium*. The latter taxon stood out, instead, for the high detected quantities of 3-*O*-caffeoylquinic acid and flavonols, compared to all the other taxa. Due to the increasing interest that surrounds the biological activities ascribed to biflavones (biapigenin and amentoflavone), it is also worth noting the high content of these, that was retrieved in *H. perfoliatum*, *H. perforatum*, *H. pubescens*,

Table 2. Loadings table of the characters in the first 2 axes of the discriminant analysis.

	Axis 1	Axis 2
Plant Height (cm)	3.2662	-3.8751
Leaf Length (mm)	3.5588	-2.4785
Leaf Width (mm)	3.1893	-9.7784
Sepal Length (mm)	8.7143	5.63
Sepal Width (mm)	3.2275	-8.4081
Petal Length (mm)	4.1146	21.052
Petal Width (mm)	0.97116	-3.147
Stamen Length (mm)	7.3005	2.1331
Stylus Length (mm)	16.896	13.029
Capsule Length (mm)	5.5245	-9.2075
Capsule Width (mm)	5.6715	7.6086

H. tetrapterum, and *H. triquetrifolium*. The cluster analysis (Figure 6) showed a branch with the specimens of *H. perforatum* subsp. *perforatum* and *H. perforatum* subsp. *veronense* mixed each other but separated from the other taxa. In the other cluster it was possible to distinguish *H. triquetrifolium* in a branch and *H. perfoliatum* and *H. pubescens* in another branch.

3.4. DNA barcoding

The *rbcL* locus showed the best performance in terms of amplification and sequencing success, while *trnH-psbA* and *matK* markers showed instead higher potential in species level resolution (Table 2). Particularly, *trnH-psbA* discriminated 100% of the taxa successfully sequenced. Therefore, a multilocus approach (*rbcL* + *trnH-psbA*), able to resolve 80% of the taxa analysed (8/10), appeared the best compromise between sequencing success and discrimination power (Table 2).

According to the results in Table 2, phylogenetic trees of each barcoding markers showed the genetic relationship between the taxa included in this study (Figures 7, 8, and 9). Only 2 subspecies *H. perforatum* subsp. *perforatum* and *H. perforatum* subsp. *veronense* were not discriminated.

4. Discussion

The integrated approach applied in this work has been able to achieve full characterization of several *Hypericum* species from Sicily. Morphological, chemical and genetic observations, offered distinct points of view of *Hypericum*'s diversity; however, a multidisciplinary procedure allowed us to point out similarities and differences among the different *Hypericum* taxa from Sicily that would have not been detected otherwise.

A combined comparison of the results from the 3 used approaches showed that *H. perfoliatum* and *H. pubescens* are close morphologically and chemically, as well as based on the results from *rbcL* marker, although belonging to different sections (Robson et al., 2013-onwards): *Hypericum* Sect. *Drosocarpium* Spach, the former, and Sect. *Adenosepalum* Spach, the latter. Similarly, also *H. calycinum* and *H. hircinum* subsp. *majus* are morphologically and chemically close, although belonging to different sections (Robson et al., 2013-onwards): *Hypericum* Sect. *Ascyreia* Choisy, the former, and Sect. *Androsaemum* (Duhamel) Godron, the latter.

Biochemical analyses confirmed the relevant amounts in bioactive metabolites of the studied taxa, assessing the high quality of the investigated materials. Furthermore, *H. perfoliatum* showed values very close to *H. perforatum*, allowing to be suggested as a potential alternative to the former. Wild populations from Sicily confirmed their suitability to straightforward cultivation, aimed to obtain high-quality plant material. It appeared necessary, however, to perform thorough biochemical screenings, extended to a larger number of populations.

Table 3. Performance of each barcoding marker tested on *Hypericum* sp. pl., in single and in multi locus. The species level resolution percentage is calculated on the successfully obtained sequences.

	<i>rbcL</i>	<i>matK</i>	<i>trnH-psbA</i>			
PCR success	100% (10/10)	60% (6/10)	80% (8/10)			
Sequencing success (contigs)	90% (9/10)	83% (5/6)	87% (7/8)			
Sequence quality (contigs)	91%	81%	84%			
Fragment length (bp average)	529	813	498	<i>rbcL+matK</i>	<i>rbcL+trnH-psbA</i>	<i>matK+trnH-psbA</i>
Specie level resolution	55% (5/9)	80% (4/5)	100% (7/7)	70% (7/10)	80% (8/10)	70% (7/10)
GD average N (K2P%)	2.1	9.5	9.5			
N (total seq compared)	23	14	26			
Variable sites	55/529	222/813	253/751			

The results indicate the effectiveness of DNA barcoding in discriminating the taxa of *Hypericum*, suggesting the possibility to build a fast and accurate molecular identification method. This finding may be greatly helpful in view of taxa identification, even from herbal formulations.

According to the principal component analysis, there was no statistically significant morphological variation in the populations originally recognized as *H. perforatum* subsp. *perforatum* and *H. perforatum* subsp. *veronense*, collected in 4 different localities. Furthermore, none of the applied techniques was able to distinguish the populations

of *H. perforatum* subsp. *veronense* from those populations that, based on their morphological traits, had been formerly attributed to *H. perforatum* subsp. *perforatum*. Hence, it is possible to attribute all the *H. perforatum* studies populations to *H. perforatum* subsp. *veronense*, and exclude the presence in Sicily of *H. perforatum* subsp. *perforatum*.

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Supplementary Information 1. The mean values of the measures of the 11 quantitative morphological characters considered.

Supplementary Information 2. Mean values of the 20 chemical determinations used for multivariate analyses and their totals.

Supplementary Information 3. Pearson correlation coefficients (r) among the 11 quantitative morphological characters measured.