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Genetic Diversity in *Hypericum* and AFLP Markers for Species-Specific Identification of *H. perforatum* L.

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Abstract

One of the top-selling medicinal products worldwide is *Hypericum perforatum* (St. John's Wort). Despite its cosmopolitan distribution and utilization, little is known regarding the relationship of the bioactive compounds in *H. perforatum* to the plants from which they are purportedly derived. In this study, amplified fragment length polymorphism (AFLP) analysis of 56 *Hypericum* accessions, representing 11 species, was conducted to gain a better understanding of diversity within *Hypericum* species, especially within cultivated accessions of *H. perforatum*, and to establish a molecular methodology that will provide breeders and regulators with a simple, affordable, and accurate tool with which to identify purported *H. perforatum* material. Utilizing four primer combinations, a total of 298 polymorphic markers were generated, of which 17 were present in all *H. perforatum* accessions and 2 were specific to only *H. perforatum*. This study demonstrates that AFLP can be utilized not only to determine the relationships of closely related *Hypericum* accessions, but as a tool to authenticate material in herbal remedies through the use of genetic fingerprinting.

Keywords

Hypericum; Hypericaceae; marker analysis; genetic diversity; St. John's wort

Introduction

The genus *Hypericum* L., family Hypericaceae, is composed of approximately 450 species of trees, shrubs, and herbs widely distributed in temperate regions across the globe [1]. Originally native to southern Europe, *H. perforatum* is commonly found throughout temperate regions of both the northern and southern hemispheres [2]. Classified within the second largest section (Hypericum) of the genus, *H. perforatum*, commonly known as St. John's wort, is the best known species of the family. *Hypericum perforatum* has been suggested to have originated from the ancient hybridization and subsequent polyploidization of two diploids (2n = 2× = 16), *H. maculatum* subsp. *maculatum* Crantz and *H. attenuatum* Choisy [3]. It is a facultative apomict, as both sexual and aposporic processes can take place on the same plant [4]. While most *H. perforatum* individuals generated through apomixis are tetraploid (2n = 4× = 32) there are hexaploid (2n = 6× = 48), diploid (2n = 2× = 16), and aneuploid individuals as well [5], [6], [7].

Hypericum perforatum's biological extracts are widely recognized as valuable phytopharmaceutical agents with antiviral capabilities [8], and the potential to treat maladies

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such as depression, skin wounds, and burns [9]. *Hypericum perforatum* contains at least ten classes of biologically active compounds [10], of which two of the more important bioactive compounds, hypericin and hyperforin, are broadly variable in biological activity in humans [11]. Research indicates that these compounds vary in concentration and or constituency depending on species origin, tissue type, genetics, and environmental factors [11]. In addition, concentrations of these compounds can vary widely between accessions derived from the same species [12], [13].

Quality control involved with the production and distribution of phytopharmaceutical medicines has not been highly regulated with respect to species of plants being used in the preparation of commercial products and the concentration of bioactive compounds. Moreover, the technology available for identification of *H. perforatum* plant material in commercially available products is not standardized and thus variation between products is an issue [14]. Because of the importance of *H. perforatum* to the phytopharmaceutical industry, it is important to develop a reliable marker system that can be used to affordably and accurately identify plant material purported to be *H. perforatum* in order to aid producers while protecting consumers from potentially adulterated products.

Studies conducted by Arnholdt-Schmidt [15] and Mayo et al. [7] demonstrated that techniques such as RAPD (random amplification of polymorphic DNA) and AFLP (amplified fragment length polymorphism) analysis, would enable the elucidation of genetic diversity in wild populations of *Hypericum* spp. In this study, AFLP analysis was used to describe patterns of genetic variation and distribution within and among wild and commercially cultivated accessions of *H. perforatum*, and additionally, to develop a suite of species-specific markers that can be used to identify *H. perforatum* plant material. AFLP analysis is a whole-genome approach that has broad applicability in determining genetic variability within and among plant populations [17], crop origins [18], and relationships among cultivars [9]. AFLP markers are highly repeatable [19], provide broad genomic coverage and a virtually limitless number of genetic markers. Using AFLP technology, we identify two monomorphic and 28 polymorphic species-specific markers that can be used to accurately identify plant material purported to be *H. perforatum*.

Materials and Methods

Hypericum spp. were obtained from the North Central Regional Plant Introduction Station in Ames, Iowa (Table 1). Fifty-six *Hypericum* accessions from three different continents were studied, including 11 different species, 38 wild-collected and four cultivated accessions of *H. perforatum*, and two accessions of the outgroup *Triadenum walteri* [20]. The taxonomic identities of these accessions follow the systematic treatment used in the Germplasm Resources Information Network database, http://www.ars-grin.gov/npgs, except that accessions of *H. perforatum* were identified to subspecies on the basis of available herbarium vouchers, digital images, living plants, and geographic origin by following Robson's (2002) key [21]. Leaf material was obtained from three individual plants per accession, flash frozen in liquid nitrogen, and stored at −80°C prior to DNA extraction.

Total genomic DNA was extracted from leaf tissue using the DNeasy Plant Mini kit (Qiagen Inc.; Valencia, CA, USA) in accordance with the supplied protocol and quantified using a Nanodrop (Nanodrop Technologies; Wilmington, DE, USA) spectrophotometer. Amplified fragment length polymorphism (AFLP) analysis was run on each sample and its technical replicate in accordance to Vos et al. [16], with modifications to include slight differences in adapter and primer sequences (Table 2). Digestion, ligation, pre-selective and selective amplifications were performed as in Hawkins et al. [22]. Following amplifications, samples

were submitted to the DNA facility of the Iowa State University and run on an ABI 3100 Genetic Analyzer (Applied Biosystems; Foster City, CA, USA).

AFLP banding patterns were visualized with Genographer 1.6.0 [23]. For analytical purposes, bands of the same size were considered homologous, even though it is possible that some bands of the same size may actually represent non-homologous genomic fragments. Visual comparisons between three biological replicates, as well as two technical replicates, were used to determine reproducibility. Bands absent from two of the three biological replicates and their corresponding technical replicates were excluded from the study. Homologous bands were scored for presence (1) or absence (0).

To visualize relationships among accessions, Neighbor-joining analysis was conducted in Paup* version 4.0 [24], using the 56 accessions of *Hypericum* spp. and rooting with two accessions of *Triadenum walteri*. Default settings were employed, except “Break ties” was set to “randomly” and distances were calculated using Nei's [25] restriction-site distances. Branch support was assessed through the implementation of 5000 bootstrap replicates. Principal coordinate analysis (PCO) was performed with NTSYS-pc [26] to obtain an additional visual representation of patterns of genetic variation in the wild and cultivated material and to explore possible relationships with geography. Genetic diversity within *H. perforatum* was hierarchically partitioned using analysis of molecular variance (AMOVA) [27] in the GenAlEx program [28].

Results

AFLP markers were generated for 56 accessions of *Hypericum* spp. and 2 accessions of *Triadenum walteri*. Four AFLP primer combinations produced a total of 298 easily scored and reproducible markers. Within the 42 *H. perforatum* accessions, 221 markers were generated, of which 204 (92%) are polymorphic and 17 (8%) are present (monomorphic) in all accessions. Of the 17 monomorphic markers, only two (IVC134 and IVC335) were specific to *H. perforatum*, while the other 15 were present in accessions outside of *H. perforatum*. However, AFLP analysis generated 28 polymorphic *H. perforatum*-specific markers (Table 3). Of these, 10 were present at a frequency of 50% or more, 9 were present in 20–49% of the accessions, and 11 were present in less than 20% of the *H. perforatum* accessions.

Neighbor-joining analysis (Fig.1) revealed a monophyletic *H. perforatum* clade supported by a bootstrap value of 80. Within the *H. perforatum* clade there is a basal monophyletic group (clade 3) composed primarily of accessions from Lithuania and supported by a bootstrap value of 99. The remainder of the *H. perforatum* accessions are sister to this basal Lithuanian group and are divided into two additional major clades (clades 1 and 2) and one minor paraphyletic group (“paraphyletic accessions”). The larger clade (clade 2) predominately contains accessions from the Czech Republic and appears to be divided into 3 groups, each with bootstrap support of 100. Three of the four domesticated *H. perforatum* accessions studied (Ames 27453–27455) are located within this clade. The remaining major clade (clade 1) is comprised of accessions representing all 4 of the sub-species found in *H. perforatum*. As expected, the *H. perforatum* clade and its sister group, the *Hypericum* spp. clade, are composed of species sharing the characteristic dark leaf glands, these containing hypericin, pseudohypericin and hyperforin.

PCO analysis on AFLP data derived from all accessions show a clear delineation between *H. perforatum* and all other accessions (Fig. 2A). Congruent with the neighbor-joining analysis, the *H. perforatum* accessions appear in a tight cluster most closely associated with other *Hypericum* spp. that produce dark glands. When only *H. perforatum* accessions are included in the PCO analysis, three separate clusters are apparent, consistent with the three major clades

recovered in the neighbor-joining analysis (Fig. 2B). Additionally, 5 accessions (Ames 27452, 27510, 27511, and 27512, and PI 325351) occupy an intermediate position in the PCO outside of the three major clusters, indicative of possible introgression. These five accessions are basal to the larger two *H. perforatum* clades in the neighbor-joining tree.

The distribution of genetic diversity within and between *Hypericum* spp. populations was explored using AMOVA. Accessions were grouped together based on region of origin and/or domestication, only polymorphic markers were employed, and the analysis examined both the global and locus-by-locus partitioning of genetic diversity (Table 4). First, AMOVA was used to evaluate partitioning of genetic diversity within and among accessions from different geographic areas. Results indicate that the majority of variation (64%) present in the *H.* spp. used for this study can be attributed to within-population differences, while 36% of the variation can be ascribed to among-population genetic variation. When only *H. perforatum* accessions are included in the AMOVA, 88% of the variation can be attributed to within-population differences due to geographic collection locations, while only 12% of the variation can explain among-populations differences. Second, if the populations are segregated by domestication, the measure of variation within-populations increased to 94%, while variation among-populations decreased to 6%. A closer look at the PCO indicates that there is a domesticated accession (Ames 27452 Elixir; Richters, Goodward, Canada) central to the 3 main groups. This could be a result of hybridization and introgression of genetic material from the other primary clusters. With this in mind, the same comparison was made while excluding Elixir. The resulting analysis indicated that the within-population variance is 11% and the among-population variance is 89%. A fourth AMOVA, consisting of only *H. perforatum* accessions and segregated into 4 populations based on relatedness as indicated by neighbor-joining analysis (accessions in clade 1 = population 1 etc.), was conducted. The among-population variance is 33% and the within-population variance is 67%.

Discussion

One obstacle facing breeders, horticulturists, researchers, and oversight agencies working with medicinals is the inability to genetically determine the source of plant material. Markers generated in this study may aid in overcoming this obstacle. Of 298 polymorphic markers generated, 17 markers are present in all *Hypericum* accessions and 30 markers are present in only *H. perforatum*. Two markers (IVC134, IVC335), are specific for *H. perforatum*, and present in all accessions studied; these may prove particularly useful for identification of *H. perforatum* plant material. Collectively, the 30 unique *H. perforatum* markers may aid breeders in determining genetic identity and source, can be employed as a tool by producers to accurately diagnose the identity of individual plant lots, and could be useful to agencies or consumer groups as a means to evaluate end-user *H. perforatum* “St. John's wort” preparations. Additionally, this molecular marker study provides the foundation for future work focused on developing species-specific primers that could be used to identify material purported to be *H. perforatum* with a single PCR reaction.

Neighbor-joining analysis supports the delineation of *Hypericum* spp. that either have or lack hypericin-containing dark glands. It is also evident from both neighbor-joining and PCO analysis that *H. perforatum* clusters tightly and separately from other species of *Hypericum*. Within *H. perforatum*, three distinct clades and one minor paraphyletic group are observed. Three of the four domesticated accessions belong to the same clade (clade 2), and two of those accessions are phylogenetically sister to one another and share boot-strap support of 100%. Interestingly, clade 2 is comprised entirely of subspecies *perforatum*, suggesting that the domesticated accessions originate from within this group. None of the domesticated accessions in our study belong to clades 1 or 3, which contain members of subspecies *perforatum* (clade 3) and a mixture of subspecies *perforatum*, *songaricum*, and *veronense* (clade 1). It is within

these clades that breeders may choose to look in order to identify new traits or increase genetic diversity within the domesticated accessions.

Accessions from regional geographic areas tend to be more closely related. However, there are multiple instances where *H. perforatum* accessions from one location are more closely associated with those from different locations. Additionally, only 12% of the total amount of genetic diversity observed can be attributed to among-population difference, indicative of high levels of gene flow between populations. For example, the presence of the California accession Ames 27490 in clade 2; given that wild populations from California are naturalized from foreign introductions, it is not surprising that this accession groups with the European, domesticated species in clade 2. Observations such as this are consistent with results previously shown in *H. perforatum* illustrating that populations from different geographic areas can and often times are more closely related [29].

Analysis of molecular variance between *H. perforatum* accessions and *Hypericum* accessions from other species indicates that there is a high level of among-population variation (36%). This indicates an abundance of variation at the genus level, which the phylogenetic and clustering analyses readily partitioned into distinctive groupings. When comparing only *H. perforatum* accessions by geographic region of collection, 88% of the variance occurs within populations. The variance due to geographic distribution was similar to variance attributed to domestication if the cultivated accession Elixir was excluded from the analysis. Elixir, which is centrally located in the PCO, is responsible for 5% of the among-population variance within the domesticated varieties. The high within-population variation exhibited in the analysis when the populations are distinguished by either geographic location or domestication, along with the findings of Maron et al. [25], encouraged us to re-analyze the data with the populations segregated in accordance with the neighbor-joining analysis. When analyzed under these conditions, the level of among-population variation is substantially increased. These findings imply dispersal of plant material outside of their original range, most likely with human assistance.

Genetic distance analysis of the AFLP data revealed that the cultivated populations studied share higher genetic identity with the Western and Central European populations (0.925) than with populations from East Europe and Asia (0.828). This could be attributed to the fact that the cultivated varieties used in this study were developed in Germany and Denmark. Additional studies involving a larger sampling of domesticated material will help distinguish these possibilities, and may shed additional light on the source(s) and number of times that *H. perforatum* has been domesticated.

While other studies have utilized a molecular approach to place *H. perforatum* within a phylogenetic framework [14], [20], [29], [30], this is the first study placing an emphasis on the relationships and diversity between both wild and cultivated accessions of *H perforatum*, within the overall phylogenetic framework of the genus *Hypericum*. This study demonstrates that there is a great deal of genetic diversity among *Hypericum* species as well as within *H. perforatum*, and that this diversity is structured phylogenetically and geographically. These data provide the foundation for future work characterizing the evolutionary history, genetic relationships, and recent domestication of St. John's wort and its closely related species.

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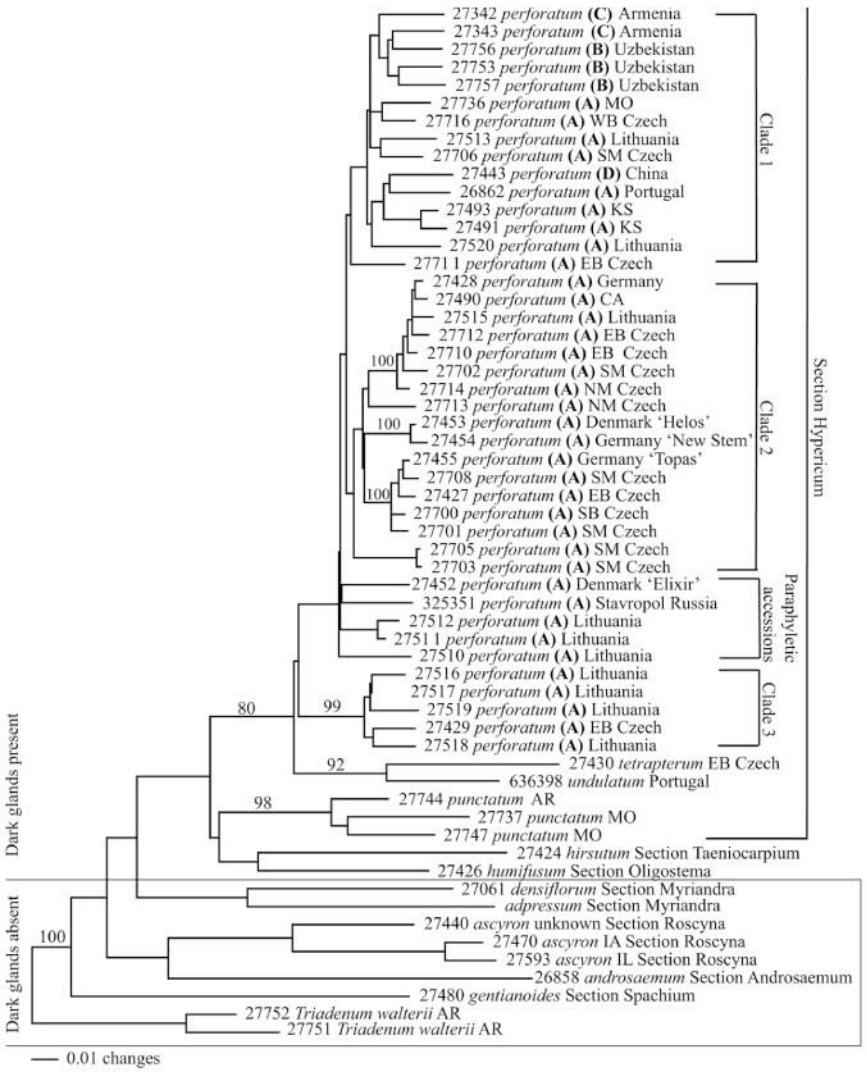

Fig. 1.

Neighbor-joining analysis of *Hypericum* spp. Bold letters represent different sub-species **A** = *perforatum*, **B** = *songaricum*, **C** = *veronense*, and **D** = *chinense*. Accessions in single quotes indicate cultivated accessions. Taxa outside of box contain dark glands. Numbers along branches denote bootstrap support.

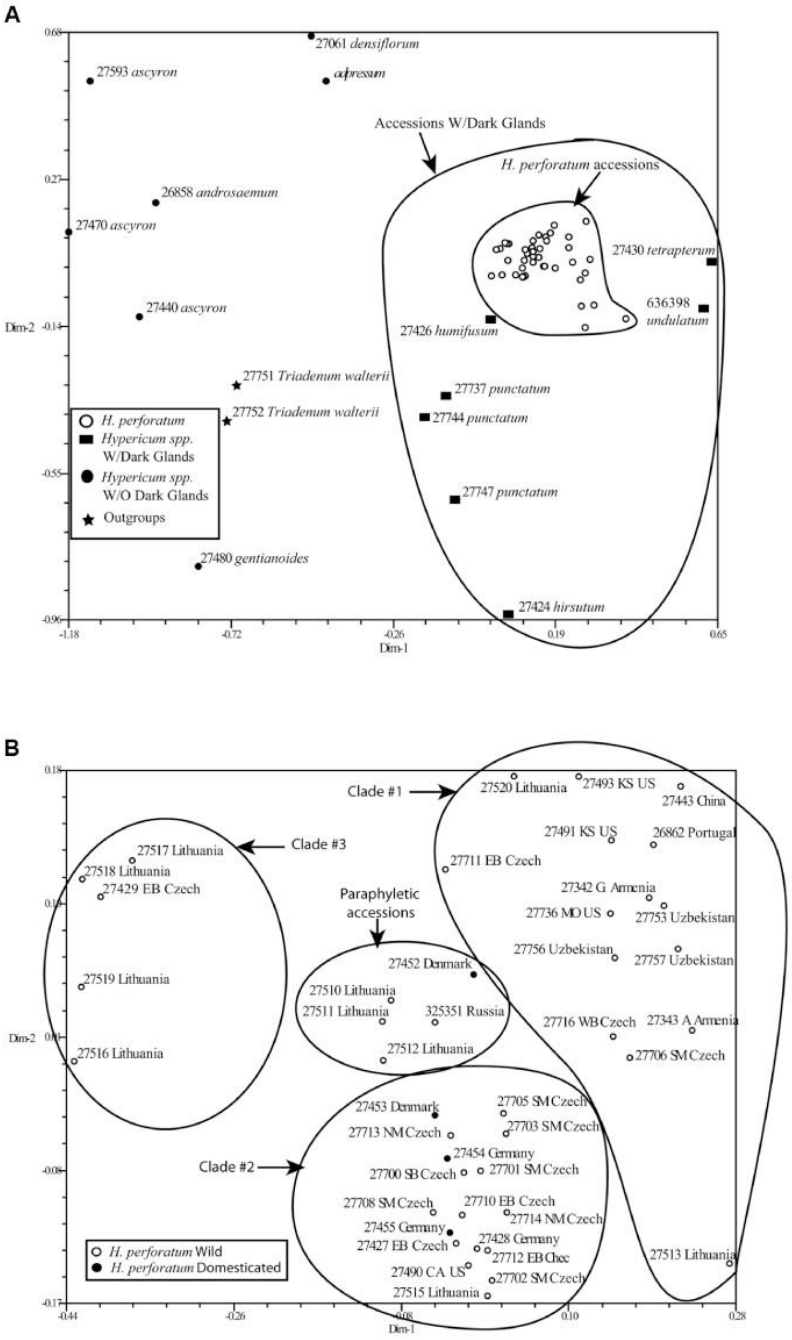

Fig. 2.

A PCO illustrating the relationship of all *Hypericum* spp. and the outgroup *Triadenum walteri*. Inner circle is composed of only *H. perforatum* accessions and outer circle contains all accessions with dark, hypericin-containing glands. **B** PCO illustrating the relationship of domesticated and non-domesticated accessions of *Hypericum perforatum*. Circles delineate accessions based upon clade designation from the neighbor-joining analysis.

Table 1

Accessions of *Hypericum* spp. studied

NCRPIS number	Species	Original location
Ames 26862	*H. perforatum* L. *perforatum*	Coimbra, Portugal 2
Ames 27342	*H. perforatum* L. *veronense*	Gegark 'unik', Armenia 3
Ames 27343	*H. perforatum* L. *veronense*	Ararat, Armenia 3
Ames 27427	*H. perforatum* L. *perforatum*	East Bohemia, Czech Republic 2
Ames 27428	*H. perforatum* L. *perforatum*	Germany 2
Ames 27429	*H. perforatum* L. *perforatum*	East Bohemia, Czech Republic 2
Ames 27443	*H. perforatum* L. *chinense*	China 3
Ames 27452	*H. perforatum* L. *perforatum* 'Elixir'	Denmark 1
Ames 27453	*H. perforatum* L. *perforatum* 'Helos'	Denmark 1
Ames 27454	*H. perforatum* L. *perforatum* 'New Stem'	Germany 1
Ames 27455	*H. perforatum* L. *perforatum* 'Topas'	Germany 1
Ames 27490	*H. perforatum* L. *perforatum*	California, United States 4
Ames 27491	*H. perforatum* L. *perforatum*	Kansas, United States 4
Ames 27493	*H. perforatum* L. *perforatum*	Kansas, United States 4
Ames 27510	*H. perforatum* L. *perforatum*	Lithuania 2
Ames 27511	*H. perforatum* L. *perforatum*	Lithuania 2
Ames 27512	*H. perforatum* L. *perforatum*	Lithuania 2
Ames 27513	*H. perforatum* L. *perforatum*	Lithuania 2
Ames 27515	*H. perforatum* L. *perforatum*	Lithuania 2
Ames 27516	*H. perforatum* L. *perforatum*	Lithuania 2
Ames 27517	*H. perforatum* L. *perforatum*	Lithuania 2
Ames 27518	*H. perforatum* L. *perforatum*	Lithuania 2
Ames 27519	*H. perforatum* L. *perforatum*	Lithuania 2
Ames 27520	*H. perforatum* L. *perforatum*	Lithuania 2
Ames 27700	*H. perforatum* L. *perforatum*	South Bohemia, Czech Republic2
Ames 27701	*H. perforatum* L. *perforatum*	South Moravia, Czech Republic 2
Ames 27702	*H. perforatum* L. *perforatum*	South Moravia, Czech Republic 2
Ames 27703	*H. perforatum* L. *perforatum*	South Moravia, Czech Republic 2
Ames 27705	*H. perforatum* L. *perforatum*	South Moravia, Czech Republic 2
Ames 27706	*H. perforatum* L. *perforatum*	South Moravia, Czech Republic 2
Ames 27708	*H. perforatum* L. *perforatum*	South Moravia, Czech Republic 2
Ames 27710	*H. perforatum* L. *perforatum*	East Bohemia, Czech Republic 2
Ames 27711	*H. perforatum* L. *perforatum*	East Bohemia, Czech Republic 2
Ames 27712	*H. perforatum* L. *perforatum*	East Bohemia, Czech Republic 2
Ames 27713	*H. perforatum* L. *perforatum*	North Moravia, Czech Republic 2
Ames 27714	*H. perforatum* L. *perforatum*	North Moravia, Czech Republic 2
Ames 27716	*H. perforatum* L. *perforatum*	West Bohemia, Czech Republic 2
Ames 27736	*H. perforatum* L. *perforatum*	Missouri, United States 4
Ames 27753	*H. perforatum* L. *songaricum*	Uzbekistan 3
Ames 27756	*H. perforatum* L. *songaricum*	Uzbekistan 3
Ames 27757	*H. perforatum* L. *songaricum*	Uzbekistan 3
PI 325351	*H. perforatum* L. *perforatum*	Stavropol Region, Russia 3
Ames 27430	*H. tetrapterum* Fr.	East Bohemia, Czech Republic
PI 636398	*H. undulatum* Schousb.	Coimbra, Portugal
Ames 27737	*H. punctatum* Lam.	Missouri, United States
Ames 27744	*H. punctatum* Lam.	Arkansas, United States
Ames 27747	*H. punctatum* Lam.	Missouri, United States
Ames 27424	*H. hirsutum* L.	Central Bohemia, Czech Rep.
Ames 27426	*H. humifusum* L.	Central Bohemia, Czech Rep.
Ames 27061	*H. densiflorum* Pursh	Tennessee, United States
Unknown	*H. adpressum* W. P. C. Barton	Unknown
Ames 27440	*H. ascyron subsp. pyramidatum* N. Robson	Unknown
Ames 27470	*H. ascyron subsp. pyramidatum* N. Robson	Iowa, United States
Ames 27593	*H. ascyron subsp. pyramidatum* N. Robson	Illinois, United States
Ames 26858	*H. androsaemum* L.	Coimbra, Portugal
Ames 27480	*H. gentianoides* L.	Florida, United States
Ames 27751	*Triadenum walteri* (J. G. Gmel.)	Arkansas, United States
Ames 27752	*Triadenum walteri* (J. G. Gmel.)	Arkansas, United States

Accession collection locations of *Hypericum* spp. are designated as follows: 1 = domesticated, 2 = Europe, 3 = East Europe/Asia, 4 = United States of America.

Table 2

AFLP primer and adapter sequences

Adapters	**5′-Sequence-3′**
EcoRI forward adapter	CTC GTA TAC TGC GTA CC
EcoRI reverse adapter	AAT TGG TAC GCA GTA
Msel forward adapter	GAC GAT GAG TCC TGA G
Msel reverse adapter	TAC TCA GGA CTC ATC
+ 1 Pre-selective primers	
EcoRI + A	TAC TGC GTA CCA ATT C – **A**
Msel + C	GAC GAT GAG TCC TGA GTA A – **C**
+ 3 Selective primers	
Msel + CAA (I)	GAC GAT GAG TCC TGA GTA A – **CAA**
Msel + CAC (III, IV)	GAC GAT GAG TCC TGA GTA A – **CAC**
EcoRI + AGC (A)	(FAM) - TAC TGC GTA CCA ATT C – **AGC**
EcoRI + ACG (B)	(HEX) - TAC TGC GTA CCA ATT C – **ACG**
EcoRI + AAC (C)	(HEX) - TAC TGC GTA CCA ATT C – **AAC**

Four + 3 selective amplifications, designated IA, IIIA, IIIB, and IVC, were performed. Roman numerals represent non-labeled selective primers and the letters “A, B, or C” represent the 5′ FAM or 5′ HEX labeled primers with bold-type indicating selective nucleotides.

Table 3

Markers detected in *H. perforatum* as revealed by AFLP analysis

Marker	% present	Accessions positive for markers
Markers present only in *H. perforatum* (30)		
IA258	Polymorphic (40 %)	27427, 27429, 27453, 27454, 27455, 27510, 27511, 27512, 27516, 27517, 27518, 27519, 27700, 27701, 27703, 27705, 27708
IA320	Polymorphic (2 %)	27515
IA321	#Polymorphic (31 %)	27427, 27428, 27455, 27490, 27515, 27700, 27701, 27702, 27708, 27710, 27712, 27713, 27714
IA323	Polymorphic (7 %)	27452, 27511, 27512
IA360	Polymorphic (21 %)	27443, 27452, 27491, 27493, 27706, 27716, 27736, 27756, 27757
IA409	Polymorphic (62 %)	All accessions except: 26862, 27429, 27443, 27452, 27453, 27454, 27491, 27493, 27516, 27517, 27518, 27519, 27706, 27711, 27713, 27753
IA415	Polymorphic (36 %)	27427, 27453, 27454, 27455, 27511, 27512, 27700, 27701, 27703, 27705, 27708, 27716, 27736, 27757, 325351
IA430	Polymorphic (7 %)	27517, 27519, 27713
IIIA321	Polymorphic (2 %)	27443
IIIA358	Polymorphic (33 %)	26862, 27428, 27490, 27491, 27493, 27513, 27515, 27520, 27702, 27710, 27711, 27712, 27714, 27736
IIIA378	Polymorphic (74 %)	All accessions except: 26862, 27429, 27491, 27493, 27516, 27517, 27518, 27519, 27520, 27736, 27756
IIIA398	Polymorphic (62 %)	All accessions except: 27342, 27427, 27443, 27452, 27513, 27520, 27700, 27701, 27702, 27706, 27716, 27736, 27753, 27756, 27757, 325351
IIIA467	Polymorphic (14 %)	27342, 27428, 27453, 27454, 27706, 27712
IIIB200	Polymorphic (83 %)	All accessions except: 26862, 27343, 27513, 27736, 27753, 27756, 27757
IIB245	Polymorphic (71 %)	All accessions except: 26862, 27429, 27452, 27491, 27493, 27510, 27516, 27517, 27518, 27519, 27704, 27711, 27757
IIIB286	Polymorphic (21 %)	27455, 27516, 27518, 27519, 27700, 27701, 27708, 27713, 325351
IIIB288	Polymorphic (36 %)	27427, 27428, 27429, 27453, 27454, 27490,
IIIB320	Polymorphic (14 %)	27427, 27428, 27490, 27702, 27710, 27712
IIIB389	Polymorphic (33 %)	27343, 27427, 27428, 27452, 27490, 27491, 27493, 27510, 27515, 27702, 27710, 27712, 27714, 27753
IIIB390	Polymorphic (52 %)	27427, 27428, 27452, 27453, 27454, 27455, 27490, 27513, 27515, 27700, 27701, 27702, 27703, 27705, 27706, 27708, 27710, 27712, 27713, 27714, 27716, 325351
IIIB404	Polymorphic (2 %)	26862
IIIB473	Polymorphic (2 %)	27490
IVC134	Monomorphic (100 %)	All accessions
IVC253	Polymorphic (29 %)	27427, 27453, 27454, 27455, 27511, 27512, 27700, 27701, 27703, 27705, 27708, 27716
IVC254	Polymorphic (5 %)	27511, 27512
IVC259	Polymorphic (16 %)	27427, 27455, 27700, 27701, 27708, 27736
IVC335	Monomorphic (100 %)	All accessions
IVC355	Polymorphic (52 %)	27427, 27428, 27455, 27490, 27491, 27493, 27510, 27512, 27513, 27515, 27519, 27700, 27701, 27702, 27706, 27708, 27710, 27712, 27713, 27714, 27716, 27736
IVC383	Polymorphic (10 %)	27453, 27454, 27736, 325351
IVC412	Polymorphic (50 %)	27342, 27343, 27428, 27453, 27454, 27490, 27491, 27493, 27513, 27515, 27701, 27702, 27703, 27705, 27706, 27710, 27712, 27714, 27716, 27736, 27753

Markers present in all *H. perforatum* accessions (17)

IA119, IA127, IA185, IA222, IA290, IIIA106, IIIA121, IIIA222, IIIA249, IIIB271, IVC114, IVC118, IVC134, IVC202, IVC217, IVC310, IVC335

Table 4

Analysis of molecular variance (AMOVA) for *Hypericum* spp.

Source of variation	d.f.	Sum of Squares	Variance components	% Total variance	Probability
Comparison between *H. perforatum* and other *Hypericum* species					
Among-population	1	454.19	19.95	36%	0.001
Within-population	54	1905.67	35.29	64%	
Total	55	2359.86			
Comparison of *H. perforatum* accessions by geographic collection locations (Domesticated, Europe, East Europe/Asia, and US)					
Among-populations	3	178.97	4.00	12%	0.002
Within-populations	38	1117.70	29.41	88%	
Total	41	1296.67			
Comparison of *H. perforatum* accessions by domestication state (Domesticated, Wild)					
Among-populations	1	46.17	2.06	6%	0.073
Within-populations	40	1250.50	31.26	94%	
Total	41	1296.67			
Comparison of *H. perforatum* accessions by domestication excluding 27452 ‘Elixir’ (Domesticated, Wild)					
Among-populations	1	52.73	3.90	11%	0.034
Within-populations	39	1210.00	31.03	89%	
Total	40	1262.73			
Comparison of *H. perforatum* accessions by distribution within neighbor-joining analysis					
Among-populations	3	403.40	11.65	33%	0.001
Within-populations	38	893.27	23.51	67%	
Total	41	1296.67			

Genetic distance within *H. perforatum* was measured using Nei's unbiased measure of genetic distance. The highest estimate of genetic distance (0.187) was between domesticated accessions and accessions from the Eastern Europe/Asia region. While the lowest estimate of genetic distance (0.077) was between domesticated accessions and European accessions.