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Chemical variation within and among six northern willow species

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Abstract

Plant tissues typically contain a diverse complement of secondary metabolites that serve as protection against various biotic and abiotic hazards. Chemical similarities are commonly used to infer phylogenetic relationships among plant taxa, but the studies are typically based on the mean concentration of each compound in each study species, thus overlooking within-species variability. In order to investigate patterns of intra- and interspecific chemical variation in plants, we measured the concentrations of condensed tannins and 36 other phenolic compounds in 120 leaf samples representing six northern *Salix* species. Multivariate clustering and ordination analyses of the data show that: (1) Despite considerable within-species variation in chemical profiles, intraspecific variability is on average lower than the variation among species. (2) Interspecific similarities are sensitive to the data analysis methods used, and different chemical classes produce partly contradictory results. (3) Compounds within each biosynthetic class tend to behave in a correlated manner and, consequently, overall chemical similarities are weakly correlated with the phylogeny of the studied species. The conclusion is that chemical data are poorly suited for phylogenetic inference, unless methods for data analysis are improved to take into account the biosynthetic routes by which the compounds are produced.

Keywords: *Salix*, Salicaceae; Willows; HPLC; Multivariate analysis; Chemosystematics; Herbivory; Phenolics; Salicylates; Condensed tannins

1. Introduction

In addition to the multitude of compounds that are needed for primary metabolic functions, plants produce a diverse array of chemicals that are collectively referred to as secondary metabolites (Seigler, 1998; Wink, 2003). Many of these compounds protect plants against abiotic hazards such as UV-radiation (Koes et al., 1993), or act as toxins and deterrents against herbivores and pathogens (Berenbaum, 1995; Hartmann, 1999; Wink, 2003). However, secondary compounds constitute a highly heterogeneous group which includes thousands of chemicals with varying structures that are produced via diverse and in many cases unconnected biosynthetic pathways (Berenbaum, 1995; Seigler, 1998; Wink, 2003; Martens and Mithöfer, 2005).

The taxonomic distribution of individual secondary compounds varies considerably. Some, such as many flavonoids, can be found in many distantly related plant groups (Harborne and Turner, 1984; Wink, 2003; Pelser et al., 2005; Martens and Mithöfer, 2005), whereas others may occur only in a few, closely related taxa or even in a single species (Julkunen-Tiitto, 1989; Jenett-Siems et al., 2005). Chemical similarities are frequently used to infer relationships among plant taxa in so-called chemosystematic studies (e.g., Julkunen-Tiitto, 1989; Cool et al., 1998; Keinänen et al., 1999a; Santos and Salatino, 2000; Nogueira et al., 2001), but the seemingly erratic occurrence of certain compounds in highly divergent plant groups has also led to doubts about the usefulness of chemical data in phylogenetic inference (Becerra, 1997; Grayer et al., 1999; Wink, 2003). A further complication is that chemosystematic analyses are typically based on the average concentration of each compound in each study species, which is potentially dangerous if the overall variation within species is

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large. Many plant populations are known to harbour considerable variation in individual chemical profiles (Cool et al., 1998; Laitinen et al., 2000, 2005; Semmar et al., 2005; Windsor et al., 2005), but the relative level of within-species chemical variation in comparison to the variation among species remains largely unexplored.

The distribution of chemical variation within and among plant species is directly relevant for the evolutionary relationships between plants and their associated herbivores and pathogens. Especially herbivorous insects tend to utilize only one or a few related plant species, which is generally though to result from chemical differences among plant taxa (Ehrlich and Raven, 1964; Strong et al., 1984; Futuyma and Keese, 1992). However, host use is a dynamic trait which may change during the evolutionary history of an insect lineage, and factors that set the limits for host use will also influence the direction of host shifts. Phylogenetic studies have in most cases found distinct differences between the phylogenies of herbivorous insects and their host plants, but in some cases it has been possible to instead link the evolutionary history of host use to the degree of chemical similarities among host taxa (Becerra, 1997; Wahlberg, 2001).

Willows (*Salix* spp.) constitute a near-ideal model group for investigating intra- and interspecific chemical variation as well as chemistry-mediated evolutionary interactions between plants and their enemies. The genus includes over 400 species, and their ecological diversity ranges from creeping tundra species to large forest trees (Argus, 1997; Skvortsov, 1999). Willow tissues characteristically contain a diverse complement of phenolic compounds such as salicylates, cinnamic acid derivatives, flavonoids, and condensed tannins (Julkunen-Tiitto, 1989; Shao, 1991), many of which have been shown to affect host selection and performance of plant-feeding insects and mammals (e.g., Tahvanainen et al., 1985a,b; Lindroth et al., 1988; Ayres et al., 1997; Roininen et al., 1999; Ikonen et al., 2002; Simmonds, 2003). The herbivore communities of individual willow species often show marked differences, possibly as a result of interspecific variation in phenolic composition (Pasteels and Rowell-Rahier, 1992; Sipura, 1999). Indeed, the mean concentrations of many phenolics differ widely among species, and several studies have attempted to infer relationships among willows based on chemical similarities (Julkunen-Tiitto, 1989; Shao, 1991). Thus far the results have generally been ambiguous or contradictory and, as in most chemosystematic studies, individual species have been represented by the mean concentration in each measured compound, effectively disregarding intraspecific variation in overall chemistry.

The purpose of this study was to gain a more detailed view of intra- and interspecific chemical variability in plants. For this, we used multivariate clustering and ordination methods to analyze a dataset consisting of measurements of the concentrations of condensed tannins and 36 low-molecular weight phenolic compounds in 120 leaf samples representing six northern willow species. Specifically, we wanted to use the dataset as a model system to explore: (1) What is the level of overall chemical variation within species in relation to the variation among species? (2) How do different data analysis methods affect the results and inferences that can be made? (3) Do different chemical classes produce consistent results with respect to among-species chemical similarities? and (4) How do patterns in overall chemical similarities conform to the phylogenetic relationships among the study species? The results are discussed in relation to chemosystematic methods and current hypotheses on the ecology and evolution of plant–herbivore interactions.

2. Results and discussion

2.1. Chemical variation within and among species

The measured compounds mainly include various flavonoids, salicylates, and cinnamic acid derivatives (Table 1), and the leaf samples represent a total of 60 willow individuals; the two leaf samples from each plant were selected so that the upper leaf had a sawfly-induced leaf gall attached to the midrib, whereas the lower leaf was ungalled (see Section 3). Regardless, galled and ungalled leaves from the same individual tend to be clustered in pairs in the Unweighted pair-group method using arithmetic averages (UPGMA) clustering dendrogram (Fig. 1(a)). The pairing is not invariable, but within-individual distances are on average lower than distances among individuals (blocked multi-response permutation procedure tests within species, all $P < 0.001$). More importantly, leaf samples representing the same willow species are grouped together (Fig. 1(a)), so that it is possible to assign each sample to the correct willow species on the basis of its phenolic profile, despite considerable within-species variation. These results are very consistent irrespective of the standardization method (maximum/z-correction) and distance measure (Euclidean/correlation) used, or inclusion or exclusion of condensed tannins (results not shown). The maximum number of samples that are misplaced with regard to the willow species is two (when a z-transformation and Euclidean distances are used).

In contrast, similarities among species are clearly sensitive to changes in data analysis parameters. *Salix phylicifolia* and *S. lapponum* are grouped together in all results, but among-species similarities in the rest of the dendrogram show considerable variability depending on the standardization method and distance measure that are used. For example, *S. glauca* is clustered together with *S. reticulata* (instead of *S. myrsinites*) if Euclidean distances are used instead of correlation distances, and other alternative groupings can also be found (results not shown). In some cases, among-species similarities in the lower part of the dendrogram also change if only ungalled leaves are included, or if condensed tannins are excluded from the dataset. However, both of these factors appear to be of minor importance in comparison to the data analysis methodology.

Table 1
Measured compounds and their correlations with the two axes in the NMS ordination in Fig. 2

Category	Compound	Axis 1	Axis 2
Condensed tannins	Condensed tannins	−0.61	0.08
Flavones	Luteolin glycoside 1	0.33	0.82
	Luteolin glycoside 2	0.77	0.12
	Luteolin-7-glucoside	0.70	0.56
	Luteolin derivative 1	0.50	0.60
	Luteolin derivative 2	0.41	0.24
	Luteolin derivative 3	0.34	0.04
	Luteolin derivative 4	0.09	0.79
	Apigenin-7-glucoside	0.66	0.68
	Apigenin derivative 1	0.57	0.08
	Apigenin derivative 2	−0.24	−0.32
Flavonols	Myricetin-3-galactoside	−0.69	−0.57
	Myricetin-3-glucoside + glucuronide	−0.80	−0.34
	Myricetin-3-arabinopyranoside	−0.78	−0.51
	Myricetin-3-rhamnoside + arabinofuranoside	−0.74	−0.23
	Quercetin-3-galactoside	−0.60	−0.59
	Quercetin-3-glucoside + glucuronide	−0.19	−0.60
	Quercetin-3-arabinopyranoside	−0.65	−0.57
	Quercetin-3-arabinofuranoside	−0.65	−0.66
	Quercetin glycoside 1	−0.57	−0.10
	Quercetin-3-rhamnoside	−0.68	−0.53
	Isorhamnetin-3-glucoside	−0.22	−0.16
Other flavonoids	Ampelopsin	−0.53	−0.01
	Ampelopsin derivative 1	−0.42	0.04
Salicylates	Salicin	0.67	−0.19
	Salicortin	0.73	−0.31
	Tremulacin	0.71	−0.07
Cinnamic acid derivatives	Chlorogenic acid	0.49	0.48
	Cinnamic acid derivative 1	0.82	0.14
	p-Hydroxycinnamic acid	0.74	0.09
	Cinnamic acid derivative 2	0.57	−0.08
Unclassified phenolics	Picein derivative 1	−0.58	0.27
	Picein	−0.06	0.56
	Gallic acid derivative 1	0.27	0.58
	Triandrin	−0.11	0.49
	Unknown 1	0.67	−0.12
	Unknown 2	−0.25	−0.36

The clustering analyses were also performed after dividing the data into separate chemical classes and, indeed, the UPGMA clustering dendrograms differ depending on whether the clustering is done on the basis of flavonoids (Fig. 1(b)) or other low-molecular weight phenolics (Fig. 1(c)). For example, the flavonoid dataset groups *S. borealis* with *S. phylicifolia* and *S. lapponum*, whereas *S. borealis* is grouped with *S. myrsinites* on the basis of other phenolics, and other changes are also evident (Fig. 1(b) and (c)). The integrity of most species clusters still remains, but especially samples from *S. phylicifolia* and *S. lapponum* tend to be intermixed if the clustering is based on only non-flavonoids.

As expected, ungalled leaf samples from the same willow species form relatively clear groups also in the Nonmetric multidimensional scaling (NMS) ordination based on Euclidean distances and a maximum correction for each compound (Fig. 2). Again, *S. phylicifolia* and *S. lapponum* are grouped together in all analyses, while the relative positions of the other species are more sensitive to changes in standardization procedures and distance measures (results not shown). Correlation coefficients of the measured concentrations with the NMS ordination axes reveal that compounds in any given compound class tend to behave in an interdependent manner: there is a positive correlation between the concentrations of most flavones and both ordination axes, whereas the correlation coefficients are negative for all flavonols (Table 1). Similarly, there is a coordinated pattern within salicylates and cinnamic acid derivatives, whereas responses are more variable in the 'unclassified phenolics', which do not form a distinct biosynthetic class (Table 1).

2.2. Implications for herbivory

Chemical variability among plant species, among individuals within species, and among tissues within individuals are probably the main factors directing diet preferences of

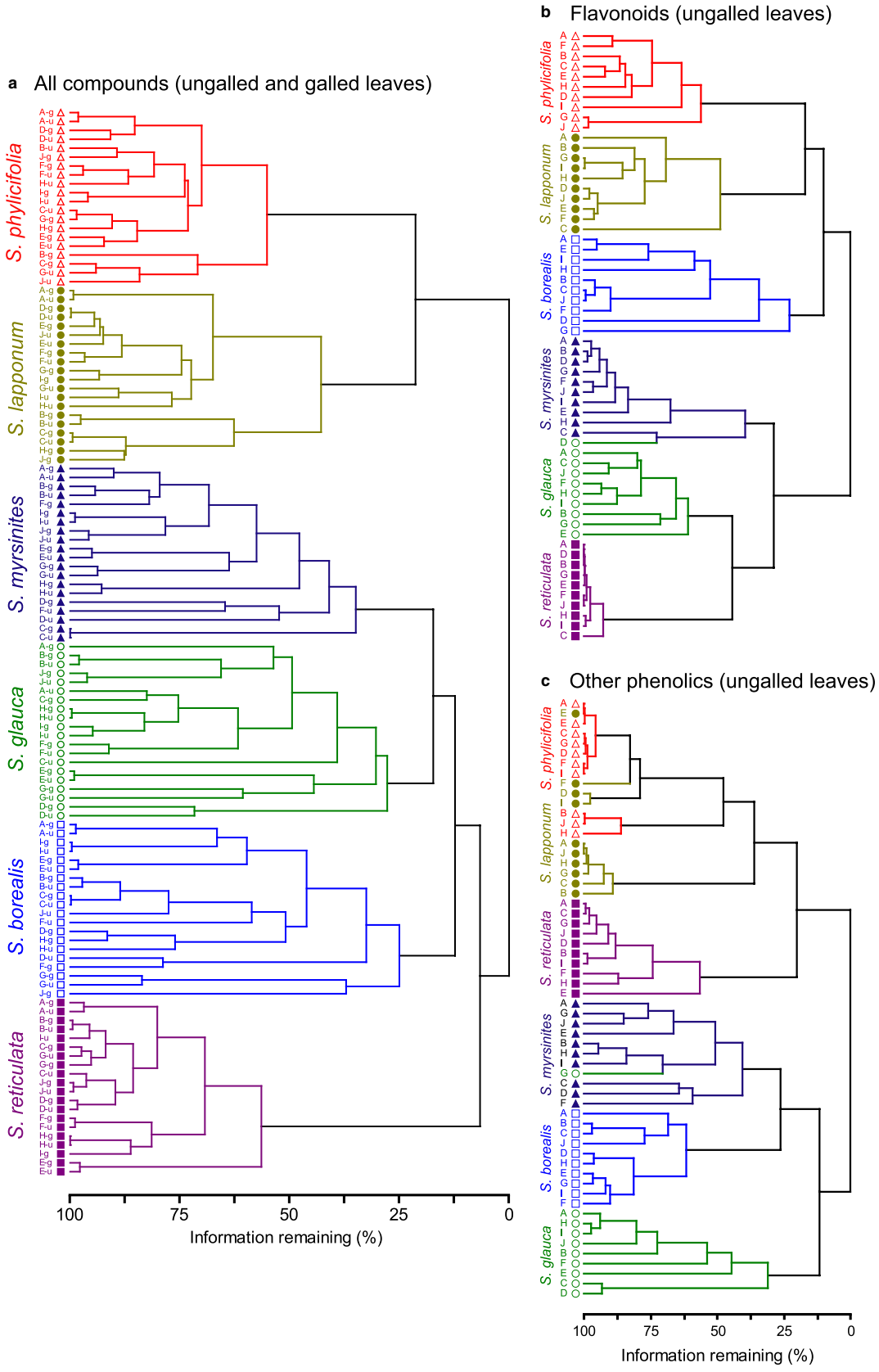

Fig. 1. UPGMA clustering dendrograms of willow leaf samples based on z-transformed data and correlation distances, (a) dendrogram of all leaf samples on the basis of all measured compounds; (b) dendrogram of ungalled leaves on the basis of flavonoids; (c) dendrogram of ungalled leaves on the basis of other low-molecular weight phenolic compounds. Capital letters refer to willow individuals (A–J within each species), lowercase letters in (a) denote sample types (g, galled leaf; u, ungalled leaf). Condensed tannins were excluded in (b) and (c).

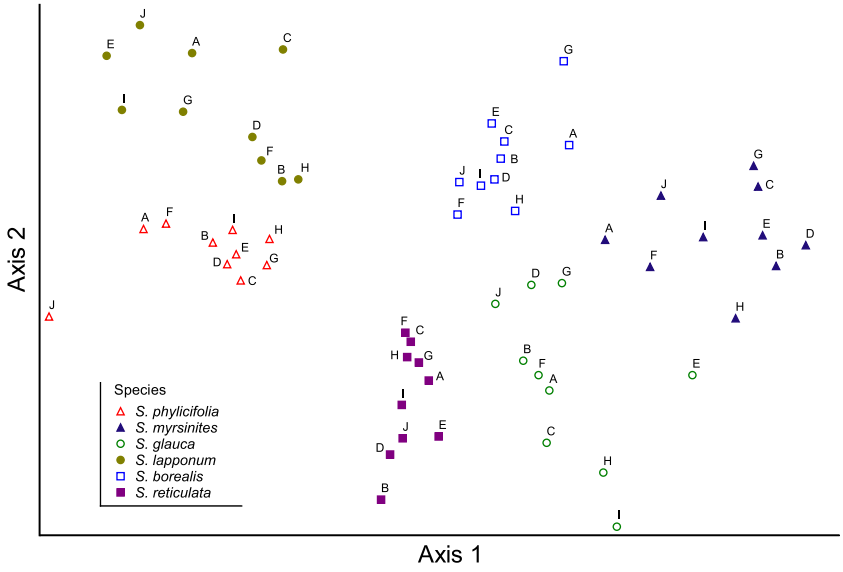

Fig. 2. Nonmetric multidimensional scaling (NMS) ordination of ungalled leaves, when concentrations of the measured compounds are scaled to the maximum (maximum = 1), and inter-sample distances are measured by Euclidean distances. Capital letters indicate willow individuals (A–J within each species). Axes 1 and 2 explain 68.3% and 19.0% of the variance in the original distance matrix, respectively (87.4% combined).

most herbivores (Ehrlich and Raven, 1964; Futuyma and Keese, 1992; Hemming and Lindroth, 1995; Ruel and Ayres, 1999). As stated above, many of the phenolic compounds analyzed in our study have been shown to influence host selection and performance of plant-feeding insects (e.g., Tahvanainen et al., 1985b; Lindroth et al., 1988; Kolehmainen et al., 1994, 1995; Ayres et al., 1997; Roininen et al., 1999; Ikonen et al., 2002; Simmonds, 2003). Consequently, the way in which the overall chemical variation is distributed should have an effect on the ecological and evolutionary interactions between willows and their natural enemies.

With regard to their foliar chemistry, each willow species in our study apparently constitutes a distinct 'chemical cluster', which could provide the basis for the distinct host specificity of many willow-associated herbivores (Pasteels and Rowell-Rahier, 1992; Nyman et al., 2000; Roininen et al., 2005). In contrast, interspecific chemical similarities are ambiguous and weakly correlated with the phylogeny of the study species (see below), which may explain why the phylogenies of willows and specialist herbivores have been found to be widely discordant (Roininen et al., 1993; Nyman et al., 2000; Nyman, 2002). However, it must be noted that attempts to relate macroevolutionary patterns of host shifts by insects to overall chemical similarities among willow species have likewise been unsuccessful (Roininen et al., 1993, 2005; Nyman et al., 2000; Nyman, 2002) although a few examples have been found in other insect–host systems (Becerra, 1997, 2003; Wahlberg, 2001). There are at least two possible reasons for this: First, overall chemical similarities can be informative for host identification if the process involves sensory input from multiple deterrent and attractant compounds (Tahvanainen et al., 1985b; Städler et al., 2002), but resistance to herbivory, as well as host identification, can also be based on only one or a few chemicals (Futuyma and Keese, 1992; Kolehmainen et al., 1994; Roininen et al., 1999). Second, among-species similarities depend on the chemical class that is under investigation (Fig. 1(b) and (c)), while the relative toxicities of compound classes may differ (Tahvanainen et al., 1985b; Berenbaum, 1995; Ayres et al., 1997; Ruuhola et al., 2001). When at the same time insect species differ in their sensitivity to various secondary compounds (Futuyma and Keese, 1992; Kolehmainen et al., 1995; Ayres et al., 1997), it may be difficult or even impossible to find general rules governing host shifts in plant-feeding insects. Nevertheless, insect communities are sometimes correlated with chemical similarities among hosts (Abrahamson et al., 2003), so the results presented here can provide useful background information for interpreting community-level patterns in willow-associated folivores in northern Fennoscandia.

2.3. Chemical similarities and phylogenetic inference

The results are more disconcerting from a chemosystematic viewpoint, because inferred among-species similarities are sensitive to changes in data analysis procedures and depend on the compound class under investigation. Consequently, it is hardly surprising that previous chemosystematic analyses of *Salix* based on different sets of compounds have led to conflicting results (Julkunen-Tiitto, 1989; Shao, 1991; see also Roininen et al., 1993). Willows have also proven to be an extremely challenging group for morphological (Argus, 1997; Skvortsov, 1999) and molecular (Leskinen and Alström-Rapaport, 1999; Azuma et al., 2000) phylogenetic analyses, but it is clear that the chemical similarities observed

in our study conflict with the relationships of the study species. For example, *S. phylicifolia* is phylogenetically closer to *S. borealis*, which exhibits a very dissimilar chemical profile, than to *S. lapponum*, which is chemically close. The similarities in the rest of the dendrograms (Fig. 1) also disagree with current classifications (cf. Argus, 1997; Skvortsov, 1999).

The loose connection between willow phylogeny and overall chemical similarities is apparently mainly explained by the production of individual compounds via fixed biosynthetic pathways in plant tissues. Especially the coordinated behaviour of most flavones and flavonols (Table 1) follows from the fact that compounds in both of these classes are formed via distinct branching points in the phenylpropanoid pathway (Koes et al., 1993; Seigler, 1998; Burbulis and Winkel-Shirley, 1999; Martens and Mithöfer, 2005). For example, a mutation that increases the activity of flavone synthase will lead to an increase in substrates for all enzymes that catalyze the production of diverse flavone glycosides (see Seigler, 1998; Keinänen et al., 1999b; Martens and Mithöfer, 2005). Such a change would simultaneously drain precursors from the production of compounds such as flavonols that are synthesized 'downstream' in the pathway. The observed opposite patterns in these two flavonoid groups (Table 1) are indeed indicative of trade-offs in the production of alternative end products along the flavonoid biosynthetic route, a result which mirrors results from previous studies on intraspecific chemical variation in plants (Keinänen et al., 1999b; Semmar et al., 2005). The same complication naturally applies to the whole phenylpropanoid pathway (and other biosynthetic routes), meaning that small genetic changes can cause large shifts in the chemical properties of plant individuals and species. Overall chemical similarities have rarely been contrasted with phylogenetic information derived from DNA sequence data, but the few studies done this far have consistently demonstrated that closely related plant species can have highly divergent chemical profiles (Becerra, 1997, 2003; Pelser et al., 2005; Windsor et al., 2005).

In conclusion, our results support Wink's (2003) notion that chemical data should be used cautiously when inferring phylogenetic relationships among plant taxa (see also Grayer et al., 1999; Erpenbeck and van Soest, 2005). Standard phylogenetic inference methods assume that the characters that are used for phylogeny reconstruction evolve independently (Sneath and Sokal, 1973; O'Keefe and Wagner, 2001), but this central assumption is directly violated by the production of secondary metabolites via coordinated biosynthetic pathways. The problem can partly be alleviated by analyzing as many compounds as possible, but the only real solution is to develop statistical corrections that take into account known biosynthetic routes and the resulting interdependencies within and among compound classes. The development of suitable methods for the analysis of chemosystematic data still in its infancy.

3. Experimental

3.1. Sample collection

Leaf samples were collected from the surroundings of the Kilpisjärvi Biological Research Station (69°3′N, 20°48′E) in Finnish Lapland. Of the six representative species, *S. phylicifolia* L., *S. borealis* (Fr.), and *S. lapponum* L. belong to the subgenus Vetrix (sections Arbuscella, Nigricantes, and Villosae, respectively), whereas *S. glauca* L., *S. myrsinites* L., and *S. reticulata* L. represent subgenus Chamaetia (sections Glaucae, Myrtosalix, and Chamaetia, respectively) (Skvortsov, 1999). The sampled species are common and ecologically prominent in northern Fennoscandia, but their habitats differ considerably: *Salix phylicifolia*, *S. lapponum*, *S. glauca*, and *S. borealis* grow mainly in the subalpine forests dominated by mountain birch (*Betula pubescens* Ehrh. ssp. *czerepanovii* (N. I. Orlova) Hämet-Ahti), whereas *S. myrsinites* mainly inhabits more open subalpine areas, and the creeping *S. reticulata* grows in the treeless *regio alpina*. Ten randomly selected but widely spaced (>50 m) willow individuals were sampled of each species, and two leaves were collected from each individual. The leaves were selected so that the upper leaf had a *Pontania* sawfly gall in the midrib, whereas the lower leaf was ungalled (Nyman and Julkunen-Tiitto, 2000). Only leaf blade samples were used for this study, so the samples do not include galled tissues. The samples were air-dried and then stored at −20 °C until analysis.

3.2. Chemical analyses

Details of compound identification and quantification are described in Nyman and Julkunen-Tiitto (2000), which also gives the mean concentration (with standard errors) of each compound in each study species. In brief, phenolics were extracted with methanol, and low-molecular weight phenolic compounds were quantified by high-performance liquid chromatography (HPLC-DAD). Compounds were identified on the basis of their retention times and UV spectra, and chromatograms were cross-aligned to confirm peak correspondence across samples and species. The measured compounds mainly represent various flavonoids, salicylates, and cinnamic acid derivatives (Table 1). In addition, the levels of condensed tannins were analyzed by a butanol/HCl-colorimetric tannin assay (Hagerman and Butler, 1994; Hagerman, 1995).

3.3. Data analysis

The full data matrix consists of the concentrations of 36 phenolic compounds and the overall level of condensed tannins in 120 leaf samples (6 species × 10 individuals × 2 samples). All multivariate analyses were performed with

PC-ORD version 4.33 (MjM Software, Gleneden Beach, OR). Because the program does not allow empty cells in the matrix, missing data (1.9% of the cells) was replaced by the mean concentration of each compound within each species. This reduces the overall variation within species slightly, but all analyses were also performed with a reduced matrix which includes only the 74 samples with no missing data; the results were qualitatively near-identical, so the replacement procedure evidently does not affect the results and conclusions of this study. Because galled willow leaves may have elevated concentrations of phenolic compounds (Nyman and Julkunen-Tiitto, 2000), most analyses were performed using only ungalled leaves (60 samples). Furthermore, the structure of condensed tannins is known to vary among willow species (Ayres et al., 1997), so all analyses were also repeated with a data set in which tannins had been excluded.

Before the multivariate analyses, concentrations were standardized either by a z-transformation (zero mean and unit standard deviation) or by standardizing to the maximum (maximum = 1) within each compound, in order to avoid giving greater weight to compounds with high average concentrations. Unweighted pair-group method using arithmetic averages (UPGMA) clustering and nonmetric multidimensional scaling (NMS) analyses were based on Euclidean or correlation distances among samples. Blocked multi-response permutation procedure (MRBP) tests were used to test whether leaf samples from the same willow individual are chemically more similar than expected by chance. Sample (galled/ungalled leaf) was used as the block variable, and willow individual as the group variable. The tests were performed separately within each species, using z-transformed data and Euclidean distances.

Before the NMS ordinations, we used the slow-and-thorough Autopilot feature of PC-ORD to determine the optimal number of axes to which adding axes does not result in a statistically significant ($P \leqslant 0.05$) improvement according to a Monte Carlo test (maximum number of iterations = 400; instability criterion = 0.00001; starting number of axes = 6; number of real runs = 40; number of randomized runs = 50). With all datasets, the optimal number of axes was two. In the final analyses, we used 100 runs with random and PCA starting coordinates, a stability criterion of 0.00001, and 100 iterations to evaluate stability of the solution.

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