Testing the boundaries of closely related daisy taxa using metabolomic profiling

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Abstract Advances in high-throughput, comprehensive small molecule analytical techniques have seen the development of the field of metabolomics. The coupling of mass spectrometry with high-resolution chromatography provides extensive chemical profiles from complex biological extracts. These profiles include thousands of compounds linked to gene expression, and can be used as taxonomic characters. Studies have shown metabolite profiles to be taxon specific in a range of organisms, but few have investigated taxonomically problematic plant taxa. This study used a phenetic analysis of metabolite profiles to test taxonomic boundaries in the *Olearia phlogopappa* (Asteraceae) complex as delimited by morphological data. Metabolite profiles were generated from both field- and shade house-grown material, using liquid chromatography-mass spectrometry (LC-MS). Aligned profiles of 51 samples from 12 taxa gave a final dataset of over 10,000 features. Multivariate analyses of field and shade house material gave congruent results, both confirming the distinctiveness of the morphologically defined species and subspecies in this complex. Metabolomics has great potential in alpha taxonomy, especially for testing the boundaries of closely related taxa where DNA sequence data has been uninformative.

Keywords chemotaxonomy; LC-MS; metabolomics; *Olearia*; species complex

Supplementary Material The Electronic Supplement (Appendix S1: LC-MS dataset validation) is available in the Supplementary Data section of the online version of this article at http://www.ingentaconnect.com/content/iapt/tax

■ INTRODUCTION

Chemotaxonomy has long been accepted as a useful tool in plant taxonomy, with many methods for species separation developed in the 1960s (Harborne, 1973, 1975; Smith, 1976; Bohm, 1998; Reynolds, 2007). While analytical methods for volatile oils, fatty acids and alkaloids have been developed (Harborne, 1973; Zidorn, 2008; Wink & al., 2010), the mainstay of chemotaxonomy has been the analysis of flavonoids (Wilt & al., 1992; Bohm, 1998; Markham, 2006). Flavonoids have been popular because they are structurally diverse and almost ubiquitous in flowering plants, meaning they can be used at all levels from higher level systematics to the taxonomy of species, populations, and hybrid taxa (Harborne, 1975; Kim & al., 2004; Ekenäs & al., 2009). Further, they are stable and easily identified, meaning they can be extracted from old material or herbarium specimens (Bohm, 1998) and that no special equipment or methods are required to handle and process samples, or identify compounds (Wink, 2003; Zidorn, 2008). Lastly, because of the roles these compounds play in defense and signaling, they are subject to natural selection and are therefore potentially informative in evolutionary studies (Harborne, 1975; Bohm, 1998; Wink, 2003; Kirk & al., 2005; Ekenäs & al., 2009; Wink & al., 2010).

Although the application of chemotaxonomy in phenetic taxonomy and systematics was once widespread, it is no longer commonly used, as there are many factors that limit its application in cladistic analyses. The inclusion of standards and outgroup taxa allows phylogenies to be constructed using chemical data based on the loss or gain of chemical compounds, but such studies do not strictly meet the requirements of cladistic analysis (Harborne, 1967; Bohm, 1998). The potential for convergent evolution and problems with quantification also makes their use in cladistic analyses problematic (Hegnauer, 1986; Wink, 2003; Reynolds, 2007; Wink & al., 2010). Further, plant chemical profiles may obscure evolutionary relationships either because some compounds are not plant-derived (taken up and stored but not synthesised), while others that could be present go undetected because they occur in trace amounts, or the genes coding them are silenced (Hegnauer, 1986; Bohm, 1998; Wink, 2003; Zidorn, 2008).

For these reasons, and the advantages offered by genetic technology, DNA sequencing has largely superseded chemical taxonomy (Ekenäs & al., 2009). DNA sequencing has been hugely successful in underpinning a natural classification system for plants. Regions such as the internal transcribed spacer region (ITS) or combinations of chloroplast regions such as *trnL-F*, *rbcL* and *matK* have been useful in constructing

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phylogenies and delimiting species (CBOL Plant Working Group, 2009; Stuessy, 2009; Kelly & al., 2010). However, despite its promise sequencing has not solved all questions in plant taxonomy. While many sequencing studies have confirmed relationships at generic and section level, others have been uninformative at species level (Clarkson & al., 2004; Brown & al., 2006; Devey & al., 2008) or in recently diverged taxa (Kress & al., 2005; Zidorn, 2008; Ekenäs & al., 2009). Further, there are myriad issues related to parallel evolution, recombination and gene duplication (Stuessy, 2009), and regions differ in the degree of variation they exhibit across plant groups. Unlike the *CO1* gene in animals, no single region has been identified in the plant genome that can be routinely used for specific or infraspecific level taxon delimitation. Instead, several regions are generally used for species-level studies, requiring several regions to be screened, or development of new markers made before suitable loci are found for any one group of plants (Kress & al., 2005; Shaw & al., 2005, 2007; Chase & al., 2007; CBOL Plant Working Group, 2009; Korotkova & al., 2011). This can make species-level work slow and expensive. Other molecular methods that provide more variation, such as microsatellite and low-copy nuclear genes have been developed for some taxa (Takayama & al., 2011). Again, these markers are typically not universal and development of new markers can be time consuming and expensive.

Chemical data can still play a vital role in alpha taxonomy, especially where common sequence data is unable to resolve taxa. Chemical data may be informative for determining taxonomic boundaries, using a phenetic approach without inferring evolutionary relationships (Nyman & Julkunen-Tiitto, 2005; Wink & al., 2010). In particular, secondary metabolites have great application for distinguishing taxa at inter- and infraspecific levels (Bohm, 1998; Kim & al., 2004; Nyman & Julkunen-Tiitto, 2005; Zidorn, 2008). They may also be useful for insights into the origin of hybrid taxa (Kirk & al., 2005; Horwarth & al., 2008; Ekenäs & al., 2009).

Advances in analytical instrumentation, data handling and processing tools have seen the development of methods to investigate a broad range of small molecules (100–1500 Da) within organisms. Now known as metabolomics (Kirk & al., 2005; Rochfort, 2005; Ivanisevic & al., 2011), this untargeted small molecule analytical approach produces metabolite profiles based on thousands of compounds per sample in many chemical families (Fiehn & al., 2000; Kirk & al., 2005; Xie & al., 2008), rather than comparatively few compounds in one group as in flavonoid analyses. Metabolomics is now being applied in a range of areas such as natural product chemistry, metabolism, biomarker discovery, functional genomics, systems biology (Rochfort, 2005; Trenerry & Rochfort, 2010), and identification and quality control of food and medicinal plants (Schaneberg & al., 2003; Ge & al., 2008; Xie & al., 2008; Kim & al., 2010). More recently, metabolomics is being used in the classification and identification of many cryptic organisms, including microbes and pathogens (Sauer & al., 2008; Fournier & al., 2009; Sauer & Kliem, 2010), fungi (Pope & al., 2007; Frisvad & al., 2008; Kang & al., 2011), and sponges (Ivanisevic & al., 2011).

To date, studies of vascular plants using metabolomic profiling methods for classification have been largely limited to the identification and quality control of crop species (Schaneberg & al., 2003; Ge & al., 2008; Xie & al., 2008; Kim & al., 2010). Other studies using metabolomic methods have shown well-defined plant species to possess different chemical profiles for select families of compounds (e.g., Nyman & Julkunen-Tiitto, 2005; Kite & al., 2007, 2009; Ekenäs & al., 2009). However, untargeted metabolomic profiling has not yet been used to test taxonomic boundaries in plants.

In this study we employed metabolomic profiling to test taxonomic boundaries in the *Olearia phlogopappa* (Labill.) DC. (Asteraceae) species complex of south-eastern Australia. The complex is here defined as comprising *O. phlogopappa*, *O. brevipedunculata* N.G.Walsh, *O. lirata* (Sims) Hutch., *O. rugosa* (F.Muell. ex W.Archer Bis) Hutch. and *O. stellulata* (Labill.) DC. Members of this complex belong in *Olearia* sect. *Asterotriche* Benth. and are those species that are often confused with *O. phlogopappa*, or included within this species in Floras and other accounts (e.g., Curtis, 1963; Willis, 1973; Lander, 1994; Walsh & Lander, 1999). A recent revision of this complex, based on morphological characters, supports the previously accepted species circumscriptions, but proposes a new infraspecific treatment for *O. phlogopappa* (Messina & al., 2013). Phylogenetic studies have confirmed section *Asterotriche* as monophyletic, and well placed in *Olearia* s.str. (Cross & al., 2002; Brouillet & al., 2009; Messina, 2013). However, attempts to support the morphology-based revision of this complex using genetic analyses have proved uninformative because of the highly conserved nature of the ITS, *rpl32*, *matK*, and *psbA* regions in this group, even between morphologically distinct taxa (Cross & al., 2002; Messina, 2013). In this study, we take some of the taxa identified by analyses of morphological characters and use metabolomic profiling as an independent test of their distinctiveness.

■ METHODS

Sample collection and extraction. — Sixty field samples representing 17 taxa (3 of *O. asterotricha* (F.Muell.) Benth., 2 *O. brevipedunculata*, 3 *O. canescens* (Benth.) Hutch., 2 *O. frostii* (F.Muell.) J.H.Willis, 2 *O. gravis* (F.Muell.) F.Muell. ex Benth., 6 *O. lirata*, 4 *O. nernstii* (F.Muell.) Benth., 6 *O. phlogopappa* subsp. *phlogopappa*, 5 *O. phlogopappa* subsp. *continentalis* Messina, 5 *O. phlogopappa* subsp. *flavescens* (Hutch.) Messina, 4 *O. phlogopappa* subsp. *gunniana* (DC.) Messina, 2 *O. phlogopappa* subsp. *insularis* Messina, 4 *O. phlogopappa* subsp. *subrepanda* (DC.) Messina, 1 *O. phlogopappa* subsp. *serrata* Messina, 2 *O. quercifolia* Sieber ex DC., 5 *O. rugosa*, 4 *O. stellulata*) were collected from locations in four states of Australia: Tasmania, Victoria, New South Wales and Queensland. However, only taxa with three replicates were included in statistical analyses. This reduced number of samples to four (of five) currently recognised species in the *Olearia phlogopappa* complex, five (of nine) infraspecific taxa, and three well-defined taxa for use in outgroup comparisons

(Appendix 1). The taxa excluded from analyses on the basis of sample size included both taxa that were morphologically well defined (*O. frostii*, *O. gravis*, *O. quercifolia*) and less well defined (*O. brevipedunculata*, *O. phlogopappa* subsp. *serrata*). Field samples were comprised of leaves pooled from across five separate plants and dried and preserved at the point of collection, thereby giving a representative "population" sample. A voucher specimen was taken from every population and deposited at MEL.

As part of a morphological study (Messina & al., 2013) cuttings were also taken from many field populations and grown in a common shade house environment at La Trobe University. An attempt was made to take cuttings from the same five individuals that contributed dried leaves to the pooled field sample for each taxon at each location, but the success rate was low and shade house samples were made from one plant per population. For this and other logistical reasons, 31 samples from 7 taxa (7 of *O. phlogopappa* subsp. *phlogopappa*, 4 *O. phlogopappa* subsp. *continentalis*, 3 *O. phlogopappa* subsp. *gunniana*, 3 *O. phlogopappa* subsp. *insularis*, 3 *O. phlogopappa* subsp. *subrepanda*, 7 *O. rugosa*, 4 *O. stellulata*) were included in the present study (Appendix 1). Plants were grown in a mesh shade house in 10 inch pots in an organic soil mixture consisting of pine bark compost, coarse sand, iron, micronutrients, iron sulphate, gypsum, and a 70-day slow-release fertilizer. Leaves were harvested in May 2011; average weather conditions for this month at La Trobe University were mean maximum temperature 15.4°C and mean minimum temperature 7.4°C, with 3.9 hrs of sunshine (BOM, 2011).

In the laboratory, approximately 0.1 g of dried and ground leaf material was weighed into 10 mL tubes, and elutants were extracted in 2.5 mL of 80% methanol. After 40 hours tubes were centrifuged and supernatant removed and stored at 4°C. A solvent mixture with moderate polarity was used in order to extract a broad range of metabolites without extracting the most lipophilic compounds such as waxes and non-polar lipids which cause contamination of the LC column. Samples were then diluted 1 : 4 v/v sample/ddH_2O to facilitate retention on the reversed phase column and then filtered through a Sartorius Minisart RC4 0.2 µm syringe filter to remove precipitants from the supernatant.

LC-MS system details and method. — Sample extracts were analysed using an Agilent 1200 series UHPLC coupled with an Agilent 6520 dual ESI quadrupole time of flight (QTOF) MS system. Deionized water (18.2 MΩ.cm, Millipore Synergy 185) and LC-MS grade acetonitrile (Merck, Kilsyth, Victoria, Australia) were used throughout this experiment. An ultra high pressure Agilent Zorbax Eclipse plus RRHD XDB C18 2.1 × 100 mm, 1.8 µm column was used for chromatographic separation. Analytes were eluted using a gradient method (0 min 1%B, 6 min 30%B, 12 min 100%B, 13 min 100%B, 13.1 min 100%B, 17 min 1%B) with the following mobile phases: (A) 0.1% formic acid in water, and (B) 0.1% formic acid in acetonitrile with a flow rate of 0.4 mL/min and a column temperature of 35°C. The sample injection volume was 5 µL.

Electrospray ionization source conditions were as follows: nebuliser pressure 40 psi, gas flow rate 10 L/min, Gas temperature 300°C, capillary voltage 4000 V, Fragmentor voltage 150 V, and skimmer voltage 65 V. The instrument was operated in the extended dynamic range mode and data was collected in the range 100–1700 mass-to-charge ratio (*m/z*). Reference ions were continuously sprayed into the source using the second nebuliser for spectral mass calibration. Only the negative ion mode was employed. This mode was used to include flavonoid compounds in the analysis, as these compounds ionize more efficiently in negative ionization mode.

Quality control (QC). — Both field and shade house analyses included a biological pooled QC (PBQC) sample, that consisted of a 5 µL aliquot of each of the sample extracts. This is an accepted means of validating metabolomic datasets (Dunn & al., 2011). Technical replicates of three randomly selected samples were also analysed, as well as a 10 µM quercetin standard (Sigma Aldrich, Castle Hill, New South Wales, Australia). The samples were injected in a random order with the PBQC and standard analysed every 15th injection. The PBQC and standard was used to check for retention time shifts, mass accuracy and sensitivity changes throughout the whole run (see Electr. Suppl.: Appendix S1). Automated mass axis recalibration was also carried out after each QC analysis to ensure minimal mass accuracy error. Principal component analysis (PCA) using mean-centered data was undertaken using Mass Profiler Professional (Agilent, Mulgrave, Victoria, Australia) to visualise any variation exhibited in QC samples across the run. See supplementary material for validation of data.

Data processing. — Chromatographic deconvolution was carried out using MassHunter Qualitative analysis software (Agilent). This software finds metabolite peaks, and produces a list containing the accurate mass, retention time and intensity of individual peaks, known as a molecular feature. The molecular feature extraction algorithm searched for peaks above a 750-count threshold and included the adduct ions Cl^-, Br^- and $HCOO^-$ and dimers (gas phase ion clusters). Data was then imported into Mass Profiler Professional (Agilent) where retention times (RTs) were aligned across all samples and peak intensities were normalised. Both field and shade house data analyses yielded datasets with thousands of features, many of which were unique to single samples thus making them uninformative in statistical analysis. They were removed from the datasets. With singletons removed, aligned field samples gave a matrix of just over 8300 features, and over 5800 features for the shade house samples. In both datatsets each sample had between 500–1600 peaks.

Data analysis. — Filtered and aligned shade house and field data matrices were imported into PRIMER (Clarke & Gorey, 2006) for separate multivariate analysis. Although comparing field-collected with shade house samples could reveal the effect of environment on underlying infra- and interspecific genetic variation in metabolite profiles, this was not an aim of the study. In any case, the field and shade house samples were collected differently (five individuals per sample in the field, versus one individual per sample in the shade house), confounding any comparisons between them. Instead, we analysed the datasets separately and looked for congruence in the outcomes, despite the differences in sample collection methodology.

Distance matrices were generated using the Bray-Curtis coefficient of similarity on log-transformed data. Multidimensional scaling (MDS, 50 random starts) was performed on the distance matrices to produce 3-dimensional ordinations that helped to visualize similarities within and differences between the metabolite profiles of the taxa, but the ANOSIM routine was used to objectively test for significant differences between them. This is a nonparametric routine that uses the ranked rather than actual distance between two samples in analyses. The test statistic is R, a measure of within-taxon versus between-taxon similarity. When $R = 1$ all replicates of a taxon are more similar to each other in their metabolite profiles than they are to any other replicate of a different taxon, whereas $R = 0$ indicates that similarities within and between taxa are the same, and taxa are not differentiated (Clarke & Warwick, 2001). Thus, R is essentially a measure of group "distinctiveness", and is useful in this case as measure of the distinctiveness of each taxon. We used $n = 10{,}000$ randomisations for the initial multi-taxon analyses, which yielded significant overall R values for both field and shade house analyses. Post hoc comparisons were then conducted for each pair of taxa. A minimum of three samples per group is needed to establish significance at $\alpha = 0.1$ in the ANOSIM routine, so only taxa with at least three replicates were included in these analyses. However, in most pairwise comparisons both taxa had four or more replicates and significance could be judged at $\alpha = 0.05$.

■ RESULTS

Differentiation of taxa. — Field samples gave unequivocal support for the hypothesis that taxa defined on the basis of their morphology also differ in their chemical profiles. All taxa formed discrete groups in the ordinations (Fig. 1A–C), and ANOSIM testing showed that all 55 pairwise taxon comparisons were significantly different, with 34 of these indicating perfect distinction ($R = 1$, Table 1).

Analyses of the shade house samples also gave robust support for the hypothesis that taxa defined by their morphology are chemically distinct entities. Most taxa formed discrete clusters in the ordinations (Fig. 1D–F), and ANOSIM testing showed that 19 of 21 pairwise taxon comparisons were significantly different ($P < 0.05$) with virtually no overlap ($R \geq 0.45$; Table 1). Typically, the distinction between taxa in paired comparisons was greatest when one taxon was a species, as opposed to comparisons between subspecies of *O. phlogopappa*. The only taxa not significantly different from each other were *O. phlogopappa* subsp. *phlogopappa* and subsp. *insularis*, and subsp. *phlogopappa* with subsp. *gunniana*. Samples of *O. phlogopappa* subsp. *phlogopappa* were spread throughout the ordination on all three axes, overlapping with several other taxa (Fig. 1D–F). Pairwise comparisons with this taxon yielded lower R values than most other subspecies of *O. phlogopappa* (i.e., R values mostly 0.4–0.8). In particular, at least some samples of *O. phlogopappa* subsp. *phlogopappa* tended to cluster with samples of subsp. *insularis* on all three axis of the ordination, reflecting the low R value of the pairwise comparison of these taxa. Conversely, samples of subsp. *gunniana* showed some differentiation from subsp. *phlogopappa* samples in the ordination, but were judged not to be significantly different in the ANOSIM test ($P = 0.083$).

***Olearia phlogopappa* s.l. —** We also tested the distinctiveness of *O. phlogopappa* s.l. against other species in our dataset by collapsing all the subspecies of *O. phlogopappa* into this single taxon, and re-running the ANOSIM analyses (Table 2). Using field-collected samples, *O. phlogopappa* s.l. differed significantly from the outgroup taxa *O. asterotricha*, *O. canescens* and *O. nernstii* (R values > 0.7), and less strongly but still significantly from *O. lirata* and *O. rugosa*. The differentiation of *O. phlogopappa* s.l. from *O. rugosa* was stronger for field-collected samples than shade house samples. *Olearia phlogopappa* s.l. did not differ significantly from *O. stellulata* for field-collected samples, but they did differ weakly for glasshouse samples.

■ DISCUSSION

Differentiation of taxa. — Previously, taxonomic differences have been quantified using metabolomic profiles for a range of well-established plant species, even where taxa are closely related (Nyman & Julkunen-Tiitto, 2005; Kite & al., 2007, 2009; Ekenäs & al., 2009; Kim & al., 2010). In this study, genetically or morphologically distinct species (and subspecies) in *Olearia* sect. *Asterotriche* were differentiated using metabolomic profiles, confirming taxon distinctions and validating the utility of this method for identification and differentiation.

Within the *Olearia phlogopappa* complex, boundaries between *O. rugosa*, *O. stellulata*, and *O. lirata* have been historically ambiguous. A recent morphological study supported the distinction of these species, with circumscriptions of the latter three slightly revised (Messina & al., 2013). Metabolomic profiling adds support to these revised concepts by providing further evidence for the appropriate placement of several populations which were poorly resolved in the morphological study and only tentatively placed in species (e.g., *O. lirata* from the southern Tablelands of New South Wales [*A. Messina 111*], *O. rugosa* from Tasmania and Flinders Island [*A. Messina 245, 248*], and *O. stellulata* from Wilsons Promontory [*A. Messina 181*]) (Walsh & Lander, 1999; Messina & al., 2013). The support is evident because all three species were still clearly differentiated when these poorly resolved populations were included (Table 1; Fig. 1).

Metabolomic profiling also gave substantial but incomplete weight to the recognition of *O. phlogopappa* s.l. as an entity distinct from other species. *Olearia phlogopappa* s.l. was quite distinct from the three outgroup taxa, and moderately but still significantly distinct from two other species in the complex, *O. lirata* and *O. rugosa*. However, there was considerable overlap of *O. phlogopappa* s.l. and *O. stellulata* in ordination space for both field and shade house samples, and only the latter samples showed a significant interspecific difference in metabolite profiles. Why these taxa should be so poorly differentiated based on their metabolite chemistry, while being quite distinct morphologically (Messina & al., 2013), is unclear.

Infraspecific variation in *O. phlogopappa*. — Defining the boundaries of *O. phlogopappa* has been a major challenge due to the large range of morphological variation in this taxon. Problems with applying a stable taxonomy have been complicated by varying and sometimes conflicting taxonomic interpretations over the last 150 years (Hooker, 1856; Hutchinson, 1917; Curtis, 1963; Walsh & Lander, 1999). As currently recognised, *O. phlogopappa* consists of nine subspecies, which are widespread throughout south-eastern Australia (Messina & al., 2013). Infraspecific taxa are morphologically variable, but readily assigned to this species on the basis of floral and vegetative characters (Messina & al., 2013). Metabolomic profiling has supported many of the currently recognised subspecies; four of the six subspecies tested had

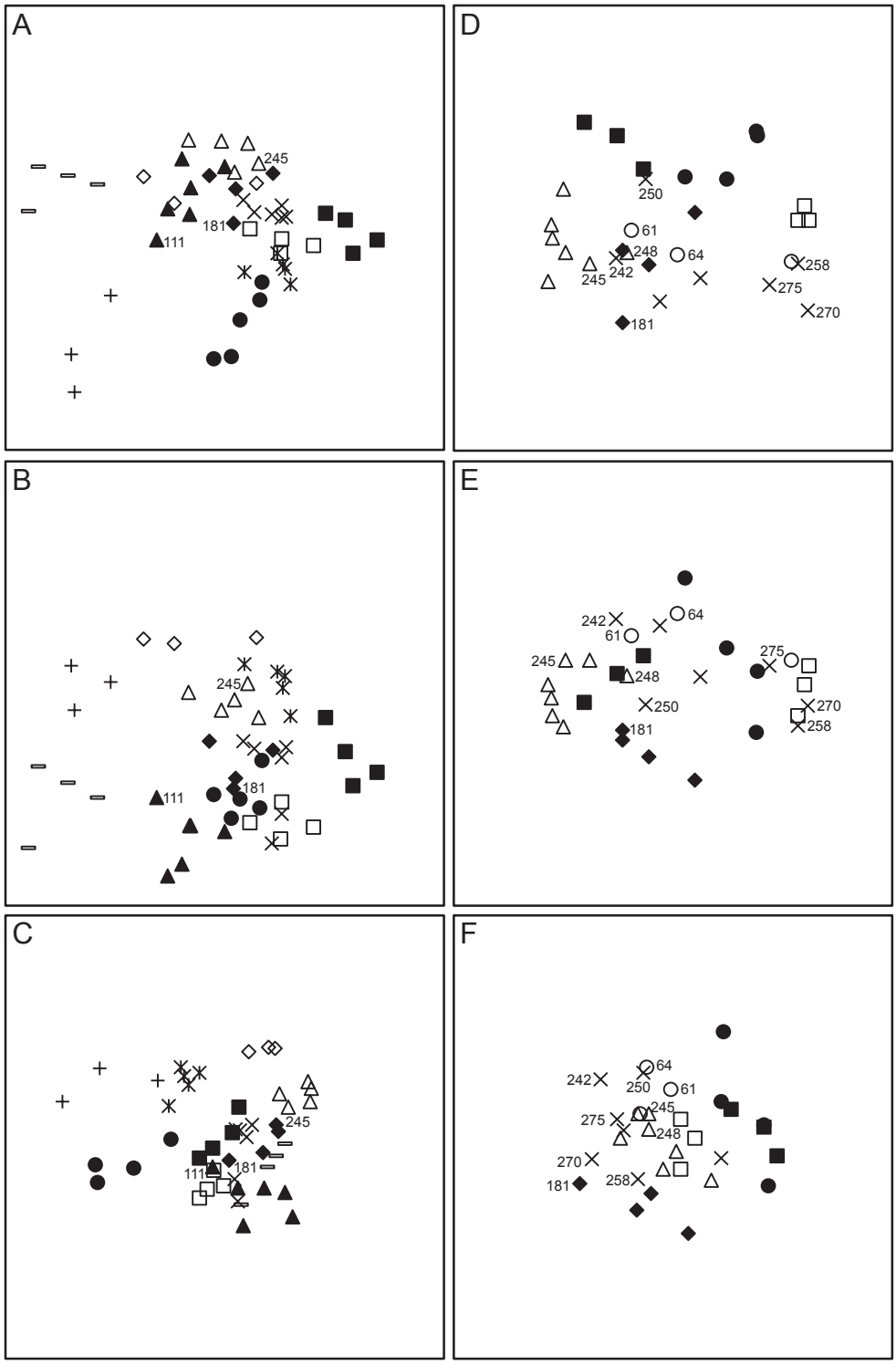

Fig. 1. Three-dimensional ordination of samples based on the Bray-Curtis dissimilarity matrix. **A–C,** field ordination, stress value = 0.1; **D–F,** shade house ordination, stress = 0.11. **Top row,** axis 1 and 2; **middle row,** axis 1 and 3; **bottom row,** axis 2 and 3. Symbols represent taxa: ◇, *O. asterotricha*; +, *O. canescens*;▲, *O. lirata*; ═, *O. nernstii*; ×, *O. phlogopappa* subsp. *phlogopappa*; ●, *O. phlogopappa* subsp. *continentalis*; ✳, *O. phlogopappa* subsp. *flavescens*; □, *O. phlogopappa* subsp. *gunniana*; ○, *O. phlogopappa* subsp. *insularis*; ■, *O. phlogopappa* subsp. *subrepanda*; ◆, *O. stellulata*; △, *O. rugosa*. Numbers next to symbols indicate collection numbers.

significantly different metabolomic profiles. Samples of subsp. *subrepanda*, subsp. *continentalis*, and subsp. *flavescens* (based on field samples only) were all significantly different (Table 1). Subspecies *gunniana* was distinct from all other taxa, but the difference between it and subsp. *phlogopappa* was not significant. However, this value was close to significant ($P = 0.083$), and replicates of subsp. *gunniana* form a distinct cluster in the ordination (Fig. 1).

Table 1. Pairwise ANOSIM comparisons of taxa.

Pairwise comparison		Field R statistic	Shade house R statistic	Pairwise comparison		Field R statistic	Shade house R statistic
AST	CAN	*1**	–	Pf	Pc	0.972**	–
AST	LIR	1*	–	Pf	P	0.976**	–
AST	NERN	1*	–	Pf	Ps	0.988**	–
AST	Pf	1*	–	Pf	Pg	1**	–
AST	Pc	1*	–	Pf	RUG	1**	–
AST	P	0.988*	–	Pf	STEL	1**	–
AST	Ps	0.981*	–	Pc	Pi	–	0.796*
AST	Pg	1*	–	Pc	P	0.976**	0.603**
AST	RUG	0.949*	–	Pc	Ps	1**	0.907*
AST	STEL	0.944*	–	Pc	Pg	0.906*	0.778*
CAN	LIR	1*	–	Pc	RUG	1**	0.987**
CAN	NERN	1*	–	Pc	STEL	0.975**	0.917*
CAN	Pf	1*	–	Pi	P	–	0.107
CAN	Pc	1*	–	Pi	Ps	–	*0.852**
CAN	P	1*	–	Pi	Pg	–	*0.593**
CAN	Ps	1*	–	Pi	RUG	–	0.81**
CAN	Pg	1*	–	Pi	STEL	–	0.648*
CAN	RUG	1*	–	P	Ps	0.817**	0.476*
CAN	STEL	1*	–	P	Pg	0.77**	0.242
LIR	NERN	1**	–	P	RUG	0.859**	0.661**
LIR	Pf	1**	–	P	STEL	0.54**	0.45*
LIR	Pc	1**	–	Ps	Pg	0.958*	*1**
LIR	P	0.972**	–	Ps	RUG	1**	0.937**
LIR	Ps	1**	–	Ps	STEL	1*	0.852*
LIR	Pg	0.956**	–	Pg	RUG	1**	1**
LIR	RUG	0.997**	–	Pg	STEL	0.948*	0.907*
LIR	STEL	0.933**	–	RUG	STEL	0.838**	0.706**
NERN	Pf	1**	–				
NERN	Pc	1**	–				
NERN	P	1**	–				
NERN	Ps	1*	–				
NERN	Pg	1*	–				
NERN	RUG	1**	–				
NERN	STEL	1*	–				

The R statistic indicates differentiation of taxa, with $R = 1$ when all replicates of a taxon are more similar to each other than any replicate of a different taxon.
* Denotes significant ($P < 0.05$), and ** highly significant ($P < 0.01$) P values. Numbers in italics represent values regarded significant where $P = 0.1$ as this is the lowest possible value due to limited sample size (see text).
Taxon coding: AST, *O. asterotricha*; CAN, *O. canescens*; LIR, *O. lirata*; NERN, *O. nernstii*; P, *O. phlogopappa* subsp. *phlogopappa*; Pc, *O. phlogopappa* subsp. *continentalis*; Pf, *O. phlogopappa* subsp. *flavescens*; Pg, *O. phlogopappa* subsp. *gunniana*; Pi, *O. phlogopappa* subsp. *insularis*; Ps, *O. phlogopappa* subsp. *subrepanda*; STEL, *O. stellulata*; RUG, *O. rugosa*.

Olearia phlogopappa subsp. *phlogopappa* and subsp. *insularis* were not separated by their chemistry, although some samples of subsp. *phlogopappa* from the south and west coast of Tasmania (*A. Messina 258, 270, 275*) loosely clustered in ordination space (Fig. 1D–F). The remaining samples were scattered throughout the ordination. Similarly, only the samples of subsp. *insularis* from Victoria (*A. Messina 61, 64*) clustered together. A similar pattern was observed in the morphological data (see Messina & al., 2013). In the morphological study both these taxa included a core group of morphologically similar populations and several atypical populations that were otherwise unplaced. For example, samples of subsp. *phlogopappa* on the east coast of Tasmania (e.g., *A. Messina 242, 250*) were not positioned closely to other populations within the subspecies in the shade house analysis. Likewise, some subsp. *insularis* samples from the Victorian coast (e.g., *A. Messina 61, 64*) did not cluster with samples from Islands in Bass Strait. These atypical populations were included in taxa on the basis of weak morphological affinities, and geographic or ecological similarities. Although metabolomic analysis does not support these two taxa as currently defined, neither does it propose a better taxonomy of these few atypical populations. Rather, in both studies these populations remain outliers. As such, no changes have to be made to the morphological treatment based on the metabolomic data. All other taxa in *O. phlogopappa* are supported, and removal of these two subspecies would upset an otherwise stable subspecific treatment. Further work may be warranted to determine where these few atypical populations should be placed.

Field versus shade house samples. — A long-standing issue in plant taxonomy is disentangling the effects of environment from underlying genetic influences on key traits. This is especially the case in chemotaxonomy, where the expression and concentration of many compounds is likely to be under a large degree of environmental control (e.g., Zidorn & al., 2004; Horwath & al., 2008; Murai & al., 2009), by both biotic and abiotic factors. The same is likely true of the metabolite profiles in this study, but differences in the sampling methodology for the field and shade house samples did not permit any conclusions about environmental influences on the metabolite profiles. However, despite the methodological differences, both datasets gave congruent results in that both field and shade house samples confirmed the distinctiveness of the morphologically defined species and subspecies in the *Olearia phlogopappa* species complex. This is especially noteworthy for the field collections, where combining leaves from several individuals into single samples for analyses probably reduced intra-taxon variability between samples, and probably also reduced inter-taxon dissimilarity. If so, using the metabolite profiles of the field collections in multivariate analyses was probably a conservative test of the distinction between the morphologically defined taxa. These data suggest that regardless of sampling strategy, metabolomic profiling is sufficiently robust to play a significant role in establishing low-level taxon boundaries.

Table 2. Pairwise ANOSIM comparisons of *Olearia phlogopappa* s.l. with other *Olearia* species.

Comparison	Field R statistic	Shade house R statistic
v AST	0.724**	
v CAN	0.980**	
v LIR	0.621**	
v NERN	0.977**	
v RUG	0.531**	0.376**
v STEL	0.119	0.235**

These analyses were created by collapsing all subspecies of *O. phlogopappa* into a single taxon. The R statistic indicates differentiation of taxa, with $R = 1$ when all replicates of a taxon are more similar to each other than any replicate of a different taxon.
** Denotes highly significant ($P < 0.01$) P values.
Taxon coding: AST, *O. asterotricha*; CAN, *O. canescens*; LIR, *O. lirata*; NERN, *O. nernstii*; RUG, *O. rugosa*; STEL, *O. stellulata*.

Logistics. — The methodology in this study was straightforward. Only small samples of leaf material (0.1 g) were required, and the use of automated LC-MS to generate metabolite profiles removed the need for laborious chromatography and subsequent manual interpretation (Kite & al., 2003). However, the equipment is expensive and requires a technician for maintenance and operation. As such, many studies would need to outsource the analysis, similar to the strong trend towards outsourcing DNA sequence analyses to high-throughput facilities. In comparison, chemical analysis is much faster than DNA sequencing, with far fewer steps, and is a much cheaper option than sequencing. Metabolomic profiling also obviates the not inconsiderable health risks associated with the use of large volumes of hexane and chloroform in traditional thin-layer chromatography.

Many chemotaxonomic studies have targeted specific compounds (e.g., Zidorn & Stuppner, 2001; Kite & al., 2003, 2007; Saito & al., 2009). One major benefit of this approach is the identification of biologically interesting compounds that may explain differences between taxa in their ecology and evolutionary history (Takemoto & Arita, 2009; Wink & al., 2010; Kang & al., 2011). By contrast, the untargeted approach we took in this study resulted in thousands of features and an extremely large dataset, making it impossible at this time to identify individual compounds and their ecological roles in *Olearia* (cf. Trenerry & Rochfort, 2010). With time, specific compounds should become more easily identifiable through the development of metabolite databases (see Werner & al., 2008). In any case, it seems logical that a bigger dataset will be more informative than a smaller one, and taking an untargeted approach removes potential biases that might result from selective data inclusion (Kirk & al., 2005).

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Appendix 1. Species names and authority, locality, altitude, date collected, and voucher number. All samples were made in Australia, and vouchers are deposited at MEL. Location abbreviations represent: NSW, New South Wales; QLD, Queensland; TAS, Tasmania; VIC, Victoria; NP, National Park; SF, State Forest; SP, State Park. Letters in parentheses after voucher number (F and S) refer to which analysis sample was included; field or shade house respectively. An asterisk (*) indicates sample not included in statistical analyses, but present in the QC sample.

Olearia asterotricha (F.Muell.) Benth., NSW, Nattai NP, 600 m, 10-Nov-07, *A. Messina 108* (F); VIC, Bunyip SF, 140 m, 28-Dec-07, *A. Messina 155* (F); VIC, Grampians NP, 600 m, 28-Oct-08, *A. Messina 224* (F, S*); VIC, Mt. Kincaid, 150 m, 29-Oct-08, *A. Messina 225* (S). ***O. brevipedunculata*** N.G.Walsh, VIC,

Appendix 1. Continued.

Mt. Buffalo, 1560 m, 3-Dec-07, *A. Messina 128* (S*); VIC, Bogong High Plains, 1725 m, 5-Dec-07, *A. Messina 133* (S*); VIC, Bogong High Plains, 1670 m, 5-Dec-07, *A. Messina 137* (F*); NSW, Kosciusko NP, 1560 m, 24-Jan-08, *A. Messina 170* (F*). ***O. canescens*** (Benth.) Hutch., QLD, Cracow, 320 m, 14-Sep-07, *A. Messina 67* (F); NSW, Oxley Wild Rivers NP, 950 m, 26-Oct-07, *A. Messina 89* (F); NSW, 5 km east of Mole River, 800 m, 3-Nov-07, *A. Messina 100* (F). ***O. frostii*** (F.Muell.) J.H.Willis, VIC, Bogong High Plains, 1670 m, 5-Dec-07, *A. Messina 136* (F*); VIC, Mt. Blowhard, 1670 m, 11-Jan-08, *A. Messina 158* (F*). ***O. gravis*** (F.Muell.) Benth., NSW, Kings Plain NP, 800 m, 28-Oct-07, *A. Messina 93* (F*); NSW, Kwiambal NP, 380 m, 2-Nov-07, *A. Messina 99* (F*). ***O. lirata*** (Sims) Hutch., VIC, Coopracambra NP, 600 m, 16-Oct-07, *A. Messina 84* (F); NSW, Belmore Falls - Mossvale, 600 m, 10-Nov-07, *A. Messina 111* (F, S*); VIC, Warrandyte SP, 300 m, 28-Nov-07, *A. Messina 124* (S*); VIC, Snake Island, 0 m, 2-Jan-08, *A. Messina 157* (F); NSW, Kosciusko NP, 700 m, 23-Jan-08, *A. Messina 166* (F); TAS, West Ulverstone, 30 m, 18-Nov-08, *A. Messina 240* (F); TAS, Eaglehawk Neck, 80 m, 3-Dec-08, *A. Messina 283* (F). ***O. nernstii*** (F.Muell.) Benth., QLD, Mount Coot-tha, 110 m, 11-Sep-07, *A. Messina 65* (S*); NSW, Way Way SF, 230 m, 21-Sep-07, *A. Messina 78* (F); NSW, Myall Lakes NP, 20 m, 21-Sep-07, *A. Messina 82* (F, S*); QLD, Lamington NP, 800 m, 31-Oct-07, *A. Messina 94* (F); NSW, Washpool NP, 1100 m, 31-Oct-07, *A. Messina 97* (F). ***O. phlogopappa*** subsp. ***phlogopappa***, TAS, Flinders Isand - Strzelecki NP, 500 m, 19-Nov-08, *A. Messina 242* (F, S); TAS, Mt. Elephant, 400 m, 21-Nov-08, *A. Messina 250* (F, S); TAS, Trial Habour, 10 m, 25-Nov-08, *A. Messina 258* (F, S); TAS, South Cape, 30 m, 30-Nov-08, *A. Messina 270* (F, S); TAS, Randalls Bay, 10 m, 1-Dec-08, *A. Messina 273* (F, S); TAS, South Bruny Island NP, 40 m, 2-Dec-08, *A. Messina 275* (S); TAS, Eaglehawk Neck, 60 m, 3-Dec-08, *A. Messina 281* (F, S). ***O. phlogopappa*** subsp. ***continentalis*** Messina, NSW, Barrington Topps, 1300 m, 6-Nov-07, *A. Messina 103* (F); NSW, Kanangra-Boyd NP, 1300 m, 8-Nov-07, *A. Messina 104* (F, S); VIC, Mt. Buffalo, 1100 m, 4-Dec-07, *A. Messina 129* (S); VIC, Mt. Buffalo, 1150 m, 4-Dec-07, *A. Messina 130* (S); VIC, Errinundra NP, 900 m, 9-Dec-07, *A. Messina 142* (F, S); VIC, Glen Allen, 950 m, 12-Dec-07, *A. Messina 146* (F). ***O. phlogopappa*** subsp. ***flavescens*** (Hutch.) Messina, VIC, Mt. Buffalo, 1612 m, 3-Dec-07, *A. Messina 127* (F, S*); VIC, Bogong High Plains, 1640 m, 5-Dec-07, *A. Messina 139* (F); VIC, Mt. Hotham, 1845 m, 6-Dec-07, *A. Messina 141* (F, S*); VIC, Baw Baws, 1480 m, 28-Dec-07, *A. Messina 153* (F); NSW, Mt. Kosciusko, 1780 m, 22-Jan-08, *A. Messina 165* (F); NSW, Kosciusko NP, 1200 m, 24-Jan-08, *A. Messina 174* (F). ***O. phlogopappa*** subsp. ***gunniana*** (DC.) Messina, TAS, Sideling SF, 560 m, 21-Nov-08, *A. Messina 247* (F); TAS, Gowrie Park, 450 m, 24-Nov-08, *A. Messina 256* (F); TAS, Bronte Lagoon, 700 m, 26-Nov-08, *A. Messina 260* (S); TAS, East Bagdad, 440 m, 4-Dec-08, *A. Messina 285* (F, S); TAS, Swansea - Lake Leake road, 520 m, 4-Dec-08, *A. Messina 286* (F, S). ***O. phlogopappa*** subsp. ***insularis*** Messina, VIC, Sandy Point, 10 m, 1-Sep-07, *A. Messina 61* (S); VIC, Waratah Bay, 15 m, 1-Sep-07, *A. Messina 64* (F*, S); TAS, Flinders Island-Emita beach, 20 m, 20-Nov-08, *A. Messina 244* (F*, S). ***O. phlogopappa*** subsp. ***salicina*** (Hook.f.) Messina, VIC, Otways NP, 300 m, 29-Oct-08, *A. Messina 230* (S*); TAS, West Ulverstone, 40 m, 18-Nov-08, *A. Messina 241* (S*). ***O. phlogopappa*** subsp. ***serrata*** Messina, VIC, Howitt High Plains, 1595 m, 26-Mar-08, *A. Messina 185* (F*, S*). ***O. phlogopappa*** subsp. ***subrepanda*** (DC.) Messina**,** TAS, Ben Lomond, 1225 m, 23-Nov-08, *A. Messina 251* (F, S); TAS, Mount Field NP, 1200 m, 26-Nov-08, *A. Messina 261* (F, S); TAS, Mt. Wellington, 1000 m, 28-Nov-08, *A. Messina 265* (F); TAS, Great Lake, 1160 m, 5-Dec-08, *A. Messina 290* (F, S). ***O. quercifolia*** Sieber ex DC., NSW, Newnes SF, 1150 m, 8-Nov-07, *A. Messina 105* (F*, S*); NSW, Wentworth Falls, 850 m, 9-Nov-07, *A. Messina 106* (F*, S*). ***O. rugosa*** (F.Muell. ex W.Archer bis) Hutch., VIC, Coopracambra NP, 900 m, 18-Nov-07, *A. Messina 116* (F); VIC, Mt. Macedon, 600 m, 27-Oct-08, *A. Messina 222* (S); TAS, Flinders Island- Darling Range, 60 m, 20-Nov-08, *A. Messina 245* (F, S); TAS, Mt. Cameron, 320 m, 21-Nov-08, *A. Messina 248* (S); VIC, Gembrook, 270 m, 28-Dec-07, *A. Messina 156* (F, S); VIC, Wilsons Promontory NP, 275 m, 22-Feb-08, *A. Messina 180* (F, S); VIC, Wilsons Promontory NP, 120 m, 22-Feb-08, *A. Messina 182* (F, S). ***O. stellulata*** (Labill.) DC., VIC, Wilsons Promontory NP, 330 m, 22-Feb-08, *A. Messina 181* (F, S); VIC, Otways NP, 300 m, 29-Oct-08, *A. Messina 232* (F, S); TAS, Tullah, 175 m, 24-Nov-08, *A. Messina 257* (S); TAS, Trial Habour, 90 m, 25-Nov-08, *A. Messina 259* (F); TAS, Ferntree, 500 m, 28-Nov-08, *A. Messina 263* (S); TAS, Randalls Bay, 10 m, 1-Dec-08, *A. Messina 274* (F).