Volatiles of High-Elevation Five-Needle Pines: Chemical Signatures through Ratios and Insight into Insect and Pathogen Resistance

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Abstract

High-elevation five-needle pine trees are a group of *Pinus* species in the subgenus *Strobus* that occur at the edges of plant growth near the alpine tree line. These species are ecologically very important and are also threatened by climate-driven insect outbreaks and an exotic pathogen. Volatile organic compounds (VOCs) play central roles in the environmental adaptation of plants and in their defense against insects and pathogens. For example, the VOCs emitted by some high-elevation five-needle pine species attract female, tree-killing mountain pine beetles (MPB, *Dendroctonus ponderosae*) in the pioneering phase whereas VOCs from other species strongly repel this foremost herbivore, but the mechanism is unknown. We collected and compared headspace VOCs from foliage of eight species of high-elevation five-needle pines in Europe and North America. Overall, VOCs differed quantitatively among species with few qualitative differences. Despite species emitting essentially the same compounds, Random Forest analysis correctly classified 117 of the 126 trees sampled by using VOCs and identified the most important compounds for species classification and for separating species resistant from those susceptible to MPB or white pine blister rust (*Cronartium ribicola*). These VOC 'fingerprints' resulted largely from species emitting distinctive ratios of compounds, rather than through presence of species-specific compounds. Importantly, these *Pinus* species vary greatly in resistance to the main herbivore (MPB) and pathogen (white pine blister rust) causing tree mortality. Thus, these findings provide insights and should guide research into understanding resistance and in developing tools to manage these important trees. For instance, studies into the functions of five-needle pine VOCs in defense against abiotic or biotic stressors should focus on blend ratios rather than on individual compounds.

Keywords Headspace · Insect and disease resistance · *Pinus* species · Random Forest · Ratios · Volatile organic compounds

Introduction

High-elevation five-needle pines (High Five pines) are a group of about a dozen related species that occur at or near the alpine treeline and are distributed patchily across the middle latitudes of the Northern Hemisphere (Tomback et al. 2011). These species belong to the *Pinus* subgenus *Strobus*, a lineage in which most species have adapted to stressful environments of poor soils and extreme temperatures (Eckert

and Hall 2006; Keeley 2012). Examples of High Five pines include Great Basin bristlecone pine (*P. longaeva* Bailey) of western North America, the oldest living tree species on Earth (Schulman 1958; Rocky Mountain Tree-Ring Research 2019), and Swiss stone pine (*P. cembra* L.), the predominate timberline tree of the Alps (Ali et al. 2005). High Five pines are keystone species that shape the communities in which they occur by stabilizing slopes, as nurse plants for other plant species, and as habitat and food for animals (Baumeister and Callaway 2006; Körner 2012; Tomback and Achuff 2010; Vogan and Schoettle 2015; Tomback et al. 2016). High Five pines also serve as important indicators of past and present climate change (e.g., Carrer et al. 2007; Kipfmueller and Salzer 2010; Millar et al. 2015; Panayotov et al. 2010; Rochefort et al. 1994; Salzer et al. 2009).

High Five pines have received increased attention in recent years because of large increases in mortality (Cleaver et al. 2015; Hansen et al. 2016; Tomback et al. 2011). The health of High Five pine species in western North America is being severely affected by several threats, most notably native

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mountain pine beetles (MPB; *Dendroctonus ponderosae* Hopkins) which are undergoing climate-driven range expansion, exotic white pine blister rust (WPBR; caused by *Cronartium ribicola* J.C. Fisch), and changes to fire regimes (Bentz et al. 2010; Gray and Jenkins 2017; Logan et al. 2003, 2010; Tomback et al. 2011). For example, a recent survey found that half of limber pines (*P. flexilis* James) in the central and southern Rocky Mountains were dead or declining due to MPB outbreaks and WPBR (Cleaver et al. 2015). Similarly, widespead MPB- and WPBR-attributed declines have occurred in whitebark pine (*P. albicaulis* Engelm.), which warranted its listing in 2011 as a candidate species under the U.S. Endangered Species Act (Goeking and Izlar 2018; Keane et al. 2012). Comparable declines in whitebark pine have occurred in the Canadian Rocky Mountains (Smith et al. 2013). Considerable variability exists among High Five pine species in their susceptibility to MPB and WPBR. For example, of the six High Five pine species in western North America, only *P. longaeva* is resistant to WPBR whereas all European High Five species are highly resistant to WPBR (Geils et al. 2010; Tomback and Achuff 2010). Moreover, while MPB infests and kills most *Pinus* species within its range (Wood 1982), recent work found that mortality due to MPB is low in foxtail pine (*P. balfouriana* Grev. and Balf.) and absent in *P. longaeva* (Bentz et al. 2017; Eidson et al. 2017, 2018). This resistance to MPB in *P. longaeva* has been proposed to be due to relatively high levels of constitutive defensive chemistry (Bentz et al. 2017). Other factors that can affect resistance in High Five pine species to MBP include the number of resin ducts (Ferrenberg et al. 2014) and inducibilty of phloem terpenes and phenolics (Raffa et al. 2017). Genetics, within-needle terpene content, and fungal endophytes are known to influence susceptibility to WPBR in High Five pines (Bullington et al. 2018; Liu et al. 2019).

A hallmark of pine species is the characteristic and strong fragrance emanating from their foliage. While aesthetically pleasing to humans, these volatile organic compounds (VOCs) have numerous ecological functions that allow plants to interact with and adapt to their environment. For example, VOCs can communicate plant identity and location to enemies like insect herbivores (Bruce and Pickett 2011) and to beneficial organisms like pollinators (Burkle and Runyon 2016; Raguso 2008). Plant VOCs also play key roles in defense by attracting natural enemies of herbivores (Kessler and Baldwin 2001; Mäntylä et al. 2017; Turlings and Erb 2018) and by directly killing pathogenic bacteria and fungi (Farré-Armengol et al. 2016; Himejima et al. 1992; Huang et al. 2012; Vainio-Kaila et al. 2017). VOCs can also function as airborne signals within and between plants, alerting distant branches and neighboring plants of imminent attack (Karban et al. 2014). A fundamental role for plant VOCs is protection against abiotic stress (Holopainen and Gershenzon 2010). For example, volatile terpenoids can protect plants from damage caused by high temperatures (Behnke et al. 2007; Copolovici et al. 2005), high light and oxidative damage (Loreto and Velikova 2001; Vickers et al. 2009) and cold stress (Cofer et al. 2018). Moreover, plant VOCs, especially terpenoids, are thought to influence the flammability of foliage and behavior of wildfires (Jenkins et al. 2014; Page et al. 2012). The VOCs of High Five pine species are poorly studied, but are known to play important roles in their fitness. For example, the VOCs emitted by susceptible *P. flexilis* strongly attract host-searching female MPB in the pioneering stage, whereas those emitted by resistant *P. longaeva* repel pioneering female MPB (Gray et al. 2015).

Given these important roles, VOCs should be sensitive to selection imposed by environmental factors like abiotic stress and herbivore interactions (Dicke and Baldwin 2010; Loreto et al. 2014). Hence, comparison of the qualitative and quantitative differences in VOCs among closely related taxa that vary in their resistance to herbivores and pathogens could provide insights into mechanisms underlying resistance. We hypothesized that there would be distinct differences in the presence/absence of compounds in VOC blends among High Five pine species, as has been found in previous studies comparing foliar VOCs across other *Pinus* species (e.g., Celiński et al. 2015; Dormont et al. 1998; Ioannou et al. 2014; Mitić et al. 2017; Roussis et al. 1995; Tsitsimpikou et al. 2001). In this study, we used multivariate analysis (Random Forest and non-metric multidimensional scaling) to compare the VOCs emitted by eight species of High Five pines (six in North America and two in Europe) and identify the compounds most important for discrimination among species.

Methods and Materials

Species and Site Selection All six species of high-elevation five-needle white pines occurring in North America (*P. albicaulis*, *P. aristata* Engelm., *P. balfouriana*, *P. flexilis*, *P. longaeva* and *P. strobiformis* Engelm.) and two species occurring in Europe (*P. cembra* and *P. peuce* Griseb.) were sampled in this study (Fig. 1 and Table 1). We define High Five pines as *Pinus* species in the subgenus *Strobus* that grow at or near alpine tree line (Tomback et al. 2011). High Five pines are probably not a monophyletic group but share a similar ecology and threats (Eckert and Hall 2006; Keeley 2012). At each site, we randomly selected average, but similarly-sized mature trees that showed no external signs of stress (e.g., herbivory or pathogen infection). Size was measured as height and mean diameter at breast height (assessed at 1.37 m; DBH) (Table 1).

Collection and Analysis of VOCs VOCs were collected *in situ* from the lower branches (≤ 3 m above ground) of trees
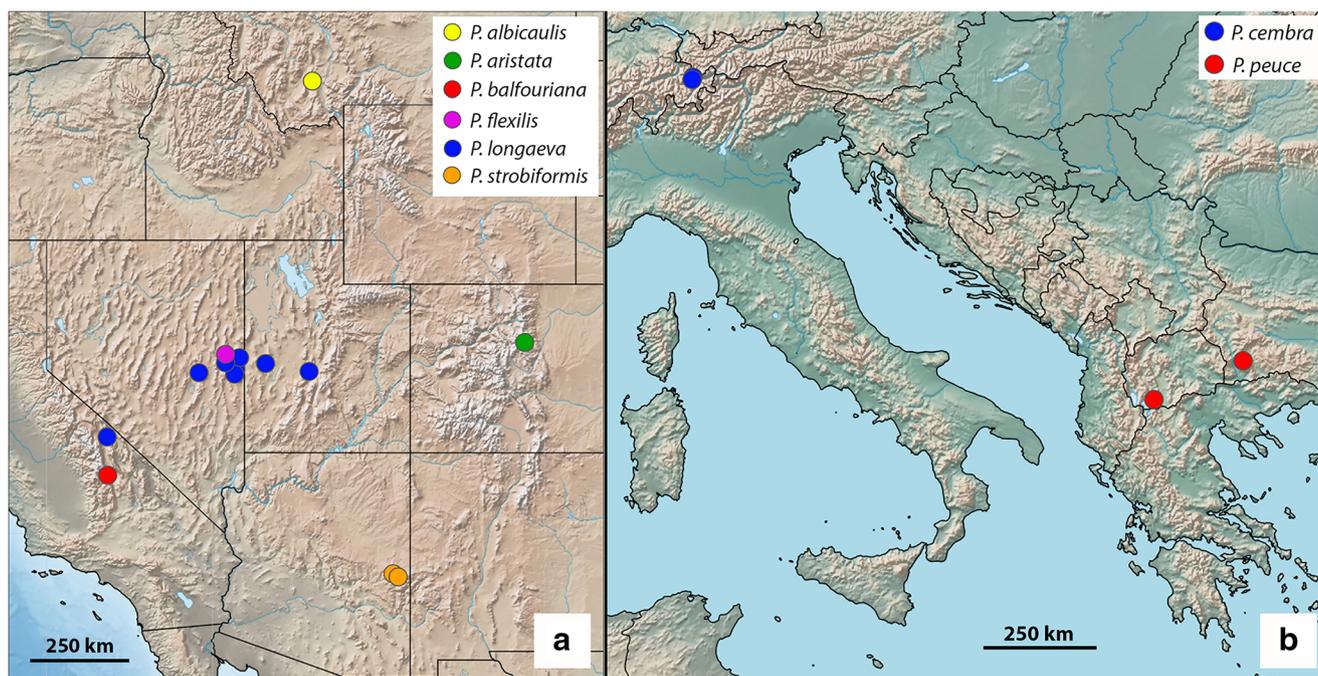
Fig. 1 Locations where VOCs of high-elevation five-needle pine species were sampled in (**a**) western North America and (**b**) Europe

following established headspace sampling methods (e.g., Burkle and Runyon 2017; Page et al. 2012; Tholl et al. 2006). The apical approximately 50 cm of a branch on each tree was enclosed in a clear Teflon bag (50 cm wide × 75 cm deep; American Durafilm Co., Holliston, MA, USA) and air was pulled for 30 min at 0.5 L/min through a volatile trap containing 30 mg of the absorbent HayeSep-Q (Restek, Bellefonte, PA, USA) by using a portable volatile collection system (Volatile Assay Systems, Rensselaer, NY, USA) or an AirLite air sampling pump (SKC Inc., Eighty Four, PA, USA). Volatile traps were kept cool (< 22 °C, stored in a cooler and/ or shipped on ice) and were processed within two weeks of sampling. VOCs were collected during the growing season (between 16 June–20 August) between 1000 and 1500 h in good weather (15–25 °C; sunny to mostly sunny). VOCs were collected from empty bags to identify and exclude potential background and/or contaminant odors. After VOC collection, needles of enclosed branches of each tree were weighed and volatile emissions were standardized on a per weight basis (ng per hour per gram FW).

VOCs were eluted from traps by using 200 μl of dichloromethane (remainder of solvent pushed out into GC vial with a gentle stream of ultra-high-purity nitrogen gas) and 1 μg of *n*-nonyl-acetate was added as the internal standard. Samples were analyzed by using an Agilent 7890A gas chromatograph (GC) coupled with a 5975C mass spectrometer (MS) and separated on a HP-1 ms (30 m × 0.25 mm i.d, 0.25 μm film thickness) column with helium as the carrier gas. The GC oven was maintained at 35 °C for 3 min and then increased by 5 °C per min to 125 °C,

then 25 °C per min to 250 °C. Quantifications were made relative to the internal standard by using ChemStation software (Agilent Technologies, Wilmington, DE, USA). Volatile compounds were identified by comparison of chromatographic retention times and mass spectra with those of commercially available standards. All standards were purchased from Sigma-Aldrich (St. Louis, MO, USA) except for (*E*)-β-farnesene which was obtained from Bedoukian Research, Inc. (Danbury, CT, USA). NIST 08 Mass Spectral Search Program (National Institute of Standards and Technology, Gaithersburg, MD, USA) was used to classify the remaining unidentified compounds as unidentified monoterpenoids (MT1, MT2, etc.) or sesquiterpenoids (ST1, ST2, etc.). Additional information on these unidentified compounds is given in Table S2 (Online Resources 1). Although we were not able to identify these unknown compounds, we verified their occurrence across species by comparing retention times, mass spectra, and by using the NIST 08 Mass Spectral Search Program.

Statistical Analysis A one-way Welch's ANOVA was used to test for a species effect on total VOCs emitted, followed by a Tukey's honest significant difference (HSD) *post-hoc* test to identify pairwise differences for each species; amounts were square-root transformed for ANOVA and HSD analysis. We used the Random Forest classification algorithm (Breiman 2001) to compare and contrast VOCs of High Five pine species and to identify the compounds most important for discriminating species. We also used Random Forest to identify the compounds most important for discriminating *Pinus* species reported to be

Table 1 High-elevation five-needle pine species, sample sizes, locations, dates, and tree sizes used in a comparative study of foliage volatiles

Pinus species	Common name	Site	Trees sampled	Latitude	Longitude	Elevation (m)	Sample Date	DBH[a] (cm)	Height (m)
P. albicaulis	Whitebark pine	Tobacco Root Mtns, Montana, USA	6	45.51	−111.99	2667	3.viii.2012	37 ± 4	8 ± 2
P. aristata	Rocky Mountain bristlecone pine	Mt. Evans, Colorado, USA	12	39.64	−105.59	3505	20.vii.2014	29 ± 2	9 ± 1
P. balfouriana	Foxtail pine	Horseshoe Meadow, California, USA	12	36.45	−118.16	3018	16.viii.2014	56 ± 6	16 ± 1
P. cembra	Swiss stone pine	Davos 1, Switzerland	4	46.76	9.89	2050	18.vii.2014	n.d.	n.d.
		Davos 2, Switzerland	4	46.80	9.90	2000	19.vii.2014	n.d.	n.d.
P. flexilis	Limber pine	Cave Mtn, Nevada, USA	15	39.16	−114.61	3230	20.viii.2013	102 ± 12	12 ± 1
P. longaeva	Great Basin bristlecone pine	Cave Mtn, Nevada, USA	15	39.16	−114.61	3230	20.viii.2013	85 ± 8	13 ± 1
		Beehive Peak, Utah, USA	6	38.96	−112.09	2682	16.vi.2014	n.d.	n.d.
		Notch Peak, Utah, USA	4	39.14	−113.40	2804	2.viii.2014	31 ± 8	4 ± 1
		Wheeler Peak, Nevada, USA	4	39.00	−114.30	3261	2.viii.2014	76 ± 14	6 ± 1
		Mt. Moriah, Nevada, USA	4	39.28	−114.19	3420	3.viii.2014	74 ± 14	5 ± 0.4
		Mt. Washington, Nevada, USA	4	38.89	−114.33	2743	3.viii.2014	17 ± 4	6 ± 2
		Currant Mtn, Nevada, USA	4	38.93	−115.41	2819	15.viii.2014	53 ± 8	8 ± 0.4
		Patriarch Grove, California, USA	12	37.38	−118.18	3083	16.viii.2014	90 ± 8	13 ± 1
P. peuce	Macedonian pine	Pirin Mtns, Bulgaria	4	41.76	23.41	2160	12.vii.2014	n.d.	n.d.
		Pelister, Macedonia	4	41.03	21.23	1470	14.vii.2014	n.d.	n.d.
P. strobiformis	Southwestern white pine	Gila National Forest, Arizona, USA	12	33.91	−109.41	2812	19.vii.2014	28 ± 2	13 ± 1

[a] Diameter of tree stem (bole) at breast height (1.37 m) ± standard error

n.d., not determined

resistant or susceptible to MPB or WPBR. Species included in the analysis as susceptible to MPB were *P. albicaulis*, *P. aristata*, *P. flexilis*, and *P. strobiformis*; species included as resistant to MPB were *P. balfouriana* and *P. longaeva* (Bentz et al. 2017; Eidson et al. 2017, 2018; Wood 1982). Susceptibility of European pine species (*P. cembra*, *P. peuce*) to MPB is unknown and were excluded from this analysis. In a separate analysis, we included species that are susceptible to WPBR as *P. albicaulis*, *P. aristata*, *P. balfouriana*, *P. flexilis*, and *P. strobiformis*; species included that are reported resistant to WPBR were *P. cembra*, *P. longaeva*, and *P. peuce* (Geils et al. 2010; Tomback and Achuff 2010). Random Forest is a machine-learning algorithm that has proven useful in analyzing large VOC data sets (Jaeger et al. 2016; Ranganathan and Borges 2010). Our analysis used RANDOMFOREST package v.4.6–14 (Liaw and Wiener 2002) under R v.3.3.3 (R Development Core Team 2016) applying the measure of importance option of predictor variables, the proximity option, a measure of the internal structure of the data used to detect outliers, the number of trees set at 100, a maximum of 5 variables tried at each split, and all other parameters set to the defaults. Random Forest returns a confusion matrix that summarizes the accuracy of the classification as well as the variable importance. The importance of each VOC for classification

was ranked by using mean decrease in Gini impurity index which is based on overfitted models (Breiman 2001). To characterize VOC dissimilarity among our *Pinus* species, a non-metric multidimensional scaling (NMDS) ordination based on a matrix of Euclidean dissimilarities (Kenkel and Orlóci 1986; Dixon 2003) was calculated on the rank order proportion of VOCs from each tree. The package VEGAN v 2.5–1 was used to employ the monoMDS function via metaMDS, which increases the likelihood of achieving a global optimum, using a maximum number of iterations of 1000. This function implements Kruskal's (1964) non-metric multidimensional scaling (NMDS) by using monotone regression and weak treatment of ties, where equal observed dissimilarities are allowed to have different fitted values.

Results

VOCs emitted by High Five pine species We sampled eight species of High Five pines (Table 1) and a total of 35 volatile compounds were identified using GC-MS analysis from the headspace of these species (Table S1 in Online Resource 1). VOCs emitted were composed entirely of terpenes: monoterpenoids made up the vast majority of the composition

Table 2 Importance ranking (Mean Decrease in Accuracy, MDA) and Mean Decrease of the Gini Index of volatile organic compounds (VOCs) in classifying eight species of high-elevation five-needle pines by using Random Forest. MT, unidentified monoterpenoid; ST, unidentified sesquiterpenoid

Volatile compound	Mean decrease accuracy	Mean decrease Gini index
o-cymene	25.62	7.82
camphor	24.99	6.54
MT 5	22.64	6.40
3-carene	21.44	5.70
limonene	20.54	3.91
β-cubebene	20.35	4.40
β-phellandrene	19.98	4.76
α-terpinene	19.96	4.30
α-phellandrene	18.06	3.41
tricyclene	17.82	3.90
bornyl acetate	17.13	3.54
(E)-β-farnesene	14.64	3.05
MT 1	14.47	2.51
camphene	14.09	2.32
α-pinene	13.97	2.66
β-myrcene	13.07	1.90
p-cymene	12.90	2.22
β-pinene	12.87	1.76
MT 9	12.16	2.00
MT 2	11.84	1.57
γ-terpinene	11.80	1.87
α-farnesene	11.60	1.78
(E)-β-ocimene	11.55	1.98
α-terpineol acetate	11.25	1.86
ST 2	11.25	1.49
MT 10	10.87	1.92
ST 1	10.52	1.68
ST 5	10.38	1.50
sabinene	10.29	1.60
terpinolene	9.37	1.26
MT 8	8.43	1.07
ST 4	6.95	0.80
ST 3	6.42	1.01
α-humulene	6.00	0.92
MT 7	5.96	0.81

of VOCs (27 compounds; 77%) followed by sesquiterpenoids (eight compounds; 23%) (Table 2). The total amount of VOCs released per gram of foliage differed among species (Welch's ANOVA, $F = 56.05$, $P < 0.0001$) (Fig. S1 in Online Resource 1). The blend of VOCs emitted by High Five pine species was qualitatively very similar with 32 of the 35 VOCs emitted by all eight *Pinus* species (Table S1 in Online Resource 1). The only compounds not emitted by all species were unidentified
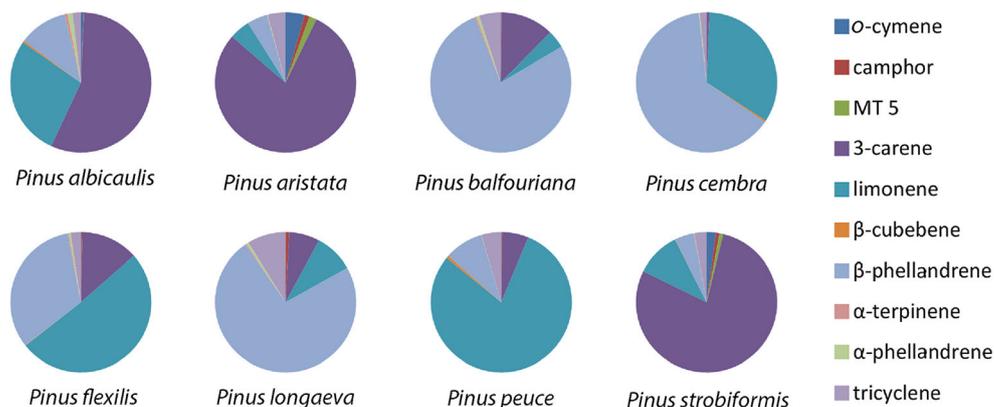
monoterpenoid 5 (MT 5), unidentified sesquiterpene 2 (ST 2), and α-terpinene. All of these were relatively minor compounds in the VOC blend that were emitted in small amounts (Table S1 in Online Resource 1). MT 5 was emitted only by *P. albicaulis* and *P. strobiformis*; ST2 was emitted by all species except *P. albicaulis*; and α-terpinene was absent only from the VOC blend of *P. peuce*. Although the compounds emitted were qualitatively similar, there were clear quantitative differences in VOCs among species. The mean amounts of many compounds varied across the species, sometimes greatly so. This resulted in large differences in ratios of VOCs in emitted blends of these *Pinus* species (Fig. 2).

Random Forest and Ordination Random Forest classification correctly differentiated between all *Pinus* species using VOCs with an out-of-bag accuracy of 92.9%, with most species having only one tree misclassified (Table 3). The Random Forest out-of-bag accuracy for distinguishing resistant and susceptible *Pinus* species using VOCs was 98.2% for MPB and 96% for WBPR (Table 4). We used NMDS ordination to visualize species differences based on VOC composition (Fig. 3). NMDS ordination of the VOC data had a high linear fit ($R^2 = 0.98$) and a low stress value (0.09), with two convergent solutions found after 566 tries. A stress of ca. 0.05 provides an excellent representation in reduced dimensions, whereas a stress of ca. 0.1 provides a good representation (Clarke 1993). The NMDS plot, in which each symbol is a tree and distances between symbols are proportional to the dissimilarity in VOCs, showed strong clustering that tended to reflect species (Fig. 3).

Discussion

The VOCs of High Five pines were similar in that all eight species examined released the same 32 compounds (of 35 total). Such qualitative similarity in VOCs was unexpected because High Five pine species have long and disparate histories. The *Pinus* subgenus *Strobus*, within which the High five pine species are scattered, is an ancient lineage that diverged around 85 Ma (Eckert and Hall 2006; Willyard et al. 2006) and High Five species have distinct biogeographic and evolutionary histories (Keeley 2012; Tomback et al. 2011). The compounds comprising VOC blends generally vary across plant species, and even closely related taxa often emit qualitatively different mixes of odors (e.g., Courtois et al. 2016; Jürgens et al. 2006; Levin et al. 2003; Luani et al. 2019). For example, the VOCs of species and subspecies of sagebrushes (*Artemisia*, subgenus *Tridentatae*), evolutionarily a relatively young group (<5 Ma), display dramatic divergence in the number and identity of compounds emitted (Jaeger et al. 2016). Moreover, the VOCs of species within the genus *Pinus* have been shown to vary qualitatively across

Fig. 2 Mean amounts (ng per hour per gram) of the 10 most important VOCs for classifying high-elevation five-needle pine species by using Random Forest (Table 2). Note the differences in ratios among the species

Pinus albicaulis   *Pinus aristata*   *Pinus balfouriana*   *Pinus cembra*

Pinus flexilis   *Pinus longaeva*   *Pinus peuce*   *Pinus strobiformis*

- *o*-cymene
- camphor
- MT 5
- 3-carene
- limonene
- β-cubebene
- β-phellandrene
- α-terpinene
- α-phellandrene
- tricyclene

species (Celiński et al. 2015; Dormont et al. 1998; Ioannou et al. 2014; Mitić et al. 2017; Roussis et al. 1995; Tsitsimpikou et al. 2001) and can take on qualitatively very different forms, for example some species emit VOCs dominated by short, straight-chain hydrocarbons (Savage et al. 1996; Adams and Wright 2012) unlike the terpene-based odors of the High Five pines.

This qualitative similarity of High Five pine VOCs, despite such old divergence times and disparate histories, suggests that these compounds could play important roles in the environmental adaptation of these trees. These High Five pine species grow at or near alpine treelines, at the limits of plant growth, and must deal with harsh environments. The mix of volatile compounds might be conserved in these species as adaptions against the abiotic challenges that accompany growth at high elevations, such as extreme UV exposure or cold temperatures. Volatile terpenoids emitted by plants can protect against such abiotic threats including high light, UV, oxidative damage, and cold stress (Cofer et al. 2018; Loreto and Velikova 2001; Loreto and Schnitzler 2010; Vickers et al. 2009). Moreover, many monoterpenes are antimicrobial (Gershenzon and Dudareva 2007; Himejima et al. 1992; Marei et al. 2012; Vainio-Kaila et al. 2017) and VOCs could

defend against snow molds, caused by deep snow cover, which can be an important mortality factor for High Five pines (Barbeito et al. 2013). Alternatively, the mix of VOCs emitted by High Five pines could be constrained by genetics or physiology, but this seems less likely since closely related *Pinus* species can vary dramatically in the makeup of VOCs (Adams and Wright 2012; Celiński et al. 2015). Total amounts of VOCs released differed for some species (Fig. S1 in Online Resource 1), but total emission rate is known to be positively correlated with air temperature (Jamieson et al. 2017; Niinemets et al. 2004). We suspect that total VOC differences of species in this study were influenced by air temperature at time of sampling, which we were unable to control. More research is needed, ideally using common gardens, to understand how genetics and environment affect VOCs and ascertain what functions these volatile compounds perform for High Five pines.

Despite large qualitative similarity, the VOCs of High Five pines can be used to reliably distinguish species. The Random Forest algorithm correctly identified 117 of the 126 trees sampled by using VOCs. Interestingly, this was achieved not through the presence of species-specific compounds in VOC blends, but rather largely via differences in the relative amounts

Table 3 Confusion matrix of Random Forest classification showing observed and predicted *Pinus* species and error rate when using VOCs for classification. Bold numbers indicate the number of individual trees

classified correctly for each species; unbolded numbers were incorrectly classified using VOCs

Observed class	Predicted class								
	P. albicaulis	*P. aristata*	*P. balfouriana*	*P. cembra*	*P. flexilis*	*P. longaeva*	*P. peuce*	*P. strobiformis*	Class error
P. albicaulis	**6**	0	0	0	0	0	0	0	0.00
P. aristata	0	**11**	0	0	0	0	0	1	0.08
P. balfouriana	0	0	**10**	0	0	2	0	0	0.17
P. cembra	0	0	0	**7**	0	0	1	0	0.13
P. flexilis	0	0	0	0	**13**	2	0	0	0.13
P. longaeva	0	0	0	0	1	**52**	0	0	0.02
P. peuce	0	0	0	1	0	0	**7**	0	0.13
P. strobiformis	0	1	0	0	0	0	0	**11**	0.08

Table 4 Importance ranking (Mean Decrease in Accuracy, MDA) of top ten volatile organic compounds (VOCs) in classifying species of high-elevation five-needle pines resistant versus susceptible to mountain pine beetle (*Dendroctonus ponderosae*) or white pine blister rust (*Cronartium ribicola*) by using Random Forest. MT, unidentified monoterpenoid; ST, unidentified sesquiterpenoid

| Susceptibility to mountain pine beetle[a]		Susceptibility to white pine blister rust[b]	
Volatile compound	Mean decrease accuracy	Volatile compound	Mean decrease accuracy
β-phellandrene	19.72	3-carene	22.58
o-cymene	19.28	*o*-cymene	21.81
3-carene	17.57	camphor	16.95
camphor	14.47	bornyl acetate	16.37
MT 5	14.40	α-pinene	15.46
α-pinene	13.76	β-phellandrene	15.25
tricyclene	12.51	MT 5	13.78
bornyl acetate	11.78	tricyclene	13.62
p-cymene	10.95	α-terpinene	12.88
ST 2	10.87	*p*-cymene	12.59

[a] Random Forest out-of-bag accuracy 98.2%. Species included that are susceptible to mountain pine beetle (MPB): *Pinus albicaulis, P. aristata, P. flexilis, P. strobiformis*; species included that are resistant to MPB: *P. balfouriana, P. longaeva*. Susceptibility of European pine species (*P. cembra, P. peuce*) to MPB is unknown and were excluded from this analysis

[b] Random Forest out-of-bag accuracy 96%. Species included that are susceptible to white pine blister rust (WPBR): *P. albicaulis, P. aristata, P. balfouriana, P. flexilis, P. strobiformis*; species included that are reported resistant to WPBR: *P. cembra, P. longaeva, P. peuce*

of compounds emitted. For instance, the three compounds whose presence/absence varied among species ranked number three, five, and fifteen in the list of compound importance for classifying species (Table 2). Thus, it is the relative amounts and ratios of compounds that varied consistently across species and permitted accurate taxonomic classification. Recent research found similar quantitative differences in VOCs within Great Basin bristlecone pine (*P. longaeva*) in which proportions of compounds emitted changed consistently with elevation (Gray et al. 2019). This shows that environment can have consistent and predictive influence on VOCs of High Five pines; however, this relatively small within-species environmental effect was overcome by larger intrinsic differences among species. For example, we sampled *P. longaeva* of a variety of

Fig. 3 Non-linear Multidimensional Scaling (NMDS) ordination plot of the first and second dimensions for 35 VOCs emitted (ng per hour per gram) from eight species of high-elevation five-needle pines. Ellipses represent the standard deviation of the centroid for each species group
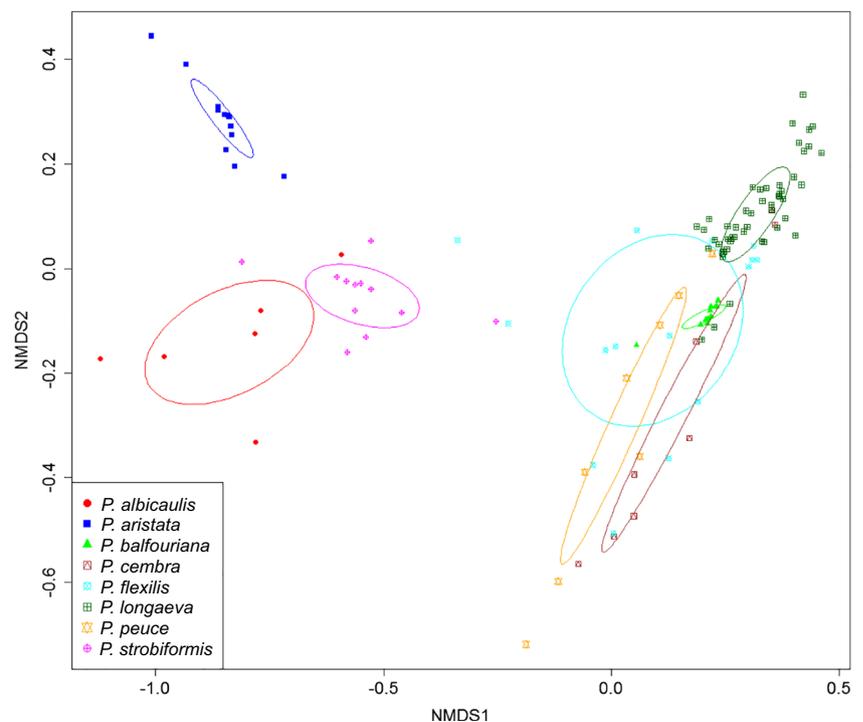
sizes at eight sites spanning its geographic range and Random Forest still correctly identified 51 of 52 *P. longaeva* trees and ordination showed strong clustering of this species, and of High Five species in general (Fig. 3). VOCs could also be used by Random Forest to consistently distinguish species resistant from those susceptible to MPB or WPBR (Table 4). Examination of High Five VOCs by using a GC column with a chiral stationary phase might reveal further consistent differences in proportions since several *Pinus* monoterpenes can occur as stereoisomers (e.g., α-pinene, camphene, limonene; Seybold et al. 2006).

Because VOCs play critical roles in the interactions of plants with their environment, understanding differences in VOCs in closely-related plant species could help deduce VOC function. This could have particular importance for the North American High Five pine species which are threatened by outbreaks of MPB and WPBR (Tomback et al. 2011). For example, susceptibility to MPB attack varies greatly across North America High Five species with *P. longaeva* and *P. balfouriana* being almost completely resistant and *P. albicaulis* and *P. flexilis* being highly susceptible (Bentz et al. 2017). Comparison of VOCs emitted by these species might provide insights into how some species avoid being killed by MPB. Indeed, a recent study found that pioneering female MPBs were strongly attracted by VOCs from *P. flexilis* but repelled by VOCs from *P. longaeva* (Gray et al. 2015). That study did not identify the bioactive compounds, but did find evidence that MPB responses were mediated by ratios of multiple compounds (Gray et al. 2015). Specific differences in amounts of VOCs could also affect attraction of MPBs in the mass attack phase, since some host VOCs can act as strong synergists of MPB pheromones (Borden et al. 2008). The findings of Gray et al. (2015), together with the results presented here, suggest that if host-seeking herbivorous insects are to distinguish High Five pine species by using olfaction, as most insect herbivores do (Bruce and Pickett 2011), then they likely rely on species-specific differences in the ratios of compounds in odor blends and not on dominant compounds that are present or absent in the specific volatile blends. Thus, understanding how *P. longaeva* repels MPB—knowledge that could be utilized to protect the other High Five species—will likely require investigation of multiple compounds, focusing first on the compounds whose quantities distinguish susceptible and resistant pine species (Table 4). Similarly, given the antimicrobial nature of many VOCs emitted by High Five pines (e.g., monoterpenes; Gershenzon and Dudareva 2007; Marei et al. 2012; Vainio-Kaila et al. 2017), comparisons of VOCs of species susceptible (most North American species) to WPBR with those resistant to infection (European species) (Table 4), focusing on ratios rather than single compounds, could offer insight into mechanisms underlying resistance to this debilitating pathogen.

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