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# Leaf Micromorphology Aids Taxonomic Delineation within the Hypervariable Genus *Metrosideros* (Myrtaceae) on O‘ahu<sup>1</sup>

Gary L. Sur,<sup>2,6,7</sup> Richard Keating,<sup>4</sup> Neil Snow,<sup>5</sup> and Elizabeth A. Stacy<sup>2,3</sup>

**Abstract:** Variation in leaf micromorphology can aid delimitation of taxonomically difficult groups. The woody genus *Metrosideros* (Myrtaceae) dominates Hawai‘i’s native forests and comprises striking, predominantly infraspecific, macromorphological variation, including many forms unrecognized in current taxonomic treatments. On taxonomically rich O‘ahu, 10 taxa (varieties or unnamed morphotypes of *M. polymorpha* and other species) occur in a predictable sequence with overlapping ranges from low to high elevation along the numerous leeward ridges of the Ko‘olau Range. We used scanning electron and light microscopy to examine mature, nonsenescent sun leaves from each of these 10 taxa. Parametric and nonparametric tests were used to compare stomatal complex (SC) traits and those of a newly described secretory structure (SS) across taxa. Correlations among traits and discriminant analyses were also done. Density and length of SCs were inversely related overall, and both measures varied significantly across taxa. Although abaxial SSs were consistently present only in the two highest-elevation glabrous taxa, adaxial SSs were present in all 10 taxa. Length and density of adaxial SSs and density of abaxial SSs varied across taxa, and densities of both SCs and SSs were greatest at high elevation. Combined, the SC and SS traits differentiated 100% of four glabrous varieties and two of three pubescent varieties of *M. polymorpha*. Variation in leaf micromorphology, including presence and density of a newly described SS, aids delimitation of closely related Hawaiian *Metrosideros* taxa and may reflect differential local adaptation across a heterogeneous landscape.

**Keywords:** elevation gradient, Hawai‘i, leaf micromorphology, *Metrosideros*, Myrtaceae, O‘ahu, ‘Ōhi‘a lehua, stomatal complexes, secretory structures, taxonomy

LEAF MICROMORPHOLOGY can be useful for the delineation of closely related plant taxa. Comprising the stoma, guard cells, and subsidiary cells, stomatal complexes (SCs) can vary significantly in size and density among closely related taxa, sometimes enabling the

separation of taxa from the ordinal to specific levels (Stebbins and Khush 1961, Moon et al. 2009, Edwin-Wosu and Benjamin 2012). Further taxonomic delineation may be possible through variation in other external leaf microstructures such as stomatal crypts

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(Goldenberg et al. 2013) or secretory structures (SSs) such as hydathodes (Jones 2011) or oil glands (Wilson 2011). Variation in leaf micromorphological traits among taxa may be associated with differential local adaptation to contrasting environments. For example, SC density may increase with increasing elevation (Kofidis et al. 2003, Kessler et al. 2007, Premoli and Brewer 2007) or precipitation (Stenström et al. 2002, Wang et al. 2011). At higher elevations, high SC density may be associated with greater tolerance to ultraviolet radiation (Günthardt-Goerg et al. 1993), desiccation (Pearce et al. 2006, Bresson et al. 2011), or reduced atmospheric levels of CO<sub>2</sub> (Garcia-Amorena et al. 2006). High SC density also may be expected in environments with fluctuating transpiration conditions, where it allows a rapid response to changing conditions (Larcher 2003, Drake et al. 2013). Compared to SCs, SSs show striking variation in morphology and function across and within plant families. External SSs can take the form of pits or flattened structures (Pascal et al. 2000, Machado et al. 2008), raised nectaries (Aguirre et al. 2013), or glandular trichomes (Machado et al. 2008). The varied functions of SSs appear to include the release of antiherbivorial compounds (Molano-Flores 2001, Barbehenn and Constabel 2011), secretion of nectar to attract herbivore-detering insects (Schoederer et al. 2010), and detoxification of heavy metals (Franceschi and Nakata 2005), among others.

*Metrosideros* (Myrtaceae) in Hawai'i is a taxonomically difficult group that dominates the native vegetation of the Islands, spanning a striking range of environments (Adee and Conrad 1990), including wet forests and bogs, deserts, subalpine forests, windy cliffs, and riparian zones (Dawson and Stemmermann 1990). If recent estimates of the arrival of *Metrosideros* in Hawai'i (3.9 Myr [2.6–6.3 Myr] [Percy et al. 2008]) are accurate, then the genus has been in Hawai'i for much of the relatively short history of the main islands. During that time *Metrosideros* has diversified into a large number of morphologically distinguishable forms, both pubescent

and glabrous, comprising five species, including the hypervariable landscape-dominant *M. polymorpha* (Dawson and Stemmermann 1990). On young Hawai'i Island (0.5 Myr old) (Carson and Clague 1995), where this group has received the most attention, studies of the dominant varieties of *M. polymorpha* have revealed significant ecological and neutral genetic divergence (DeBoer and Stacy 2013, Morrison and Stacy 2014, Stacy et al. 2014), as well as partial reproductive isolation (Stacy et al. 2017) among forms, suggesting that *Metrosideros* is a rare example of incipient ecological speciation in trees. On the older island of O'ahu (3.7 Myr old) (Carson and Clague 1995), extensive field observations of morphological variation within *Metrosideros* suggest the presence of 10 widespread taxa in the Ko'olau Range, including four species, two recognized varieties of *M. polymorpha*, and five additional morphotypes that we treat here as provisional varieties of *M. polymorpha* (see Figure 1); each of the five provisional varieties is consistently diagnosable through leaf macromorphological characters and elevation range. *Metrosideros* on O'ahu occurs continuously above 250 m on the island's heavily eroded Ko'olau Volcano, and these 10 taxa occur with overlapping ranges in a predictable sequence from low to high elevation across the volcano's many leeward ridges (see Table 1 and Figure 2). One taxon, *M. tremuloides*, is restricted predominantly to the windy sides of the leeward ridges along steep slopes (Dawson and Stemmermann 1990). High cross-fertility among forms (E.A.S., unpubl. data) and the presence of morphologically intermediate individuals of *Metrosideros* throughout the Ko'olau Range (E.A.S., pers. obs.; J. Lau, pers. comm.) suggest that hybridization among these closely related forms is not uncommon. With its northwest-southeast orientation, the Ko'olau backbone receives the brunt of the northeast trade winds, causing environmental conditions to vary sharply from drier [precipitation: 1,000 mm/yr (Giambelluca et al. 2013)] and generally still conditions at low elevation to wetter [precipitation: 6,000 mm/yr (Giambelluca et al. 2013)] conditions at high

TABLE 1

Traits of 10 *Metrosideros* Taxa That Occur Commonly across the Leeward Ridges and Backbone of the Ko'olau Range, O'ahu

Taxon	Taxon Code	Elevation	Approximate Elevation Range in Ko'olau (m above sea level) <sup>a</sup>	Presence of Abaxial Pubescence	Frequency of Abaxial SS (%)	Frequency of Adaxial SS (%)
<i>M. p.</i> var. <i>incana</i>	I	Lowest	240–550	Yes	NA	100
<i>M. p.</i> var. C	C	Low to Middle	550–650	Yes	NA	100
<i>M. p.</i> var. <i>glaberrima</i> <sup>b</sup>	G	Low to Middle	250–650	No	0	20
<i>M. tremuloides</i> <sup>b</sup>	T	Low to Middle	125–700	No	0	100
<i>M. macropus</i> <sup>b</sup>	M	Low to Middle	240–800	No	0	80
<i>M. p.</i> var. L	L	Middle to High	450–920	No	10	100
<i>M. p.</i> var. Z	Z	High	640–950	No	100	100
<i>M. p.</i> var. F	F	High	640–800	Yes	NA	100
<i>M. p.</i> var. B	B	Backbone	680–960	No <sup>c</sup>	100	100
<i>M. rugosa</i>	R	Backbone	700–960	Yes	NA	100

Note: *M. p.* is the dominant species, *M. polymorpha*, and vars. C, L, Z, F, and B are infraspecific taxa not yet recognized in any taxonomic treatment. Shown for each taxon is its relative position (Elevation) within the full elevation range of *Metrosideros* forest along the leeward ridges, with backbone taxa occurring at the highest elevation. For each taxon, the presence of abaxial pubescence and the frequencies of individuals examined via light microscopy in this study that possessed abaxial and adaxial leaf secretory structures (SS) are also shown. Abaxial surfaces of pubescent leaves were not observed via light microscopy and are labeled as not applicable (NA).

<sup>a</sup> Approximate elevations within the middle Ko'olau Range based on field observations and Dawson and Stemmermann (1990). Many ranges extend lower where the Ko'olau backbone is more eroded and thus lower.

<sup>b</sup> Three taxa with broad elevation ranges.

<sup>c</sup> Emerging leaves of *M. polymorpha* var. B are pubescent, but adult leaves are glabrous, a condition common in many Myrtaceae.

elevations (i.e., along the Ko'olau backbone), where cloud cover is higher (Leopold 1949, Burroughs and Larson 1979), and gusts can exceed 44.7 m/sec (Chock et al. 2005). Thus, the taxa of *Metrosideros* distributed along the leeward ridges are exposed to dry, calm conditions at low elevation and increasingly more variable, but often wet and windy conditions with increasing elevation up to the backbone.

In this study we used scanning electron and light microscopy to examine and compare leaf micromorphology among 10 *Metrosideros* taxa from O'ahu: *M. macropus* Hook. & Arn., *M. rugosa* A. Gray, *M. tremuloides* (Heller) P. Kunth, and seven morphotypes or varieties of the hypervariable *M. polymorpha* Gaud. We aimed to determine the utility of leaf micromorphology for the delimitation of these closely related taxa, especially for the unnamed forms of *M. polymorpha*. Although the four pubescent taxa are easily distinguished using macromorphological characters, the glabrous taxa are particularly difficult to distinguish consistently, likely due

to hybridization. We also noted associations between micromorphology and environment. In particular, we predicted an increase in the density of SCs with elevation within this group in response to more-variable weather conditions at high elevation relative to lower elevations on the Ko'olau's leeward ridges.

#### MATERIALS AND METHODS

##### Leaf Collection and Site Description

Ten taxa of *Metrosideros* (six glabrous and four pubescent) (Figure 1) were sampled from five leeward ridges of the heavily eroded Ko'olau Volcano on the island of O'ahu (Figure 2). Species of *Metrosideros* make up the dominant woody vegetation on the ridges of the Ko'olau Range, and the sampled taxa include all the morphotypes that are widespread in the range. For convenience we use "var." to include formally named varieties and unnamed forms, with the latter receiving a designation of a capital letter. Two leaves from each of two

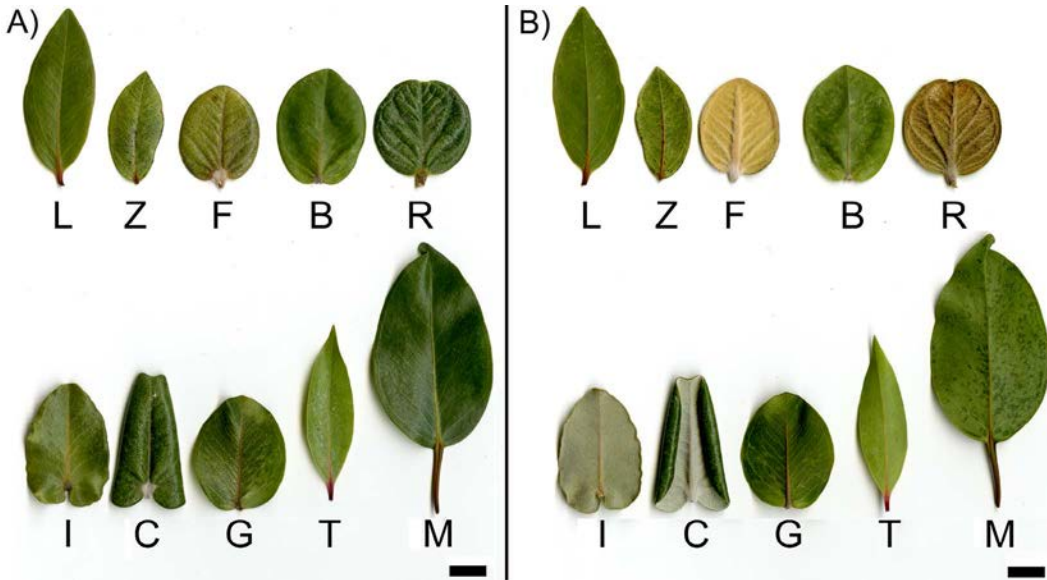


FIGURE 1. Variation in leaf morphology among 10 *Metrosideros* taxa of O'ahu's Ko'olau Range: (A) adaxial leaf surfaces; (B) abaxial leaf surfaces (scale bars = 10 mm). Taxon codes are shown in Table 1.

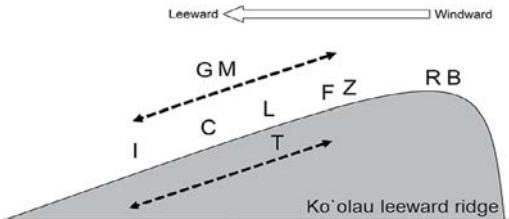


FIGURE 2. Schematic of a leeward ridge of O'ahu's Ko'olau Volcano. Ten *Metrosideros* taxa (codes are in Table 1) are distributed largely in a predictable sequence with overlapping ranges along the leeward ridge, and T is distributed broadly on the windy slopes of the leeward ridges. Dashed arrows below G, M, and T indicate the broad elevation ranges of these three taxa.

trees for nine of these taxa (exception: *M. polymorpha* var. L) were sampled for scanning electron microscopy (SEM); leaves of L were not available when the SEM observations were made. One leaf from each of 10 additional trees for each of the 10 taxa (six trees of *M. polymorpha* var. F) was sampled for light microscopy. All collections were of mature, but not senescing, sun leaves; apparent hybrids were avoided. Sampling within each

taxon was spread across multiple ridges to capture natural variation. The leaves were transported to the laboratory and stored at 4°C in sealable bags.

#### Scanning Electron Microscopy (SEM)

Leaf samples were examined within 2 weeks of collection, with the exception of one taxon (*M. polymorpha* var. C), which was imaged after 25 days. The leaves were cleaned with water, and a single section from each was removed with a razor blade under a dissecting scope (Olympus SZ51, Olympus Corporation, Tokyo, Japan) and affixed (abaxial surface up) by adhesive carbon tabs to a Deben cold-stage mount (Deben UK Ltd., Suffolk, United Kingdom). An SEM (Hitachi S-3400N, Hitachi Ltd., Tokyo, Japan) was used to examine the leaf sections (cooled to -15°C) under the microscope's variable-pressure program to minimize electron charging in the still-hydrated samples (Pathan et al. 2010). At 350× magnification (area: 92.2 μm<sup>2</sup>), five micrographs per leaf section were created, and each was examined for SC length based on

20 haphazardly chosen SCs (i.e., 100 SCs per leaf, or 200 SCs per tree) using Quartz PCI software (Quartz Imaging Corporation, Vancouver, Canada).

### *Light Microscopy (LM)*

Leaf samples were examined within 2 weeks of collection. Each leaf was rinsed with water, air-dried, and coated with a thin layer of clear nail polish. Nail polish was applied to glabrous surfaces only, including both abaxial and adaxial leaf surfaces of the six glabrous taxa and adaxial leaf surfaces of the four pubescent taxa (because the abaxial pubescence was incompatible with the nail polish). The dried polish layers were removed with packaging tape and positioned under coverslips on microscope slides. Slides were viewed under a light microscope (Leica DM500, Leica Camera AG, Wetzlar, Germany), and micrographs were taken through the optical scope with a digital camera (Canon PowerShot SD980 IS, Canon Inc., Tokyo, Japan).

Leaf micromorphological traits were observed at magnifications of 40×, 100×, and 400×, and the resulting micrographs had circular areas of 1,590  $\mu\text{m}^2$ , 636  $\mu\text{m}^2$ , and 159  $\mu\text{m}^2$ , respectively. Abaxial SC densities and lengths of the six glabrous taxa were recorded at 100× and 400× magnification, respectively. In addition, purported SSs (see Discussion for explanation) were observed under the microscope and examined, where present, on the adaxial and abaxial leaf surfaces of the glabrous taxa and only the adaxial surfaces of the pubescent taxa (no SSs were present on the abaxial surfaces of any of the pubescent-leaved taxa). The densities and lengths of the purported SSs were recorded from micrographs at 40× and 100× magnification, respectively. SC and SS lengths were first measured as pixels in the Quartz PCI software program, and the pixels were downloaded as a Microsoft Excel file in the CSV (comma separated values) format. Although viewed at different magnifications, the lengths of SCs and SSs were measured identically end to end, using an optical micrometer with unit lengths that changed between magnifications (i.e., 100×: 1 unit = 10  $\mu\text{m}$ ; 400×: 1 unit =

2.5  $\mu\text{m}$ ) (Supplemental Appendix Figure S1). Given the micrograph's magnification, conversion from pixels to micrometers involved (1) multiplying the actual micrometer-unit length with the microstructure's pixel length, and (2) dividing the product by the micrometer-unit's pixel length. Lengths were measured for 2,674 SCs across six taxa (mean = 44.6/tree), 2,015 adaxial SSs across 10 taxa (mean = 20.1/tree), and 678 abaxial SSs across three taxa (mean = 24.6/tree).

**Authors' Note:** Supplemental materials available online at BioOne (<http://www.bioone.org/toc/pasc/current>) and Project MUSE (<http://muse.jhu.edu/journal/166>).

### *Anatomy of Leaf Stomata and Purported Secretory Structures*

To better characterize the leaf stomata and purported SSs, fresh leaf samples from four taxa were fixed in FPA<sub>50</sub>. These four taxa, including two glabrous (*M. polymorpha* var. B, *M. tremuloides*) and two pubescent (*M. polymorpha* var. *incana* (Lev.) Skottsberg, *M. rugosa*), captured the range of SC and SS sizes observed across the 10 taxa through LM. Thin hand sections were prepared and mounted in calcium chloride solution (Keating 2014). Observations were made using a microscope (Leitz Ortholux, Leica Camera AG, Wetzlar, Germany) and photographed using a Nikon camera (Coolpix 5700, Nikon Corp., Tokyo, Japan).

### *Data Analysis*

Analyses were done using Minitab 17 (Minitab Inc., State College, Pennsylvania), and RStudio (RStudio Inc., Boston, Massachusetts). Residual plots were made to check for normality and equal variances in all datasets. One-way analyses of variance (ANOVAs) with Tukey's post hoc pairwise comparisons were used for among-taxon comparisons of LM-derived SC density and SS length. Due to the small sample sizes used for the SEM measures of SC length and density and the unequal variances of LM-derived SC length and adaxial SS density, each was compared (untransformed) among taxa using a Kruskal-

Wallis test. Nemenyi post hoc tests (Pohlert 2014) were done to reveal pairwise differences among taxa in LM-derived SC length and purported SS density. The densities and lengths of abaxial SSs were compared between two taxa using *t* tests. Pairwise correlations involving all length and density measures were calculated using Pearson (*r*) or Spearman ( $\rho$ ) statistics, as appropriate, and linear discriminant analysis was used separately for the SEM and LM measures to help delineate taxa, with an emphasis on the four morphologically similar but distinct glabrous varieties of *M. polymorpha*.

## RESULTS

### Stomatal Complex Length

SCs were restricted to the abaxial surfaces of all leaf samples, and SC length varied significantly among taxa (Figure 3). The more precise SEM-based measures of SC length (1,704 total) were highly similar within taxa with two exceptions: *M. polymorpha* vars. B and Z (Table 2). The SEM measures ranged from a mean of  $14.55 \pm 0.35$  (SE) and  $15.90 \pm 1.7$   $\mu\text{m}$  for *M. polymorpha* vars. *glaberrima* (Levl.) St.

John and B to  $27.65 \pm 0.55$   $\mu\text{m}$  for *M. tremuloides*, all of which are glabrous taxa ( $H = 15.98$ ,  $df = 8$ ,  $P = .043$ ), and LM-based measures (2,674 total) of the six glabrous taxa were roughly similar but showed a smaller range among taxa (i.e.,  $4.46$   $\mu\text{m}$ ;  $H = 19.95$ ,  $df = 5$ ,  $P = .001$ ) (Table 2). Post hoc tests of the LM-based measures revealed significant differences among taxa with similar ecologies, namely *M. polymorpha* var. B and *M. tremuloides*, both of which occur in high-wind habitats (Ko'olau backbone and steep slopes of leeward ridges, respectively). Last, the mean SC length of leaves of backbone-restricted *M. polymorpha* var. B was significantly smaller than that of one of the other high-elevation glabrous taxa, *M. polymorpha* var. L ( $24.13 \pm 1.13$   $\mu\text{m}$ ), but not the other, *M. polymorpha* var. Z. Examined at  $250\times$ , the leaf stomata were level with the leaf surface and contained roughly  $4$   $\mu\text{m}$  of substomatal air space. Externally, the guard cells had cuticular flanges that curved toward the stomatal slit.

### Stomatal Complex Density

The SEM-based and LM-based measures of SC density were congruent. Mean SC den-

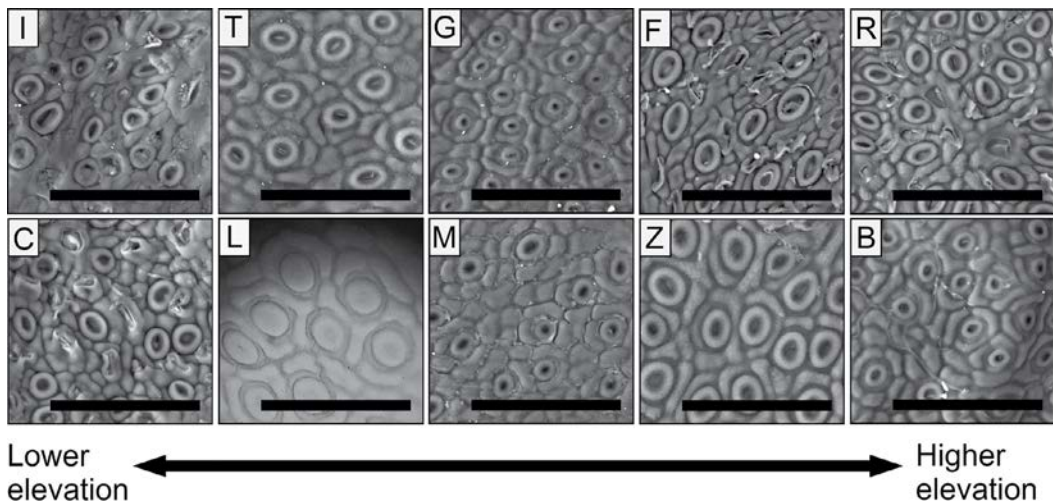


FIGURE 3. Variation among leaf SCs of 10 *Metrosideros* taxa arranged roughly from low (left) to high (right) elevation. Abaxial leaf surfaces are shown (see text; all scale bars = 0.1 mm). The image for *M. polymorpha* var. L is an LM micrograph ( $400\times$  magnification), and the other taxa were micrographed under a variable-pressure SEM ( $350\times$  magnification).

TABLE 2  
Mean ( $\pm 1$  SE) SC Lengths and Densities Measured Using Scanning Electron Microscopy (SEM) and Light Microscopy (LM)

Taxon	SEM Mean SC Length ( $\mu\text{m}$ )	SEM Mean SC Density (SCs/ $10 \mu\text{m}^2$ )	LM Mean SC Length ( $\mu\text{m}$ )	LM Mean SC Density (SCs/ $10 \mu\text{m}^2$ )
<i>M. p. var. incana</i>	21.60 $\pm$ 0.20	3.57 $\pm$ 0.23	NA	NA
<i>M. p. var. C</i>	24.35 $\pm$ 0.55	2.48 $\pm$ 0.55	NA	NA
<i>M. p. var. glaberrima</i>	14.55 $\pm$ 0.35	5.85 $\pm$ 0.08	23.03 $\pm$ 0.15	5.07 $\pm$ 0.33
<i>M. tremuloides</i>	27.65 $\pm$ 0.55	2.93 $\pm$ 0.26	24.70 $\pm$ 0.63	4.39 $\pm$ 0.23
<i>M. macropus</i>	20.45 $\pm$ 0.25	5.27 $\pm$ 0.31	23.09 $\pm$ 0.99	5.31 $\pm$ 0.34
<i>M. p. var. L</i>	NA	NA	24.13 $\pm$ 1.13	4.31 $\pm$ 0.26
<i>M. p. var. Z</i>	23.95 $\pm$ 2.45	5.56 $\pm$ 0.34	22.41 $\pm$ 1.18	5.66 $\pm$ 0.34
<i>M. p. var. F</i>	22.15 $\pm$ 0.75	4.75 $\pm$ 0.06	NA	NA
<i>M. p. var. B</i>	15.90 $\pm$ 1.70	8.65 $\pm$ 2.04	20.26 $\pm$ 0.51	7.17 $\pm$ 0.26
<i>M. rugosa</i>	19.45 $\pm$ 0.45	6.35 $\pm$ 0.18	NA	NA

Note: Abaxial leaf pubescence precluded LM measurements of SC traits in *M. rugosa*, and *M. p.* vars. *incana*, C, and F.

sity varied significantly across taxa from  $2.48 \pm 0.55$  and  $2.93 \pm 0.26/\mu\text{m}^2$  for *M. polymorpha* var. C and *M. tremuloides* to  $6.35 \pm 0.18$  and  $8.65 \pm 2.04/\mu\text{m}^2$  for the backbone-restricted *M. rugosa* and *M. polymorpha* var. B (SEM of nine taxa:  $H = 16.49$ ,  $df = 8$ ,  $P = .036$ ) (Table 2, Figure 3). LM measures of the six glabrous taxa showed the same pattern ( $F = 12.42$ ;  $df = 5, 54$ ;  $P < .001$ ;  $R^2 = 53.49\%$ ). The post hoc comparison of glabrous taxa (LM data) revealed significant differences in SC density among the three high-elevation glabrous taxa, *M. polymorpha* vars. B, Z, and L ( $P < .05$ ) (Figure 3). With all samples pooled, there was a strong negative correlation between SC density and SC length [SEM measures of nine taxa:  $r = -0.753$ ,  $P < .001$  (Figure 4); LM measures of six glabrous taxa:  $r = -0.727$ ,  $P < .001$ ]. Neither measure varied consistently with elevation in the full data set, but a trend was present in the pubescent taxa. Unlike the glabrous taxa, some of which are broadly distributed, the four pubescent taxa occur in order along the full elevation gradient of *Metrosideros* in the Ko'olau Range from the low, dry end to the windy, wet Ko'olau backbone. Grouping the four taxa into low-elevation (*M. polymorpha* vars. *incana* and C) and high-elevation (*M. polymorpha* var. F and *M. rugosa*) groups, SC density was significantly greater in the higher-elevation group ( $T = 4.12$ ,  $df = 5$ ,  $P = .009$ ).

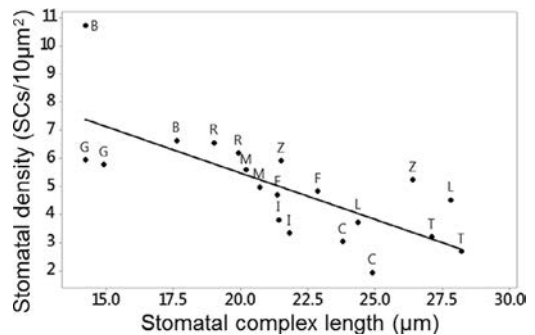


FIGURE 4. Correlation between abaxial SC length and density for 20 individuals representing 10 widespread *Metrosideros* taxa from O'ahu's Ko'olau Range. Measures were obtained using SEM for all taxa except L (for which measures were obtained via LM). Taxon codes are shown in Table 1.

### Secretory Structures

The purported SSs were observed in a majority (90%) of the leaves (individuals) examined adaxially via LM, with the 10 leaves lacking the structures restricted to two taxa: *M. polymorpha* var. *glaberrima* ( $n = 8$  of 10 examined) and *M. macropus* ( $n = 2$  of 10 examined) (Table 1). In contrast, only 21 of the 60 glabrous leaves (individuals) examined abaxially via LM had the SSs, including all 10 *M. polymorpha* var. B, all 10 *M. polymorpha* var. Z, and a single *M. polymorpha* var. L. The SEM micrographs



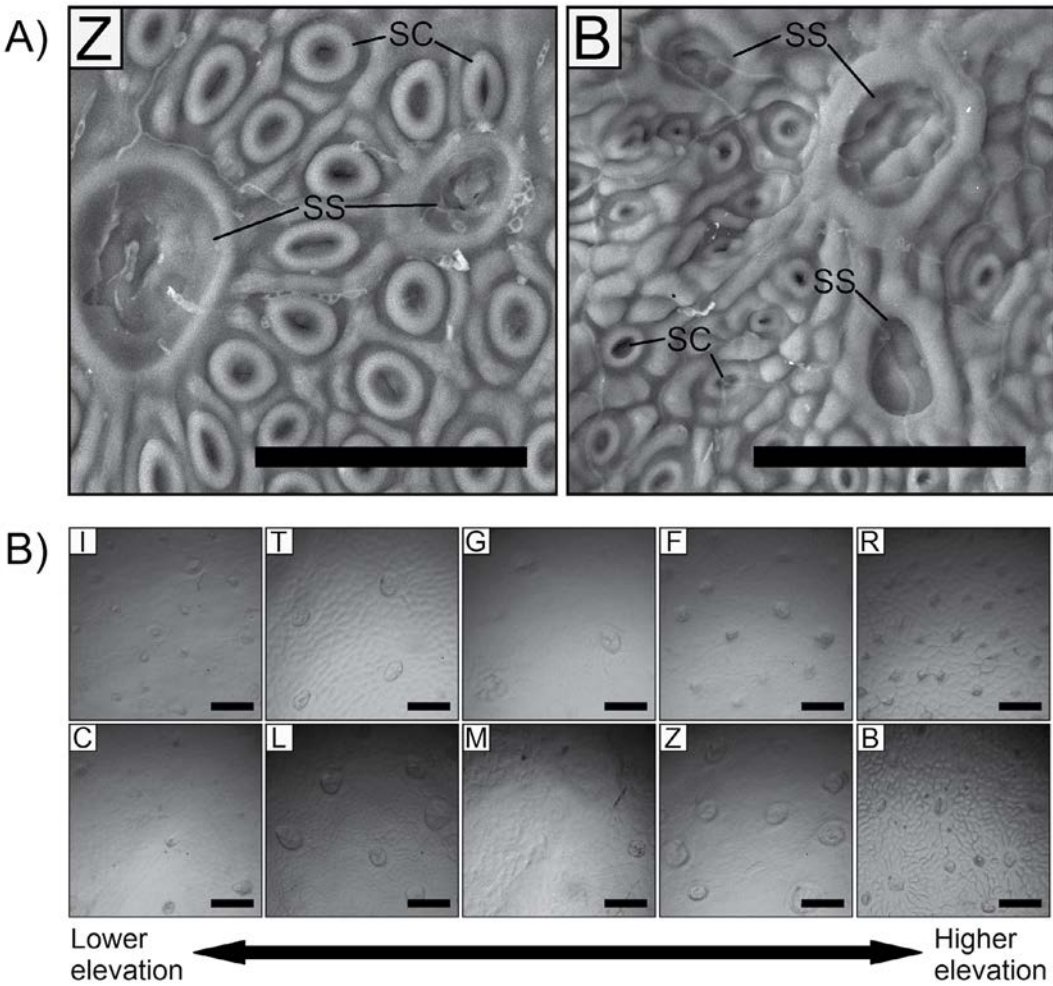


FIGURE 5. Leaf SS variation in *Metrosideros* on O'ahu: (A) Variable-pressure SEM micrograph (350×) of abaxial SSs on *M. polymorpha* vars. Z and B surrounded by smaller SCs (scale bars = 0.1 mm). (B) LM micrographs (100×) showing adaxial SSs present in all 10 taxa (scale bars = 0.1 mm).

(Figure 5A) of nine taxa consistently showed abaxial SSs in all samples of *M. polymorpha* vars. B and Z, and an absence of these structures in all other taxa.

Examination of the leaf anatomy of a subset of four taxa confirmed the purported SSs as subepidermal secretory structures or cavities. These conspicuous structures were elliptical or spherical and varied from 56 to 116  $\mu\text{m}$  in height and up to 64  $\mu\text{m}$  in width; each had a single internal epithelial layer, and

amorphous yellow contents were visible within the cavity (Figure 6).

*Secretory Structure Length*

Viewed via LM, the 2,015 adaxial SSs examined varied across the 10 taxa ( $F = 16.2$ ;  $df = 9, 86$ ;  $P < .001$ ;  $R^2 = 62.9$ ) and were  $2\times-7\times$  the size of the SCs. The mean lengths of the adaxial SSs of the four pubescent taxa (40.3–50.0  $\mu\text{m}$ ) were generally lower than those of

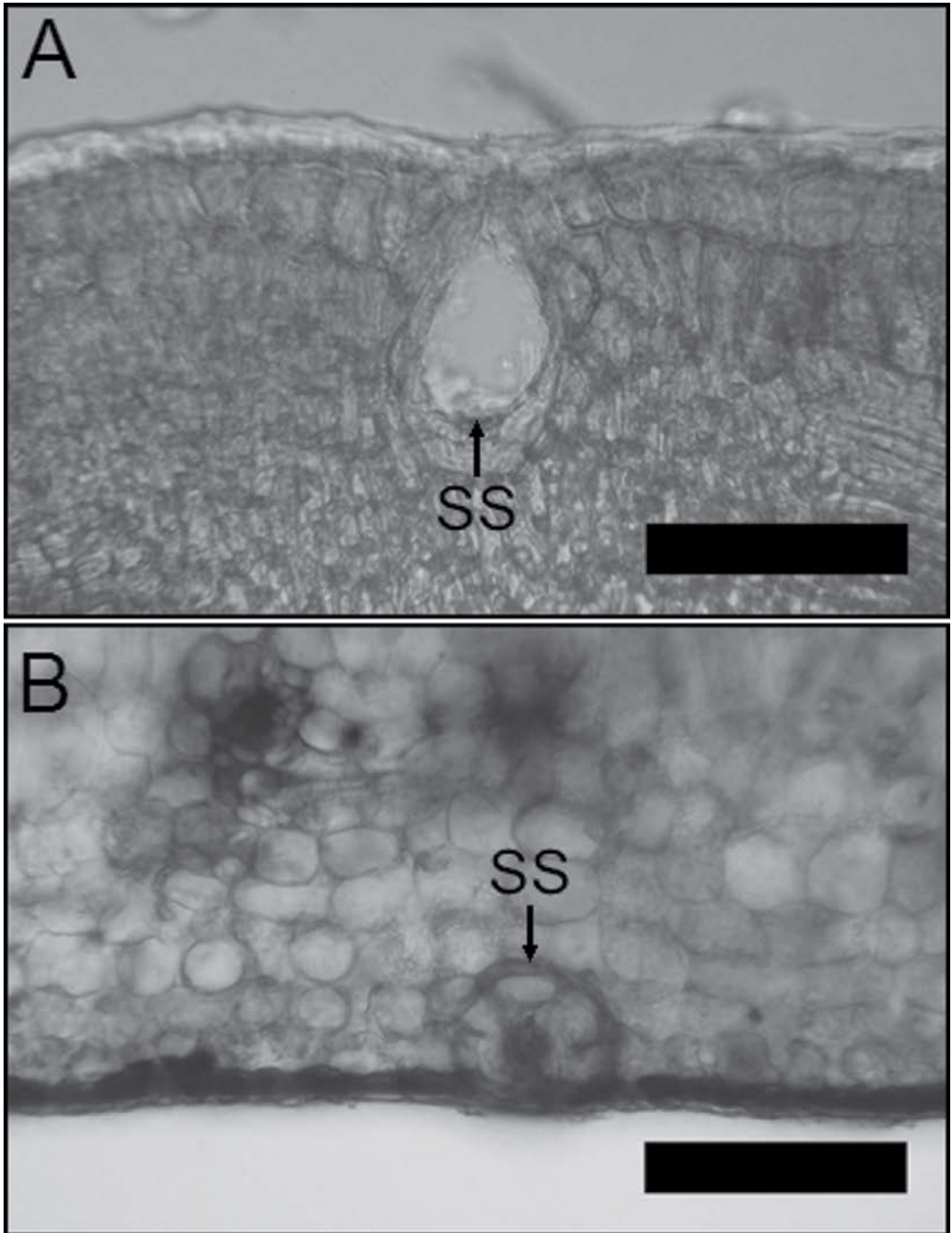


FIGURE 6. LM micrographs (250 $\times$ ) of leaf cross sections of Hawaiian *Metrosideros* showing: (A) a typical adaxial SS with contents, and (B) a representative abaxial SS with surrounding epithelial cells visible (scale bars = 0.1 mm).

TABLE 3

Mean ( $\pm 1$  SE) Length and Density of Adaxial and Abaxial SSs for Each of 10 *Metrosideros* Taxa on O'ahu Measured Using LM; Median Adaxial SS Densities ( $\pm$  Quartile Ranges) Are Also Shown

Taxon	Mean Adaxial SS Length ( $\mu\text{m}$ )	Mean Adaxial SS Density (SSs/ $\text{mm}^2$ )	Median Adaxial SS Density (SSs/ $\text{mm}^2$ )	Mean Abaxial SS Length ( $\mu\text{m}$ )	Mean Abaxial SS Density (SSs/ $\text{mm}^2$ )
<i>M. p.</i> var. <i>incana</i>	43.3 $\pm$ 2.5	29.4 $\pm$ 5.4	24 $\pm$ 17.5	NA	NA
<i>M. p.</i> var. C	50.0 $\pm$ 1.4	19.5 $\pm$ 2.6	20 $\pm$ 13.8	NA	NA
<i>M. p.</i> var. <i>glaberrima</i>	13.1 $\pm$ 8.8	2.5 $\pm$ 1.8	0 $\pm$ 2	Absent	Absent
<i>M. tremulooides</i>	62.0 $\pm$ 2.1	3.2 $\pm$ 0.5	2.8 $\pm$ 1.3	Absent	Absent
<i>M. macropus</i>	43.0 $\pm$ 7.8	2.3 $\pm$ 1.0	1.9 $\pm$ 1.6	Absent	Absent
<i>M. p.</i> var. L	69.9 $\pm$ 2.7	16.4 $\pm$ 3.9	14.5 $\pm$ 14.8	5.9 $\pm$ 5.9	13.4 $\pm$ 13.4
<i>M. p.</i> var. Z	74.3 $\pm$ 3.5	27.9 $\pm$ 2.7	27.3 $\pm$ 19.0	76.7 $\pm$ 4.1	11.8 $\pm$ 3.2
<i>M. p.</i> var. F	49.4 $\pm$ 2.5	46.3 $\pm$ 3.1	47.0 $\pm$ 15.5	NA	NA
<i>M. p.</i> var. B	57.6 $\pm$ 2.1	32.5 $\pm$ 2.82	33.8 $\pm$ 16.8	71.0 $\pm$ 2.8	30.5 $\pm$ 6.3
<i>M. rugosa</i>	40.3 $\pm$ 1.7	48.8 $\pm$ 7.3	50.0 $\pm$ 43.8	NA	NA

Note: *M. p.* = *M. polymorpha*.

the six glabrous taxa (13.1–74.3  $\mu\text{m}$ ) ( $T = 2.23$ ,  $df = 72$ ,  $P < .05$ ) (Table 3). As a result, the post hoc pairwise comparisons failed to reveal differences between macromorphologically similar taxa, with one exception: the mean adaxial SS length of *M. polymorpha* var. B (57.6  $\pm$  2.1  $\mu\text{m}$ ) was significantly smaller than that of either of the similar glabrous forms, *M. polymorpha* vars. L (69.9  $\pm$  2.7  $\mu\text{m}$ ) or Z (74.3  $\pm$  3.5  $\mu\text{m}$ ) (Table 3, Figure 5B). The mean lengths of SSs on the abaxial leaf surfaces (678 examined) were among the larger values observed for SSs overall and did not differ between the two taxa that possessed them consistently, *M. polymorpha* vars. B (71.0  $\pm$  2.8  $\mu\text{m}$ ) and Z (76.7  $\pm$  4.1  $\mu\text{m}$ ) (Table 3).

#### Secretory Structure Density

The density of adaxial SSs also varied strongly across taxa from 0, 1.9, and 2.8/ $\text{mm}^2$  (medians; maximum = 17.0) for the broadly distributed glabrous taxa, *M. polymorpha* var. *glaberrima*, *M. macropus*, and *M. tremulooides*, to 47.0 and 50.0/ $\text{mm}^2$  (maximum = 82.9) for the highest-elevation pubescent taxa, *M. polymorpha* var. F and *M. rugosa*, respectively, with the next-highest densities observed in the two highest-elevation glabrous taxa, *M. polymorpha* vars. B and Z (33.8 and 27.3/ $\text{mm}^2$ ;  $\bar{H} =$

73.01,  $df = 9$ ,  $P < .000$ ) (Table 3, Figure 5). Post hoc tests revealed significant differences in adaxial SS density between the morphologically similar glabrous taxa, *M. polymorpha* vars. B and L (14.5/ $\text{mm}^2$ ), as well as between two morphologically similar pubescent taxa, *M. polymorpha* vars. F and C (20/ $\text{mm}^2$ ). In addition, significant differences were found between taxa that are easily distinguished based on multiple morphological characters. The density of SSs on the abaxial surface differed significantly between the two taxa that consistently possessed these structures, *M. polymorpha* vars. B (30.5  $\pm$  6.3/ $\text{mm}^2$ ) and Z (11.8  $\pm$  3.2/ $\text{mm}^2$ ) ( $T = 2.64$ ,  $df = 13$ ,  $P = .020$ ) (Table 3). With all samples pooled, SS length and density were significantly negatively correlated on the abaxial surface only ( $r = -0.487$ ,  $P = .025$ ;  $n = 21$ ). Neither SS length nor density varied consistently with elevation using the full data set, except that adaxial SS density was highest amongst the four highest-elevation taxa (as described earlier), and abaxial SSs were restricted predominantly to the two highest-elevation glabrous taxa. With the four pubescent taxa combined into low-elevation (*M. polymorpha* vars. *incana* and C) and high-elevation (*M. polymorpha* var. F and *M. rugosa*) groups, mean SS density was significantly greater in the higher-elevation group ( $T = 4.12$ ,  $df = 27$ ,  $P < .001$ ).

### SC and SS Correlations

Micromorphological features of the six glabrous taxa measured via LM were somewhat correlated between the two sides of the leaf. Significant positive correlations were found between SS lengths on the abaxial and adaxial surfaces ( $r = 0.511$ ,  $P = .018$ ;  $n = 21$ ), between (abaxial) SC density and abaxial SS length ( $r = 0.526$ ,  $P < .001$ ;  $n = 60$ ), and between (abaxial) SC density and adaxial SS density ( $r = 0.542$ ,  $P < .001$ ;  $n = 60$ ). No other correlations were found between SC traits and any SS trait.

### Discriminant Analysis

Linear discriminant analyses were done using the SEM and LM data separately, because these data sets involved different individuals and numbers of taxa. Using the SEM-based values of SC length and density alone, 83.3% of individuals were assigned to the correct taxon; a single individual each of *M. polymorpha* vars. B, F, and Z was misclassified (Supplemental Appendix Table S1, Table S2). Using just the LM-based lengths and densities of abaxial SCs and adaxial SSs of the six glabrous taxa resulted in 76% correct classification (Supplemental Appendix Table S3, Table S4), and inclusion of the presence and density of the abaxial SSs improved the correct assignments to 87.1% (given that only *M. polymorpha* vars. B and Z have abaxial SSs and differ significantly in abaxial SS density). Last, repeating this exercise minus the two morphologically distinct glabrous species, *M. macropus* and *M. tremuloides*, yielded 84.4% (Supplemental Appendix Table S5) and 100% correct classification of the four glabrous varieties of *M. polymorpha* (i.e., *glaberrima*, B, L, and Z).

### DISCUSSION

The broad range of leaf micromorphologies observed in this study of 10 O'ahu taxa of *Metrosideros* is consistent with the high macromorphological and ecological diversity apparent in Hawaiian members of the genus (Dawson and Stemmermann 1990). This

group of trees is hypostomatal, and the length and density of their SCs varied significantly across the 10 taxa examined, which span a range of environmental conditions within O'ahu's Ko'olau Range. Some Hawaiian *Metrosideros* also have what appears to be a unique leaf SS found in most trees from the Ko'olau Range, predominantly on the adaxial leaf surface, but also abaxially in a limited number of taxa. They are designated as SSs given their anatomy and the presence of amorphous yellow contents visible at 250× magnification. Putative secretory cavities in leaf and petiole cross sections also were observed in *M. polymorpha* on Mauna Loa Volcano, Hawai'i Island (Corn 1979), although the leaf surface(s) involved was not specified. Additional observations that we made of abaxial leaf surfaces of *Metrosideros* from O'ahu's older Wai'anae Volcano revealed SSs occurring in a majority of the trees examined (28 of 32 trees of three taxa), yet the structures occurred in only one of three trees examined on Kaua'i (all authors, unpubl. data). These findings suggest that the variability in leaf micromorphology in *Metrosideros* across volcanoes may match that observed in gross morphology in this especially diverse group.

The SSs observed in this study are morphologically different from those documented in other Myrtaceae (Wilson 2011; R. Keating and N. Snow, pers. obs.). Further, these structures were not present on the leaves of a close relative, *M. collina* (J. R. Forst. & G. Forst.) A. Gray from the Marquesas Islands (growing in University of Hawai'i's Lyon Arboretum), or the more distant relatives *M. excelsa* Sol. ex Gaertn. from New Zealand or the New Caledonian species *M. operculata* Labill. and *M. tetrastichus* Guillaumin (one to two individuals of each examined) (all authors, unpubl. data). These observations suggest that this micromorphological feature has arisen within the Hawaiian lineage within its roughly 4-million-yr history in the Islands (Percy et al. 2008).

### Taxonomic Implications

As currently circumscribed, *Metrosideros* on O'ahu comprises four recognized species

and multiple varieties of the most abundant species, *M. polymorpha*. The three other species, *M. macropus*, *M. rugosa*, and *M. tremuloides*, are easily recognized through their macromorphological characters. Our study reveals additional differences among these closely related taxa in their leaf micromorphologies, as is commonly seen in close plant relatives (Moon et al. 2009, Edwin-Wosu and Benjamin 2012). Here, we focused on leaf micromorphological variation among the seven purported varieties of the landscape-dominant *M. polymorpha*, some of which are not yet officially recognized, to determine whether these characters support formal taxonomic recognition of these variant forms. We focused especially on the four glabrous forms for which we have additional LM-based measures of abaxial leaf characters.

The glabrous forms of *M. polymorpha*, vars. *glaberrima*, B, L, and Z, inhabit overlapping ranges and hybridize (i.e., morphologically intermediate trees are common), yet appear to be nearly fully discernable from each other based on their leaf micromorphology. Of these four taxa, the study reported here shows that the two highest-elevation forms, vars. B and Z, can be distinguished by the presence of abaxial SSs on their leaves. Our observation of abaxial SSs also in just one of the 10 trees of var. L examined is consistent with the occasional appearance of these structures in var. L by way of hybridization with either var. B or var. Z. Further, our results show that vars. B and Z can be distinguished from each other in three other micromorphological traits: the densities of abaxial SCs and SSs and adaxial SS length. These differences exist despite vars. B and Z occurring in sympatry at high elevation, and the unusually high variability within each of these varieties in the SEM measures of SC length (Figure 4). An exceptionally high density of SCs also separates var. B from three other glabrous taxa: *M. macropus*, and *M. polymorpha* vars. *glaberrima* and L. The consistently diagnostic morphological traits of *M. polymorpha* var. B (N.S. and E.A.S., unpubl. data) suggest that it may warrant recognition as another variety of *M. polymorpha* (Moon et al. 2009).

It is interesting that the only glabrous form of *M. polymorpha* that is recorded on all of the main islands, var. *glaberrima*, showed a leaf micromorphology unique within the group: a relatively low density of relatively small SCs and a very low density of variably sized adaxial SSs. The sparse SSs were observed on the leaves of only two of 10 trees examined, and no SSs were present on either leaf surface of a few individuals of var. *glaberrima* examined from Hawai'i Island (all authors, unpubl. data). These observations suggest that absence of these structures may be characteristic of this widespread form and that SSs appear in var. *glaberrima* by way of hybridization with forms that have them. Nonetheless, var. *glaberrima* is a morphologically and genetically variable taxon (Stacy et al. 2014), and further study is needed to determine if the micromorphological leaf traits observed in this taxon in O'ahu's Ko'olau Range occur throughout the Islands.

Compared to the significant micromorphological variation observed among the glabrous varieties of *M. polymorpha*, less variation occurs among the three pubescent forms. None of the pubescent leaves examined ( $n = \text{two per taxon}$ ) possessed abaxial SSs, so this character was not useful in distinguishing taxa. Further, in SC length and density (SEM measures), the pubescent taxa grouped in the middle of the distributions for both measures (Figure 4). The only significant difference found among the pubescent varieties of *M. polymorpha* was a significantly lower density of adaxial SSs in middle-elevation var. C relative to its higher-elevation neighbor, var. F. Adaxial SS density may therefore be a useful character in the delineation of these unnamed forms. It is interesting that the differences observed among taxa (whether glabrous or pubescent) in SS density appear in spite of the notably high within-taxon variability in SS density (both abaxial and adaxial; mean coefficient of variation: 71.9; range across taxa: 21.3–227.3) relative to the variability observed in the other measures (mean coefficient of variation: 11.8; range across taxa: 1.3–33.3).

### Ecological Insights

Despite their highly contrasting macromorphologies, the two *Metrosideros* taxa that co-occur along the high-elevation Ko'olau backbone share the trait of very high densities of microstructures in their leaves. Pubescent *M. rugosa* and glabrous *M. polymorpha* var. B had the greatest densities of (small) SCs and the greatest density of adaxial SSs, averaging  $1.26\times$ – $1.72\times$  the mean SC density as well as  $1.5\times$ – $2.3\times$  the median SS density of the other taxa. *Metrosideros polymorpha* var. B also had the greater density of abaxial SSs of the two taxa that possessed them. The similarities between these taxa are striking given their disparate gross morphologies and may suggest parallel micromorphological evolution in response to the often-extreme conditions along the Ko'olau backbone. Other observations also were consistent with a high density of both SCs and SSs at high elevation. Grouping the four pubescent taxa into low-elevation (*M. polymorpha* vars. *incana* and C) and high-elevation (*M. polymorpha* var. F and *M. rugosa*) groups, the densities of both SCs and SSs were significantly greater in the latter. Further, the SSs on the abaxial leaf surface were restricted to the two highest-elevation glabrous taxa. In her study of *Metrosideros* on Hawai'i Island, Corn (1979) also noted a relatively greater abundance of SSs (SC density was not examined) in leaves at high elevation and at the high end of the rainfall gradient she examined.

The high density of small SCs observed in high-elevation taxa on O'ahu may allow better control of water use under the fluctuating weather conditions that are more common along the backbone relative to low-elevation sites. High densities of smaller SCs are associated with high stomatal conductance and increased transpiration rates, as well as with fluctuating transpiration conditions (Drake et al. 2013). Smaller SCs are able to respond more quickly to changing conditions, such as rapid closing in response to desiccation (Bosabalidis and Kofidis 2002, Larcher 2003, Drake et al. 2013, Giday et al. 2013). Desiccation would be expected during

the strong wind gusts that frequent the Ko'olau backbone (Chock et al. 2005). Last, although the dense leaf pubescence of the backbone resident *M. rugosa* typically is thought to be associated with reduced stomatal conductivity (Wuenschel 1970), this effect may be small (Ehleringer and Mooney 1978, Skelton et al. 2012, Amada et al. 2017). Instead, the longer trichomes characteristic of this species may help to maintain gas exchange during wet conditions by repelling water from the leaf surface (Brewer et al. 1991, Brewer and Smith 1997).

Studies from Hawai'i Island suggest a complex relationship among SC density, leaf pubescence, and stomatal conductivity. On the island's Mauna Loa Volcano, stomatal conductance of *M. polymorpha* was similar across five elevations (107 m–2,469 m) despite increasing leaf pubescence with elevation (Cordell et al. 1998), and similar between glabrous and pubescent trees in a mixed, high-elevation population during the wet season (Hoof et al. 2008). During the dry season in the same high-elevation population, however, the glabrous trees had nearly twice the stomatal conductance rate of the pubescent trees, a difference that may be due to the significantly greater density of SCs or the lack of leaf pubescence on the glabrous trees (Hoof et al. 2008). Understanding how stomatal conductance may differ in relation to SC densities and leaf pubescence among Hawai'i's *Metrosideros* taxa will require further study.

The unequal distribution of SSs across taxa is more difficult to explain given the lack of information on their function. Secretory cavities are common in Myrtaceae (Gomes et al. 2009, Wilson 2011). For example, in *Eucalyptus* (Wilson 2011), leaf secretions ward off pathogenic or herbivorous attacks by insects (De Moraes et al. 2001) or microbes (Friedman et al. 2002). The greater densities of SSs at high elevation may suggest another adaptive explanation, namely, the reduction of water loss by glandular secretions (Wagner 1991, Mercadante-Simões and Paiva 2013) during unstable weather conditions punctuated by high winds (Chock et al. 2005). Alternatively, high densities of SCs and SSs may

be under a common genetic control in *Metrosideros*, as is observed for other traits in other plant taxa (e.g., Glover et al. 1998). The reasons for the smaller SSs in the pubescent taxa relative to the glabrous taxa or the uniformly low SS density in the three widely distributed glabrous taxa are unknown. More field and laboratory research is needed to characterize the SSs documented in this study, and additional population sampling of specimens is warranted before new taxonomic varieties are formally named.

#### CONCLUSIONS

Our findings reveal significant variation in leaf micromorphology across the many *Metrosideros* taxa in O'ahu's Ko'olau Range in spite of frequent hybridization among these largely incipient forms. Given the ecological and morphological divergence observed among the better-studied varieties of *M. polymorpha* on Hawai'i Island, the significant variation in leaf micromorphology seen here among O'ahu taxa may suggest that with additional population sampling (e.g., see Davis and Nixon 1992, Snow 1997) the formal taxonomic recognition of the unnamed forms may be warranted.

*Metrosideros polymorpha* is an intriguing and still under-studied natural experiment in plant speciation in Hawai'i. This landscape-dominant species faces severe threats, however, with the recent arrival in the Hawaiian Islands of the Myrtle rust (Uchida et al. 2006) and the highly lethal *Ceratocystis fimbriata* wilt (Mortenson et al. 2016). Recognizing rare *Metrosideros* taxa with unique associations within Hawai'i's variable landscape may be key to understanding differential disease susceptibility within *Metrosideros*. Further studies to characterize the impressive diversity within this landscape-dominant group are therefore important and may aid the management of the state's native forests.

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