RADseq resolves the phylogeny of Hawaiian Myrsine (Primulaceae) and provides evidence for hybridization

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Abstract The Hawaiian radiation of Myrsine (primrose family, Primulaceae) is the only one among the ten most species-rich Hawaiian plant lineages that has never been included in a phylogenetic analysis. Our study is based on a RADseq dataset of nearly all Hawaiian Myrsine species and a Sanger sequencing dataset based on a worldwide sampling of Myrsine and related genera. Myrsine as a whole might be paraphyletic with respect to the monotypic Macaronesian genera Heberdenia and Pleiomers, whereas Hawaiian Myrsine is resolved as monophyletic. The Sanger sequencing proved to be insufficient to resolve the Hawaiian lineage, whereas RADseq fully resolved the relationships with high support. Hawaiian Myrsine consists of three main lineages, of which one contains the majority of species and is mainly confined to Kauai, and the other two lineages primarily consist of few widespread species. Although phylogenetic reconstructions delivered fully resolved and supported tree topologies, Quartet Sampling and HyDe analyses reveal phylogenetic incongruence throughout the phylogeny and provide the first molecular evidence of extensive hybridization in the lineage.

Key words: HyDe, island biogeography, Kōlea, Myrsinaceae, Pacific Ocean, Quartet Sampling.

1 Introduction

The Hawaiian Islands have fascinated biologists for a long time due to their unique fauna and flora and the extremely high percentage of endemic taxa (about 90% of flowering plant species are endemic; Wagner et al., 1999; Keeley & Funk, 2011). Phylogenetic studies based on Hawaiian plants have shed light on the evolution of the Hawaiian flora. Their most important results include the refinement of the numbers of successful colonization events of the islands and the assessment of the geographical origin and age of the Hawaiian lineages. In the Hawaiian flora, \(+/−1200\) native vascular plant species are derived from about 259 successful colonization events (Price & Wagner, 2018). The most important source areas for successful colonists are the Americas (Baldwin & Wagner, 2010), the Indo-Pacific, and Australsia. In addition, a considerable number of Hawaiian species are derived from widespread, pantropical lineages (Keeley & Funk, 2011; Price & Wagner, 2018). Most elements of the Hawaiian flora colonized the archipelago after the formation of Kauai and Ni‘ihau (4.7–5.1 MA; Price & Clague, 2002), which are the oldest of the current main islands.

The Hawaiian Islands are a part of the Hawaiian–Emperor Seamount chain, which produced islands on the leeward side of the current main islands from the Late Cretaceous onward. However, a bottleneck for dispersal has been proposed for the time between 18 and 8 MA due to the absence of larger islands and the relatively long distance between islands (Price & Clague, 2002; Price & Wagner, 2018). Apparently, only few colonizers overcame this bottleneck, for example, the Hawaiian Lobeliads (Campanulaceae) and Melicope J.R. Forst. & G. Forst. (Rutaceae), for which molecular dating suggested an age older than that of the current high islands (Givnish et al., 2009; Appelhans et al., 2018).

Another major finding of phylogenetic studies based on Hawaiian taxa is the “progression rule,” which describes a pattern of subsequent colonizations from older to younger islands (Funk & Wagner, 1995; Cowie & Holland, 2000; Johnson et al., 2019). The progression rule pattern may be partly overshadowed by back colonizations to older islands, local extinction, and vicariance events on Maui, Moloka‘i, Lāna‘i, and Kaho‘olaw, which used to form a single island (Maui Nui) during most of their history, but were subjected...
to repeated splitting and fusion during glacial and interglacial periods with changing sea levels (Funk & Wagner, 1995; Price & Elliott-Fisk, 2004).

Phylogenetic studies based on Sanger sequencing of few loci have often failed to resolve Hawaiian plant radiations at the species level (e.g., Oh et al., 2013; Roy et al., 2013; Appelhans et al., 2014). The combination of their relatively recent age and their rapid speciation, as well as hybridization and incomplete lineage sorting (ILS), has been identified as a major reason for low phylogenetic resolution in Hawaiian lineages (e.g., Johnson et al., 2019; Stallman et al., 2019). The wide array of high-throughput sequencing (HTS or Next-Generation Sequencing) techniques undoubtedly stimulates phylogenetic and biogeographical research on the Hawaiian Islands and is enabling researchers to study radiations at or below the species level in great detail. A growing number of studies utilizing HTS methods for Hawaiian plants are becoming available. For example, Pillon et al. (2015) used transcriptome data to identify phylogenetically informative single-copy nuclear genes in Clermontia Gaudich. (Campanulaceae) and Cyrtandra J.R.Forst. & G.Forst. (Gesneriaceae). Welch et al. (2016) sequenced whole plastomes of Hawaiian mints (Lamiaceae); Jennings et al. (2016) used restriction site-associated DNA sequencing (RADseq) in a population-level study of two species of Hawaiian Lobeliads. Izuno et al. (2016, 2017) and Choi et al. (2020) used whole-genome sequencing to study population genomics of the most iconic Hawaiian tree species Metrosideros polymorpha Gaudich. (‘Ōhi‘a, Myrtaceae). Dupuis et al. (2019) used amplicon sequencing to study the Hawaiian radiation of Metrosideros; Kleinkopf et al. (2019) studied incomplete lineage sorting and hybridization in Hawaiian Cyrtandra using target enrichment; and Paetzold et al. (2019) applied RADseq to resolve the phylogeny of Hawaiian Melicope. In this study, we also focus on the RADseq method, because it is particularly well suited for young lineages (e.g., Darwell et al., 2016). RADseq and other restriction-digest methods deliver sequence information (RAD loci) adjacent to restriction enzyme cutting sites throughout the genome. The alignment of loci relies on shared restriction enzyme recognition sites between species. With increasing evolutionary distance between taxa, mutations may accumulate at recognition sites, possibly resulting in null alleles and problems with homology assessment (Rubin et al., 2012). Consequently, these methods are well suited for population genomic studies or phylogenomic studies of young species complexes such as recent and rapid island radiations.

Here, we investigate the phylogeny and putative hybridization events of the Hawaiian lineage of the pantropical distributed Myrsine L. (Köele), one of the largest genera in the primrose family (Primulaceae). Myrsine (including Rapanaea Aubl. and Suttonia A.Rich.) comprises about 300 species (Ståhl & Anderberg, 2004), and no phylogenetic study focusing on the genus has been published so far. Hosaka (1940) wrote a revision of the Hawaiian species focusing on morphological characters and made Suttonia and Rapanaea synonymous with Myrsine. One or few species of Myrsine have been included in family-level phylogenies of Primulaceae or as outgroups. Several genera including Aegiceras Gaertn., Ardisia Sw., Badula Juss., Grammadenia Benth., Hymenandra (A.DC.) Spach, Monoporus A.DC., Oncostemon A.DC., and Styloigne A.DC. have been identified as close relatives of Myrsine (Källersjö et al., 2000; Hao et al., 2004; Strijk et al., 2014).

With currently 19 or 20 accepted species, the Hawaiian lineage of Myrsine ranks among the ten largest Hawaiian plant radiations (Wagner et al., 1999, 2012). It is the only one among these largest radiations that has never been included in a phylogenetic study, and not a single DNA sequence of a Hawaiian Myrsine species has been deposited at Genbank before this study. Unlike many other Hawaiian plant lineages (Welch et al., 2016), no Myrsine species is currently believed to be extinct. However, five species are federally listed as endangered and one is listed as threatened (https://ecos.fws.gov/ecp/). Although Myrsine mezii Hosaka was previously only known from two collections made on Kaua‘i by Heller in 1895 (Hosaka, 1940; Wagner et al. 1999), a single individual was rediscovered on western Kaua‘i in 2015 by the staff at the National Tropical Botanical Garden (NTBG). Hawaiian Myrsine are adapted to several vegetation types including dry, mesic, and wet forests as well as open bogs at high altitudes (Wagner et al., 1999). Whereas the majority of species are single-island endemics, six species occur on two to six islands and, as of yet, no species have been recorded on Ni‘ihau or Kaho‘olawe (Fig. 1). One species that occurs on six islands, Myrsine lessertiuna A.DC., is extremely variable, especially regarding the shape, size, and venation of leaves and the arrangement of leaves on branches (clustered at tips vs. spread out). To categorize the variability, several subspecies, varieties, and forms have been described, and several species names, which are currently regarded as synonyms of M. lessertiuna, have been published (Hosaka, 1940; Wagner et al., 1999). Wagner et al. (1999) identified three distinct morphological “trends” within the species, but they highlighted that the breeding system and morphological variations are not known well enough to establish a solid intraspecific classification. Some of the variation has been interpreted as a sign of hybridization or may be due to environmental factors (Hosaka, 1940; Wagner et al., 1999; Lau, pers. comm.)

As Hawaiian Myrsine have never been included in a phylogenetic study before, our first goal (i) was to ascertain the monophyly of the Hawaiian Myrsine lineage. We sequenced external transcribed spacer (ETS) and (internal transcribed spacer (ITS) regions for Myrsine species from all continents and closely related taxa to achieve this goal. This dataset was also used to (ii) test if the genus as presently delimited is monophyletic. Regarding the Hawaiian lineage, we aim to (iii) test if RADseq is a suitable method to resolve the species-rich and putatively very young island radiation of Hawaiian Myrsine at the species level, (iv) to reconstruct the biogeographic pattern and the inter-island dispersal within the Hawaiian Island system, (v) to evaluate if the widespread species, especially the variable M. lessertiuna, represent monophyletic entities, and (vi) to test if hybridization has played a role in the evolution of the lineage.

2 Material and Methods

2.1 Taxon sampling
The Sanger sequencing dataset contained Myrsine species from all continents and several Pacific archipelagos. Representatives of Aegiceras, Ardisia, Badula, Embelia Burm.f., Heberdenia Banks ex A.DC., Monoporus, Oncostemon, Pleiomeris A.DC., and Styloigne were sampled to test the monophyly of Myrsine and the relationships among genera. Lysimachia L. was selected...
as the outgroup on the basis of previous studies (Källersjö et al., 2000; Martins et al., 2003; Anderberg et al., 2007; Strijk et al., 2014). The Sanger sequencing dataset contained 71 samples, of which 42 belonged to Myrsine and 19 to the Hawaiian radiation of Myrsine (Appendix I). All, but two (M. degeneri Hosaka and M. linearifolia Hosaka), species of Hawaiian Myrsine species were sampled for the RADseq study. Six specimens of the variable M. lessertiana have been sampled from Lānaʻi, Maui and Oʻahu (Appendix I). For 15 species, only a single sample could be included, mainly due to collection permit restrictions and rareness of the species. One non-Hawaiian species of Myrsine (M. africana L.) and two species of the related Ardisia were sampled as outgroups. The RADseq dataset consisted of 31 samples (Appendix I).

2.2 Sanger sequencing: lab work
Total DNA was extracted from silica-dried leaf material using the Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) or a standard CTAB protocol (Doyle & Doyle, 1987). The nuclear markers ITS and ETS were PCR-amplified using the primers ITS2, ITS3, ITS4, and ITS5 (White et al., 1990), and 18S-IGS and Burt (Baldwin & Markos, 1998; Becerra, 2003), respectively. PCR conditions were as follows: Initial denaturation of 5 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 52°C, and 0.40 min at 72°C; and final elongation of 7 min at 72°C. PCR products were cleaned using ExoSAP-IT (affymetrix USB; Cleveland, OH, USA) and were sequenced at Microsynth Seqlab (Goettingen, Germany) on an ABI 3100 sequencer. All sequences have been deposited at Genbank (Appendix I).

2.3 Sanger sequencing: phylogenetic analyses
Alignments were constructed in CLC genomics workbench version 12 (Qiagen, Aarhus, Denmark) using the MUSCLE algorithm (Katoh & Toh, 2008) and edited manually in Mesquite 3.40. (Maddison & Maddison, 2015; Data S1). Phylogenetic analyses were performed using Bayesian Inference (BI; MrBayes 3.2.6; Ronquist et al., 2012) and Maximum likelihood (ML; RAxML 8.2.4.; Stamatakis, 2014). The use of the Akaike Information Criterion (AIC) and jModelTest 2.1.3 (Darriba et al., 2012) revealed TIM2+G and TVM+G as the best-suited substitution models for ITS and ETS, respectively. As these models are not implemented in MrBayes and RAxML, GTR+G was used in the phylogenetic analyses, as it was the best-scoring implemented model. BI analyses consisted of four independent MCMC runs observed for 10 million generations, sampling every 1000th generation. All runs reached stationarity (standard deviation of split frequencies <0.01), and effective sample sizes proved to be sufficient (all values above 200; Tracer 1.7.1; Rambaut et al., 2014). Also, 50% majority-rule consensus trees were calculated in MrBayes after discarding the first 25% of the
trees as burn-in. Statistical support of branches was measured as posterior probability (PP) values, and only values of ≥0.95 PP were considered as strong support for clades. Results of ML tree search were evaluated with 1000 bootstrap replicates. Bootstrap (BS) values of 50%–69% were considered to indicate low support, values of 70%–89% as moderate support, and values of ≥90% as strong support.

2.4 RADseq: lab work
DNA was extracted as described above. Quality and quantity of the DNA extractions were checked using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA USA) in combination with the Qubit® dsDNA Broad Range assay kit. The DNA concentration was normalized to 30 ng/µL. For several samples, DNA had to be extracted twice to obtain the minimum DNA quantity, as requested by Floragenex (Eugene, OR, USA), and the extractions were pooled and concentrated using Eppendorf Concentrator Plus (Eppendorf AG, Hamburg, Germany). The normalized samples were sent to Floragenex for library preparation, using $5$/iB1 as restriction enzyme and following the protocol outlined in the study of Baird et al. (2008). Sequencing was performed on an Illumina GAIIx sequencer with a read length of 100 bp (single end). The libraries were sequenced together with 73 plant libraries of another project (Paetzold et al., 2019), and 32 libraries were pooled on one Illumina lane (three lanes were sequenced in total). Several libraries had low DNA quantities and were sequenced on a separate Illumina lane (samples marked in bold in Appendix I). These libraries were based on DNA extractions with only 1 μg of DNA and were also sequenced together with 14 low-concentration libraries from another project (Paetzold et al., 2019). Here, 24 libraries were pooled on a single Illumina lane. Demultiplexed raw data for all samples have been deposited at the Sequence Read Archive (SRA; https://www.ncbi.nlm.nih.gov/sra; BioProject number PRJNA614459; see Appendix I for individual accession numbers).

2.5 RADseq: ipyrad pipeline and phylogenetic analyses
FastQC (Andrews, 2010) was used for an initial quality check. One mismatch in the barcode sequence was allowed for demultiplexing. Read trimming was done using cutadapt v.1.9.1 (Martin, 2011), which consisted of adapter removal, deleting bases with Phred Scores <30, and removing trimmed reads shorter than 35 bp. The trimmed reads were assembled using ipyrad 0.7.28 (Eaton, 2014) using default parameters, with the exception of the clustering threshold and filtering parameters (see below). Clustering is done in two steps: within and across samples. During within-sample clustering, the individual sequence reads are clustered to form RAD loci, and the consensus sequences of the RAD loci are then clustered across samples. Two thresholds (85% and 90%) for clustering (c) within and across samples were used. Four datasets were assembled, differing in the minimum (min) number of samples recovered per RAD locus. In the min4 dataset, all RAD loci were included that had been recovered in at least four of the 31 samples. This dataset contained the highest number of loci, but also the highest proportion of missing data. The other datasets contained only RAD loci for which sequences for at least nine (min9), 14 (min14), and 19 (min19) samples were present in the final dataset, respectively. The different settings of clustering threshold and filtering resulted in eight datasets (min4c85, min4c90, min9c85, min9c90, min14c85, min14c90, min19c85, and min19c90), which were used for further analyses.

The full alignments (instead of only SNPs) were used for phylogenetic reconstruction. BI was performed using ExaBayes 1.5 (Aberer et al., 2014), applying the GTR + G model. All analyses consisted of four independent MCMC chains, which were observed for 100.000 generations, sampling every 100th generation. Majority-rule consensus trees were calculated in ExaBayes after deleting the first 25% of trees as burn-in (Data S1). As for the Sanger datasets, RAxML was utilized for ML analyses, using the GTR + G model and 500 bootstrap replicates (Data S1). The same values as described for Sanger sequencing results were used to interpret the support values for PP and BS.

2.6 RADseq: phylogenetic concordance and hybridization
It has been shown that resolution and support of phylogenetic trees, based on concatenated analyses of phylogenomics datasets, can rely on few genes, sites, or loci, so that a highly supported phylogeny might not reflect the true evolutionary history of a lineage (Shen et al., 2017). As a hybrid origin of several taxa of Hawaiian Myrsine has been assumed on the basis of morphologically intermediate specimens (Hosaka, 1940; Wagner et al., 1999; Lau J, pers. comm.), processes such as hybridization or introgression need to be considered as well. In addition, rapid speciation may lead to ILS, which can lead to low resolution or erroneous phylogenetic reconstruction. We evaluated the concordance and phylogenetic informativeness of our datasets using Quartet Sampling v.1.2.1 (QS; Pease et al., 2018). QS analyses were carried out using the ExaBayes consensus trees as input, and we performed 100 QS replicates per branch (Data S1). QS delivers three branch-specific values that indicate how many QS replicates produced a quartet topology concordant with the input phylogeny (Quartet Concordance [QC]), estimate if the frequencies of the two possible discordant topologies are equal or skewed toward one discordant topology (Quartet Differential [QD]), and specify the percentage of informative QS replicates (Quartet Informativeness [QI]).

To evaluate topological incongruence between datasets with respect to the position of M. mezii (see Section 3.2.), we tested the model fit of the three possible topologies using Bayes Factors through stepping stone (SS) sampling (Xie et al., 2011), as implemented in MrBayes 3.2.6. (Ronquist et al., 2012). Topological constraints were imposed as monophyly of the respective clade comprising M. mezii. Constraints regarding the relationships within that clade or other parts of the topology were not enforced. Stepping stone sampling was computed with 50 steps in four runs of 100.000 generations, sampling every 100th generation, with a GTR + G model of substitution, and discarding the first step as burn-in.

Putative hybridization events and potential hybrid and parental taxa were identified using HyDe v.0.4.3 (Blischak et al., 2018). HyDe has mainly been applied to datasets, based
on whole-genome shotgun and transcriptome sequencing (Blischak et al., 2018; Zhang et al., 2019), but it has also been successfully applied to RADseq data (Duran Castillo, 2019). We ran HyDe analyses based on the full alignments (Data S1). HyDe allows testing for hybridization and introgression at a population/specimen level or at the species level. As M. lessertiana is largely polyphyletic (see below) and only a single specimen each represents most other species, we decided to perform the analysis at a specimen level using the run_hyde.py script (Blischak et al., 2018). HyDe tests all possible combinations of input taxa as putative hybrids and parents. Bonferroni-corrected P-values are used to assess significance of results (0.05/number of tests; Blischak et al., 2018). HyDe estimates the amount of admixture (γ). Although a 50:50 hybrid is characterized by a γ-value of about 0.5 (we used a γ-range of 0.4–0.6 to account for stochasticity of results), very low levels of admixture (e.g. 0.01 = close to parent P1; 0.99 = close to parent P2) may be indicators for several processes such as ILS and more ancient hybridization. Intermediate γ-values (γ-ranges used here: 0.1–0.4 and 0.6–0.9) may also indicate older hybridization events that involved the ancestors of the tips in the phylogeny or recent hybridization events where at least one parent represents a hybrid species.

3 Results

3.1 ITS/ETS phylogeny

The ITS/ETS consensus tree shows a close relationship of Myrsine and the two monotypic Macaronesian genera, Heberdenia and Pleiomeris (Fig. 2). These three genera are united in a polytomy. Low support of internal branches (e.g., the clade of Heberdenia, M. semiserrata, M. africana, and M. spec 2) does not allow a conclusion as to whether Heberdenia and Pleiomeris are nested within Myrsine or if they represent sister lineages to a monophyletic Myrsine. The Hawaiian radiation of Myrsine was resolved as monophyletic (1.00 PP, 85% BS). As expected, the resolution within the Hawaiian clade was very low, and only revealed a sister group relationship of M. lanaiensis and the remainder of the Hawaiian species (1.00 PP, 88% BS). As the backbone of the Myrsine phylogeny could not be resolved, the geographic origin of the Hawaiian colonizer could not be assessed.

3.2 RADseq phylogeny

Illumina sequencing produced heterogeneous results and yielded a minimum of 179 545 and a maximum of 28 870 450 raw reads per sample. An initial ExaBayes run based on the min4c90 dataset did not produce a well-resolved consensus tree, very likely due to the samples with a low number of sequence reads. We, therefore, excluded all samples with less than 10% locus coverage in the min4c90 dataset (<6548 loci) from further analyses. In total, seven samples (marked with “low yield” in Appendix I) were excluded, leaving 24 high-yield samples for downstream analyses. These high-yield samples showed an average of 6 315 747 reads after trimming (min: 434 887, max: 28 864 497, four samples with less than 1 million reads; Table S1). The numbers of RAD loci retained after filtering in the different datasets were as follows: 65 447 (min4c85, min4c90), 53 015 (min9c85), 53 027 (min9c90), 41 745 (min14c85), 41 767 (min14c90), 12 984 (min9c85), and 12 980 (min9c90) (Table S1).

The excluded low-yield samples had 179 501–854 302 reads after trimming, and only 891-5090 RAD loci were recovered in these samples (min4c90 settings). To place the seven low-yield samples in the phylogeny, we performed seven additional ipyrad assemblies with the 24 high-yield samples and one of the low-yield samples at a time (Fig. S1). The least stringent setting (min4c90) was used for these analyses to include as many RAD loci as possible from the low-yield samples. The resulting datasets were analyzed using ExaBayes and RAxML, as described above.

The phylogenetic analyses based on the 24 high-yield RADseq samples produced fully resolved and highly supported trees (Fig. 3). The different settings for minimum presence of loci and the clustering threshold resulted in highly similar tree topologies, but the placement of one species (M. mezii) varied in the different analyses. The consensus tree based on the ExaBayes analysis of the min4c90 dataset is used to present the results, and the other settings are mentioned only regarding the placement of M. mezii. Support values were 1.00 PP and 100% BS for nearly all clades, and all cases of support values of <0.95 PP and <90% BS are displayed in Fig. 3 and Table 1.

The Hawaiian Myrsine lineage is subdivided into three main clades. Clade A consists of the Kauaian endemics M. alyxifolia Hosaka, M. denticulata (Wawra) Hosaka, M. fernseei (Mez) Hosaka, M. helleri (Degener & I.Degener) St. John, M. mezii, M. petiolata Hosaka, and M. wawraea (Mez) Hosaka, as well as an undescribed species from Kaua‘i. The trees including the seven low-yield samples (Fig. S1) revealed that the Kauaian endemics M. kauaiensis Hillebr., M. knudsenii (Rock) Hosaka as well as M. fosbergii Hosaka and M. punctata (H.Lév.) Wilbur from O‘ahu and Kaua‘i also belong to Clade A, so that it comprises all Kauaian endemics and two species from O‘ahu. Clade A can be further divided into two subclades (A1 and A2), one of which (Subclade A2) includes all species with linear or narrow lanceolate leaf shapes (M. fosbergii, M. helleri, M. petiolata), plus the small-leaved M. denticulata. Subclade A1 contains the only case of incongruence between datasets. In all analyses of the min4 and min4 datasets, M. mezii is resolved as sister to M. alyxifolia and the undescribed species (node 2 in Fig. 3). The support for this placement is strong (0.98–1.00 PP) in the BI analyses, whereas the ML analyses revealed only low-to-moderate support (51%–79% BS). In the analyses based on the min datasets, M. mezii is resolved as sister to the remainder of Subclade A1 (M. alyxifolia, M. fernseei, M. wawraea, and the undescribed species; node 6 in Fig. 3). This placement is strongly supported (0.95 PP) only in the BI analyses of the min9c85 dataset. In the min9 datasets, M. mezii is resolved as sister to M. fernseei and M. wawraea, with maximum support in the BI analyses and low support in the ML analyses (59%–61% BS).

Clade B includes four out of six sampled M. lessertiana specimens and M. pukooneosis (H.Lév.) Hosaka from O‘ahu, Maui, Moloka‘i, and Lāna‘i, M. vaccinioides W.L.Wagner, Herbst & Sohmer from West Maui, the widespread M. sandwicensis A.D.C. (all islands except Kaua‘i and Ni‘ihau), M. juddii Hosaka from O‘ahu, and M. emarginata (Rock) Hosaka.
from Oʻahu, Maui, and Lānaʻi (Figs. 3, S1). Whereas the latter three species form their own subclade, *M. pukooensis* and *M. vaccinioides* are nested within *M. lessertiana*. The distinctions between *M. emarginata* and *M. lessertiana* across many populations are not distinct, so that the complex was treated as one highly variable species recently (Wagner et al., 2012). As the sample referred to *M. emarginata* is here resolved as distinctive, a population-based analysis needs to be done to study the entire complex.

Clade C contains the remaining two specimens of *M. lessertiana* that form the two successive sister lineages to the widespread and monophyletic *M. lanaiensis* Hillebr.

### 3.3 Phylogenetic concordance/discordance and hybridization

As the different clustering thresholds (c) produced nearly identical numbers of assembled loci and did not have any influence on the topology and support of the phylogenies, the QS and HyDe analyses were based only on the c90
datasets (min4c90, min9c90, min14c90, min19c90). QS produced highly similar results across datasets (except for the aforementioned incongruent placement of *M. mezii* in Subclade A1 (left: min9c90; right: min19c90). Most clades have maximum or strong support (≥0.95 PP, ≥90% BS) in all phylogenetic analyses, and these nodes are marked with an asterisk (*). Support values for seven nodes that had lower support values in one or several analyses are shown in Table 1. The distributions of the species are shown in brackets after the species names, and the island on which the respective specimens have been collected is highlighted in bold face in case of multi-island species. BS, Bootstrap; H, Hawai‘i; K, Kaua‘i; L, Lāna‘i; Ma, Maui; Mo, Moloka‘i; O, O‘ahu; PP, posterior probability.

**Fig. 3.** RADseq phylogeny of Hawaiian *Myrsine*. The majority-rule consensus tree of the ExaBayes analysis based on the min14c90 dataset is shown. The insets on the upper right area represent incongruent topologies of *M. mezii* in Subclade A1 (left: min9c90; right: min19c90). Most clades have maximum or strong support (≥0.95 PP, ≥90% BS) in all phylogenetic analyses, and these nodes are marked with an asterisk (*). Support values for seven nodes that had lower support values in one or several analyses are shown in Table 1. The distributions of the species are shown in brackets after the species names, and the island on which the respective specimens have been collected is highlighted in bold face in case of multi-island species. BS, Bootstrap; H, Hawai‘i; K, Kaua‘i; L, Lāna‘i; Ma, Maui; Mo, Moloka‘i; O, O‘ahu; PP, posterior probability.
lessertiana specimens that form the consecutive sister clades to M. lanaiensis show low QC and QD scores, indicating support for one of the two discordant quartet topologies. The QS scores for M. mezii indicate that only a weak majority of the QS replicates support the placement of the species as sister to M. alyxifolia and the new species (QC = 0.19; node 2 in Fig. 3), and the discord is skewed toward one alternative topology (QD = 0.51). The results based on the min19c90 dataset are nearly identical with QC = 0.18 and QD = 0.47 for this node (Fig. S2). Contrastingly, the QS scores for M. mezii, based on the min9c90 dataset, provide full support (QC = 1; QD = N.A./QI = 1; Fig. S3) for the alternative placement of this species, as shown in node 6 in Fig. 3. The third possible placement of M. mezii, as estimated using the min19c90 dataset, receives no support from the QS analysis (Fig. S4).

The QC score for node 7 in Fig. 3 is −0.05, meaning that a slight majority of QS replicates resolved the alternative topologies. The QD score of 0.59 indicates that one alternative topology is resolved more frequently. The QI scores for the nodes that lead to M. mezii are similar across all datasets (min4c90: 0.80; min9c90: 0.68; min14c90: 0.82; min19c90: 0.70).

The results of the Bayes factors through SS analyses are shown in Table 2. The mean marginal likelihoods calculated for the three possible placements of M. mezii are highly similar for all datasets (min4c90, min9c90, min14c90, min19c90). Topology one (node 2 in Fig. 3) received the highest marginal likelihood values in the min9c90 and m9c90 datasets, whereas the marginal likelihood was highest for the second topology (node 6 in Fig. 3) in the min14c90 and min19c90 datasets. Similar to the QS analyses, topology three (node 7 in Fig. 3) is not resolved as the most likely topology in any of the analyses.

HyDe analyses entailed 4620 tests (all possible combinations of two parental and one hybrid in 22 ingroup samples), of which 1269 (27.47%, min19c90 dataset) to 1406 (30.43%, min4c90) reported significant levels of hybridization (Bonferroni-corrected P-value: 0.05/4620 = 1.08 × 10⁻⁹; Blischak et al., 2018; Table S2). Considering only significant results, many showed γ-values between 0.0–0.1 or 0.9–1.0, characterizing low levels of admixture. Between 159 (min4c90) and 171 (min19c90), significant results had γ-values between 0.4 and 0.6, indicating 50:50 hybrids (Table S2). Also, 527 (min19c90) to 558 (min14c90) significant results showed intermediate levels of admixture (γ: 0.1–0.4 and 0.6–0.9; Table S2), which may be caused by various events such as more ancient hybridization, backcrossings, or hybridization in which at least one parent is of the hybrid origin. The specimens tested herein fall into four categories regarding their HyDe results: they are either nearly exclusively putative parents, exclusively putative hybrids (considering γ = 50:50), both putative hybrids as well as putative parents, or neither (Fig. S5). Most species and specimens were identified as both putative hybrids and parents in at least some significant tests (Fig. S5; Table S3). Seven samples—both specimens of M. lanaiensis, M. lessertiana 3, M. mezii, M. pukoeoensis, M. sandwicensis, and the new species—were never or only once or twice identified as 50:50 hybrids (Fig. S5; Table S3). Only M. lanaiensis 1 was never identified as a hybrid using the intermediate γ-range (Fig. S5; Table S3). The other six
specimens and *M. alyxifolia* 2 and 3, *M. helleri*, and *M. lessertiana* 1 and 6 showed up to 20 significant results for hybridization using the intermediate $\gamma$-range, considerably less than for the other specimens (Fig. S5; Table S3). The specimens never identified as 50:50 hybrids were often identified as parental taxa, with the exception of *M. lanaiensis* 1 and *M. mezii*. *Myrsine helleri*, *M. lessertiana* 1 and 2, and *M. mezii* were never or only once or twice identified as parents of 50:50 hybrids (Fig. S5; Table S3). In contrast, *M. helleri* and *M. mezii* are among the samples that were most frequently identified as parents when applying the intermediate $\gamma$-range (Fig. S5; Table S3).

### Table 2
Mean marginal likelihood values of the Bayes factors through stepping stone analyses

| Dataset   | Topology 1   | Topology 2   | Topology 3   |
|-----------|--------------|--------------|--------------|
| min4c90   | −8671166.98  | −8671200.77  | −8671396.88  |
| min9c90   | −6951557.73  | −6951570.64  | −6951626.94  |
| min14c90  | −5530789.64  | −5530787.75  | −5530871.73  |
| min19c90  | −1787025.43  | −1786965.65  | −1786986.05  |

The highest likelihood value for each dataset is marked in bold. The topologies are displayed in Fig. 3.

4 Discussion

#### 4.1 Notes on the monophyly of Hawaiian Myrsine and the genus as a whole

The Hawaiian radiation of *Myrsine* was resolved as monophyletic in our ITS/ETS phylogeny. However, the ITS/ETS dataset failed to resolve relationships within the Hawaiian clade. *Myrsine* as a whole might be paraphyletic with respect to the monotypic Macaronesian genera *Heberdenia* and *Pleiomeris*. A strong uniting character for a close relationship of the three genera is the arrangement of flowers in axillary fascicles, whereas most Myrsinoideae (Primulaceae) have racemes, panicles, or solitary flowers (Ståhl & Andersberg, 2004). Future studies based on HTS methods and an increased taxon sampling are needed to assess the relationships of the three genera. These studies will also shed light...
on the biogeography of the widespread genus Myrsine and might allow assessing the biogeographic origin of Hawaiian Myrsine.

4.2 Phylogeny and biogeographic patterns of Hawaiian Myrsine

Our analyses show that RADseq is very well suited to resolve the evolutionary relationships of Hawaiian Myrsine at and below the species level, which demonstrates the massive improvement of RADseq as compared with Sanger sequencing of few loci, especially for recent radiations and lineages, in which hybridization occurs (Figs. 2, 3). RADseq studies often report different degrees of tree resolution and/or varying statistical support with respect to settings for filtering the minimum number of samples per locus. Often a low minimum number that maximizes the number of included loci as well as the amount of missing data yielded the consensus trees with highest support and resolution (Wagner et al., 2013; Wang et al., 2017). In our study, this effect was minuscule, but still the average branch support was slightly higher in the trees based on the min4 datasets as compared with the other datasets (Fig. 3).

Our RADseq study resulted in a largely resolved and supported phylogeny, which reveals interesting morphological groups and biogeographic patterns. The new species from Kaua‘i is morphologically similar to M. mezii, except for the leaf base being acute to obtuse versus rounded to obtuse, the leaf margin being serrate versus rounded to obtuse, and the leaf base being acute to obtuse versus rounded to obtuse. The new species is currently being monitored in the framework of the Hawaiian Plant Extinction Prevention Program (PEPP), so that hopefully flowering and fruiting characters can be collected, which will allow a full description of the species. The West Maui endemic Myrsine vaccinioides is described as a small shrub with small leaves and a serrate leaf margin, which is found in bog habitats at an elevation of about 1500 m (Wagner et al., 1989). It is morphologically similar to M. punctata and M. sandwicensis (Wagner et al., 1989), but it is nested within the main clade of the polyphyletic M. lessertiana, despite sharing no apparent morphological similarities. Myrsine vaccinioides grows in an extremely wet bog habitat, which is dominated by sedges, grasses, Metrosideros polymorpha (‘Ōhiʻa), and Vaccinium spp. (Ericaceae). ‘Ōhiʻa plants can be large trees, but they grow as prostrate shrubs (Metrosideros polymorpha var. pseudouroga Skotts.) in the habitat where M. vaccinioides is found (Wagner et al., 1999, personal observations). Similar to the habitat-related growth form shown by ‘Ōhiʻa, M. vaccinioides might be a dwarf form of M. lessertiana, and the small size of the plants might be triggered by the habitat. Smaller leaves of bog species, compared to related species from other habitats, have been documented for Hawaiian Plantago L. (Plantaginaceae; Dunbar-Co et al., 2009) and to some degree in Hawaiian Geranium L. (Geraniaceae; Carquist & Bissing, 1976). The first author collected seeds of M. lanaiensis and grew plants at the Botanical Garden in Goettingen, Germany. He observed that the leaves of the seedlings had a serrate margin, and that the serration became less pronounced in older seedlings and the leaf margins became fully entire when the plants reached a height of about 30 cm. Taking this observation into account, we hypothesize that the serrate leaf margin in M. vaccinioides might represent a juvenile leaf form, and fully developed leaves do not form as a result of the dwarfism.

The phylogeny shows an interesting biogeographic pattern. Myrsine is most diverse on Kaua‘i, with 15 species including 11 endemics. O‘ahu is home to eight or nine species (three endemics), five or six species (one endemic) grow on Maui Nui, and only the three widespread species occur on the Big Island (Wagner et al., 1999, 2012, plus the new species from Kaua‘i). All Kauanian endemics are members of Clade A (Figs. 1, 3), which suggests that the only major radiation of Hawaiian Myrsine occurred on this island. This pattern is not surprising, as Kaua‘i has a highly variable physical geography with deeply eroded drainages and well-defined canyons, along with coastal and inland cliff systems, and it is by far the oldest of the current high islands (Price & Clague, 2002), so that a longer time span was available for the evolution of a higher number of species. In contrast, all, except two (M. juddii, O‘ahu, and M. vaccinioides, Maui), species of Clades B and C occur on more than two islands (Figs. 1, 3; Wagner et al., 1999). These large differences in dispersibility are surprising, because all species have similar inconspicuous, insect-pollinated flowers and similar sized, dark-colored drupes that are presumably dispersed by birds (Sakai et al., 1995; Wagner et al., 1999, 2017; Price & Wagner, 2017). The widespread species generally have a much wider elevational range and occur in different vegetation types as compared with the single-island endemics (Wagner et al., 1999). The wider ecological niche might, therefore, account for the differences in distribution range between Clade A on the one side and Clades B and C on the other. If so, Hawaiian Myrsine fits well into the model of species-rich, ecologically specialized lineages with narrow distributions versus widespread, species-poor, ecologically generalist lineages (Price & Wagner, 2004).

4.3 Polyphyly of Myrsine lessertiana and hybridization

The Q5 analyses revealed multiple cases of phylogenetic incongruence across the phylogeny, and HyDe identified numerous significant results for hybridization. A high number of significant tests for hybridization revealed low levels of admixture (below 0.1 and above 0.9) as well as a considerable amount of high-level admixture (intermediate and 50% hybrids). Low levels of admixture may represent traces of more ancient hybridization events, but they could also imply unrelated processes such as ILS. So far, hybridization in Myrsine has been demonstrated for the Lord Howe Island species (Papadopulos et al., 2011); however, large-scale studies are lacking. Our study provides the first molecular evidence for hybridization in the Hawaiian Myrsine lineage. The high number of significant results for hybridizations and the involvement of nearly all sampled taxa suggest that hybridization has played a major role in the evolution of the lineage. A complete taxon sampling as well as a population sampling for several species is needed to draw conclusions about the amount of hybridization events through time and to reliably infer hybrid taxa and their parental species. However, the taxon sampling used herein allows first
estimations. On the basis of morphology, it has been hypothesized that hybridization has played a role in the evolution of several Hawaiian species including *M. emarginata*, *M. mezii*, *M. pukooensis*, *M. sandwicensis*, and *M. lessertiana* (Wagner et al., 1999).

One of the two specimens of *M. lessertiana* (specimen 2) that were resolved as sister to *M. lanaiensis* is a representative of the morphotype with a more prominent leaf venation (Wagner et al., 1999). The other specimen (*M. lessertiana* 1) was taken from a small sapling with juvenile leaves, so that it is not sure if it also represents this morphotype. The HyDe analyses (50:50; min4c90, min9c90, min14c90, min19c90) revealed that in 76% of the significant tests identifying one of these two specimens as a hybrid, *M. lanaiensis* was estimated as one of the parental taxa. The HyDe analyses (50:50; min4c90, min9c90, min14c90, min19c90) revealed that in 76% of the significant tests identifying one of these two specimens as a hybrid, *M. lanaiensis* was estimated as one of the parental taxa. Among the remaining *M. lessertiana* samples, specimen 3 stands out as the only one frequently estimated as a parent, but it is identified as a hybrid taxon for a single time (50:50 hybrids). A similar pattern can be observed for the two specimens of *M. lanaiensis*, *M. pukooensis*, *M. sandwicensis*, and the new species, so that these taxa might be involved in recent hybridization events as parents, but not as hybrids. All of these specimens have been identified as hybrids to a small degree using the intermediate γ-range, which might represent traces of more ancient hybridization events in the backbone of the Hawaiian *Myrsine* phylogeny. Interestingly, Wagner et al. (1999) mentioned specimens of *M. lessertiana* that are transitional toward *M. pukooensis* and *M. sandwicensis*, respectively. The high number of significant HyDe tests in which these two species are resolved as parental taxa might indicate that the two species are parental to the transitional specimens; however, a denser taxon sampling including the transitional morphotypes is needed to draw further conclusions.

Most of the Kauaian endemics (Clade A) are frequently identified as hybrids using both the 50:50 γ-range and the intermediate γ-range. Several of the Kauaian species occur sympatrically (own observation), and there might be ongoing hybridization events among these species. It is not known whether the putative hybrid species are homoploid or polyploid hybrids; however, there is currently no evidence for ploidy shifts in the genus. The chromosome number and ploidy level are known only for a single Hawaiian *Myrsine* species (*M. sandwicensis*; 2n = 46; Kiehn, 2005), and the numbers are congruent with the few known numbers in *Myrsine* species from the Neotropics (Carvalho et al., 2017) and New Zealand (Dawson, 1995). *Myrsine mezii* stands out as the only species that is neither identified as hybrid nor as parent using the 50:50 γ-range. However, it is often identified as a parent using the intermediate γ-range. *Myrsine helleri* shows a similar increase in significant parental HyDe results with regard to the two γ-ranges. In both cases, this could mean that the two species are involved in hybridization events in which one parent is a hybrid species itself, so that the genome of the resulting hybrid is not a 50:50 mix between two “pure” species. Another explanation is that the detection of the two species as parents using the intermediate γ-range reflects the signal of more ancient hybridization events in the Hawaiian lineage.

This paper presents the first molecular evidence for hybridization in Hawaiian *Myrsine*. An increased taxon sampling including all species and more samples of the widespread species *M. lanaiensis*, *M. lessertiana*, *M. pukooensis*, and *M. sandwicensis* throughout the distribution range, and including all morphotypes of *M. lessertiana*, is needed to assess the role of hybridization in the evolution of Hawaiian *Myrsine* more precisely. Instead of RADseq, focusing on Target Enrichment might be a good strategy for a successive study, as this method allows allele-based hypothesis testing as well as exploring gene tree/species tree conflicts, and thus more direct tests for hybridization, as has recently been demonstrated by Kleinkopf et al. (2019) for Hawaiian *Cyrtoandra*.

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in bold face in case of multi-island species. All trees have been rooted with the two Ardisia species as outgroups, and the outgroups have been pruned due to space constraints. H, Hawai‘i; K, Kaua‘i; L, Lāna‘i; Ma, Maui; Mo, Moloka‘i; O, O‘ahu.

**Fig. S2.** Results of the Quartet Sampling of the min4c90 dataset plotted on the majority-rule consensus tree of the ExaBayes analysis based on the min4c90 dataset. The values for Quartet Concordance (QC), Quartet Differential (QD), and Quartet Informativeness (QI) are displayed next to each node (QC/QD/QI), whereas the Quartet Fidelity (QF) score is shown next to the specimen names. Nodes with strong or full support are highlighted in bold.

**Fig. S3.** Results of the Quartet Sampling of the min9c90 dataset plotted on the majority-rule consensus tree of the ExaBayes analysis based on the min9c90 dataset. The values for Quartet Concordance (QC), Quartet Differential (QD), and Quartet Informativeness (QI) are displayed next to each node (QC/QD/QI), whereas the Quartet Fidelity (QF) score is shown next to the specimen names. Nodes with strong or full support are highlighted in bold.

**Fig. S4.** Results of the Quartet Sampling of the min19c90 dataset plotted on the majority-rule consensus tree of the ExaBayes analysis based on the min19c90 dataset. The values for Quartet Concordance (QC), Quartet Differential (QD), and Quartet Informativeness (QI) are displayed next to each node (QC/QD/QI), whereas the Quartet Fidelity (QF) score is shown next to the specimen names. Nodes with strong or full support are highlighted in bold.

**Fig. S5.** Presentation of the HyDe results per specimen. The charts show the ratios of how often a specimen has been estimated to be a hybrid (dark blue: 50:50 hybrid [γ-values between 0.4 and 0.6]; light blue: with intermediate levels of admixture [γ-values between 0.1–0.4 and 0.6–0.9]) or a parental taxon (red: parent of 50:50 hybrids [γ-values between 0.4 and 0.6]; light red: with intermediate levels of admixture [γ-values between 0.1–0.4 and 0.6–0.9]) among the significant HyDe results.

**Data S1.** ETS and ITS alignments, phylogenetic trees of all datasets as well as Quartet Sampling and HyDe results, available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.6wwpzgmvs
### Appendix I. Voucher information, SRA, and Genbank accession numbers for specimens used in this study

| Genus      | Species                  | Coll. & Nr. | Locality          | Herbarium & Barcode | SRA Accession for RADseq data | Genbank Accession for ITS | Genbank Accession for ETS |
|------------|--------------------------|-------------|-------------------|---------------------|-----------------------------|--------------------------|--------------------------|
| Aegericas  | corniculatum             | QA121101    | China (Hainan)    |                     | -                           | -                        | -                        |
| Aegericas  | corniculatum             | Ku 007      | China (Taiwan)    | O‘ahu               | BISH, GOET019815            | SAMN14425894             | -                        |
| Ardisia    | brevicaulis              | Appelhans   | China (Taiwan)    |                     | -                           | -                        | -                        |
| Ardisia    | crenata                  | Appelhans   | O‘ahu             |                     | -                           | -                        | -                        |
| Ardisia    | crenata 2                | TDNA058     | Réunion           |                     | -                           | -                        | -                        |
| Ardisia    | elliptica                | Appelhans   | China (Taiwan)    |                     | -                           | -                        | -                        |
| Ardisia    | elliptica 2              | Bone 26     | Mauritius         |                     | -                           | -                        | -                        |
| Ardisia    | japonica                 | Ku 008      | China (Taiwan)    |                     | -                           | -                        | -                        |
| Ardisia    | maclurei                 | Ku 015      | China (Taiwan)    |                     | -                           | -                        | -                        |
| Ardisia    | squamulosa               | Ku 024      | China (Taiwan)    |                     | -                           | -                        | -                        |
| Ardisia    | violacea                 | Ku 018      | China (Taiwan)    |                     | -                           | -                        | -                        |
| Badula     | balfouriana              | Bone 51     | Mauritius         |                     | -                           | -                        | -                        |
| Badula     | borbonica                | DS236,      | Réunion           |                     | -                           | -                        | -                        |
| Badula     | crassa                   | Bone 22     | Mauritius         |                     | -                           | -                        | -                        |
| Badula     | insularis                | Bone 04     | Mauritius         |                     | -                           | -                        | -                        |
| Badula     | platyphylla              | Bone 02     | Mauritius         |                     | -                           | -                        | -                        |
| Embelia    | ribes                    | Thiv 4578   | Canary Islands    |                     | -                           | -                        | -                        |
| Heberdenia | excelsa                  | A. Santos-  | Canary Islands    |                     | -                           | -                        | -                        |
| Heberdenia | excelsa 2                | Thiv 4578   | Canary Islands    |                     | -                           | -                        | -                        |
| Lysimachia | engleri                  | Peng s.n.   | China (Yunnan)    |                     | -                           | -                        | -                        |
| Lysimachia | ephemeralis              | Douglas s.n.| Cult. Dublin,     |                     | -                           | -                        | -                        |
| Monosporus | bipinnatus               | DS7,        | Mayotte           |                     | -                           | -                        | -                        |
| Myrsine    | africana (low-yield)     | Appelhans   | cult. Bot. Gard.  |                     | GOET019817                 | SAMN14425908             | MT231612                 |
| Myrsine    | alyxifolia               | Appelhans   | Goettingen,       |                     | GOET019819, PTBG100057491  | SAMN14425905             | MT231613                 |
| Myrsine    | alyxifolia 2             | Appelhans   | Kaua‘i           |                     | GOET019820, PTBG100057454  | SAMN14425903             | MT231614                 |
| Myrsine    | alyxifolia 3             | Appelhans   | Kaua‘i           |                     | GOET019818, PTBG100057451  | SAMN14425901             | MT231615                 |
| Myrsine    | australis                | Cooper &    | New Zealand       |                     | -                           | -                        | -                        |
| Myrsine    | australis 2              | Nickerson   | New Zealand       |                     | -                           | -                        | -                        |
| Myrsine    | cheesemanii              | Whistler 5902 | Mitiaro (Cook     |                     | US01232928                  | -                        | MT231615                 |
| Myrsine    | coriacea                 | Wasum 1113  | Brazil            |                     | GOET022991                  | -                        | MT231617                 |
| Myrsine    | denticulata              | Appelhans   | Kaua‘i           |                     | -                           | -                        | -                        |
| Myrsine    | faberi                   | Hao 269     | cult. China       |                     | -                           | -                        | -                        |
| Myrsine    | emarginata               | Appelhans   | O‘ahu             |                     | GOET019822, PTBG100057443  | SAMN14425916             | -                        |

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### Appendix I. Continued

| Genus | Species | Coll. & Nr. | Locality | Herbarium & Barcode | SRA Accession for RADseq data | Genbank Accession for ITS | Genbank Accession for ETS |
|-------|---------|------------|----------|---------------------|------------------------------|--------------------------|--------------------------|
| **Myrsine** | *fernseei* | Wood 17116 Appelhans MA661 | Kaua‘i, National Tropical Botanical Garden | PTBG1000062447 silica only | SAMN14425922 | MT231617 | - |
| **Myrsine** | *forsbergii* [low-yield] | Perlman 19765 | Marquesas | US01202153 | - | MT231618 | MT231218 |
| **Myrsine** | *gracilissima* | Perlman 19765 | Marquesas | PTBG | - | MT231619 | MT231220 |
| **Myrsine** | *grantii* | Wood 10342 | Marquesas | PTBG | - | MT231620 | MT231219 |
| **Myrsine** | *helleri* | Wood 16262 | Kaua‘i | PTBG | - | DQ499108 | - |
| **Myrsine** | *howittiana* | Brambach et al. 868 | Sulawesi | GOET | - | MT231621 | MT231222 |
| **Myrsine** | *involucrata* | Brambach et al. 731 | Sulawesi | GOET | - | MT231645 | MT231221 |
| **Myrsine** | *juddii* [low-yield] | Oppenheimer HO0906 | O‘ahu | PTBG | - | SAMN14425910 | - |
| **Myrsine** | *kauaiensis* [low-yield] | Wood 17117 | Kaua‘i | PTBG1000062389 | - | SAMN14425911 | - |
| **Myrsine** | *knudsenii* [low-yield] | Wood 17074 | Kaua‘i | PTBG1000062719 | - | SAMN14425923 | - |
| **Myrsine** | *lanaiensis* | Appelhans MA685 | Kaua‘i | GOET019826, PTBG1000057448, US | - | SAMN14425904 | - |
| **Myrsine** | *lanaiensis 2* | Appelhans MA699 | Hawai‘i | BISH, GOET019825, PTBG1000057515, US | - | SAMN14425907 | MT231622 |
| **Myrsine** | *lanaiensis 3* [low-yield] | Oppenheimer HO0927 | Lāna‘i | PTBG | - | SAMN14425909 | - |
| **Myrsine** | *ledermannii* | Pelman 21478 | O‘ahu | PTBG1000033866 only silica | - | SAMN14425895 | - |
| **Myrsine** | *lessertiana* | Appelhans MA607 | Pohnpei | GOET019831, PTBG1000057518, US | - | SAMN14425896 | - |
| **Myrsine** | *lessertiana 2* | Appelhans MA612 | O‘ahu | GOET019832, PTBG1000057940, US | - | SAMN14425899 | MT231627 |
| **Myrsine** | *lessertiana 3* | Appelhans MA649 | Maui | BISH, GOET019835, PTBG1000057531, US | - | SAMN14425898 | MT231625 |
| **Myrsine** | *lessertiana 4* | Appelhans MA626 | Maui, Olinda rare plant facility (cult.) | only silica | - | SAMN14425897 | - |
| **Myrsine** | *lessertiana 5* | Oppenheimer HO0928 | Lāna‘i | PTBG | - | SAMN14425921 | MT231629 |
| **Myrsine** | *lessertiana 6* | Appelhans MA625 | O‘ahu | GOET019835, PTBG1000057531, US | - | SAMN14425897 | MT231627 |
| **Myrsine** | *lessertiana 7* | Appelhans MA611 | O‘ahu | GOET019833 | - | MT231566 | - |
| **Myrsine** | *lessertiana 8* | Appelhans MA618 | O‘ahu | GOET019834 | - | MT231624 | MT231225 |
| **Myrsine** | *lessertiana 9* | Appelhans MA627 | Maui | silica only | - | MT231626 | - |
| **Myrsine** | *lessertiana 10* | Appelhans MA671 | Kaua‘i | GOET019828 | - | MT231628 | MT231226 |
| Genus      | Species     | Coll. & Nr. | Locality      | Herbarium & Barcode | SRA Accession for RADseq data | Genbank Accession for ITS | Genbank Accession for ETS |
|-----------|-------------|-------------|---------------|---------------------|-----------------------------|--------------------------|--------------------------|
| Myrsine   | leucantha   |             | Papua New Guinea | PTBG                | DQ499107                  |                          |                          |
| Myrsine   | mezii       | Wood 16832  | Kaua'i        | GOET                | SAMN14425913              | MT231630                 | MT231228                |
| Myrsine   | minutifolia | Brambach et al. 1776 | Sulawesi |                    |                            |                          |                          |
| Myrsine   | novocaledonica | Baumann-Bodenheim 6764 | New Caledonia | GOET022990          | MT231646                  |                          |                          |
| Myrsine   | oliveri     |             | New Zealand   |                     |                            |                          |                          |
| Myrsine   | petiolata   | Appelhans MA674 | Kaua'i |                     |                            |                          |                          |
| Myrsine   | pukooensis  | Appelhans MA622 | O'ahu |                     |                            |                          |                          |
| Myrsine   | punctata (low-yield) | Appelhans MA681 | Kaua'i |                     |                            |                          |                          |
| Myrsine   | rawacensis (cf) | Culmsee r2150 | Sulawesi | GOET               | SAMN14425906              | MT231567                 | MT231229                |
| Myrsine   | sandwicensis | Appelhans MA619 | O'ahu | GOET019840,         |                            |                          |                          |
| Myrsine   | sandwicensis 2 | Appelhans MA620 | O'ahu | GOET019841         |                            |                          |                          |
| Myrsine   | sandwicensis 3 | Appelhans MA640 | Maui | GOET019842         |                            |                          |                          |
| Myrsine   | sandwicensis 4 | Appelhans MA647 | Maui | GOET019844         |                            |                          |                          |
| Myrsine   | sandwicensis 5 | Appelhans MA694 | Hawai'i | GOET019845        |                            |                          |                          |
| Myrsine   | sandwicensis 6 | Oppenheimer H91620 | Maui | PTBG0000061779     |                            |                          |                          |
| Myrsine   | sandwicensis 7 | Oppenheimer H00928 | Lāna'i | PTBG              |                            |                          |                          |
| Myrsine   | semiserrata  | Srisangao 97466 | Myanmar | US01325900     | MT231638                 | MT231234                |
| Myrsine   | sp. nov.    | Appelhans MA669 | Kaua'i |                   |                            |                          |                          |
| Myrsine   | spec 2      | Harbath 642  | Brazil |                    |                            |                          |                          |
| Myrsine   | stolonifera | Lin 436     | China (Tibet) |                    |                            |                          |                          |
| Myrsine   | trinitatis  | Carrington 2318 | St. Kitts |                    |                            |                          |                          |
| Myrsine   | vaccinioide | Appelhans MA658 | Maui | PTBG0000057503    | SAMN14425917              |                          |                          |
| Myrsine   | wawraea     | Appelhans MA673 | Kaua'i | GOET019847,        |                            |                          |                          |
| Oncostemum| ankifensiie | P. Fritsch 1504 | Madagascar | CAS                |                            |                          |                          |
| Oncostemum| elephantipes| De Nevers 11557 | Madagascar | CAS                |                            |                          |                          |
| Oncostemum| evonymoides | FA8088      | Madagascar | CAS                |                            |                          |                          |
| Oncostemum| gracile     | PF1736      | Madagascar | CAS                |                            |                          |                          |
| Oncostemum| seyrigii    | PF1742      | Madagascar | CAS                |                            |                          |                          |
Species/specimens that yielded low DNA quantities and were sequenced on a separate Illumina lane are marked in bold. In the case of Myrsine sp. nov. Appelhans MA690, permits did not allow the collection of a herbarium sample, but a specimen from the same individual is available at PTBG (Perlman 24020, PTBG1000050740, under M. mezii). For the silica sample M. fosbergii Appelhans MA661, a voucher of the mother plant is Tangalin 3964 (PTBG). Seven low-yield samples which have been excluded from the main analyses are indicated by the term “low-yield.” ITS and ETS sequences that were generated for this study are marked with an asterisk (*). Sequences downloaded from Genbank have been published by Anderberg et al. (2000), Hao et al. (2004), Kondraskov et al. (2015), Strijk et al. (2014), and Wright et al. (2006). SRA, Sequence Read Archive.