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Perspective

Ex situ conservation of threatened plant species in island biodiversity hotspots: A case study from Hawai'i

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ABSTRACT

Global plant extinction rates have increased dramatically over the past 50 years. Conservation efforts are especially challenging across the Hawaiian archipelago, where habitat loss and competition with invasive species threaten the existence of native plant species. Currently, 238 endemic Hawaiian plant species have fewer than 50 individuals remaining in the wild. To counteract this daunting statistic, conservationists apply a suite of *ex situ* techniques to effectively safeguard the many threatened plant species from imminent extinction. This perspective piece highlights how an integrated conservation approach that utilizes a co-located seed bank, micropropagation laboratory, and greenhouse, has led to the successful rescue and conservation of many threatened Hawaiian plant species. We draw on specific examples from the long history of Hawaiian plant conservation to detail successes and ongoing challenges associated with the implementation of *ex situ* conservation techniques. In doing so we discuss how plant micropropagation has emerged as a critical *ex situ* conservation tool, and how this underutilized tool fits into plant conservation as a whole. We also emphasize the essential roles that partnerships with external organizations play in ensuring that effective conservation efforts are implemented. Last, using lessons learned from these examples we detail and discuss an *ex situ* plant conservation decision tree that is widely applicable to other plant diversity hotspots of conservation concern, in order to help ensure regional and global zero-extinction goals are met.

1. Introduction

Evidence indicates that rapid human alteration of global processes and the degradation of ecosystems worldwide are driving a sixth mass-extinction event (Ceballos et al., 2015). The loss of plant biodiversity has profound impacts on human livelihoods by negatively affecting ecosystem services (Balvanera et al., 2014; Cardinale et al., 2012) that play integral roles in the function of food production and natural systems (Tilman et al., 2014; Vitousek et al., 1997). By current estimates tropical plant species are generally twice as threatened as temperate species, due in part to high rates of anthropogenic habitat conversion (Brummitt et al., 2015). To counteract these effects, botanical gardens and arboreta around the world have led the charge in the conservation of threatened species (IUCN, 2019) through *in situ* (in natural habitat; Chen et al., 2009; Havens et al., 2014) and *ex situ* (outside natural habitat; Havens et al., 2006; Li and Pritchard, 2009; Mounce et al., 2017) programs that have successfully safeguarded thousands of plant species across the United States (Oldfield et al., 2019) and worldwide (Wyse Jackson and Kennedy, 2009). These programs have also pioneered the development of cutting-edge techniques that have led to insights on *in* and *ex situ* plant conservation (Donaldson, 2009; Smith, 2016), while also supporting plant reintroduction and restoration efforts (Kawelo et al., 2012; Miller et al., 2016). Nonetheless, there is still a critical need and great potential to expand the focus of these conservation efforts (Miller et al., 2016), and to conduct national (Havens et al., 2014) and global assessments (Pelletier et al., 2018) of the conservation status of plant species, to improve threatened plant conservation efforts worldwide (Corlett, 2016).

Although a diverse set of techniques are used to conserve threatened species, many *ex situ* plant conservation programs have traditionally focused on preserving these species in seed banks (Havens et al., 2004; O'Donnell and Sharrock, 2017). Conventional seed banking is used extensively as a conservation strategy because it is the simplest method for long-term germplasm storage. Seed storage is also relatively low

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maintenance and does not require specialized equipment when implemented on small scales. However, seed banking is not a viable option for some plant species (Fant et al., 2016), which are referred to in the literature as "exceptional species" (Pence, 2011). There is ongoing debate about how best to define exceptionality, so in this article we refer to plant species as "exceptional" if (a) they produce recalcitrant (desiccation-sensitive) or freeze-sensitive seeds that cannot be stored conventionally; or (b) existing populations produce few or no viable seeds (Pence, 2013). These factors make *ex situ* conservation very challenging and plant tissue culture, or micropropagation, has emerged as an important *ex situ* storage alternative for these species (Pence, 2011). Using these techniques, explants (*i.e.,* small pieces of living plant tissue that have been removed for culturing), are placed *in vitro* and used to culture viable plantlets or clones (Fay, 1992). These clones are subsequently stored to maintain plant species' genetic lines. Over the past five decades these *in vitro* techniques have been developed and refined with the goal of putting exceptional species into a stable environment where they can be propagated and grown to complete full life cycles (Pence, 2013; Sugii and Lamoureux, 2004). Plant micropropagation has therefore become a key component of the "integrated plant conservation" approach (Falk, 1990), which uses a combination of *in* and *ex situ* approaches to ensure persistence of threatened plant species (Kramer et al., 2011).

Using this integrated approach to leverage all available conservation tools is especially important in island ecosystems, such as the Hawaiian archipelago, due to the fact that a majority of recorded plant extinctions occur on islands, where species tend to have small populations and are more vulnerable to disturbance events (Humphreys et al., 2019). For example, 45% of the species on the United States federal Threatened and Endangered species list are endemic to the state of Hawai‘i (424 out of 946 species; U.S. Fish and Wildlife Service, 2019). Some 238 Hawaiian endemic plant species (close to 25% of all native plants in the state) have fewer than 50 individuals (<100 for dioecious species) remaining in the wild (referred to as PEPP species throughout; Plant Extinction Prevention Program, 2019). These factors make plant conservation in Hawai‘i particularly pressing, but this has also promoted a surge of innovative developments for *in* and *ex situ* conservation techniques, and the creation of large collaborative organizational networks working towards common conservation goals (Keir and Weisenberger, 2014). These conservation networks focus a great deal of effort on the collection of propagules of threatened species for *ex situ* storage and propagation. Furthermore, seed banking (Weisenberger and Keir, 2014) and micropropagation (Sugii and Lamoureux, 2004) have been integrated seamlessly as critical components to *ex situ* plant conservation in this system.

The overall objective of this perspective piece is to detail the integrated and highly collaborative approach to threatened plant conservation that has been implemented in Hawai‘i. In doing so we highlight how micropropagation has emerged as a critical tool for plant conservation; the benefits and challenges associated with the application of these underutilized techniques; and future directions for their application. We draw on examples and historical perspectives from the Lyon Arboretum Hawaiian Rare Plant Program (HRPP) at the University of Hawai‘i, Mānoa and its partner organizations to underscore that combining multiple *ex situ* approaches has been essential to ensure the conservation of many threatened Hawaiian plant species. Last, we detail how micropropagation and other emerging approaches such as cryopreservation fit into an *ex situ* plant conservation decision tree that is widely applicable to other regions with high concentrations of threatened species. In this article we refer to plant species as threatened if they are experiencing rapid population decline and have ongoing threats to their existence; however, unless otherwise specified, not all these species have been assessed for the IUCN Red List or are currently listed on the United States Fish and Wildlife Service Threatened and Endangered species list.

2. *Ex situ* conservation of plant species: examples from Hawai‘i

2.1. Threats to the Hawaiian flora

As the most isolated oceanic landmass in the world, the Hawaiian archipelago has some of the highest percentages of plant endemism. Overall, 89% of native Angiosperms and 71% of Pteridophytes are endemic, and these species face serious threats such as habitat loss and competition with invasive species (Sakai et al., 2002). Widespread ecosystem degradation due to human introduction of invasive plants (Loope and Mueller-Dombois, 1989), vertebrates (Nogueira-Filho et al., 2009; Shiels and Drake, 2011), and invertebrates (Joe and Daehler, 2008) has occurred throughout Hawai‘i. These threats have already led to the extinction of ~10% of native Hawaiian plant species in the wild (Wagner et al., 1999), and over half of Hawaiian plant are considered threatened (Weisenberger and Keir, 2014). Many local and federal organizations focus significant effort to recover native species and to restore native habitat in this system (Keir and Weisenberger, 2014), and the collaboration between the HRPP and external organizations is a clear example of how networks of conservation organizations work collaboratively to ensure threatened Hawaiian plants are conserved.

2.2. Unique aspects of the HRPP and the Hawaiian conservation model

The success of the HRPP, and Hawaiian plant conservation in general, was built upon long-term partnerships with >25 conservation organizations, including federal and state agencies as well as local NGOs and other stakeholders, working collaboratively to ensure the success of conservation efforts (Table 1). Furthermore, all *ex situ* conservation strategies (seed bank, micropropagation, greenhouse, and living plant collections on arboretum grounds) are housed in one location at the Lyon Arboretum. These units work closely together, alongside other *ex situ* conservation facilities such as the National Tropical Botanical Garden and the Hawaii Island seed banks, and with many other government and non-government partners that have a vested interest in the success of plant conservation as a whole. In addition to providing guidance, partners supply plant material for preservation and propagation, and pick up material for outplanting into *in situ* restoration efforts. Due to the rarity of many threatened species and subsequent limited seed production, it is sometimes necessary to rely on vegetative propagules or immature fruit to save plant species with few surviving individuals in order to secure collections. In an effort to save everything and anything possible, in many cases propagules come from collections made under suboptimal conditions often due to the challenges associated with collecting material, such as difficult to reach remote populations, collectors who are unable to return at a more optimal time, or species experiencing an unexpected and rapid decline. Additionally, many Hawaiian plant taxa (Chau et al., 2019) cannot be banked using conventional drying and cold storage methods. Therefore, one major focus of the HRPP has been on the development of micropropagation techniques to propagate and preserve species when seed storage is not a viable option (Sugii, 2011; Sugii and Lamoureux, 2004). Finally, previous work has highlighted the importance of botanic gardens and arboreta in preserving threatened species (Mounce et al., 2017), and one of our objectives is to show how plant micropropagation fits into this broader scope by detailing how it has become an integral tool for Hawaiian plant conservation.

2.3. Early development of plant micropropagation techniques and the importance of these tools for Hawaiian plant conservation

Plant tissue culture was conceptualized in the early 1900s and since then has been expanded for use in a broad range of applications (Preece, 2003; Thorpe, 2007). Surface disinfestation techniques and early micropropagation media formulations were first developed by Knudson (1922) and these early insights led to the development of

Table 1
Roles of major organizations involved in Hawaiian plant conservation and their ongoing partnerships with the HRPP.

Organization	Type	Conservation role
Botanic Gardens Conservation International (BGCI)	Non-profit	Coordinates plant conservation efforts and shares knowledge across a network of >600 institutions (*e.g.,* botanic gardens, seed banks). Provides the HRPP with outreach guidance and a venue to share information with other gardens and the public.
Center for Plant Conservation (CPC) and participating institutions	Non-profit, State, Private	The CPC's goal is to protect the most threatened plant species by collaboratively developing conservation best-practices and promoting cutting-edge plant conservation research. There are 62 participating institutions and Hawaiʻi has four gardens (Honolulu Botanical Gardens, Lyon Arboretum, National Tropical Botanical Garden, Waimea Arboretum) and the Laukahi HPCN as members. The HRPP maintains 100 CPC National Collection species.
Cincinnati Zoo and Botanical Garden CREW (Conservation and Research of Endangered Wildlife)	Non-profit	Shares knowledge on *ex situ* conservation techniques, particularly micropropagation and cryopreservation. CREW and the HRPP have an ongoing collaboration to conduct cryogenic research on 12 native Hawaiian species that have been deemed exceptional.
Hawaiʻi Dept. of Land and Natural Resources - Division of Forestry and Wildlife (DLNR-DOFAW)	State	Manages and protects the watersheds, native ecosystems, and cultural resources of Hawaiʻi. Conducts plant restoration and enhancement for 400 threatened Hawaiian species.
Hawaiian Rare Plant Program (HRPP)	State	Tissue culture and seed repository for the state of Hawaiʻi. Conducts research on *ex situ* storage of threatened Hawaiian plant species.
Hawaiʻi-specific seed banks (*e.g.,* Hawaii Island Seed Bank, National Tropical Botanical Garden)	Non-profit	Maintains seed bank of threatened and culturally important Hawaiian species. The HRPP collaborates with these seed banks to rescue seeds that cannot be stored because they are compromised (*e.g.,* immature, damaged, low viability) or considered exceptional.
International Union for Conservation of Nature (IUCN)	Non-profit	Operates the Red List of Threatened species and supports the Hawaiian Plant Specialist Group which helps to prioritize species-level conservation efforts. The HRPP contributes *ex situ* species status and inventory updates and is part of the specialist group.
Kew Gardens	International	Maintains the Millennium Seed Bank which stores many Hawaiian species. Provides training programs on *ex situ* conservation best-practices.
Laukahi: The Hawaiian Plant Conservation Network (HPCN)	Non-profit	Voluntary alliance of federal and state agencies, non-profit organizations, and individuals working to safeguard Hawaiʻi's flora and native ecosystems through coordinated conservation efforts by implementing the Hawaiʻi Strategy for Plant Conservation (Keir and Weisenberger, 2014). The HRPP collaborates with the HPCN to implement projects that improve living collections, data management, and coordination and communication with partners.
Oahu Army Natural Resource Program (OANRP)	Federal	Environmental program organized by the U.S. Army in Hawai'i to oversee U.S. Army compliance with the Federal Endangered Species Act. Manages 51 threatened Hawaiian species. The HRPP collaborates closely with OANRP to bank clonal genetic lines of founders (*e.g.*, *Eugenia koolauensis*), germinate exceptional seeds (*e.g.*, *Pritchardia kaalae*), salvage immature seeds for *in vitro* germination (e.g, *Cyanea grimesiana* spp. *obatae*), and propagate plants for restoration (*e.g.*, *Phyllostegia kaalaensis* and *hirsuta*).
Plant Extinction Prevention Program (PEPP)	State	Manages wild plants, collects seeds, and establishes new populations of 190 threatened Hawaiian plant species that have <50 individuals remaining in the wild. The HRPP works with each island-specific PEPP manager to coordinate field collections and to strategize species conservation plans.
United States Fish and Wildlife Service	Federal	Recover and prevent the extinction of Hawaiian plant and animal species. Administers the U.S. Endangered Species Act. Provides funding to conduct controlled propagation and storage research on endangered species (*e.g.*, *in vitro* protocol development for the 10 exceptional endangered Hawaiian species managed by HRPP).
United States National Parks (*e.g.,* Haleakalā, Maui and Volcanos National Park in Hawaiʻi)	Federal	Conserve large tracts of land and protect many threatened Hawaiian plant species. Conduct primary research on the management of threatened ecosystems and plant populations. The HRPP assists National Parks in the initiation of germplasm banking of founder plants clonal lines (*e.g.*, *Schiedea haleakalaensis*).
University of Hawaiʻi (*e.g.,* College of Natural Sciences, College of Tropical Agriculture and Human Resources)	State	Implement and communicate results of basic and applied research on Hawaiian species to further conservation efforts. Collaborate with HRPP researchers and students. For example, using fungal symbionts to improve restoration outcomes (*Phyllostegia* sp.*;* Zahn and Amend, 2017), genetic studies of threatened species (*e.g.*, *Kokia cooke*i; Sherwood and Morden, 2014).

media to culture tobacco (Murashige and Skoog, 1962). This proved to be an effective method for clonal propagation and is still used widely today in agriculture. The innovation spurred the development of new techniques for clonal propagation and *in vitro* germplasm storage in the mid-1960s (Thorpe, 2007) with the creation of standardized protocols for plant micropropagation (Bhojwani and Dantu, 2013; Pierik, 1997), as well as for specific groups such as trees (Bonga and von Aderkas, 1992). Equipped with this solid base of knowledge, vegetative micropropagation techniques gained traction for use in propagating and storing plants for conservation efforts (Fay, 1992), which led to the development of additional methods to sterilize and culture explants in the field (Pence, 2005). While the plant tissue culture methods discussed here are applicable in many contexts, system-specific knowledge is very important when using these approaches for plant conservation efforts.

The HRPP was established at Lyon Arboretum in 1991 and started with a focus on the propagation of native and ornamental plants, as well as threatened plant species, using micropropagation methods. A seed bank was established soon after, integrating a second key germplasm storage method. An early success using tissue culture to propagate *Cyanea pinnatifida*, a critically endangered Hawaiian species (Bruegmann and Caraway, 2003), spurred further interest in the use of micropropagation and pointed to its viability as an important *ex situ* technique in this system. The program rapidly expanded, and in 1998 the HRPP was awarded a contract to

propagate 54 native species for the Oahu Army Natural Resource Program (Table 1) using tissue culture techniques. With the establishment of the Plant Extinction Prevention Program (PEPP) in 2003, there was additional focus on locating and collecting increasingly rare plant populations.

The creation of PEPP greatly increased the number of species and accessions directed towards the HRPP. Moreover, this marked a shift towards funding efforts that focus on returning to locations to secure germplasm from threatened plant species and then send material to the HRPP for preservation, among other institutional collections. This enabled the HRPP to focus additional efforts on developing *in vitro* techniques to store and propagate some of the most threatened Hawaiian plant species, which remains the main focus of the program today. Through this process the HRPP continued to expand, and the micropropagation lab currently houses >30,000 plants representing >200 native plant taxa; 150 of which are federally listed as Threatened and Endangered by the United States Fish and Wildlife Service. As of May 2019, 13 of the native species in the HRPP micropropagation collection are believed to be extinct in the wild (Wood et al., 2019), and five of those species are listed as PEPP species (Table A.1). Almost all plants are held in the micropropagation collection, but the HRPP also maintains a small greenhouse that is used for transitioning plants from *ex situ* storage to partner greenhouses that propagate plants for reintroduction programs. While micropropagation is now considered an essential tool for plant conservation in this system there are many important aspects to consider in its implementation.

2.4. Important considerations when implementing a plant micropropagation program

The HRPP and other micropropagation pioneers have developed and refined micropropagation protocols which detail methods to effectively propagate and conserve threatened plant species (Pence, 2013; Sugii, 2011). Many factors must be carefully considered when initiating a culture of plant tissue, including the selection of proper sterilization procedures (*e.g.,* bleach, gas, ethanol dip, flame); culture conditions (temperature, light, airflow); and a suitable culture media formulation (Sarasan et al., 2006). Moreover, procedures must be further tailored to the types of propagules used (*e.g.,* immature/mature seed, meristems, stem internodes, leaves, inflorescences; (Bhojwani and Dantu, 2013) and their condition of those propagules, which often arrive in poor condition and/or highly contaminated due to the less than ideal field collection conditions they are collected under (Pence, 2005). Despite these challenges, effective tissue culture protocols have been developed for >300 threatened Hawaiian plant species (Weisenberger and Keir, 2014), and in many cases relatively standard tissue culture methods can be used. However, a great deal of trial and error is associated with the development of micropropagation protocols for certain species, and some simply cannot be propagated using any known techniques. Furthermore, while standard protocols are sometimes applicable within genera, more effort is necessary to determine which components of these methods are generalizable across phylogenetic and/or functional groups. Internal databases used to track plant accessions hold great potential to elucidate these patterns, in addition to being essential for the long-term conservation value of botanical collections (Cibrian-Jaramillo et al., 2013).

The HRPP and its conservation partners have transitioned to common data collection and storage methodologies that increase the efficiency of data exchange between different botanical databases, and allow for the curation of large living *ex situ* collections (Berendsohn, 1997). Curation of these databases has become a sophisticated process, and within each botanical collection plant provenance is standardized and clearly identifiable using standardized fields and individual plant reference codes. In addition to standardized fields for genotypic (taxon), and environment and source history of field collections (provenance, landowner, collection date and number, plant material type, maturity, quantity, observational field notes), the HRPP micropropagation database stores extensive data on the status of *ex situ* collections. This includes a lab accession number, propagule type and quantity, detailed tissue culture procedure, observational notes, research results, and inventory. These data are used as references for tissue culture attempts with new species, to ensure knowledge is effectively transferred with employee turnover, and to preserve the genotypic and phenotypic value of accessions by tracking plant founders, ensuring their value for conservation applications (Rae, 2011).

Compared to conventional seed banking, micropropagation programs have higher start-up costs associated with procuring specialized equipment to create sterile environments for culture maintenance (*e.g.,* transfer hoods). Therefore, when initiating a new micropropagation program it is best to focus on storing the most threatened taxa *in vitro* before expansion. Furthermore, because of the specialized knowledge required to perform micropropagation procedures, investing time and resources in staff training is critical to long-term program success (Kyte et al., 2013), though, in our experience, a small program can be maintained with one full-time staff member supplemented by the help of student interns. Additional considerations include the costs associated with the continuous maintenance of a germplasm collection in a highly controlled environment (*e.g.,* climate control, glassware), and ongoing creation of culture media (Abeli et al., 2019; Sarasan et al., 2006). While the specialized training and equipment associated with curating a micropropagation collection may create a barrier to entry, the long-term cost-benefit of maintaining exceptional species that are critically endangered or extinct in the wild in stable *ex situ* storage may far outweigh the initial costs of implementation. This makes these approaches particularly beneficial in tropical biodiversity hotspots experiencing rapid species decline. Moreover, micropropagation can effectively store large living collections in compact spaces, *e.g.,* the entire HRPP collection is housed in a 75 m^2 room, and can yield significant cost savings in the long run by decreasing the labor costs associated with maintaining collections in the greenhouse or on arboretum grounds. Below we outline species-specific case studies to highlight how these *ex situ* conservation techniques have proven effective in the rescue and conservation of threatened Hawaiian plant species, and showcase others where additional research is still necessary to overcome barriers to plant reproduction.

2.5. Ex situ plant conservation case studies from the HRPP

We highlight several case studies from the HRPP where threatened species have either been effectively conserved using *ex situ* techniques, or still face challenges related to their stable long-term storage and survival (Table 2). For clarity, we refer to plants as 'seedlings' if they were produced *in vitro* from seeds or embryos, or 'plantlets' if they were produced from cuttings rooted *in vitro*. Through these examples and others, the following key insights have emerged which highlight why micropropagation is a critical tool for *ex situ* plant conservation in Hawai'i, and holds great potential for use in other systems where it has not yet been integrated.

- Propagation of immature and mature seeds: Micropropagation approaches can be used to germinate immature seeds (*e.g.*, *Kanaloa kahoolawensis*), or to perform embryo rescue for those that do not develop properly due to inbreeding depression (*e.g.*, *Kokia cookei*; Fig. 1; Sugii, 2011). Additionally, *in vitro* approaches can be used to increase germination rates of mature seeds when available seeds are very rare and/or when previous greenhouse germination attempts have failed (*e.g.*, *Cyanea grimesiana* subsp. *grimesiana*). Another extension of this approach not discussed in the case studies is that harvesting propagules early and using tissue culture to aid in germination has helped to conserve palms in the genus *Pritchardia* as the fruit is very vulnerable to predation from rats in Hawai'i.
- Maintenance of clonal lines: Dioecy is prevalent in the Hawaiian flora (Sakai et al., 1995) and is a large obstacle for many species that only persist as a few individuals. Micropropagation is an efficient method to store clones and assist in maintaining parental lines that could be otherwise lost for future breeding efforts. Therefore, for all of the case studies in Table 2 and for many other species, micropropagation fills the critical role of holding clonal lines while collectors continue to search for additional founders for future breeding programs.

- Production of plants for restoration: Tissue culture methods allow for early cloning of plants that would otherwise take months or years to replicate, or for the multiplication of material when few seeds are available. This allows for the mass production of plantlets, derived from tissue culture, for restoration programs for use as replicates in experimental reintroductions when plants cannot be produced by other means (*e.g., Cyanea pinnatifida*; Fig. 1). This approach was used for all the effective case studies highlighted in Table 2.
- Protection from pathogens: Micropropagation is an effective approach to shelter particularly vulnerable plants from virulent pathogens, (*e.g.*, many Hawaiian species in the Lamiaceae). This approach can therefore be used to store groups of species that have experienced rapid decline due to pathogen introduction, in anticipation of future introduction of agents that affect plant health.

In addition to these important roles played by micropropagation, the case studies outlined highlight that by co-locating *ex situ* storage options the HRPP is able to maximize the use of all plant material that arrives, enabling rescue of all collected material (*e.g.,* tissue culture, seed bank, greenhouse). Additionally, following initial collection attempts HRPP researchers continue an open dialog with collectors to improve the condition of future collected material. In other words, if material was collected when immature, HRPP researchers can inform collectors who can amend future procedures, resulting in improved collections and increased propagation success. Ultimately, this process leads to a fine tuning of propagule management that can provide suggestions on how to best utilize each *ex situ* technique to maximize germplasm quality and minimize long-term storage costs. Collaborations such as these, and partnerships with a multitude of external organizations, enable the HRPP to apply an integrated plant conservation model (Falk, 1990) to Hawaiian plant conservation by operating at the interface between *in* and *ex situ* conservation programs.

2.6. Partnerships with external organizations are essential for successful outcomes

The co-location of *ex situ* conservation facilities at the HRPP is only one component that has fostered success. Partnerships developed with external federal and state agencies, non-profit conservation organizations, and local plant material collectors are also a critical component of Hawaiian plant conservation (Table 1). Recent efforts to develop a statewide conservation strategy have highlighted these important relationships which have developed organically between *ex situ* micropropagation, seed bank, and nursery facilities (Keir and Weisenberger, 2014). This development has helped to bridge the 'Academic-Agency divide' (Farnsworth, 2004) by employing basic science techniques to develop solutions to applied conservation problems. In doing so, both the HRPP and its partner organizations are able to meet their sometimes disparate goals. For example, the goal of academic research programs (*e.g.,* University of Hawai'i) is typically to produce unique scientific findings, while federal (*e.g.,* United States Fish and Wildlife Service, Oahu Army Natural Resource Program) and state agencies (*e.g.,* Hawai'i Department of Land and Natural Resources) have a mandate to prevent plant extinction and are required to secure and maintain *ex situ* collections of threatened species to meet recovery goals. Projects undertaken by the HRPP fuse these two goals not only by conserving threatened species in partnership with state and federal agencies, but also by performing basic research that has led to the development of best practices for *ex situ* conservation that furthers the understanding of the biology of threatened species. An important added benefit of this approach is that funding from unrelated sources can be combined and leveraged for maximum gain.

In addition to continued funding, the long-term success and expansion of *ex situ* collections at the HRPP is heavily dependent on collaborations with external collectors from many organizations (*e.g.*, PEPP, other local botanic gardens, state and federal agencies) that provide plant material and information about the status and biology of each species. In turn, these organizations rely on the HRPP as the main *ex situ* storage facility for the state of Hawai'i, and the only facility in the state that incorporates micropropagation into conservation efforts. In addition to storing plant germplasm in tissue culture and/or seed banking, the HRPP develops *ex situ* storage protocols/recommendations that can be applied more broadly, with the ultimate goal of supporting threatened plant restoration efforts. To fill this critical need the HRPP propagates threatened Hawaiian plant species that are initially grown in the HRPP greenhouse then transferred to conservation nurseries such the mid-elevation rare plant facilities operated by the Hawai'i Department of Land and Natural Resources for off-site propagation and/or outplanting by partners such as PEPP. Finally, to facilitate communication between all *in-* and *ex situ* conservation partners the HRPP hosts regular meetings of the Hawai'i Rare Plant Restoration Group (also the IUCN Hawaiian Plant Specialist group) in an informal forum where threatened plant conservation efforts can be coordinated, and common priorities and goals can be defined. This interorganizational coordination is an essential component of plant conservation in Hawai'i and has great potential to be applied to other global hotspots of plant biodiversity loss.

3. An *ex situ* plant conservation decision tree for threatened plant species

Creating tools to facilitate *ex situ* conservation decisions for threatened species can streamline the decision-making process. Using knowledge gleaned from the outcomes of many collaborative plant conservation efforts across Hawai'i we have developed a decision tree for *ex situ* plant conservation, which is widely applicable to other *ex situ* conservation challenges around the world (Fig. 2). Given the type of material collected (seeds or cuttings/divisions), this decision tree can also assess which *ex situ* storage method(s) are ultimately feasible (seed banking, micropropagation, cryopreservation, greenhouse, and/or collection and storage of living plants), with the goal of maintaining a germplasm collection that serves as a repository for controlled breeding and/or future outplanting programs. This decision tree can also identify where key gaps remain in our understanding of factors limiting processes such as seed germination (*e.g.,* pollinators, genetics, health of mother plants, seed dormancy) that necessitate further research. Furthermore, when a lack of available materials precludes *ex situ* storage, this decision tree points to the potential use of air layering, which has enabled the propagation of woody threatened species by rooting plant branches *in situ* (Moreira et al., 2009) to produce propagules for *ex situ* storage. Last, in cases when no propagules can be collected, and when air layering is not feasible, pollen can be collected and stored for future research or whole plant harvests can be considered a last resort.

This decision tree represents a comprehensive view of all *ex situ* plant conservation approaches and technologies currently available, and it can be used to screen regional flora to determine which approaches should be prioritized for development and application. For example, an extensive study of the storage longevity of 295 Hawaiian plant species found that ~11% of species are short-lived in conventional storage and have recommended re-collection intervals between 1 and 5 yr (Chau et al., 2019). Such species can be stored conventionally but could also benefit from placing material in cryopreservation (*see* Section 4). Additionally, we consider a large percentage of threatened Hawaiian plant species to be exceptional (~25%; unpub. data), much higher than global estimates of exceptionality [~8% for desiccation-sensitive seeds (Wyse and Dickie, 2017); ~1% for freeze-sensitive or no viable seeds, (V. Pence, pers. comm.)], underscoring the importance of developing micropropagation and cryopreservation approaches in this system. This approach therefore provides a guiding framework not only for practitioners initiating *ex situ* conservation plans for specific species, but also to inform how *ex situ* conservation needs vary regionally, taxonomically, or functionally.

Table 2
Case studies from the Hawaiian Rare Plant Program highlighting the role of micropropagation as an *ex situ* conservation tool.

Species & Hawaiian name // IUCN rank & reference	Background	Outcomes and importance of micropropagation
Effective efforts		
Cyanea grimesiana subsp. *grimesiana* (Haha) // Critically endangered	Only three wild plants are known to exist. Whole inflorescences were collected from two plants growing in different locations that never cross-pollinated. Seeds were germinated using routine tissue culture (TC) methods and both immature and mature seeds were used because of seed rarity and a lack of success in prior greenhouse germination attempts. The *in vitro* seed sowings produced seedlings that were multiplied through cloning (microcuttings, no hormones) to establish clonal lines and increase the number of plants, some of which were eventually outplanted for restoration. Restored populations produce seed that is now collected and stored at HRPP but all wild plants have died.	Species has gone full circle from collection to reintroduction and seed is now collected from reintroduced plants. Demonstrated that establishment of germplasm collection with multiple clonal lines can be successful. Over 10 yr passed between the initial establishment of the germplasm collection and reintroduction efforts, and some parental lines were lost over time in the *in vitro* cultures, emphasizing the need for cryopreservation for long-term storage of clonal lines.
Cyanea pinnatifida (Haha) (Fig. 1a-d) // Extinct in the wild (Bruegmann and Caraway, 2003)	At the time of collection only one plant, was known in the wild and it was threatened by a precariously hanging boulder. Two side shoots were collected from the base and tissue was easily transferred to *in vitro* culture using standard protocols. Clones were propagated in the greenhouse where researchers observed the leaf morphology change from juvenile to mature (see section to the right). Greenhouse plants eventually produced seed from which additional seedlings were grown and outplanted next to the source plant.	This example was the first *in vitro* storage success at the HRPP and demonstrated that routine TC techniques can be used to germinate many Hawaiian species, and that there is some tolerance to inbreeding. Moreover, it highlights the importance of studying ontogenetic stages as researchers eventually realized the source plant was still a juvenile after growing plants to maturity in the HRPP greenhouse. This species also went full circle and outplanted populations now produce seed that is banked. Original material is still stored at HRPP and could potentially be stored in cryopreservation.
Cyanea truncata (Punaluu cyanea) // Critically endangered (Bruegmann et al., 2016)	Declared extinct in the wild in the 1970s. The species was rediscovered in the wild in 1998 (two individuals) and seeds were collected and germinated at HRPP using TC. A subset of cloned seedlings (germinated *in vitro*) were outplanted at a private location (Kooloa Ranch) with PEPP/state partnership. Restored populations produce seed that is collected and stored at HRPP. Another wild population was later discovered on State lands but no original wild plants remain.	Another species that went full circle from collection to a reproductive population of outplants. Nonetheless, personnel changes led to challenges in determining provenance for *in vitro* collections. This was sorted out and led to the development of standardized protocols used by HRPP and partners to ensure that provenance of all collections could be cross-referenced across the multiple organizations involved in initial material collection, *ex situ* storage, and eventual reintroduction.
Ongoing challenges		
Kanaloa kahoolawensis (Ka palupalu o Kanaloa) // Critically endangered (Portner et al., 2016)	This species was very widespread according to historic pollen records but was only ever observed in one location and now no known wild plants remain. Two mature plants grown from seeds collected from the wild are still in cultivation, but collections for this species were generally of low quality (*e.g.*, immature or broken seeds; delay between maturation and harvest; compromised by insects/fungus) because collecting required difficult helicopter access. HRPP was able to germinate two plantlets with TC, but the plants were not healthy. One callus culture has been maintained for seven years but with no regeneration success. Cuttings have been collected but high endogenous contamination resulted in no TC success from those collections.	Improved health of mother plants and improved TC protocols have increased success of propagation attempts recently but most success has been from cuttings used in greenhouse propagation. This example highlights the ability of micropropagation to germinate immature seeds (or material sitting for too long) and to maintain material until suitable TC methods are developed. However, it also points to the importance of coordinating collection efforts to ensure high quality material is collected, and that plant material transportation networks need to be improved to ensure the timely arrival of material for plant propagation and storage attempts.
Kokia cookei (Koki'o) (Fig. 1e-h) // Extinct in the wild (World Cons. Monit. Centre, 1998)	At the time of collection this species only existed as a grafted plant. Fruit almost always aborted prior to maturation, or if fruit matured it did not produce viable seeds. HRPP researchers coordinated with collectors to obtain fruit that was nearly mature and were able to perform embryo rescue on fully formed embryos before they aborted by excising and germinating embryos using standard TC protocols. Cloning attempts have not been successful but true seedlings taken out of *in vitro* culture have been successfully outplanted. Genetic diversity is very low (Sherwood and Morden, 2014).	This is a good example of where TC methods can produce viable plants from immature collections but this species has faced a severe genetic bottleneck and does not produce viable seeds. Current efforts are being made to cross the progeny with each other and the original maternal line in a garden collection. Continued efforts to process new fruit in TC to produce more plants are necessary. This highlights that micropropagation can be used to mass produce clones for reintroductions when no seed sources exist.
Hibiscadelphus woodii (Wood's hau kuahiwi) // Extinct; but see background (Clark, 2016)	When collections for the HRPP were made only three known plants existed on a cliff face that required dangerous repelling to access, which also put plants in danger of rock falls. High quality cuttings were obtained but had high endogenous contamination and did not have TC success. Wild plants stopped flowering eventually and died. The species was believed to be extinct but in early 2019 the species was rediscovered in the wild (four individuals) using drone (unmanned aerial vehicle) technology, offering hope for future propagation success.	Communication challenges between institutions hampered the conservation process in this instance as the lack of initial propagation success led to a decreased effort to collect material to try additional propagation methods and develop new protocols. New drone technology, that can help with difficult surveys, rediscovered individuals of this species in the wild. If material can be collected new TC protocols could potentially be utilized to conserve this species.

4. New and future *ex situ* plant conservation initiatives

Our proposed *ex situ* conservation decision tree (Fig. 2) includes approaches not currently integrated into the HRPP at Lyon Arboretum, such as the use of cryopreservation for germplasm storage and the integration of a pollen storage program. Cryopreservation, or the storage of living plant tissue in liquid nitrogen, offers the potential for long-term stable storage of plant material (Normah et al., 2019), extending the storage interval well beyond the time frame afforded by *in vitro* tissue culture methods (typically up to 10 yr before material recollection is suggested; unpub. data). New cryopreservation techniques offer their own challenges and require additional specialized equipment, but have the potential to fill gaps in the storage of exceptional species (Pence, 2013). Furthermore, the material costs to start a small cryopreservation program within an existing tissue culture lab are relatively low (Abeli et al., 2019; Pence, 2013), and less than $20,000 USD by our estimate.

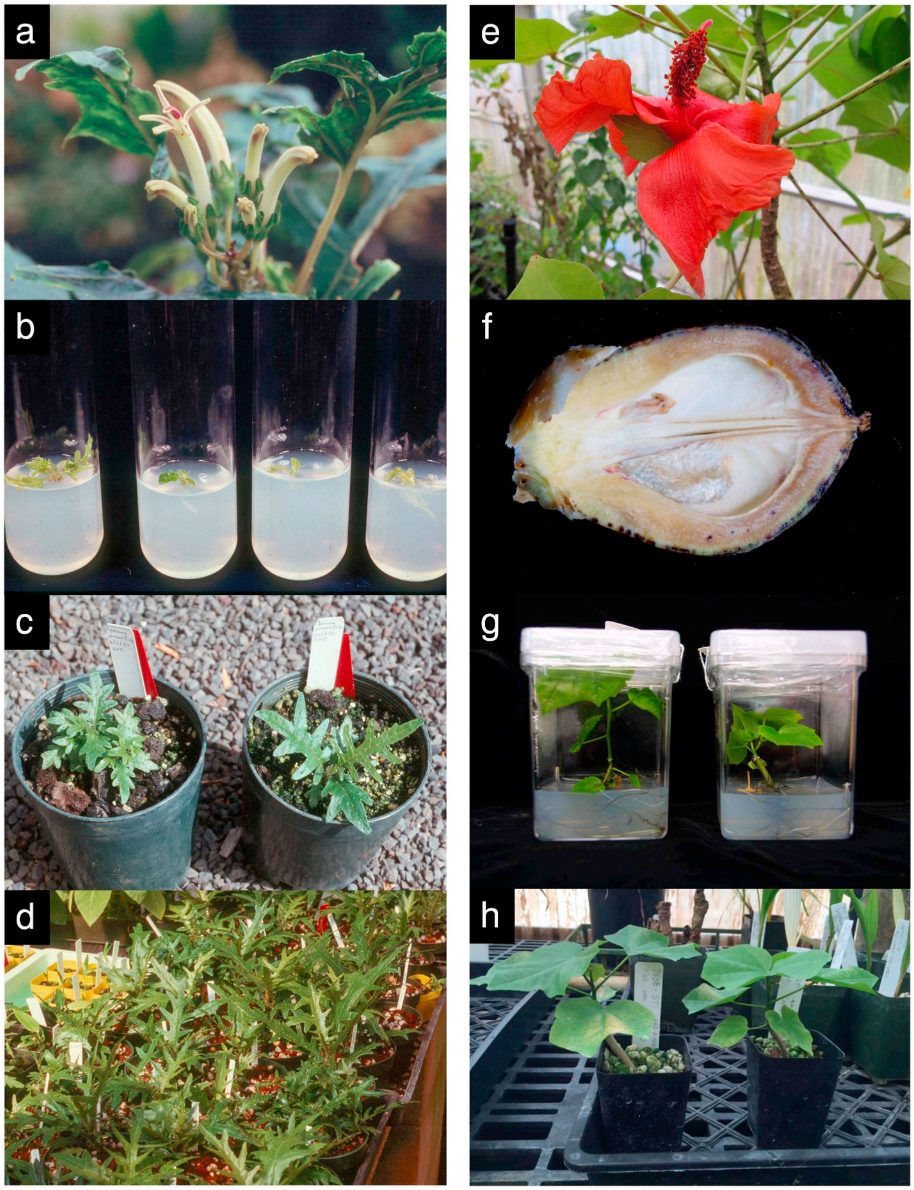

Fig. 1. Approaches used to conserve two Hawaiian plant species that are extinct in the wild, *Cyanea pinnatifida* (Haha; left) and *Kokia cookei* (Koki'o; right). Left panels: (a) inflorescence; (b) *in vitro* propagation of clones; (c) clones in the HRPP greenhouse; (d) clones generated with micropropagation for reintroduction efforts. Right panels: (e) inflorescence; (f) fruit used for embryo rescue; (g) seedlings grown *in vitro*; (h) seedlings in the HRPP greenhouse. Photo credit panels a-d: G. Koob; panels e-h N. Sugii.

Pollen storage is an additional promising *ex situ* conservation strategy that can be used to complement seed storage efforts. While pollen does not conserve the whole plant genome in its haploid state, it can be an important source of genetically diverse and heritable character traits in plant breeding programs that utilize artificial pollination (Nadarajan et al., 2018). Pollen can tolerate both short- and long-term storage with careful monitoring and regulation of temperature and humidity (Wang et al., 1993). Cryopreservation is also a promising tool for long-term pollen storage as it slows down metabolic activity and decreases pollen viability loss (Rajasekharan et al., 2013). Similarly, the storage of fern spores has proven to be an effective and economical method to preserve the germplasm of endangered ferns (Li and Shi, 2014).

In addition to the development and integration of new *ex situ* storage methods into plant conservation programs (*e.g.,* cryopreservation, breeding programs, pollen storage, reintroductions on site), there is the potential to extract additional information about the biology of the many threatened species stored in *ex situ* collections to improve restoration outcomes given that there is large variation in plant reintroduction success, both in Hawai'i (Kawelo et al., 2012) and globally (Albrecht et al., 2019; Godefroid et al., 2011). For example, living botanical collections provide a rich resource for the study of tropical plant functional ecology (Perez et al., 2018) and plant functional characteristics extracted from these collections have great potential to predict threatened plant reintroduction outcomes. Standardized plant functional traits can be used to understand plant habitat preference and stress-tolerance, among other processes (Violle et al., 2007), and these traits have effectively predicted the outcome of restoration initiatives in both tropical wet (Ostertag et al., 2015) and dry forests (Werden et al., 2018). Moreover, plant functional traits can be extracted non-destructively from *ex situ* collections, *e.g.,* traits collected from seed bank accessions. Leveraging existing collections in this manner has the potential to advance our understanding of how plant characteristics dictate the ability of specific threatened species to survive and persist after outplanting, thereby improving outcomes. Integrating such additional

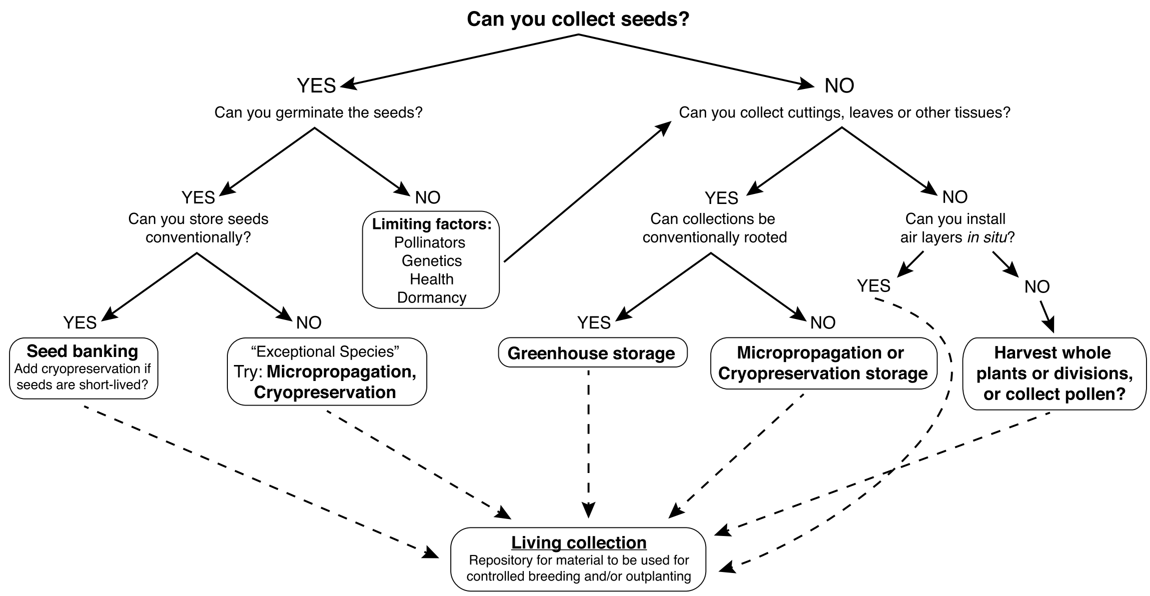

Fig. 2. Decision tree for *ex situ* plant conservation.

initiatives would further the effectiveness of integrated plant conservation programs that use a mixture of *in* and *ex situ* conservation methods to ensure the rescue and persistence of threatened plant species.

5. Conclusions

Applying the integrated plant conservation approach by involving many agency, non-profit, and individual conservation stakeholders has led to the effective conservation of many threatened Hawaiian plant species. Within this conservation matrix, micropropagation has emerged as a critical tool that not only supplements conventional seed banking methods, but also can be used to conserve the globally increasing number of threatened plant species considered exceptional due to their increased rarity. Micropropagation is currently underutilized in plant conservation plans even though it can serve essential functions including the germination of immature and/or rare mature seeds, propagation of material when seeds are not available, maintenance of clonal lines for future breeding and outplanting, shelter of species vulnerable to pathogens in a highly controlled growing environment, and the mass production of clones for restoration efforts. Moreover, the cost-benefits of holding species in stable long-term *in vitro* storage can outweigh the start-up costs associated with establishing a micropropagation program. Co-locating *ex situ* conservation facilities is only one component that has fostered success, and the HRPP also owes its effectiveness to alliances formed with a suite of external organizations and stakeholders. While challenging to implement, this integrated approach could be expanded to other geographic hotspots of biodiversity that are of conservation concern, in order to help ensure that regional and global zero-extinction goals are met.

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Declaration of competing interest

The authors have no conflicts of interest to declare.

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Glossary

HRPP: Hawaiian Rare Plant Program
PEPP: Plant Extinction Prevention Program
Exceptional species: Plant species than cannot be stored in seed banks using standard methods or easily propagated by seed