Gago,
Guam Ironwood Tree,
Casuarina equisetifolia

Past, Present, Future

Edited by Robert L. Schlub

University of Guam
Guam Cooperative Extension
2013

Appendix update
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Preface:

In 2002, roughly twenty years ago, a farmer in Guam noticed a few of his 10-year-old ironwood windbreaker trees (*Casuarina equisetifolia*) were dead or dying. A few trees exhibited wilt symptoms, which consisted of a rapid yellowing and death of branchlets, while many more had symptoms of decline, which consisted of thinning of foliage and dieback of branches. Following a quick search of ironwood stands on Guam, the sudden death disease or wilt appeared to be an anomaly, whereas decline was a serious condition and widespread. At that point, research into the etiology of decline began. With each research project comes new information about ironwood decline and with it the reshaping of my belief as to its cause or causes. After years of research and realizing that many questions regarding the how's and why's of ironwood decline remained unresolved, I decided to keep this manual as a dynamic compilation of information related to the care of the ironwood trees and ironwood decline, starting in 2002 and continuing to this revision. The earliest information, which is part of the 2013 Ironwood manual, is found in Part 1 of this revision. Part 2 was added in 2020 and Part 3 was added in 2022.

UOG clinic number: 22-342, Survey date: 06-06-2022, GPS: 14.16807, 145.22358, Location: Sinapalu 3, Disease severity (0-4): 3, Height: 13.41m, DBH: 20.21cm, No sporocarps, No termite activity, Site Condition: site incorporating a moderate level of management practices, Agdia ImmunoStrip® for *Ralstonia solanacearum* (Rs testing date: 06-06-2022, Rs + (Strong)

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Part I.
2013 Original Manual

2013 Introduction: This guide serves as an introductory text on plant health care for the Gago or Guam's ironwood (*Casuarina equisetifolia*). It contains some general information about the tree including its history on Guam and its importance to the region. It explains ironwood decline and its underlying causes. Finally, it provides some tree health care recommendations and suggestions for future research.

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Acknowledgements: Little would have been accomplished if it were not for postdoctoral researcher Dr. Zelalem Mersha and personnel at the US Forestry Service, Land Grant Universities, CSIRO Australia, Arbor Global, and The Davey Institute. Ultimately, none of this would have been possible without the initial funding from the Western Sustainable Agriculture Research and Education Program (WSARE). Other sources of funds or support included Guam Cooperative Extension, Western International Forest Disease Work Conference, American Phytopathological Society, International *Casuarina* Workshop and various United States Government Programs (USDA, NIFA, RREA, US Forest Service, APHIS, and NOAA).

Summary

Despite the myriad of utilities and merits of the ironwood tree (*Casuarina equisetifolia* subsp. *equisetifolia*) to the Pacific island of Guam, its future is in doubt because of its deteriorating health and survival rate. Ironwood trees (*C. equisetifolia*), like all trees, have a natural finite life span within a given ecosystem; however, Guam's trees are dying at unexpected rates. What is happening on Guam fits the classic definition of tree decline: symptoms are nonspecific such as the thinning of branches; tree health gradually deteriorates leading to tree death over the course of several years; decline is attributed to a complex environment of infectious and non-infectious agents. However, Guam's trees deviate from the classic model wherein mature trees are more prone to decline.

Decline was first noticed in 2002 by a local farmer. The trees at that site were less than 10 years old and planted in single-row windbreaks of several hundred trees. Less than 5 trees were characterized as wilted with the following symptoms: acropetal progression of chlorosis, tip-burn of lower branchlets giving the tree a singed appearance, and tree death within 6 months. Roughly 15 trees had symptoms of decline, which included internal wood discoloration, thinning of branches, and tree death after several years. By 2004, one third of all the ironwood trees at the naval station (Commander Navy Region Marianas (COMNAVMAR)) were dead. By 2005, Ironwood Tree Decline (IWTD) was widespread on Guam. In January 2009, a five-day IWTD conference was held with participants from Guam and off- island. Six off-island experts and other participants visited healthy and declined tree sites, collected samples, and reviewed research related to *C. equisetifolia* production worldwide and its growth on Guam. Participants concluded that a complex of biotic and abiotic factors were responsible for the decline and subsequently advanced the theory that an opportunistic conk-producing fungus like *Ganoderma* and/or *Phellinus* in association with wounding could explain the majority of Guam's declining trees.

To assess the level of ironwood tree decline on Guam, photographs of 44 randomly selected trees with varying levels of decline were categorized into two lots, one small (CBH ≤ 100 cm) and one large (CBH >100 cm) based on their circumference at breast height (CBH). Then each lot was visually cataloged into a five-scale decline severity (DS) rating. On subsequent surveys, trees with different DS ratings were characterized visually for branch thinning and quantitatively for branchlet ("needle") biomass. As DS increased from 0 (healthy tree) to 4 (nearly dead tree), branch thinning progressively increased from 0 to 95.0% and 0 to 92.5% for small and large trees, respectively. There was no significant difference between branchlet biomass for DS 0 and DS 1 nor between DS 2 and DS 3 trees. The greatest branchlet weight loss, at 95.3%, occurred in DS 4 trees. Internal symptoms included various patterns of discoloration in trunks and a white soft-rot in roots. Discoloration was consistently traced into branches through cross-sectioning at the branch-trunk interface. In branches, the presence of discoloration was only 100% consistent in DS 3 and 4 trees. External symptoms start at the top of the tree and progress downward; whereas internal discoloration starts at the tree's base and diminishes acropetally.

To determine the status of the decline problem and to seek possible causes, a survey of 1,427 trees was conducted in 2008 and 2009. A highly significant ($p=0.0001$) linear function ($r^2 = 0.997$) between the presence of basidiocarps and decline severity emerged from the survey. Basidiocarps were either flat (resupinate) or shelflike (conk). Sixty-five percent of the trees at the most severe level of decline (nearly dead) had basidiocarps. Thirty-five conks were collected from the survey area under different stages of tree decline. Species from five

basidiomycete genera of the class Agaricomycetes, belonging to the orders Polyporales (*Ganoderma, Favolus, Pycnoporus*), Hymenochaetales (*Phellinus*) and Thelephorales (*Sarcodon*) were identified based on macro- and micromorphology and DNA sequencing. The most common species observed was in the genus *Ganoderma.* Diagnostics were based on the prolific production of double walled basidiospores from sporocarps (a characteristic feature of members of the Ganodermataceae). DNA sequencing, using the nuclear ribosomal internal transcribed spacer (ITS) region confirmed Guam's species as a member of the *G. australe* species complex. The second most frequently collected conk belonged to the genus *Phellinus.*

Various modeling techniques were applied to the 1,427 tree survey data set. For each sampled tree, the level of decline was measured on an ordinal scale consisting of the five-decline levels ranging from healthy (DS=0) to near dead (DS=4). Several predictors were also measured including tree diameter, fire damage, typhoon damage, presence or absence of termites, presence or absence of basidiocarps, and various geographical or cultural factors. The five decline response levels can be viewed as categories of a multinomial distribution, where the multinomial probability profile depends on the levels of these various predictors. Such a data structure is well-suited to a proportional odds model; thereby leading to odds ratios, involving cumulative probabilities which can be estimated and summarized using information from the predictor coefficient. The logistic model used dieback as a variable, which was derived from the decline severity variable. A healthy tree (DS=0) was assigned a dieback value of 0, all other decline severity trees (DS=1, 2, 3, or 4) were assigned a dieback value of 1. Various modeling techniques were applied to address data set issues: reduced logistic models, spatial relationships of residuals using latitude and longitude coordinates, and a correlation structure induced by the fact that trees were sampled in clusters at various sites. Among the findings, factors related to ironwood decline were found to be "conks", termites, and level of human management. A conk-producing species in the *Ganoderma australe* complex was identified as the primary wood-rotter. This fungus is commonly found on Guam where IWTD is widespread and rarely on Saipan, a nearby island where the majority of trees are considered healthy. With the addition of GIS map derived variables to those of the original model, it was found that trees are less likely to exhibit ironwood tree decline symptoms when there is adequate soil moisture holding capacity, as in a forest setting or in a properly managed landscape such as a golf course. Likewise, the level of declining trees at a given site can be expected to intensify with increases in the occurrence of "conks", termites, altitude, or tree size. When tree circumference and dieback maps were compared, tree site productivity could not explain the high level of IWTD predicted in central Guam. The increased presence of termites, conks, and storm damage with increased tree size suggests that under ideal tree stand conditions, these variables are part of the normal process of tree senescence.

Bacterial colonization of the xylem is seen in trees with thinning foliage, which is indistinguishable from those attributed to ironwood tree decline. Three bacteria were consistently isolated: *Ralstonia solanacearum, Klebsiella oxytoca,* and *Klebsiella variicola.* We believe *Klebsiella* spp. are responsible for the wetwood symptom associated with Guam's declining trees and that both *R. solanacearum* and *Klebsiella* spp. play a role in tree decline. In the future, the current model will be strengthened, with the addition of *Ralstonia* and *Klebsiella* survey data.

Background

History: *Casuarina equisetifolia*, locally known in English as the ironwood tree and in the Chamorro language as "gago," is known to be indigenous to Australia, the Malayan Islands, the east side of the Bay of Bengal, and occurs on many islands of the Pacific, extending eastward to the Marquesas Islands and northward to the Mariana Islands (Safford, 1905). Pollen records indicate that the ironwood tree has grown on Guam for hundreds of years (Athens and Ward, 2004) and is likely native to Guam (Fosberg *et al.*, 1979; Stone, 1970). It has been continually propagated on Guam since the 1600's, possibly due to its usefulness and low maintenance requirements. As a result of its tolerance to salt spray and typhoon damage, its ability to support nitrogen-fixing *Frankia*, and endo-and ectomycorrhizae and the presence of cluster roots (also known as proteoid roots), the tree is able to thrive in the Mariana Islands where typhoon and coral sand beaches and other nutrient-poor soils are commonplace.

Botanical characteristics: The tree is an evergreen angiosperm. Its needle-like, jointed branchlets bear anatomical minute, tooth-shaped leaves. As a result of limited leaves and floral structures, the tree has the ability to conserve moisture and tolerate drought. Within the Mariana Islands, the average lifespan of an ironwood tree is estimated to be 35 to 90 years, with a maximum height and circumference at breast height of 13.7 and 2.9 m, respectively. Due to damage from typhoons in the Mariana Islands, exposed trees are often topped with prolific, epicormic shoots, resulting in a shorter tree with a wider crown than what is typically seen in Hawaii, an area with few typhoons.

Ecology: Ironwood tree thickets are a component of Guam's forestland where it is considered a secondary forest species (Liu and Fischer, 2006). Ironwood trees do not compete with native tree species in undisturbed limestone forests (Moore, 1973), although they grow nearly everywhere: beaches, landfills, road shoulders, cleared land, and vacant lots. In the Mariana Islands it grows both in the clay volcanic soils of savanna grasslands and calcareous and loamy sands of coastal strands. In large dense stands, trees produce a thick, slowly decomposing, allelopathic litter layer that eliminates nearly all understory vegetation.

Several prominent forest features of ironwood on Guam were mentioned in a 2002 Guam Forest Bulletin (Donnegan *et al*., 2004). Ironwood trees were reported to be among the healthiest trees on island with an estimated population of 115,924 for trees greater than 5 inches in diameter at breast height. *C. equisetifolia* was mentioned as a prominent member of the halophytic (sea-salt adapted) vegetation type. This vegetation is found along beaches in the north and south, where it may be composed solely of ironwood or a mixture of other species including *Cocos nucifera*, *Guettarda speciosa*, *Hernandia sonora*, *Pandanus tectorius*, *Scaevola taccada*, *Thespesia populnea*, and *Tournefortia argentea*. On the sandy beaches of the Mariana Islands, it has become an important perching tree for the white-collared kingfisher *(Halcyon chloris)* and the Mariana fruit-dove, *Ptilinopus roseicapilla* (Marshall, 1949). The white tern, *Gygis alba*, commonly lays eggs in ironwood trees.

Materials and Methods

Conference: Participants and attendees included administrators, researchers, students, the general public, and six off-island experts. Fourteen sites were visited during the 5-day conference period, where samples in the form of branches, cross-sections (roots, trunks, and branches) and sporocarps were collected and brought to the laboratory at the University of Guam's science building.

Ironwood tree decline (IWTD): Photographs of 44 randomly selected trees with varying levels of decline were categorized into saplings to small trees (DBH ≤ 32 cm) or large trees (DBH > 32 cm). These were then visually categorized based on a five-scale decline severity (DS) rating. Percent bare branches (PBB) was determined by analyzing the photographs. Cross-sections of 5 small and 3 large tree trunks and of branch trunk intersections from 34 small and 26 large trees were examined for evidence of discoloration or wood rot. Four to five branches from randomly selected trees were removed (30 cm from branch tip) and growth parameters measured. The branch sections were stripped and branches and branchlets ("needles") weighed. Cones were counted, weighed, and placed in 20-cm diameter Petri dishes on the laboratory bench (temperature 24 – 25 °C and 50 – 55 % relative humidity) to promote seed release.

Nematode extraction: Ten grams of roots were collected from the top 10 centimeters of soil. Eight trees were surveyed: four were in decline and four appeared healthy. Roots were rinsed to remove soil. Roots were cut into sections of a centimeter in length. Ten grams of roots were placed in a flask with 200 ml of water and placed in a shaker at 200 rpm for a total of 57 hours of shaking. The water and roots were passed through a 140-mesh sieve to collect the roots, and a 400-mesh sieve to collect the nematodes. The 400-mesh sieve was flushed, and nematodes were collected in 20 ml of water. Two ml of nematode suspension were placed in Petri dishes and identified under an inverted compound microscope. Nematode numbers are per one gram of root tissue.

One hundred ml of soil was collected from the top ten centimeters of soil associated with *Casuarina* roots. The soil samples were processed using a modified Jenkins (1964) centrifugation and flotation technique, using 100 ml subsamples. Twenty ml of the nematode suspension was placed in tubes and a 2.0 ml aliquot was placed in a cover slip-bottom dish and all the nematodes present were identified to the lowest taxon possible. The resulting data were recorded as nematodes per 10 ml of soil.

DeLey's and Blaxter's (2002) system of nematode classification was used. Photographic images were taken of many of the nematode taxa found in this study. An inverted Nikon compound microscope and a Leica DM1000 compound microscope were used for taxon identification. A Motic 2.0 camera and an imaging program were used for the pictures.

Gall wasp damage: The longest branches of a tree attainable by a ladder and/or modified rope system were cut 30 cm from branch tip. Four branches from each of 5 declined trees (DS=0,1,2,3,4) were removed and the proportion of "needles" damaged by the gall wasp determined.

Tree survey: In 2008 and 2009, GPS-assisted surveys were conducted along Guam's major thoroughfares, coastal intersecting roads to farmers' fields, agricultural experimental stations, parks, beaches, cliffs, and golf courses. For each sample tree, a set of measurements were taken and selected for analysis. Sites were evaluated for stand origin (natural and planted) and management (slight, moderate and high). Slight management practices were those associated with tree stands (natural or planted) that were allowed to develop unattended. Moderate management practices were those associated with tree stands in parks and cemeteries. High management practices reflect conditions around ironwood trees on golf courses and campuses. The GPS receiver (GPSmap 76CSx, Garmin International Inc.) was read 1 m above ground level held against the north-side of the tree. Each tree was given a decline rating by two researchers using the five-scale IWTD severity rating (**Figure 8**). A total of 1398 trees at 38 sites were surveyed for decline from October 2008 to June 2009 (Survey I). From July 2009 to December 2009, a follow-up survey of the original trees was conducted (Survey II). This survey was expanded to include additional characteristics as well as 29 additional trees and 6 sites.

Statistical modeling: Modeling was used to evaluate a set of data from 1427 individual trees, 44 sites, and 16 GIS maps. The primary objective of using statistical models was to find possible factors that could explain tree decline, in other words to find the parameters that have a positive or negative impact on the tree (K. Schlub, 2010). Various modeling techniques were applied to address data set issues. The logistic model, which used dieback as the response variable, was found to be the best fit with the data.

Tree sites were examined using the original tree explanatory variables plus those derived from 16 GIS map characteristics (Kennaway, 2010): cemetery buffer, FIA trees with conks (multi-ring buffer), fire risk, fires per year, proximity to golf courses, land cover, management areas, school buffer, soil available water at 150 cm, available water at 25 cm soil depth, soil depth to restrictive layer, soil series, and vegetation. Some maps were dropped from the analysis because of correlations between regressors. A multiplicative change in the odds ratio of unhealthy vs healthy was calculated one regressor at a time by increasing the regressor one unit and holding all remaining regressors constant.

Sporocarp survey: Trees were only surveyed for sporocarps of basidiomycetes “conks” due to infrequent sporocarps of non-basidiomycetes wood rotters. A tree survey was conducted to quantitatively and qualitatively document existing basidiocarps of wood decay fungi on ironwood trees in Guam and Saipan in January and February of 2012. The methodology used to document existing basidiocarps was developed, in part, to be consistent with previous surveys of ironwood trees on Guam (R. Schlub *et al.,* 2010). Tree surveys were conducted in areas where trees were moderate to large in size, easily accessible and where their health was in question. Three areas on Guam and six on Saipan were surveyed. One hundred three ironwood trees were inspected in three different locations on Guam and 44 trees in six locations on Saipan.

Pests and Diseases

Guam's ironwood tree insects and pathogens are generally considered incidental or opportunistic. Damage by incidental pests is precluded primarily by abiotic disorders. Drought periods, especially during the dry season, primarily affect plants in poor planting sites where the trees become stressed and consequently become vulnerable to insects and pathogens. Some pathogens may be agents of latent infections; therefore, the infection precedes environmental changes that trigger symptom production.

Scarab beetles: Scarab larvae of the subfamily Cetoniinae, the group to which the beetles *Protaetia pryeri* and *Protaetia orientalis* belong, feed on organic matter in the soil, and some species damage the roots of plants (Borror *et al*., 1989). *P. orientalis* was first noted on Guam in 1972 (Schreiner and Nafus 1986). The discovery of a beetle matching the description of *P. pryeri* was first published in 1990 (Schreiner, 1991). Beetle larvae were found under *C. equisetifolia*, *Pithocellobium dulce* Roxb. And *Leucaena leucocephala* (Lam.) de Wit, and in one instance under turfgrass. Larvae and frass were found under healthy and diseased *Casuarina*. Preliminary results from field research conducted by Campora in 2005 at the naval station and naval magazine in Guam, showed no connection between the invasive beetles *P. pryeri* (Janson) and *P. orientalis* and dying ironwood trees.

Termites: In India, termites feed on underground roots and stems of live *C. equisetifolia.* This type of damage is believed to be occurring in Guam as well. From past entomological surveys and reports, there are at least six species of termites in Guam (Su and Scheffrahn, 1998). Colonies of *Nasutitermes* sp. and *Microtermes* sp. were found feeding on dead ironwood trees (Moore, A., personal communication, 2010). The Philippine milk termite *Coptotermes gestroi* was responsible for killing ironwood trees transplanted onto a new golf course (Yudin, L.S., personal communication, 2009). The hollowing of trees by termites is often seen in sites with a high decline incidence (**Figure 1**). In some instances, it appears that old conks serve as a food source and entry point for termites. It is also possible that termites are contributing to the high incidence of xylem residing bacteria and *Ganoderma* in declining trees through transmission and or the creation of points of entry for the pathogens.

Figure 1. A cross-section of a small, declined windrow tree (DS=3) infested with termites. Bacterial ooze positive for *Ralstonia solanacearum* was present on the cut surface. No basidiocarps were present

Gall wasp: Damage to branchlet tips (**Figures 2, 3 and 4**) by an unidentified gall wasp (**Figure 5**) is known to reduce branchlet length and total benchlet mass (Mersha *et al*., 2009). The impact on tree health is probably negligible but may be significant on trees with thinning foliage (**Figure 6**). The wasp reared from branchlet tip galls was identified as belonging to the genus *Selitrichodes* (E*ulophidae: Tetrastichinae)* by John LaSalle, CSIRO, Australia.

Figure 2. Healthy branchlet tip of *C. equisetifolia* (top) and a tip further magnified with gall wasp damage (bottom)

Figure 3. *Casuarina* wasp exit hole on damaged branchlet tip of *C. equisetifolia*

Figure 4. Witches' broom symptom on ironwood branch caused by infestation of gall wasps (foreground) in comparison to healthy branches (background)

Figure 5. Unidentified *Casuarina* miniature gall wasp belonging to the genus *Selitrichodes* (E*ulophidae: Tetrastichinae)* resting on branchlet of *C. equisetifolia*

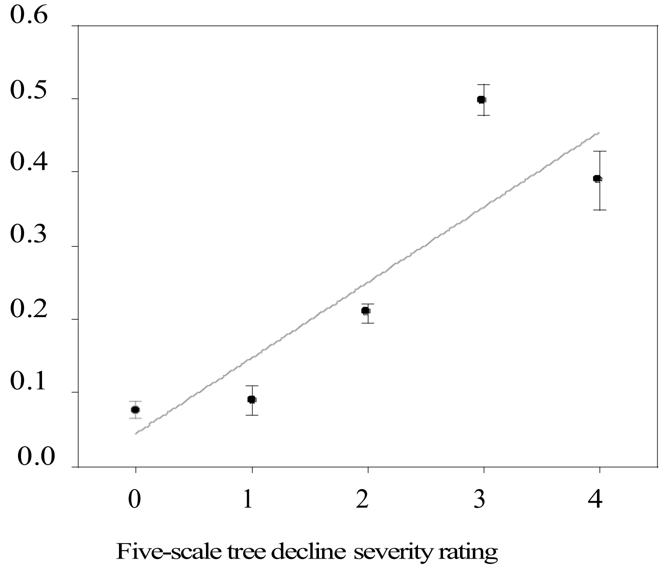


Figure 6. The proportion of ironwood tree branchlet tips damaged by the *Casuarina* gall wasp across the five-scale tree decline severity rating: 0 (healthy) to 4 (nearly dead)

Xylem residing bacteria: *Ralstonia solanacearum*, the cause of bacterial wilt, is among the most common worldwide reported pathogens of *Casuarina*. It is a xylem-resident bacterium mainly entering via roots. Occasionally reported as serious, bacterial wilt has emerged as the most serious disease of *Casuarina* in China (Huang *et al*., 2011) after its discovery in 1964.

Based on culturing from symptomatic tissues, immunostrip data, LAMP data, and other tests, *R. solanacearum* has now been confirmed on Guam. In addition, two companion bacteria (*Klebsiella oxytoca* and *K. variicola*) were found to be associated with the wetwood symptom, which is common in declined trees. Thus, two xylem-resident bacterial genera are associated with IWTD, *Ralstonia* and *Klebsiella*. In Guam, trees that harbor these bacteria do not manifest the same symptoms as those observed in China. In China, the field symptom is rapid tree death (**Figure 7**), which is triggered by severe environmental stress such as that caused by a typhoon or drought. On Guam, bacterial colonization of the xylem results in trees with thinning foliage, which is indistinguishable from symptoms associated with IWTD (**Figure 8**).

Figure 7. Bacterial wilt of *C. equisetifolia* sapling in China (photo provided by Dr. Chonglu Zhong)

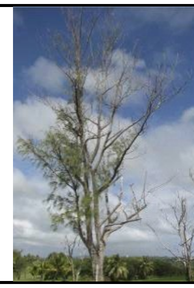
					
DS	0	1	2	3	4
PBB	0	9	49	67	98
					
DS	0	1	2	3	4
PBB	0	13	51	79	96

Figure 8. Representative photographs of small (above) and large (below) solitary trees from locations around Guam depicting five-levels of decline severity (DS) and percentage of bare branches (PBB).

Differences between China and Guam diseases can also be seen in symptoms revealed in cross-sections of the trunks and limbs. In China, xylem vessels of trunk cross-sections contain diffused areas of slightly darker tissue and yield copious amounts of bacterial ooze (**Figure 9**).

Figure 9: Cross-section of a tree in China with bacterial wilt reveals copious amounts of bacterial ooze and tissue discoloration. (From a presentation of Huang Jinshui, He Xueyou, Ke Yuzhu, Cai Shouping, Chen Duanqin, and Tang Chensheng of Fujian Academy of Forestry Sciences at International *Casuarina* Workshop Haikou, China 21-25 March 2010)

On Guam, cross-sections of infected trees revealed uncontained areas of dark discoloration or "wetwood", with sharply defined borders that radiated from the center of the tree. Droplets of bacterial ooze may or may not appear and are generally restricted to the "wetwood" which has a high moisture content (**Figure 10**).

Figure 10. Cross-sections of infected *C. equisetifolia* tree revealed expanding areas of moist discolored wood (wetwood) that radiated from the center of the tree accompanied by droplets of bacterial ooze composed of *Ralstonia solanacearum* and *Klebsiella* spp.

Nematodes: Not a great deal is known regarding the effects of nematodes on *C. equisetifolia*. However, certain species of nematodes do infect its roots: *Helicotylenchus cavenessi, Radopholus similis, Rotylenchulus reniformis, Tylenchus* sp., *Xiphinema ifacolum;* and Angiospermae: *Cuscuta campestris, Dendrophthoe falcata,* and *Dendrophthoe lanosa.* Nematode infections rarely result in the death of infected hosts, but it is not uncommon for certain root disease fungi to infect nematode-damaged roots, resulting in further damage, including mortality in some cases.

To determine if there is a linkage between the presence of nematodes and ironwood tree decline, Dr. Marisol Quintanilla extracted nematodes from ironwood roots and associated soils. *Helicotylenchus* sp. was the only herbivore recovered from healthy trees' roots. *Tylencholaimellus* sp., *Aphelenchoide*s sp., and one unknown were recovered from trees with dieback. *Helicotylenchus* sp. and *Tylenchus* sp. were consistently collected from healthy and dieback soil (**Table 1**). It was concluded that *Helicotylenchus* was the only nematode that was isolated with enough consistency to be remotely implicated in ironwood tree decline.

Table 1. Nematode counts per 10 ml soil samples from healthy ironwood trees and those with dieback

	HEALTHY					DIEBACK									
SAMPLE	**11-9**	**11-10**	**11-28**	**11-38**	**Average**	**11-8**	**11-31**	**11-29**	**11-35**	**11-33**	**11-15**	**11-41**	**11-42**	**11-25**	**Average**
Acrobeles	0	0	0	10	2.5	0	0	0	10	0	0	0	0	0	1.1
Aphelenchoides	0	10	0	0	2.5	0	0	0	0	0	0	0	0	0	0
Aphelenchus	10	0	0	0	2.5	0	0	0	0	0	0	0	0	0	0
Cephalobida	0	0	0	0	0	0	0	10	0	0	0	0	0	0	1.1
Eucephalobus	0	0	0	0	0	0	0	0	30	0	0	0	0	0	3.3
Helicotylenchus	10	30	0	0	10	30	170	0	10	90	10	10	0	30	38.9
Leptonchus	0	0	10	0	2.5	0	0	0	0	0	0	0	0	0	0
Meloidogyne	0	0	0	0	0	0	0	0	0	20	0	0	0	0	2.2
Mesocriconema	0	10	0	0	2.5	0	0	0	0	0	0	0	0	0	0
Monhystera	0	0	0	0	0	0	0	0	0	10	0	10	0	10	3.3
Paratylenchus	0	0	0	0	0	0	0	0	0	0	0	10	0	0	1.1
Plectus	0	0	0	10	2.5	0	0	0	0	0	0	0	0	0	0
Pratylenchus	0	0	0	0	0	0	10	0	0	0	0	0	0	0	1.1
Prismatolaimus	0	0	0	0	0	0	10	20	0	0	0	0	0	0	3.3
Rhabditidae	0	0	0	0	0	0	0	20	0	0	0	0	0	50	7.8
Rhabditis	0	0	0	0	0	0	0	0	0	10	0	0	0	0	1.1
Tylenchus	0	0	10	20	10	30	10	20	10	0	0	0	0	0	7.8
Wilsonema	0	0	0	0	0	0	0	0	0	20	0	0	0	0	2.2

Fungal wood-rot: There are many fungi involved in wood rot or decay. One group is the basidiomycetes. The fruiting bodies or sporocarps of these fungi are called basidiocarps. The basidiocarps found in Guam and Saipan were either flat (resupinate) (**Figure 11**) or shelflike (conk) (**Figures 12** and **14**). Though usually present, the sporocarp does not have to be present for wood rot to occur. To date, five conk-forming basidiomycete genera have been identified from ironwood trees on Guam, all in the class Agaricomycetes: *Ganoderma*, *Favolus*, *Pycnoporus*, *Phellinus*, and *Sarcodon* (R. Schlub *et al*., 2011). Distinguishing features for Guam's *Ganoderma* sp. sporocarp include an unvarnished, gray to brown fan-shaped cap, with a white pored undersurface that easily bruises brown when young (**Figure 12**). *Ganoderma* invades woody tissue through an unrestricted mycelial network while sustaining itself on cell and cell wall components (**Figure 13**). Descriptors for Guam's *Phellinus* sp. sporocarp are often formed in overlapping shelves with golden-brown pubescent cap margins when young and a yellow-brown undersurface (**Figure 14**).

Figure 11. Multiple sporocarps of an unidentified resupinate polypore on an ironwood tree (*Casuarina equisetifolia*), on the campus of the University of Guam, Mangilao, Guam

Figure 12. Sporocarp (conk) of *Ganoderma australe* species complex on *Casuarina. Equisetifolia.* On Guam, many of the trees in decline sites may have conks on their roots or butts

Figure 13. Cross-section of rotted ironwood tree butt infected with *Ganoderma australe* species complex. Note the expanding network of white mycelial strands. This sample was negative for *R. solanacearum*

Figure 14. Sporocarp (conk) of a *Phellinus* sp. on *C. equisetifolia* trees. This fungus is likely a part of the normal decay process of the ironwood trees in the Mariana Islands and not a contributor to IWTD

As a result of surveys in January and February 2012, there were mainly two species of basidiomycetes on most affected trees*:* *Ganoderma* sp. (*australe* group) which fruits on tree roots and butts and less commonly on the trunk (**Figure 12**) and *Phellinus* sp., which primarily fruits on the butt (**Figure 14**) (R. Schlub *et al.,* 2012). Both are common on Guam (**Figure 15**) and infrequent on Saipan (**Figure 16**). The presence of *Ganoderma* is a consistent indicator of a tree in decline and its occurrence is irrespective of tree size. *Phellinus* is found in association with *Ganoderma* or by itself on very large mature trees. On its own, *Phellinus* does not appear to be a contributor to ironwood decline**.**

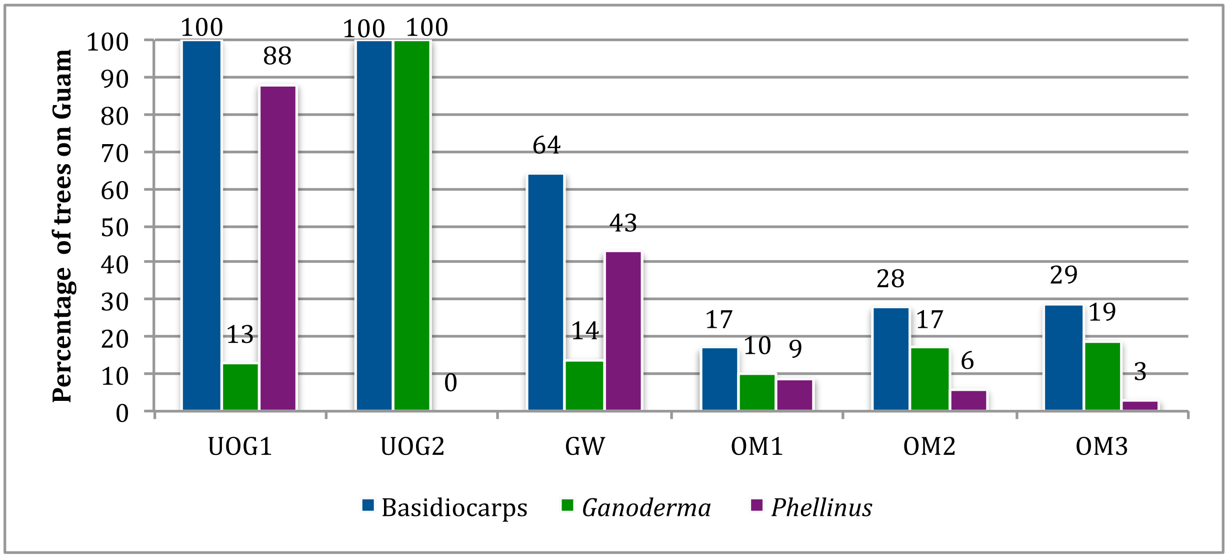


Figure 15. Percentage of trees on Guam with root, butt, or lower trunk basidiocarps, and those trees with identifiable conks of *Ganoderma* (*australe* complex) or *Phellinus*. The survey area and sites include trees flanking sidewalks on the University of Guam campus (UOG 1 & 2), a woodlot at George Washington High School (GW), and windbreaks at Onward Mangilao Golf Course (OM 1, 2, & 3)

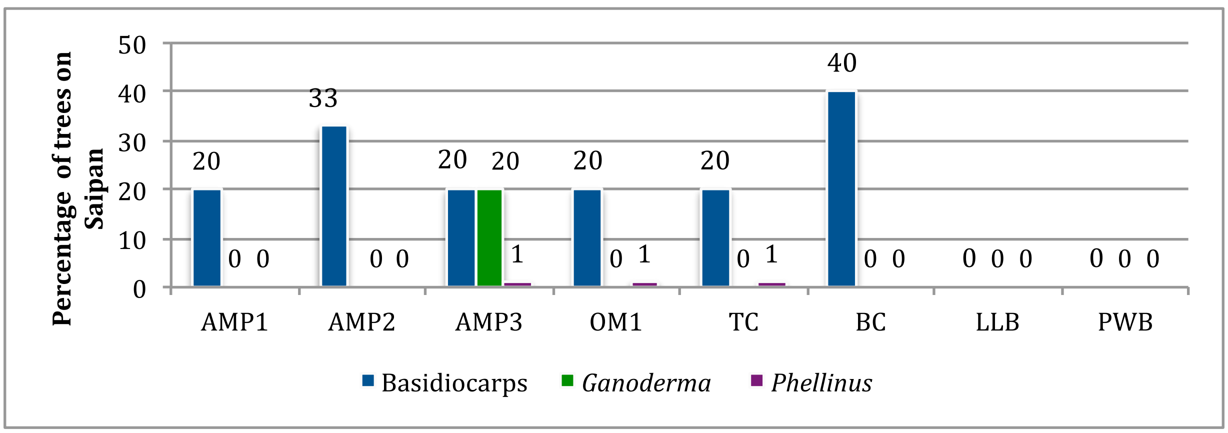


Figure 16. Percentage of trees on Saipan with root, butt, or lower trunk basidiocarps, and those trees with identifiable conks of *Ganoderma (australe complex)* or *Phellinus*. The survey area and sites on Saipan include trees in landscaped areas at American Memorial Park (AMP 1, 2, & 3), Fisherman Memorial (FM), Tennis courts (TC), Banzai Cliff (BC), Lau Lau Bay (LLB), and Public Works Beach (PWB)

Ironwood Tree Decline

Ironwood trees on the island of Guam are in the midst of a decline that was first noticed in 2002 by a local farmer (Mersha *et.al.,* 2009). The trees at that site were less than 10 years old and planted in single-row windbreaks of several hundred trees. Less than 5 trees were characterized as wilted with the following symptoms: acropetal progression of chlorosis, tip-burn of lower branchlets giving the tree a singed appearance, and tree death within 6 months. Roughly 15 trees had symptoms of decline, which included internal wood discoloration, thinning of branches and tree death after several years (**Figure 8**). By 2005, Ironwood Tree Decline (IWTD) was widespread on Guam (Campora, 2005). In January 2009, a five-day IWTD conference was held with participants from Guam and off-island (**Figure 17**). Six off-island experts and other participants visited healthy and declined tree sites (**Figure 18**), collected samples, and reviewed research related to *C. equisetifolia* production worldwide and its growth on Guam (Mersha *et al.*, 2010a, Mersha *et al*., 2010b; K. Schlub, 2010; R. Schlub *et al.*, 2010). Findings of the conference were reported at the 4th International *Casuarina* Workshop (R. Schlub *et al.,* 2011).

Figure 17. Participants from the 2009 five-day IWTD conference

Figure 18. Ironwood Decline Conference attendees visit a declined tree site at Andersen Air Force Base, Yigo, Guam

Tree survey: Thirty-eight sites (1398 trees) were surveyed for decline from October 2008 to June 2009 (Survey I) (**Figure 19**). From July 2009 to December 2009, a follow-up survey of the original trees was conducted (Survey II) (**Figure 19**). For each tree and site, explanatory variables of decline were measured including tree circumference, fire damage, typhoon damage, presence or absence of termites, presence or absence of "conks", and various geographical or cultural conditions.

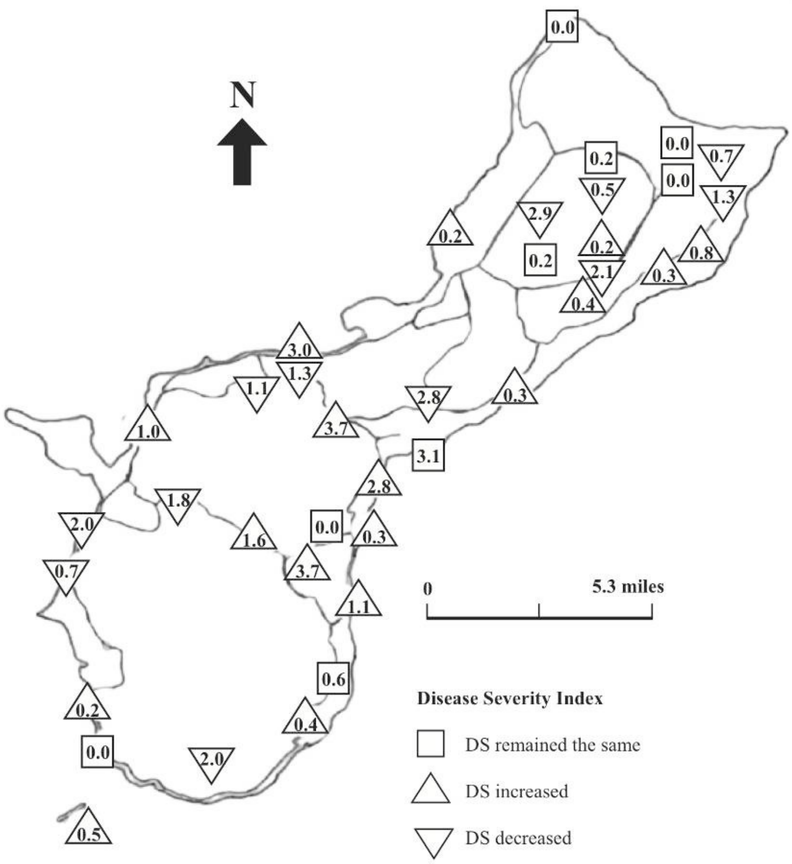
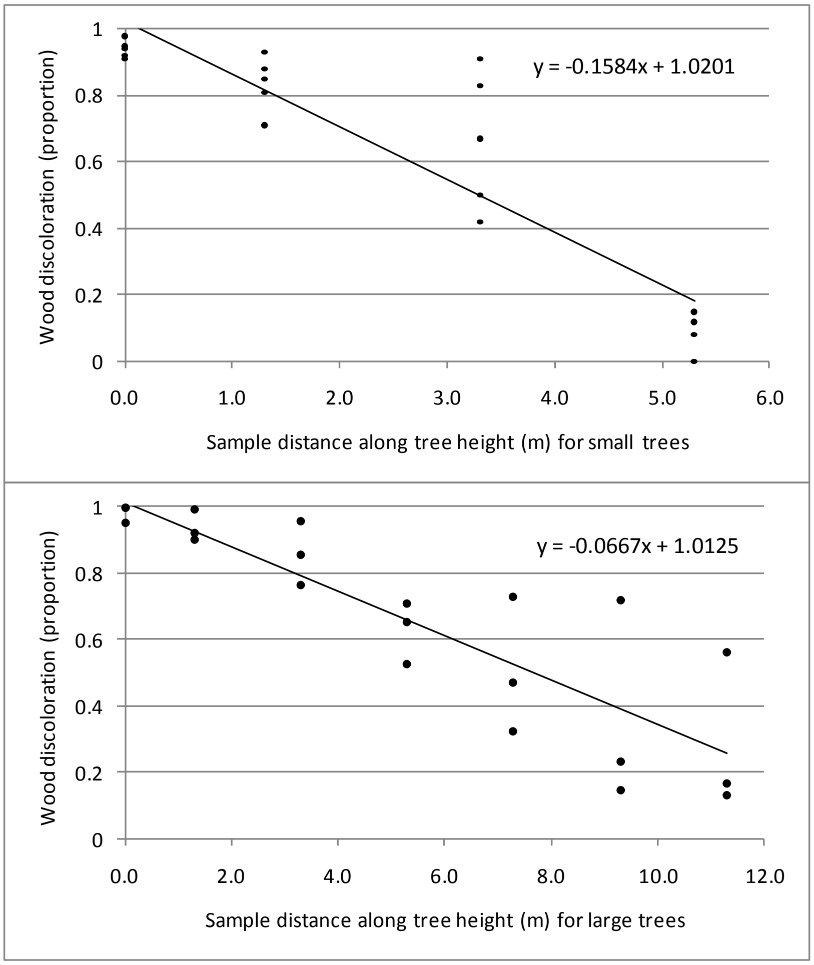


Figure 19. Means of decline severity (DS) found at sites during Survey II (July to December 2009). Values in comparison to Survey I (October 2008 to June 2009) remained nearly the same (square), increased (up- triangle) or decreased (down- triangle)

Symptoms: The presence of discoloration at the branch juncture of large branches of declining trees was consistent for large trees at all DS levels, where 80% to 100% of the branch cross-sections were discolored but was inconsistent for small trees at 1 and 2 DS levels. In healthy small trees, the cuts were clean and non-discolored. In large trees, discoloration due to mature heartwood was occasionally observed. There was a clear, consistent gradient of discoloration within the tree trunk of declining trees (**Figure 21**). Linear functions derived from the average proportion of discolored wood at each sampling distance describe well the actual acropetal wood discoloration gradients recorded within small and large trees (**Figure 20**). Wood rotting fungi that produce conks are known to cause the internal discoloration and white soft rot commonly found in DS 3 level trees (**Figure 13**). The importance of these fungi in IWTD is also supported by the fact that the percentage of trees with "conks" increased with IWTD from 2, 18, 35, 47, and 66 % for DS 0, 1, 2, 3, and 4 level trees, respectively.



F

igure 20. Proportion of wood discoloration in trunk cross-sections fitted to a linear decay function for small (upper) and large (lower) trees

Base (0 m) **1.3 m** **3.3 m** **5.3 m**

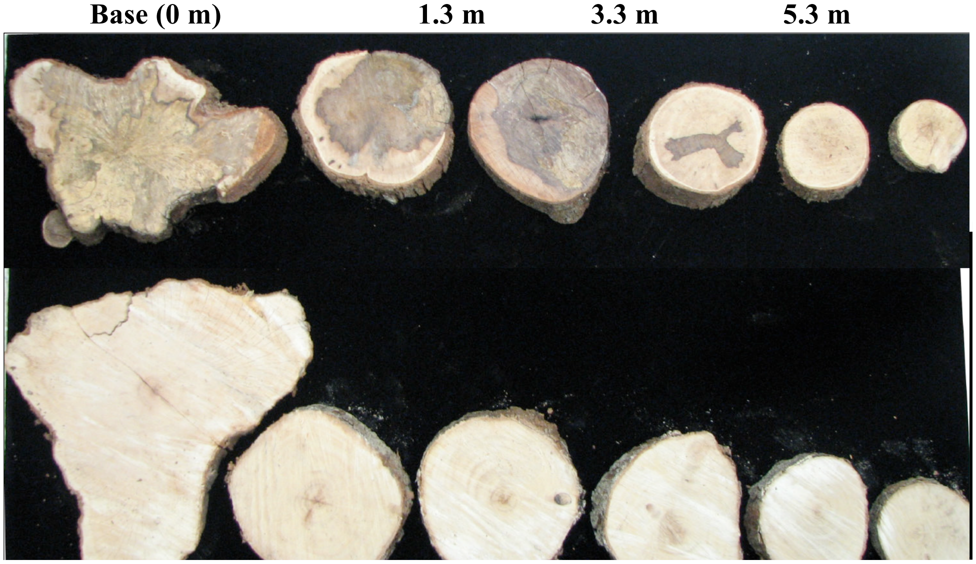


Figure 21. Trunk cross-sections from two small trees, one declined (top) and one healthy (bottom)

Internal symptoms (as seen in trunk cross-sections) vary from tree to tree and with decline severity. DS=0 trees usually have no bacterial ooze and only a small area of centrally-located discoloration. The majority of DS=1 trees generally have bacterial ooze with centralized discoloration. DS=2 trees usually have bacterial symptoms (**Figure 10**), and less common signs of wood rots caused by *Ganoderma* (**Figure 13**) and termites. Trees in a severe state of decline harbor one or all of the following: bacteria, termites, various resupinate sporocarps (**Figure 11**) and conks of *Ganoderma australe species complex* (**Figure 12**), *Phellinus* (**Figure 14**), and other Agaricomycetes. Conks and termites are more common in large trees than in small ones.

Analysis of individual trees: For each sample tree, measurements were taken and selected for analysis (**Table 2**). The primary objective of using statistical models with the ironwood tree data is to find possible factors that could be related to tree decline (K. Schlub, 2010). Various modeling techniques were applied to address data set issues. The logistic model, which used dieback as the response variable, was found to be the best fit with the data. Three explanatory variables were found to be significant and therefore could explain the ironwood's state of health (**Table 2**). Among the three regressors, presence of "conks" had the largest coefficient value at 3.31. The impact of each individual regressor was determined numerically by holding all other regressors constant. The odds favoring decline is 27.3 times greater for a tree with 'conks' than without.

Table 2. Grouping and descriptions of ironwood tree variables; those in **bold** were found to be the most suitable for predictive purposes

<table>
<tr><th colspan="5">Response Variables</th></tr>
<tr><td>Decline severity</td><td colspan="4">Severity ranking DS=0,1,2,3,4</td></tr>
<tr><td>Tree Dieback</td><td colspan="4">Healthy or unhealthy</td></tr>
<tr><th colspan="5">Explanatory Variables</th></tr>
<tr><td>Structure</td><td>Number of trunks per tree</td><td>Circumference of tree at 1.3 m height</td><td colspan="2">Site density: trees per square meter at site</td></tr>
<tr><td>Stress</td><td>Fire damage: present or not</td><td>"Conks" present or not *</td><td>Storm damage: present or not</td><td>Termites present or not</td></tr>
<tr><td>Geographic</td><td>Latitude</td><td>Longitude</td><td>Altitude</td><td>Site</td></tr>
<tr><td>Miscellaneous</td><td>Level of lawn management none, moderate, or high</td><td colspan="3">Tree origin: natural or planted</td></tr>
</table>

*** "Conks"** refer to any resupinate or shelflike sporocarp of a basidiomycete appearing on a lower trunk (< 0.25 tree height) or roots of an ironwood tree (*Casuarina equisetifolia)*

Analysis of tree sites: Tree sites were examined using the original tree explanatory variables (**Table 2**) plus those derived from 16 GIS map characteristics (Kennaway, 2010): cemetery buffer, FIA trees with conks (multi-ring buffer), fire risk, fires per year, proximity to golf courses, land cover, management areas, school buffer, soil available water at 150 cm, available water at 25 cm soil depth, soil depth to restrictive layer, soil series, and vegetation. Some maps were dropped from the analysis because of correlations between regressors. A multiplicative change in the odds ratio of unhealthy vs healthy was calculated one regressor at a time by increasing the regressor one unit and holding all remaining regressors constant.

There were six positive dieback predictors: increasing circumference, increasing altitude, presence of "conks", presence of termites, planted stand vs natural stand, and urban land location.

There were four negative dieback predictors: increasing water availability at 25 cm soil depth, golf course location, forest location, and decreases in latitude.

In summary, the most beneficial variable identified was soil moisture. Trees in areas with the highest moisture were 3.3 times less likely to be declined. Likewise, the most deleterious variable was the presence of basidiocarps. Trees with "conks" were 27 times more likely to be in a declined state.

Predicting tree size: As a result of multi-linear modeling, several factors were identified that may positively (+) or negatively (-) predict the average size of trees at a site (cm CBH). The size of a tree is restricted by tree stand density, altitude, and soil depth.

Sites with large trees are more likely to be found in urban, forest, national parks, and fire prone areas than in sites at golf courses or in close proximity to a school. It was also found that increased circumference is associated with trees having termites, "conks", typhoon damage, and multiple trunks. This suggests that large highly-vigorous trees are able to tolerate stresses to which less vigorous trees would have succumbed.

Linking dieback with site productivity: Based on the premise that tree circumference in 2008 and 2009 is an indicator of site productivity, an association between IWTD and circumference was sought. The circumference map supports the concept that nearly the entire island is suitable for the growth of small trees (**Figure 22**). However, as the size of the trees increases the area suitable for sustained growth decreases. When the map for dieback (**Figure 23**) is visually compared to the map for circumference (**Figure 22**), dieback appears poorly linked to site productivity (circumference) and strongly linked to the central area of Guam. This suggests that IWTD is not a natural progression of tree maturation and death. Many factors have been evaluated as possible causes or contributors to ironwood decline. Factors that are perceived by the authors to have relevance are listed in **Table 3**.

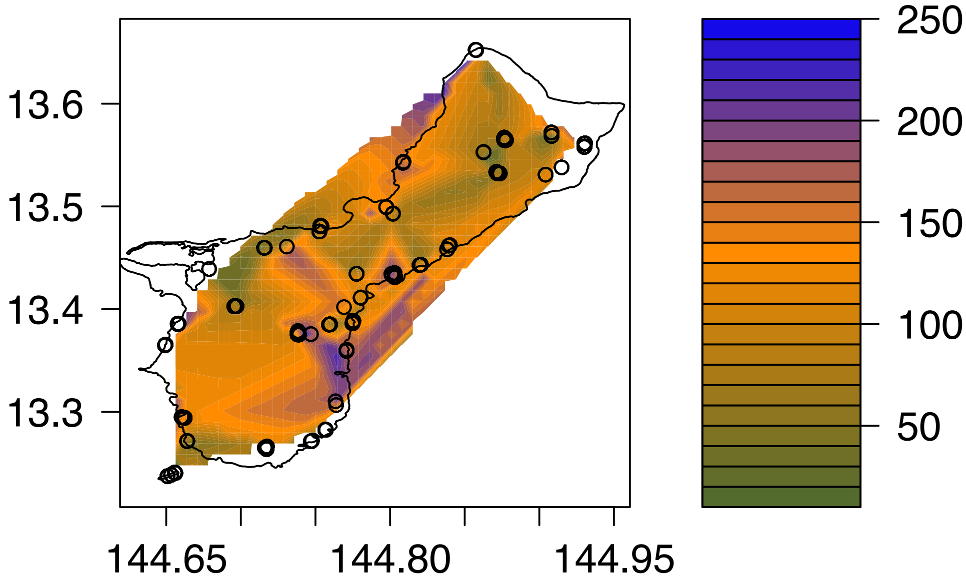


Figure 22. Map of observed tree circumference in cm (CBH) over a longitude-latitude grid of the island of Guam; hence, areas of large trees sites (purple color) have habitats more suitable for ironwood growth irrespective of the presence of IWTD

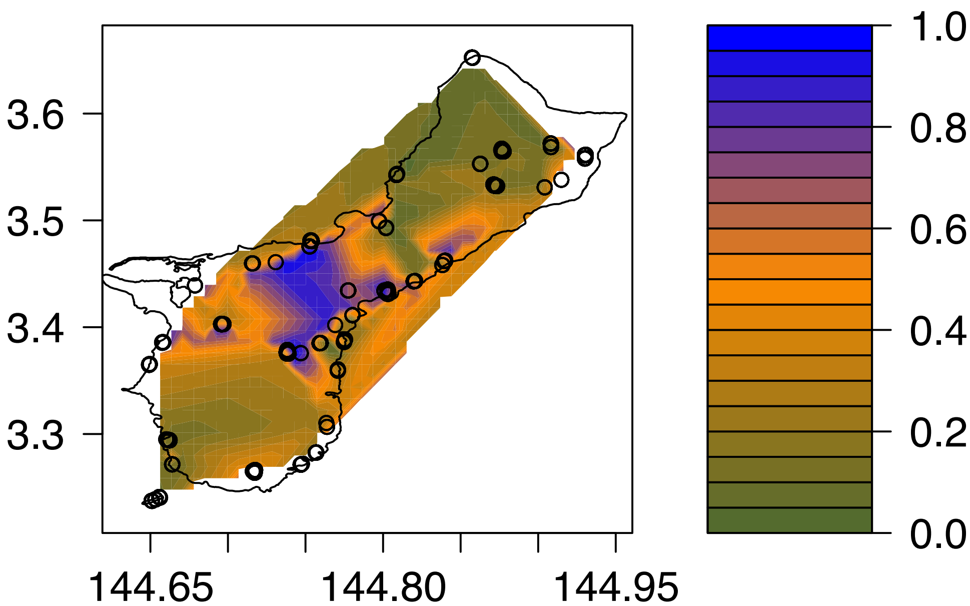


Figure 23. Map of the predicted probability of dieback using a logistic model. Areas in blue indicate regions where dieback is most likely to occur

Table 3. Likely contributors to ironwood decline and their perceived relevance from low * to high ****

Biotic factors	Emerging factors	Relevance
Branch dieback	*Pestalotiopsis*	*
Root rot	*Fusarium,*	*
Wood rot	*Ganoderma*	****
Xylem residing bacteria	*Ralstonia solanacearum* *Klebsiella oxytoca, K. variicola*	*** **
Nematodes	*Helicotylenchus*	*
Insects	Termites Gall wasp	** *
Abiotic factors	**Emerging factors**	**Relevance**
Weather	Typhoon damage	*
Management	Poor tree care practices	*
Site environment	Poor site selection	**
Host genetics	Lack of genetic diversity	**

Recommendations

Due to the slow progression and general sporadic nature of IWTD on Guam, it is likely IWTD could be reduced substantially through cultivar selection and adoption of cultural practices that promote healthy growth and preclude conditions favorable for pests (termites) and pathogens (wood-rots, root-rots and bacteria).

Cultivar selection: It was concluded by attendees of the IWTD conference held on Guam in 2009, that the severity of the ironwood tree decline was likely exacerbated by the lack of genetic diversity of Guam's ironwood tree population. Khongsak Pinyopusarerk recommended the evaluation of seedlots used in the 1991-1993 International Provenance trials of *Casuarina equisetifolia* along with a couple of others (Pinyopusarerk *et al*., 2004). Though Guam's tree was planted in 21 countries at that time, the actual trial was never conducted on Guam. As a result of funding from the US Forestry Service, a scaled-down version of the international trial was planted at Bernard Watson's farm (N 13.56545; E 144.87790). This trial was planted in late July 2012 in an area of severe IWTD with the hope that in the future superior trees will be identified. The replicated trial (3 blocks) consisted of 11 paired seedlots (similar geography) of 4 trees each from 12 countries including Guam, with 8 ft. tree spacing (**Figures 24** and **25**).

C2	C1	K1	K2	V1	V2	T1	T2	P2	P1
C2	C1	K1	K2	V1	V2	T1	T2	P2	P1
C2	C1	K1	K2	V1	V2	T1	T2	P2	P1
C2	C1	K1	K2	V1	V2	T1	T2	P2	P1
M1	M2	S1	S2	G1	G2	A1	A2	I1	I2
M1	M2	S1	S2	G1	G2	A1	A2	I1	I2
M1	M2	S1	S2	G1	G2	A1	A2	I1	I2
M1	M2	S1	S2	G1	G2	A1	A2	I1	I2
K1	K2	V2	V1	M2	M1	A2	A1	G2	G1
K1	K2	V2	V1	M2	M1	A2	A1	G2	G1
K1	K2	V2	V1	M2	M1	A2	A1	G2	G1
K1	K2	V2	V1	M2	M1	A2	A1	G2	G1
S1	S2	C1	C2	I2	I1	T2	T1	P1	P2
S1	S2	C1	C2	I2	I1	T2	T1	P1	P2
S1	S2	C1	C2	I2	I1	T2	T1	P1	P2
S1	S2	C1	C2	I2	I1	T2	T1	P1	P2
P1	P2	V2	V1	A1	A2	S2	S1	C1	C2
P1	P2	V2	V1	A1	A2	S2	S1	C1	C2
P1	P2	V2	V1	A1	A2	S2	S1	C1	C2
P1	P2	V2	V1	A1	A2	S2	S1	C1	C2
M2	M1	K1	K2	I1	I2	T2	T1	G2	G1
M2	M1	K1	K2	I1	I2	T2	T1	G2	G1
M2	M1	K1	K2	I1	I2	T2	T1	G2	G1
M2	M1	K1	K2	I1	I2	T2	T1	G2	G1

Pairs	Geography	Prov #
M1	Malaysia	18348
M2	Malaysia	18375
P1	Papua New Guinea	20586
P2	Papua New Guinea	18153
S1	Solomon Islands	18402
S2	Vanuatu	18312
T1	Thailand	18297
T2	Thailand	18299
A1	Australia	19821
A2	Australia	18378
I1	India	18015
I2	India	18119
K1	Kenya	18141
K2	Kenya	18144
C1	China	18267
C2	China	18268
V1	China	18586
V2	Vietnam	18152
G1	Guam, Inarajan	
G2	Guam, Ritidian	

Figure 24. Plot diagram of Guam's *Casuarina equisetifolia* provenance trial, with CSIRO's international trial numbers (Pinyopusarerk *et at*., 2004)

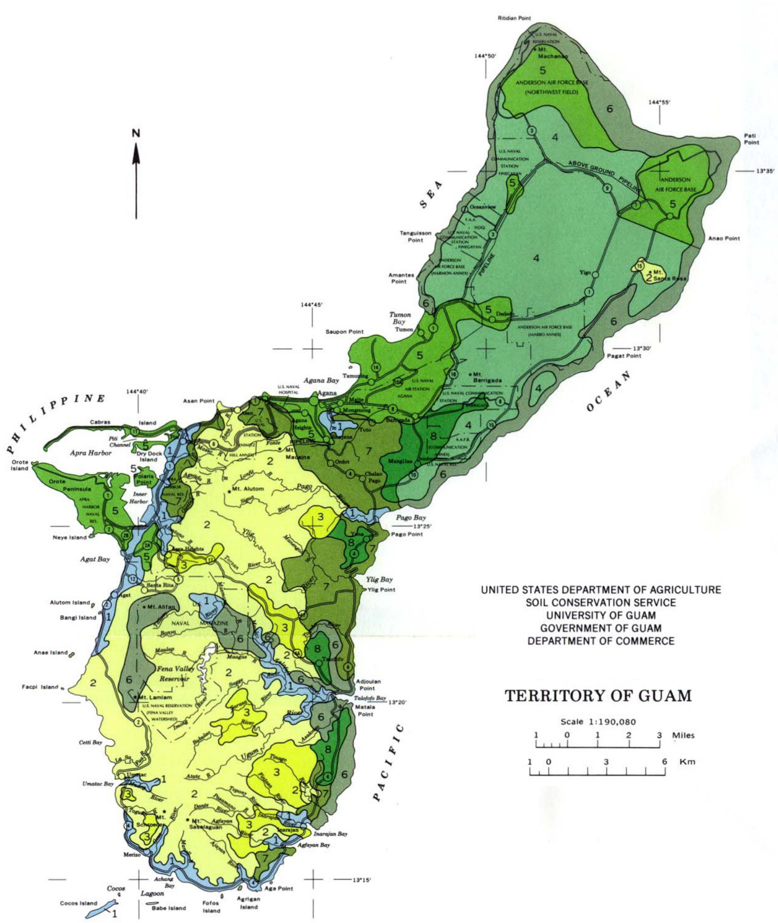
Figure 25. Provenance trial 3.5 months after transplanting

Site evaluation and soil attributes: Site evaluation and soil care before planting ensures a healthy transplanted plant with increased tolerance to transplant shock as well as a tree that will reach its full maturity. Ironwood trees are suited for a range of sites and locations. To allow for maximal growth, that it be planted 40 feet from houses and 20 feet from each other. In urban, windrow, and agro-forestry situations where small trees are desirable, tree space of 10 should be used.

The island of Guam has three broad landform categories each with their own set of soil parent materials, which are responsible for the formation of 8 major soil units each with unique chemical and physical attributes (**Figure 26**). Chemical attributes of a soil are those related to the activity of ions within the soil solution; measurements include pH and Cation Exchange Capacity (CEC). Though ironwood trees can grow across Guam's wide range of soil pH, soil nutrients are maximized between pH 6-7. Cation Exchange Capacity is a measure of the soil's ability to hold on to nutrients, which increases with a soil's fertility. Low CEC soils (<11) have a low capacity to hold on to nutrients and are subject to leaching of mobile "anion" nutrients. Landscape trees in low CEC soils are subject to nutrient deficiencies and will benefit with the addition of a slow- release fertilizer with micronutrients.

The physical attributes of a soil are those related to the size and arrangement of its solid particles. Measures of physical properties include soil bulk density, soil texture, soil porosity or percolation. Bulk density is an indicator of soil compaction, which is an indicator of root growth and soil porosity or percolation. The majority of the island of Guam has clay soils with bulk densities of 0.60-1.0 g/cm^3, which are ideal for clayey soil. Unfortunately, the soil is shallow and is often no deeper than 16 cm. The permeability or percolation rate for Guam's soils vary widely from poor (0.1 inches or less / hour) to rapid (5.0 inches or more). Poor soils should be avoided or modified as they promote shallow rooting, poor growth and root rots. Rapid soils are fine for ironwood trees, provided their roots can reach the water table, which will be critical for their survival in the dry season. Soil in an ideal state for tree growth contains 50% solids (45% mineral material and 5% organic matter) and 25% each of air and water.

Site remediation: Compacted soil in or near a planting pit should be remediated as necessary. The detrimental effects of compacted soil may include inadequacies in infiltration, aeration and water holding capacity. These factors could contribute to decreased root penetrability and thus increased susceptibility to drought and transplant shock. Remediation methods include soil aeration and incorporation of organic matter to improve porosity. Aeration is normally conducted using an air-tool or air-spade. Because Guam's productive layer is thin, vertical mulching also may benefit new planting sites. Vertical mulching consists of using an air-tool or drill to make vertical holes in the soil into which conditioned porous soil is added.



Key	Soil	Horizon depth (cm)	Clay (%)	Bulk density (g / cm³)	pH	CEC
SOILS ON BOTTOM LANDS						
1.1	Inarajan-Inarajan	0–13	50–70	0.90–1.10	5.1–7.3	51
1.2	Shioya	0–25	0–3	1.10–1.25	7.4–8.4	7
SOILS ON VOLCANIC UPLANDS						
2	Akina-Agfayan	0–10	45-80	0.80–0.95	5.1–7.3	23
3	Akina-Togcha-Ylig	0–13	45–70	0.85–1.10	5.1–6.5	36
SOILS ON LIMESTONE UPLANDS						
4	Guam	0–25	35–55	0.60–0.90	6.6–7.8	22
5	Guam-Urban land-Pulantat	0–25	35–80	0.60–1.10	6.6–7.8	27
6	Ritidian-Rock outcrop	0–10	35–40	0.70–0.90	6.6–7.8	——
7	Pulantat	0–16	70–90	0.90–1.10	6.1–7.3	31
8	Pulantat-Kagman-Chacha	0–20	40–80	0.90–1.20	6.1–7.8	26

Figure 26. General Soil Map of Guam (Young *et. al*., 1988)

Tree installation: Plants should be installed in saucer-shaped holes/pits that allow for expansion of the root zone with minimal substrate resistance (**Figure 26**). Soil should be removed with as little disturbance of the soil's profile as possible. Due to Guam's poor subsoil, mixing of topsoil and subsoil should be avoided. When backfilled, the site's profile should match with the original. To enrich the topsoil, amend with organic material. Large rocks on the side or bottom of the pit should be removed with a backhoe or cracked with an air-tool or auger. The planting area should be free of rocks and debris. It is a misconception that adding rocks or gravel in the bottom of the planting hole improves drainage. Care should be taken to avoid planting in holes with steep sides or made with a corer that compresses the sidewalls. In this scenario the roots could encircle among themselves leading to girdling roots. Balled or container trees must be carefully placed in the hole without disturbing the root ball. After installation, the tree should be staked.

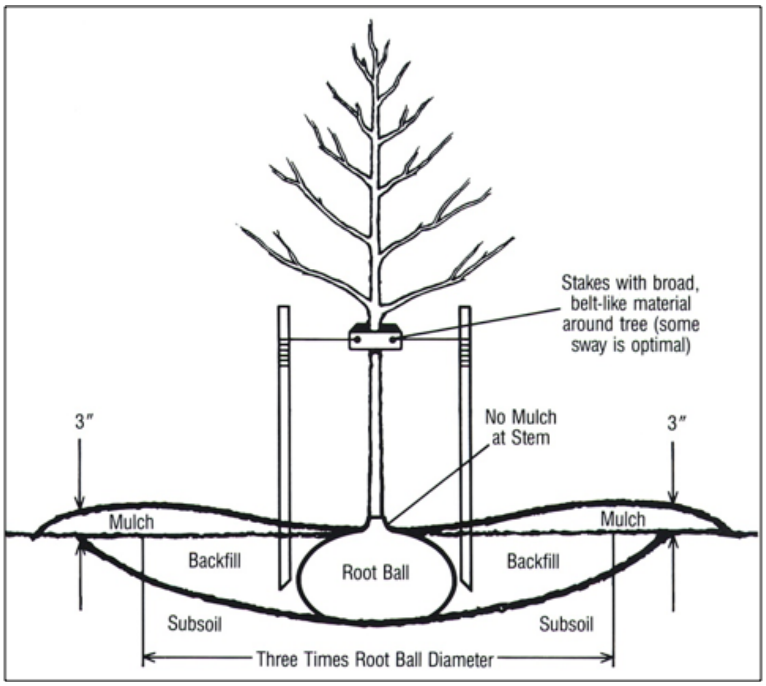


Figure 27. General hardware guidelines for tree installation

Planting bare root plants: After planting bare rooted trees, gently tap the soil and backfill with water to remove air pockets. Additional staking may be required for bare rooted trees. Bare root plantings, although limited to smaller ironwood plants, allow for earlier adaptation to the new site and faster transplant recovery. However, a drawback in using this technique is that initially roots, and the planting pit must be kept sufficiently moist to prevent roots from drying out. It is estimated that in Guam during the dry season, early care should be administered for at least three months and about one month in the wet/rainy season. Early care consists of providing tree transplants a stress-free environment, which may include daily watering.

Nutrient management: Guam's soils benefit from nutrient augmentation especially in sandy soil and areas where soil has been disturbed. The soils of northern Guam are calcareous. Trees in these soils will likely benefit from the addition of chelated iron throughout their lifetime. Fertilizer should be used sparingly as the development of nitrogen fixing *Frankia* and beneficial mycorrhizal will be held back with over application. A low nitrogen, slow-release fertilizer with micronutrients is ideal. Alternatively, apply a small amount (50 to 100 g) of a low analysis complete fertilizer such as 10-10-10 at transplant.

Mulching: Mulching or placement of organic material around the base of a new plant can be one of the most beneficial cultural practices for young ironwood trees. Mulch is anything used to cover the soil's surface for the purpose of improving plant growth and development. To be suited for plant growth, mulch must allow the exchange of air between the soil and the atmosphere and allow water to infiltrate into the soil profile. The selected mulch (e.g. ironwood needles) should be placed a few inches from the trunk and between 1-2 inches deep. Benefits of mulching include: conservation of soil moisture, moderation of soil temperature, improvement of soil quality (organic mulches), suppression of weeds, enhancement of landscape appearance, reduced maintenance, and protection of plants from damage caused by maintenance equipment.

Fertilizing: Fertilizing (also see nutrient management), especially in the early stages of planting, helps root development and may improve drought tolerance, thereby reducing transplant shock.

Watering: Watering or irrigation needs should be a part of the planning process, especially if planting is to occur in the dry season. Any irrigation program implemented should be based on knowledge of the soil percolation rates for the site. Excess moisture could lead to root rot.

Pruning: For optimal structural strength and health of a young tree pruning may be necessary. This involves the judicious removal of plant tissue in a manner, as much as possible, consistent with minimal invasiveness to the plant. Proper pruning practices will enhance the overall health of the plant and should be guided by established standards. Tool sterilization is critical in ensuring sanitation and reducing the potential transfer of pathogens. Wind damaged trees should be correctly pruned as quickly as possible to reduce the amount of deadwood and reduce the surface area of branches ripped in strong wind. Removal of deadwood reduces the establishment of termites and wood-rotting fungi that contribute to hazardous trees in Guam's urban landscape. Trees broken from typhoons should be felled by excavation instead of sawing where their colonization by a wood rotting fungus could possibly lead to infecting the root systems of neighboring healthy trees.

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Revised on August 6, 2013

PART II.
2020 Update

Appendix A

Forward: This appendix serves as an update to Robert L. Schlub's August 6, 2013, revised Gago, Guam Ironwood Tree, *Casuarina equisetifolia* Past, Present, Future guide (Guam Ironwood Tree Manual). It contains advances in the research of Ironwood Tree Decline (IWTD), expanded tree survey information, and cultivar evaluations. Finally, it provides suggestions for future research.

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Findings and Discussion

Casuarina equisetifolia shoreline protector

Casuarina equisetifolia subsp. *equisetifolia*, often referred to as ironwood, is indigenous to Southeast Asia, Malaysia, Northern Australia, Oceania and most likely to Guam and the Northern Mariana Islands. It has been introduced into a large number of countries and is now a common feature of the coastal landscape. It is commonly used in agroforestry systems for coastal protection and rehabilitation and soil stabilization reclamation. Since it is salt tolerant and grows in sand, *C. equisetifolia* is used to control erosion along coastlines, estuaries, riverbanks and waterways. It is a very hardy tree and is useful in protecting shorelines from storm surges and tsunamis. The *Casuarina* can withstand a tsunami wave better than the coconut tree, while a young forest of *Casuarina* can provide even more protection. Ironwood is a popular tree species for shelterbelt plantations. The rapid growth of *Casuarina equisetifolia* attracts world climate change practitioners with its potential for climate change mitigation and adaptation in the coastal zones. A special interest in the arena of climate change mitigation lies in this specie's storage of carbon in its rapidly grown biomass.

The tsunami on December 26, 2004 was a major natural disaster, killing some 229,866 men, women and children and causing billions of dollars in damage (United Nations, 2007). Waves crashed into 14 countries and some were 30 meters high. Many *Casuarina* shelterbelts in India, Sri Lanka and Thailand were established to protect coasts from cyclones, tsunamis and other coastal hazards, which proved effective against the 2004 Indian Ocean tsunami as well. Post-tsunami field surveys in Sri Lanka and Thailand showed that older *Casuarina equisetifolia* shelterbelts withstood the tsunami, but failed to provide protection. The tsunami passed through the shelterbelt without resistance from lower-level branches or undergrowth, a condition typical of the species. For a coastal forest of mature *Casuarina* (e.g., 80 cm dbh) the mitigation effect is marginal and only slightly more than *Cocos nucifera*. Very young stands, on the other hand, less than 10-15 cm diameter were uprooted and washed away providing no mitigation. Field observations and laboratory research have established several key parameters that determine the magnitude of tsunami mitigation offered by various types of coastal forests. These parameters include forest width, tree density, age, tree diameter, tree height, and species composition. Forest age (the average age of trees of the dominant size class) is directly correlated with both tree height and diameter. Increases in age, diameter and height generally enhance the mitigation effects of coastal forests. Diameter growth also enhances the breaking strength of trunks and branches. It also raises the resistance of the forest being toppled, up to a point, after which resistance falls.

In Sarawak, Indonesia the species is protected because of its importance in controlling coastal erosion. Since 1954, vast plantings of *Casuarina equisetifolia* have been established along the coast fronting the South China Sea. Much of the coast there is comprised of bare dunes that formerly were constantly moving inland, destroying arable land. This belt of *Casuarina* (mainly *C. equisetifolia)* covers more than 1 million hectares and stretches for 3,000 km and varies from 0.5 to 5 km in width. The importance of this tree on the beaches of the world's tropical islands will increase as sea levels rise and storms increase in intensity.

The coastline of Bangladesh is mostly exposed to extreme meteorological and hydrological conditions where cyclones and storm surges cause devastating effects including loss of human life and destruction of property. Young, dense C. *equisetifolia* is found more effective to reduce storm surge energy than other species previously tested. It was found that shelterbelt *C. equisetifolia* reduced wind speed, increased the size of sand dunes, improved the aesthetic value, increased the protection facilities against cyclones, and enhanced the attractiveness of the beach for tourism. Although *Casuarina* trees have inhibited the undergrowth of native species, the shelterbelt has increased the supply of fuel-wood for local people.

In Hawaii 12 different species of *Casuarina* have been introduced, estimated to occupy 3,800 hectares. Locally known as "ironwood," *Casuarina* have been planted for erosion control, dune stabilization, windbreaks, fuelwood plantations, beautification, and watershed cover. In the lowlands, the most extensively planted species has been *Casuarina equisetifolia*. In the uplands, *Casuarina glauca* has been most commonly used, primarily for erosion control. In addition, there are plantings of *Casuarina angularis, Casuarina nodiflora*, and an unknown species from Timor. *C. equisetifolia* is listed as one of the exceptional trees of the city and county of Honolulu. The trees are located along Kalakaua Avenue from Kapahulu Avenue to Poni Moi Road, 52 Robinson Lane, and a grove of double row parallel to the Kapiolani Park Bandstand, at Monsarrat Avenue's Waikiki Shell parking lot Makai entrance.

Pros and Cons of *Casuarina equisetifolia*

Casuarina equisetifolia and *Casuarina glauca* are both listed in the National Invasive Species Strategy as plants that should be eradicated or controlled. The Bahamas National Trust has long been on record as supporting the removal of *Casuarina* from island coastlines. Extensive research supports that removal of *Casuarinas* from coastal areas and replanting of the dune ridge with native vegetation will restore the dune and provide an effective barrier against wave action.

Provenance trial: It was concluded by attendees of the IWTD conference held on Guam in 2009, that the severity of the ironwood tree decline was likely exacerbated by the lack of genetic diversity of Guam's ironwood tree population. Khongsak Pinyopusarerk recommended the evaluation of seed lots used in the 1991-1993 International Provenance trials of *Casuarina equisetifolia* (Pinyopusarerk *et al*., 2004). As a result of funding from the US Forestry Service, a scaled-down version of the international trial was planted at Bernard Watson's farm (N 13.56545; E 144.87790). This trial was planted in late July 2012 in an area of severe IWTD. The replicated trial (3 blocks) consisted of 11 paired seed lots (similar geography) of 4 trees each from 12 countries including Guam, with 8 ft. tree spacing (**Figures 24** and **25**).

After one year, the fastest growing provenances were nearly twice as tall as the average seed lot with nearly 6 times the average biomass. The fastest three geographically paired provenances were those from Solomon-Vanuatu, Malaysia, and China; the slowest were from Australia, Kenya, and Guam (**Figure 28, 29 and 30**). The block effect was significant with the growth of most provenances increasing from block 1 to block 3 (**Figure 28**). This difference was attributed to increasing soil depth from block 1 to block 3.

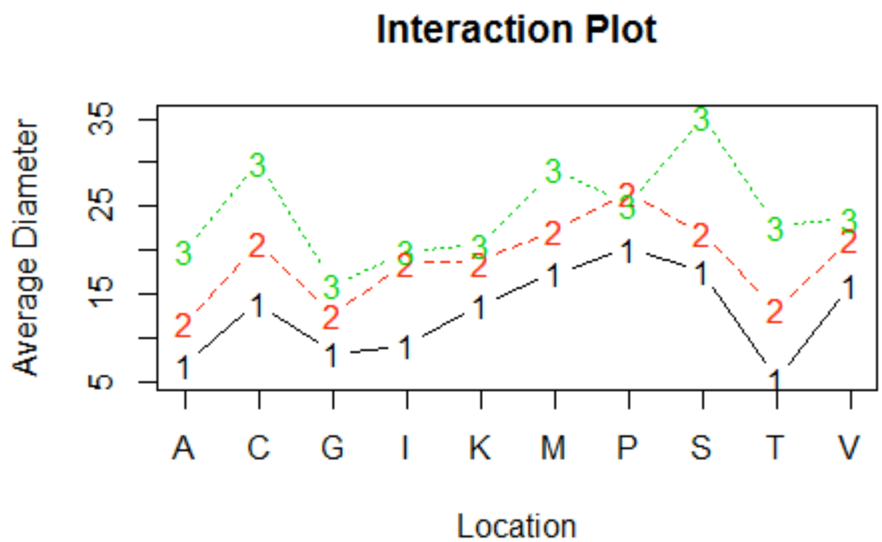

Figure 28. Diameter in millimeters of three plots of Guam ironwood provenance trial trees one year after transplant: A (Australia), C (China), G (Guam), I (India), K (Kenya), M (Malaysia), P (Papua New Guinea), S (Solomon Islands & Vanuatu), T (Thailand), V (Vietnam & China)

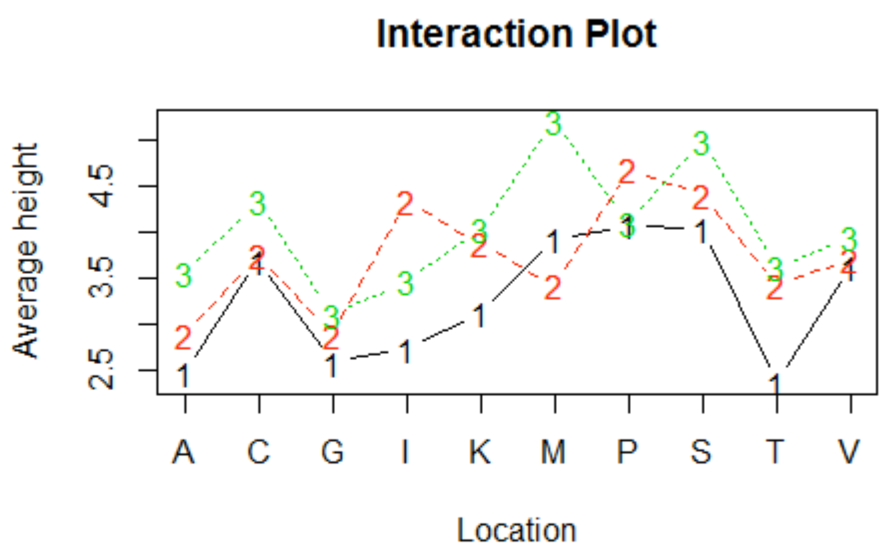

Figure 29. Height in meters of three plots of Guam ironwood provenance trial trees one year after transplant: A (Australia), C (China), G (Guam), I (India), K (Kenya), M (Malaysia), P (Papua New Guinea), S (Solomon Islands & Vanuatu), T (Thailand), V (Vietnam & China)

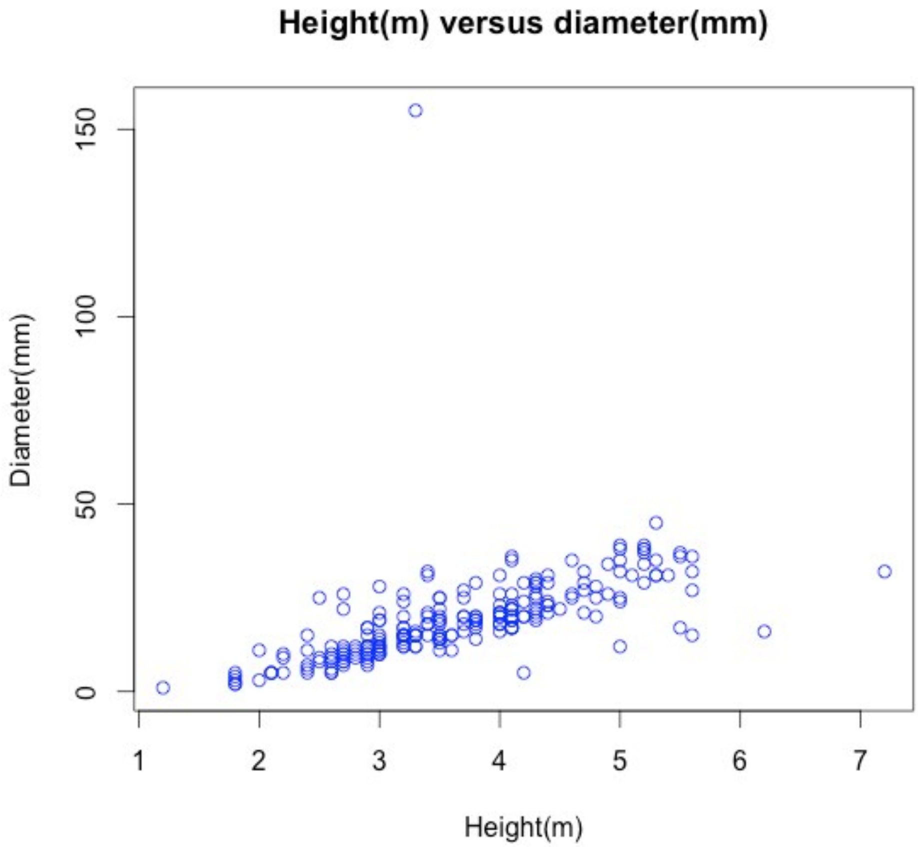

Figure 30. Scatter plot of original data collected on 6/20/2013 consisting of 219 data sets

By year two the varieties had gained in height by roughly 80% **(Figure 31).** In early September of 2013, Dr. Phil Cannon visited the provenance trial and assisted in selecting trees that were to be thinned from the trial. Subsequently, half of the trees (120 trees) were cut down and taken out of the field, thereby leaving four trees in every paired plot. In late September and mid-October, two typhoons developed near Guam where high winds and heavy rain caused damage to the trial. Tropical storm Pabuk passed near Guam on September 19-20, 2013. Nine uprooted trees were removed from the field **(Figure 32),** and 24 bent trees were straightened out and secured with ropes.

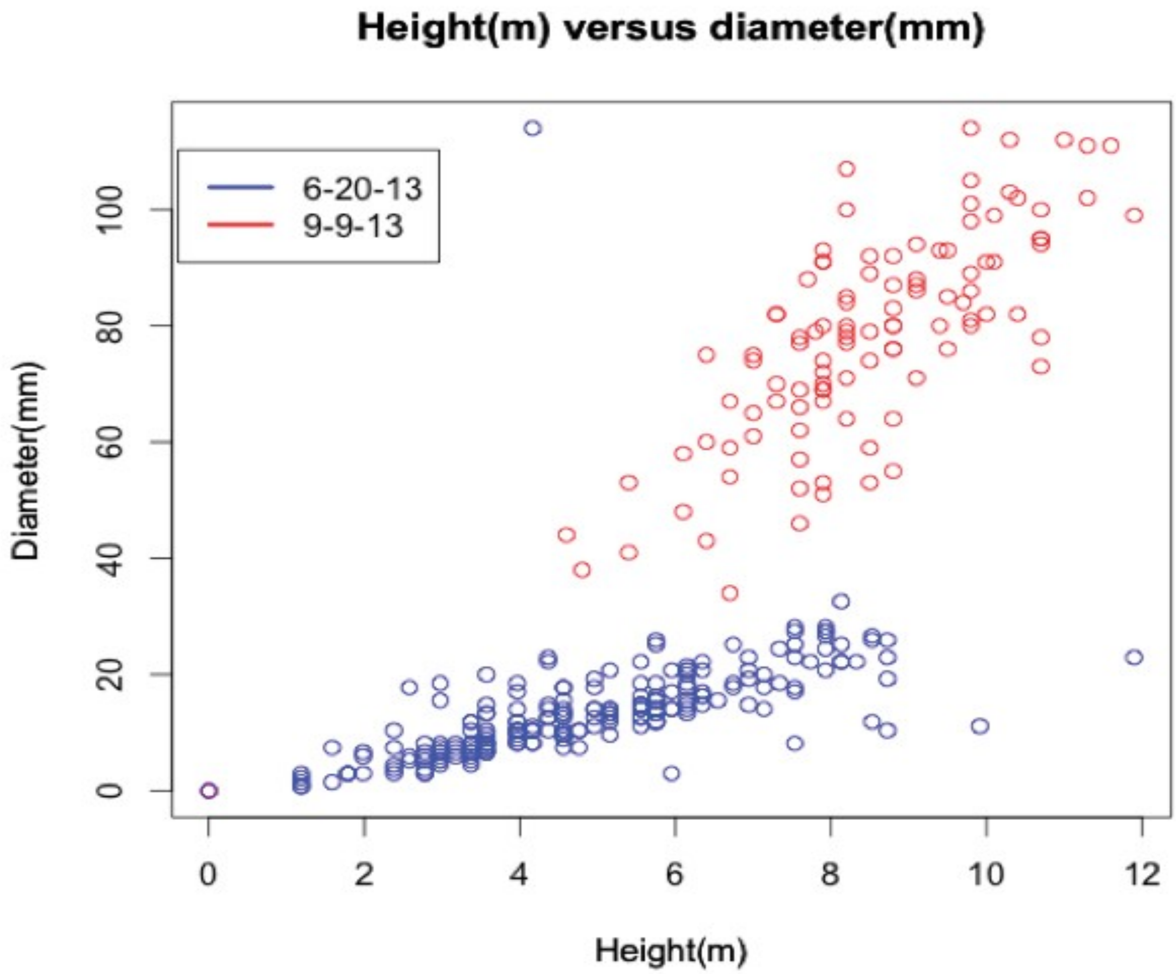

Figure 31. Scatter plot ironwood tree measurements after the first year (blue) and second year (red)

Figure 32. Uprooted tree due to high wind and rain on 9/19/2013

In the third year, the trial had to be terminated due to tree damage. Typhoon Dolphin's eye passed through the Rota Channel between Guam and Rota Island on May 15, 2015 delivering the typhoon's strongest winds in the eyewall to both locations. Andersen Air Force Base (AAFB) on the northeast side of Guam peak wind gust clocked 106 mph just before 7 p.m. local time. One hour later, AAFB was reporting peak sustained winds of 84 mph in the southern eyewall of the typhoon. In addition to the wind, we received 16 inches of rain in two days. In the ironwood provenance trial plot, 44 of 60 trees in the replicated trial and 11 of the 19 trees in the border rows were blown-over and uprooted **(Figure 33 and 34)**. One other tree in the replicated trial was snapped in half**.** As much data as possible was collected on remaining trees as well as the felled trees **(Table 4)**. At this time trees were still gaining in height; however, the rate of growth had slowed **(Figure 35).** This was particularly true for the trees from Guam and Australia. Their slower growth, reduced tree height, and their low, irregularly branched internodes may have contributed to their higher survival level (**Figure 33**).

Figure 33. Trees fallen by Typhoon Dolphin in 2015

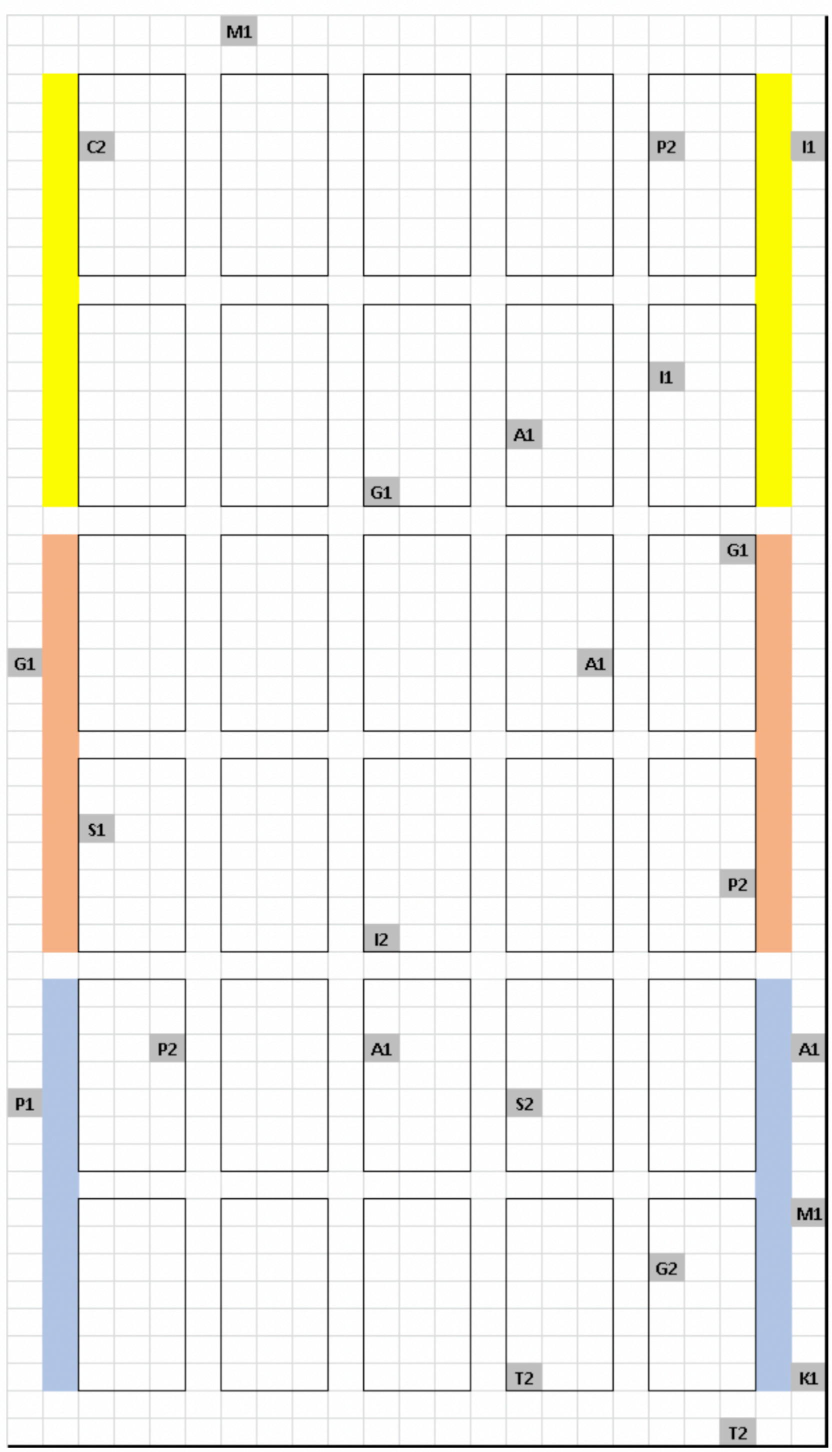

Figure 34. Trees remaining after Typhoon Dolphin struck Guam on May 15, 2015

Table 4: Tree assessment criteria and data collected after Typhoon Dolphin. The notations in green indicate what is considered favorable for a windbreak tree

<table>
<tr><td>Stem Height (Ht) higher value is better</td><td>Height of the tree based on tallest stem</td></tr>
<tr><td>Stem Diameter (dbh) higher value is better</td><td>Trunk/stem diameter at 1.3 m above ground</td></tr>
<tr><td colspan="2">Stem axis persistence: Ability of tree to retain its primary stem axis. Forking is when two or more arising branches emerging from the junction are of near equal diameter. 1= Multiple stems formed at ground level, 2= Forking in first (lowest) quarter of stem, 3= Forking in second quarter of stem, 4= Forking in third quarter of stem, 5= Forking in fourth quarter of stem, 6= Persistence complete (no forking) higher value is better</td></tr>
<tr><td colspan="2">Tree volume (V): Tree volume = $(G/4)^2 \times H$ higher value is better
G = girth (circumference) of tree at breast height in meters (or feet)
H = tree height in meters (or feet)</td></tr>
<tr><td colspan="2">Branch habit: Is an indication of how dense the “needles” are on the tree. Tree’s maximum orders: first-order branch originates from main stem, second-order branch originates off first-order branch, etc. (do not count deciduous branchlets which is where the need-like foliage originates) higher value is better</td></tr>
<tr><td colspan="2">Internodes: Distance between permanent branches taken in lower, middle, upper portions of tree.</td></tr>
<tr><td colspan="2">Branch density rating: 1= Very high, regularly branched, majority internodes <15 cm (6 in), 2= High, irregularly branched, internode around 15 cm (6 in), 3= Low, irregularly branched, internode around 30 cm (12 in), 4= Very low, sparsely branched, internode > 30 cm (12 in) lower value is better</td></tr>
<tr><td>Branch, max. length, max. diam.</td><td>Length and diameter of longest branch</td></tr>
<tr><td>Branch length rating</td><td>1= long-generally > one-quarter of tree height lower value is better
2= short-generally < one-quarter of tree height</td></tr>
<tr><td colspan="2">Branch thickness rating: ration of permanent branch and adjacent stem (point where stem is joined with branch)
1= Very heavy, more than three branches, diameter > 1/3 of adjacent stem.
2= Heavy, one to three branch, diam. > 1/3 or adjacent stem
3= Light, branch diameter up to 1/3 of adjacent stem
4= Very light, branch diameter less than 1/3 of adjacent stem lower value is better</td></tr>
<tr><td colspan="2">Branch angle rate of permanent branches: 1= Upright, < 60° 2= Horizontal > 60°</td></tr>
<tr><td colspan="2">Branchlet habit: Is an indication of how thick the “needles” are on a branch
1= Branching common, 2= majority are without branches lower value is better</td></tr>
<tr><td>Branchlet length</td><td>1= long > 15 cm (6 in), 2= short < 15 (6 in)</td></tr>
<tr><td>Cones</td><td>1= none, 2= yes presence of cones is good</td></tr>
<tr><td>Flowers and sex</td><td>1= none, 2= yes (sex), (male, female, both) presence of flower is good</td></tr>
<tr><td colspan="2">Root damage, typhoon: 1= none, 2= slight (soil lifted, no roots exposed), 3= moderate (tops of some roots exposed, not broken), 4= severe (some exposed and broken, tree would likely survive if left alone), 5= up-rooted, tree is not likely to survive
lower value is better</td></tr>
<tr><td colspan="2">Stem damage, wind: 1= none, 2= slight (15-45 off vertical), 3= moderately (45-80 deg off vert.), 4= severely (greater than 80 deg off vert.), 5= snapped killing above portion lower value is better</td></tr>
<tr><td colspan="2">Branch damage, wind: 1= no damage or stress injuries, 2= not normal, some bent, none snapped, 3= one or more branches snapped or severely bent, 4= more than one large branch snapped lower value is better</td></tr>
<tr><td colspan="2">Ref.
Ravichandran VK, BalaSubramaniam TN & Jeyachandran M. 2003. Development of volume table for Casuarina equisetifolia. The Indian Forestry 26: 7–10.

Pinyopusarerk, K., Kalinganire, A., Williams, E.R., Aken, K.M. 2004. Evaluation of International Provenance Trials of Casuarina equisetifolia. ACIAR Technical Reports Canberra, N. 58, 106pp.</td></tr>
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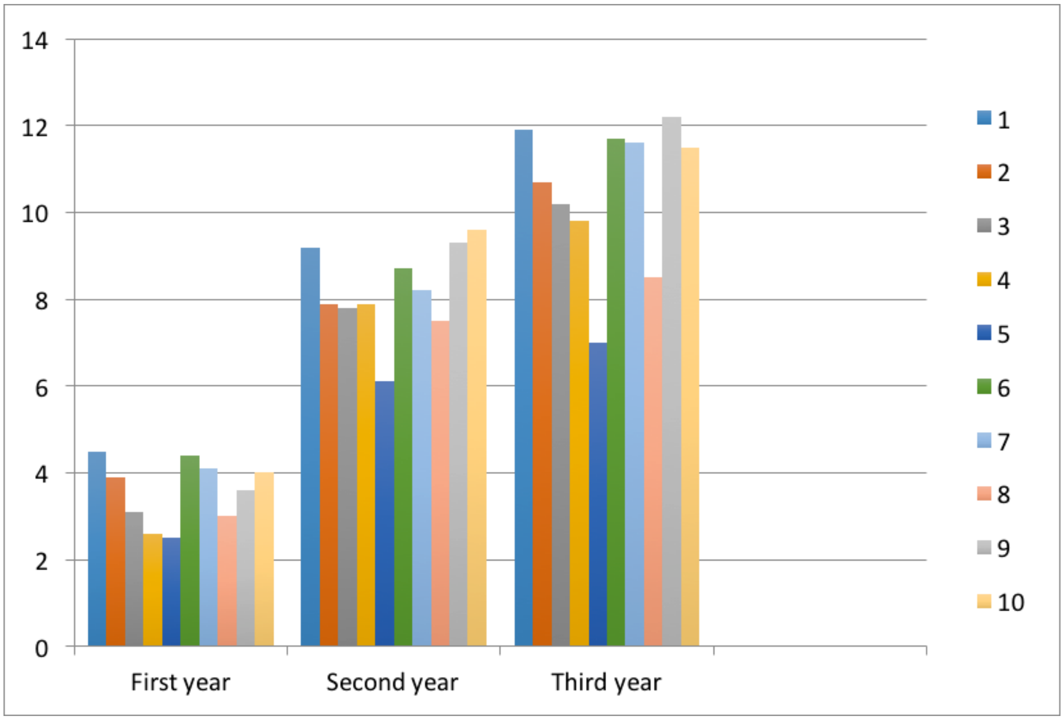

Figure 35. Average height in meters of geographic pairs at end of provenance trial May 2015: 8 (Australia), 10 (China), 5 (Guam), 3 (India), 2 (Kenya), 1 (Malaysia), 6 (Papua New Guinea), 9 (Solomon Islands & Vanuatu), 4 (Thailand), 7 (Vietnam & China)

Contributors to decline

As of 2018, four variables have emerged as useful predictors of ironwood tree decline (IWTD): Presence of *Ralstonia solanacearum* (Rs) and *Ganoderma australe* (Ga), percent cross-sectional area with wetwood (WW), and the production of ooze. Rs and Ga are the only two known pathogens of ironwood that have been identified on Guam. Presence of Rs in some healthy trees and the lack of Ga in many severely declining trees, provides anecdotal evidence that IWTD is best considered a complex of these two causal agents (**Table 5**).

Ironwood trees are healthy in Hawaii and Saipan. Groups of trees in various stages of die-back and decline, which is common on Guam, doesn't occur on Saipan or Hawaii. Rs is easily detected in drill shavings from trees on Guam; however, not a single tree tested positive on Saipan over the course of two days of sampling island wide in 2017 nor was bacteria detected in ironwood trees in Hawaii after a morning of sampling trees. On Saipan, the average number of fruiting bodies found at 8 locations was 2.5% whereas on Guam average number at 6 locations was 28%. From casual observations made in Hawaii, the presence of Ga in Hawaii is like that of Saipan.

Table 5. Range of growth characteristics of n=77 ironwood trees (*Casuarina equisetifolia*) and percentages of trees at various levels of decline which were positive for *Ralstonia solanacearum* and or *Ganoderma australe* on the island of Guam

Decline Severity *	Tree CBH **	Tree Height***	*Ralstonia solanacearum*	*Ganoderma australe*
DS=0 (n=17)	47 – 218	10.2-21	18%	0%
DS=1 (n=12)	41 -310	9.6-18	42%	8%
DS=2 (n=13)	41-152	4-15	85%	23%
DS=3 (n=15)	44-239	10-13	50%	50%
DS=4 (n=20)	38-249	6-18	84%	37%

*Decline severity based on visual comparisons of trees to a set of photographic standards depicting varying levels of bare branches and thinning foliage as shown in **Figure 1**.
** CBH: Circumference in cm at breast height (1.3 m) was found to be non-significant
***Height in meters was excluded from statistical model due to high collinearity with CBH

Termites: In India, termites feed on underground roots and stems of live *C. equisetifolia* trees. This type of damage is believed to be occurring in Guam as well.

Ironwood trees were surveyed on Guam for termites in 2015-2016. Site and tree information collected included site condition (windrow, landscape, woodlot, etc.) GPS location, tree height and girth, ironwood decline severity rating, presence or absence of basidiocarps of *Ganoderma*, termite colony formation, and relative number of termites present.

The role possibly played by termites in IWTD is still under investigation. Three ironwood tree termite surveys were conducted. The survey conducted in June 2015 consisted of 9 healthy trees, 15 in decline, 2 dead trees and 2 stumps. Of the 24 live trees, 8.3% had basidiocarps of *Ganoderma*, 41.7% tested positive for *R. solanacearum,* and 0% had both. The survey conducted from December 2015 to January 2016 consisted of 6 healthy trees and 13 in decline. Of the 19 live trees, 36.8% had basidiocarps of *Ganoderma*, 36.8% tested positive for *R. solanacearum,* and 15.7% had both. In March 2016, the December survey trees were revisited and samples were collected from 15 trees at 14 sites.

Four species of wood-feeding "higher" termites were found to attack ironwood trees in the areas of collection. Those in the family Termitidae include *Nasutitermes takasagoensis, Microcerotermes crassus,* and a yet to be identified *Microcerotermes species.* Morphological identification showed that the vast majority of ironwood infesting termites were most likely *N. takasagoensis* an arboreal species with a distribution that includes China. Taiwan, Japan, and Christmas Island (Australia). Two of the samples were morphologically identified as *M. crassus* Snyder, another species known to occur in many countries of Southeast Asia (including China, India, Malaysia, Myanmar, Thailand, and Vietnam). Nesting habits of this species range from subterranean to arboreal. This species is found around rural and suburban dwelling and sometimes enters buildings. The unknown *Microcerotermes* species has a 96% best match to

Microcerotermes biroi. The Rhinotermitidae family only includes *Coptotermes gestroi. C. gestroi,* known in Asia as the Philippine milk termite, is endemic to Southeast Asia (China, Taiwan, Indonesia) but has spread to other parts of the world including Madagascar, USA, Brazil, Cuba, Jamaica, Mexico, Puerto Rico, India, Myanmar, Sri Lanka, and French Polynesia. *C. gestroi* is a very damaging termite and a threat to wooden structures wherever it occurs.
The large presence of *Nasutitermes takasagoensis* on ironwood trees across Guam, suggests that this termite was primarily responsible for the termite colony formations that were recorded during the 2008-2009 island wide survey of 1,427 ironwood trees and subsequently included in Karl Schlub's 2010 multinomial model. At the time of the survey it was known that termites were present in 32 of the 44 sites surveyed; but nothing was known about the identification of the termite or termites involved. With the identity of *N. takasagoensis* as the primary colonizer of ironwood trees, it is now known that the presence of termites in an ironwood tree regardless of its condition presents low risk to standing structures.

The risk posed to homes and other structures by the presence of termites in ironwood trees is due to the occasional appearance of *Coptotermes gestrori.* Though *C. gestrori* was only found in 2% of the trees surveyed, these termites are voracious feeders and therefore constitute a threat to nearby wood containing structures. They feed on all sorts of cellulose-containing materials and drill holes in such materials as rubber, plastic, and Styrofoam in their search for food. Though they are reported to attach and consume heartwood of living trees in other locations in the world, this remains to be determined in Guam.

***Ralstonia solanacearum* (Rs):** Rs has now been confirmed to occur in Guam's ironwood trees. Rs is a known bacterial pathogen of more than 200 hosts, comprising of 53 botanical families. Genetic diversity in global collections of Rs strains has led to the characterization of Rs as a "species complex". Strains of Rs have been reported to cause bacterial wilt of ironwood in several countries where *C. equisetifolia* is propagated including India, China, and Mauritius. In 2012, a survey showed an association of ironwood IWTD with Rs. Results of this study showed that *Ralstonia* strains isolated from diseased ironwood trees on Guam were similar to GMI1000, they had similar BOX-PCR profiles and belonged to phylotype I and biovar 3. Pathogenicity tests revealed that *Ralstonia* was able to cause wilt in tomato and ironwood seedlings. There were no differences in pathogenicity between Guam *Ralstonia* and control strains, when used to inoculate into tomato plants and ironwood trees from Hawaii. Additionally, there were no observable differences in susceptibility of ironwood trees from Guam and Hawaii to all strains, suggesting that the association of Guam *Ralstonia* with Guam ironwood tree is not specific.
Phylotype multiplex PCR showed that all Guam Rs strains, along with the Rs reference strain (GMI1000), had bands identical with phylotype I (Asia), and all of the tested *R. solanacearum* strains contained the 280 bp amplicon, which is specific to the Rs species complex.

***Ganoderma* (Ga):** Ga is a common heart rotting fungus and normally appears in old trees of many tree species. It has been reported to have killed young trees in plantation situations with ironwood trees, as well as other trees. The widespread appearance of Ga in ironwood trees across the island, seems to point to something very unusual. Ga, a genus of more than 300 species of wood-decaying fungi has been reported as a wood decay fungi of *C. equisetifolia* throughout its range including Mexico, India, Pacific islands, South Africa, Indonesia and Malaysia. In 2015, DNA of a *Ganoderma* sample cultured from ironwood trees from Guam and Saipan was extracted and partial sequences of the internal transcribed spacer region were amplified and edited at the

Moscow Forestry Sciences Laboratory (USDA Forest Service-Rocky Mountain Research Station). Sequences were compared to the GenBank® database using the nucleotide BLAST®. Tentative ITS-based taxon identification: Guam, *Ganoderma austral* complex, score 99% to GU213473; Guam, *Ganoderma* sp., score 98% to AY569452; Saipan, *Ganoderma austral* complex, score 99% to FJ 392286.

Wetwood (WW): Wetwood is a type of heartwood in standing trees which has been internally infused with water. In some tree species, wetwood often has a water-soaked translucent appearance. In other species, the typical water-soaked appearance may be absent. In this case, WW has the appearance of either normal heartwood or it has an unusually dark color. WW occurs in both conifers and hardwoods, but its frequency can vary by species (from none to common), age, and the tree's growing conditions (Ward, 1980). Other than causing a reduction in the value of effect lumber, the condition is generally considered benign. Though the causes and mechanisms of wetwood are controversial, most investigators agree that wetwood is the result of either microbial activity (bacterial), injury, or normal aging. Some believe wetwood bacteria colonize the tree well before maturity and only produce the wetwood symptoms when the tree undergoes stress. The best information available on WW in ironwood trees comes from Mauritus (Orian, 1961).

The presence of dark stained wood and ooze is commonly found in declining trees in Guam **(Figure 36).** It consists of droplets or puddles of fluid of various viscosities and colors and may result from an infection by *R. solanacearum* or the colonization of tissue by wetwood bacteria **(Figure 37)**.

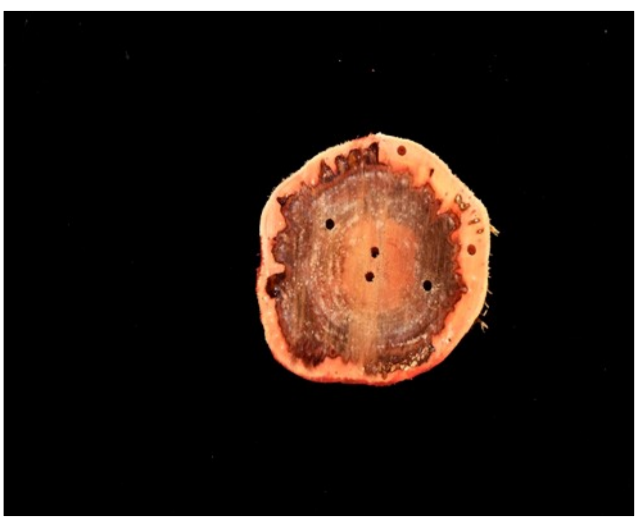

Figure 36. Cross-section of a declining *Casuarina equisetifolia* tree 24 hours after sectioning. Areas of moist discolored "wetwood" radiate from the center. An abundance of creamy, off- white ooze is observed on the cut surface. Drill holes indicate sampled sites; shavings from six holes were combined into a single sample for analysis of bacterial endophytes

The possible role of wetwood bacteria in IWTD began to emerge in 2014, when *K. oxytoca* and *K. variicola* were identified in Guam's trees. Since then, six different genera of wetwood bacteria were identified *Kosakonia*, *Enterobacter*, *Pantoea*, *Erwinia, Citrobacter*, and *Klebsiella*. Wetwood symptoms were present in 93% of tree cross-sections, of which 17% had no outward symptoms of IWTD. We are not sure as to the causes of each type of ooze. It appears that the white creamy one is due to Rs however, wetwood bacteria are always isolated as well. The watery substance may be the result of the plant's vascular system failing and the leaking of cellular contents brought on by the growth of wetwood bacteria.

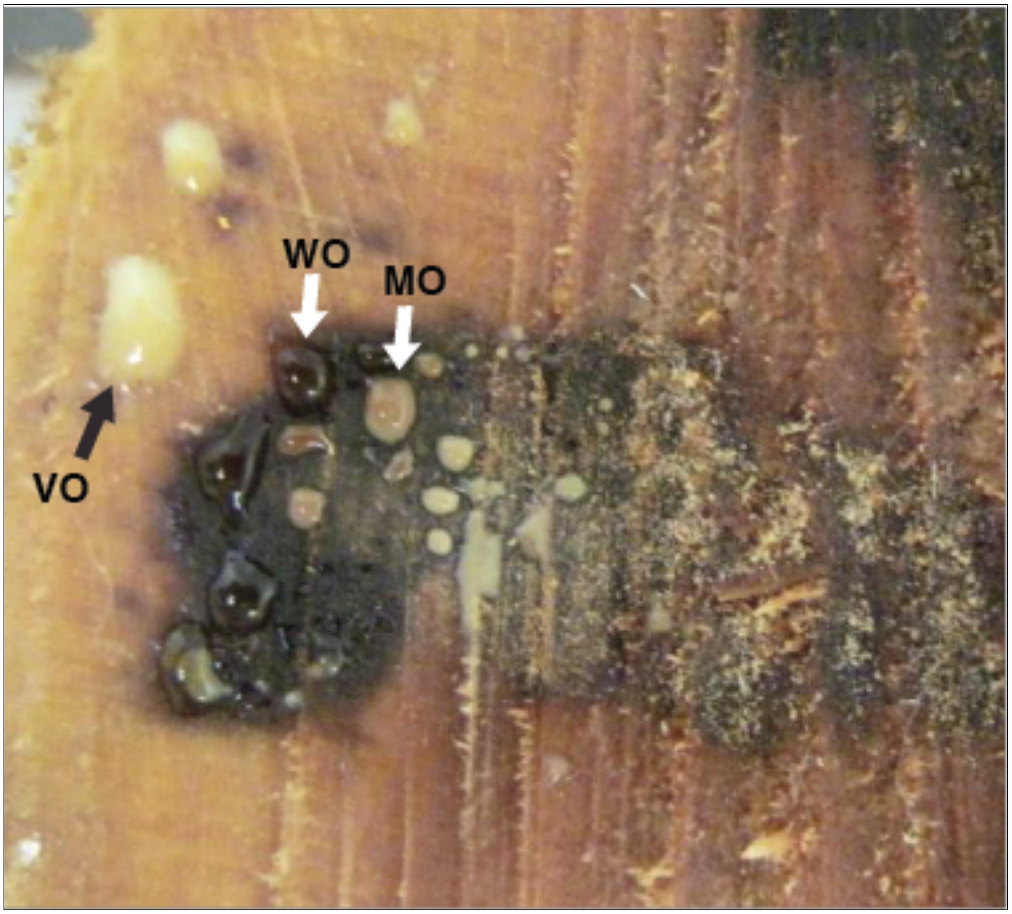

Figure 37. Three types of ooze occur in cross-sections of *C. equisetifolia:* a viscous, white to off-white substance (VO); a watery, amber substance (WO); and a mixture of the viscous and watery ooze (MO)

Figure 38 (reprint). Cross-section of rotted ironwood tree butt infected with *Ganoderma austral* species complex. Note the presence of white rot, areas of dark stained wetwood and the expanding network of white mycelial strands

Findings and Discussion

The best predictor of IWTD on Guam is the percentage of a cross-sectional area with wetwood. All trees cut down in Guam that had moderate to severe decline were positive for wetwood but not always positive for Rs or Ga. From tree cross-section, it appears that WW promotes Ga colonization (**Figure 38**). Even though the association between wetwood and decline on Guam is very high, its causality has not been established. We currently know nothing about the occurrence of wetwood in Saipan trees, which are very healthy. There are several issues that must be taken into account when determining the possible role of wetwood bacteria in IWTD: there are several species present, their initial population is high, some have grown similarly to Rs on a tetrazolium medium. Caleb Ayin was able to reproduce wilt symptoms in young seedlings with Rs but not *Klebsiella.* From the literature termites are known to cause damage to ironwood tree roots in plantations; therefore, we think it is reasonable that they may have the ability to spread Rs or wetwood bacteria around Guam. An Rs culture obtained from China by the University of Hawaii was determined it to be phylotype 1 (Asia), the same as Guam and Hawaii.

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Diseases of Plants
Tropical Forest Pathology

Decline of *Casuarina equisetifolia* (ironwood) trees on Guam: Ganoderma and Phellinus

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Phytopathology 101:S216

Ironwood trees (*Casuarina equisetifolia*), on the island of Guam have been in a state of decline for the past ten years. To determine the status of the decline problem and to seek possible causes, a survey of 1427 trees was conducted. A highly significant ($p = 0.0001$) linear function ($r^2 = 0.997$) between the presence of conks and decline severity emerged. Sixty-five percent of the trees at the most severe level of decline (nearly dead) had conks. Species from five basidiomycete genera of the class Agaricomycetes, belonging to the orders Polyporales (*Ganoderma*, *Favolus*, *Pycnoporus*), Hymenochaetales (*Phellinus*) and Thelephorales (*Sarcodon*) were identified based on macro- and micromorphology and DNA sequencing. The most common species observed was in the genus *Ganoderma*. Diagnostics was based on the prolific production of double walled basidiospores from sporocarps (a characteristic feature of members of the Ganodermataceae). Nuclear ribosomal (ITS) DNA sequencing confirmed Guam's species as a member of the *G. australe* species complex. The second most frequently collected conk belonged to the genus *Phellinus*. These two known genera of *Casuarina* wood rotting fungi are most likely playing a prominent role in the decline of Guam's ironwood trees. Due to the high association between levels of management and decline, it is believed that tree wounds from lawn equipment serve as a point of entry for the two fungi.

Diseases of Plants
Tropical Forest Pathology

Decline of *Casuarina equisetifolia* (ironwood) trees on Guam: Symptomatology and explanatory variables

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Phytopathology 101:S216

Guam's Ironwood trees (*Casuarina equisetifolia*) are dying at rates that far exceed the norm for the region. The problem fits the classic definition of tree decline: symptoms are nonspecific such as the thinning of branches; tree health gradually deteriorates leading to tree death over a course of several years; and decline is attributed to a complex of infectious and non-infectious agents. However, Guam's trees deviate from the classic model where mature trees are more prone to decline. Internal discoloration of the trunk and juncture of large branches was often traced to root and butt rot. By applying various modelling techniques to a set of 1427 individual trees, it was concluded that the presence of basidiocarps, termites, and improper tree care were significant explanatory variables for the decline. A data set created by GIS mapping was also evaluated; however, a reliable model has not yet immerged. At least 5 basidiocarp genera have been identified, of which *Ganoderma* and *Phellinus* are most likely contributing to the tree's decline. Termites reported on Guam's ironwood trees include species of *Nasutitermes*, *Microtermes* and *Coptotermes*. Other explanatory factors under study include typhoons Chata'an and Pongsona, a species of wasp belonging to the genus *Selitrichodes*, nematodes in the genera *Helicotylenchus* and *Aphelenchoides*, and the bacterium *Ralstonia solanacearum*.

Poster Session: New and Emerging Diseases-Fungi and Oomycetes

416-P

Survey of wood decay fungi of *Casuarina equisetifolia* (ironwood) on the islands of Guam and Saipan.

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As a result of statistical modeling of data from individual trees and tree sites, the occurrence of basidiocarps consistently emerged as the dominant explanatory variable for Guam's declining ironwood trees (*Casuarina equisetifolia*). A survey was conducted in February 2012 in the Mariana Islands to elucidate which of the known basidiocarp-forming genera are most consistently correlated with the decline. Species from five basidiomycete genera of the class Agaricomycetes, belonging to the orders Polyporales (*Ganoderma*, *Favolus*, *Pycnoporus*), Hymenochaetales (*Phellinus*) and Thelephorales (*Sarcodon*) were previously identified from Guam based on macro- and micromorphology and DNA sequencing. As a result of the February survey, *Ganoderma* sp. (*G. australe* complex) was the basidiocarp found to be most frequently associated with unhealthy trees. Conks of the fungus were commonly found on Guam where they appeared on roots and butts of declining and stumps of dead trees. On Saipan where decline does not exist and where the trees are considerably healthier, *Ganoderma* sp. was rarely found. In contrast, *Phellinus* sp. was the most widespread fruiting basidiocarp on Guam and Saipan. Though the actual species of *Phellinus* remains to be determined, it does not appear to represent *P. noxious*, and is not consistently associated with trees in decline. These and other species associated with ironwood trees in the Mariana Islands will be discussed.

Poster Session: **New and Emerging Diseases - Bacteria 422-P**

Identification of bacteria associated with decline of ironwood trees (*Casuarina equisetifolia*) in Guam

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Decline of ironwood (Casuarina equisetifolia) in Guam was previously attributed to termite feeding and Ganoderma australe. Recently, we found that bacteria are involved in the disease complex. Ralstonia solanacearum (Rs) and two Klebsiella species were consistently isolated from declining trees that showed no evidence of Ganoderma or termite damage. Discolored wet wood and bacterial ooze gave positive results with Rs-specific Immunostrips (Agdia, Inc. SK 33900/0025) and loop-mediated isothermal amplification. Presumptive Rs cultures isolated from host tissues produced the same positive results. 16S rDNA sequence analysis of presumptive Rs strains showed maximum identity (MI) values of 99% with Rs (strain LMG 2299; K60) and Rs (strain GMI 1000). Klebsiella strains isolated from bacterial ooze and wet wood tissues from the same trees showed 99% MI with two Klebsiella species. Cultures from three trees were identified as K. variicola (strains F2R9 and At-22); cultures from a fourth tree showed 99% MI with K. oxytoca (ATCC 13182). Neither Klebsiella nor Rs were detected in healthy trees. Ironwood and tomato seedlings co-inoculated with Klebsiella and Rs showed distortion, wilt and tissue discoloration. Klebsiella and Rs were reisolated from stems 20 cm above the inoculation point. Identification and pathogenicity tests indicate that the bacterial component of ironwood decline is far more significant than previously suspected.

Poster Session: **New and Emerging Diseases - Bacteria 423-P**

Casuarina equisetifolia decline in Guam linked to colonization of woody tissues by bacteria.

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Ironwood trees (Casuarina equisetifolia) on the island of Guam are in decline due to a combination of biotic and abiotic factors. Bacteria associated with wet-wood and vascular wilt are emerging as significant biotic factors in addition to those previously established, which include the wood-rotting fungus, Ganoderma australe species complex, and termites. Symptoms include thinning of foliage and dark discoloration of the tree's central core, which are associated with the onset of ironwood tree decline. Ralstonia solanacearum and two other bacterial species were consistently recovered in mixed culture when initial isolations were made from discolored wood tissue and from droplets of bacterial ooze, which often form on stem cross-sections of declined trees.
R. solanacearum and one of the unidentified bacterial species were translocated through xylem vessels of young tomato and C. equisetifolia plants following wound inoculation with the bacterial mixture that oozed from infected wood. Confirmation of R. solanacearum was based on cultural characteristics, Agdia immunostrip SK 33900/0025 and loop-mediated isothermal amplification data. Healthy tissues were negative for both the immunodiagnostic and the LAMP assays. This study presents the first evidence that bacteria are involved in the ironwood decline disease complex.

Early results of Casuarina equisetifolia provenance trial in Guam and advances in research on its decline

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(3) USDA Forest Service, Vallejo, CA, U.S.A.

In 2009 attendees of a conference held on Guam to address Guam's declining ironwood trees (Casuarina equisetifolia) recommended the evaluations in Guam of seedlots used in a 1991-1993 International Provenance trial. Consequently, in July 2012 a trial was established which contained 3 blocks, consisting of 10 geographically paired seedlots of 4 trees with 8 ft. tree spacing. Trees were from 11 countries, including Guam. All provenances quickly established in Guam. After one year, the fastest growing provenances were nearly twice as tall as the average seedlot with nearly 6 times the average's biomass. The fastest three geographically paired provenances were those from Solomon-Vanuatu, Malaysia, and China; the slowest were from Australia, Kenya, and Guam. The block effect was significant with the growth of most provenances increasing from block 1 to block 3. This difference was attributed to increasing soil depth from block 1 to block 3. The lack of fit between maps of ironwood circumference and predicted dieback ruled out ironwood tree decline (IWTD) as a mere response of trees to poor site conditions. This and other research and observations support the cause of IWTD as a cascade of biotic events likely starting with root damage and the establishment of bacterial wilt (*Ralstonia solanacearum*) and then quickly followed by establishment of wetwood (*Klebsiella* spp.), basidiocarps of *Ganoderma australe*species complex, and termites.

Identification and characterization of bacteria associated with decline of ironwood (C*asuarina equisetifolia*) in Guam

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Ironwood (*Casuarina equisetifolia* subsp. *equisetifolia*) is a nitrogen-fixing tree of considerable social, economic and environmental importance that commonly occurs in tropical/subtropical zones of Asia, the Pacific, Africa, and Central America. Ironwood decline was first noticed on Guam in 2002 and is now affecting thousands of trees and impacting the ecosystem. In 2012, a survey showed that *Ralstonia solanacearum* and *Klebsiella* spp. were associated with wetwood symptoms of declining trees. *R. solanacearum* strains isolated from diseased ironwood in Guam were similar to *R. solanacearum* strain GMI1000, having similar BOX-PCR profiles and belonging to phylotype I and biovar 3. Two *Klebsiella* species (*K. variicola* and *K. oxytoca*) were recovered, with *K. variicola* being the more prevalent species . Pathogenicity tests revealed that *R. solanacearum* caused wilt in tomato and ironwood seedlings, whereas neither *Klebsiella* spp. produced symptoms. There were no differences in virulence between Guam *R. solanacearum* and control strains following inoculation into tomato and ironwood from Hawaii. Additionally, no observable differences in ironwood susceptibility to *Ralstonia* strains from Guam or Hawaii, were observed, suggesting that the association of Guam *R. solanacearum* with Guam ironwood is not specific. Co-inoculation studies with both *R. solanacearum* and *Klebsiella variicola* and *K. oxytoca* revealed that *Klebsiella* sp. did not affect symptoms produced by *R. solanacearum* alone. In planta studies were feasible only on seedlings and young trees in Hawaii; thus, possible interactions between *R. solanacearum* and *Klebsiealla* sp. in adult trees remain to be investigated. A new in-field survey of declining ironwood is needed to better understand the role of *Klebsiella* and *Ralstonia* in ironwood tree decline in Guam.

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Morphological and Molecular Species Identification of Termites Attacking Ironwood Trees, *Casuarina equisetifolia* (Fagales: Casuarinaceae), in Guam

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Abstract

Ironwood trees (*Casuarina equisetifolia* subsp. *equisetifolia* L.) are ecologically and economically important trees in tropical and subtropical regions of the Indo-Pacific. Ironwood is one of the dominant tree species in Guam, but since 2002, this tree has been declining dramatically. A previous study showed that numerous sick or dead trees were under termite attack. However, the species of termites were not identified. As a first step to investigate causal relationships between termites and ironwood tree death, we assigned termites collected from ironwood trees to species using a combination of morphological characters and DNA barcoding of the 12S, 16S, COI, COII, and ITS2 regions. Based on morphology and comparisons to reference sequences in NCBI GenBank, the most likely species assignments were *Nasutitermes takasagoensis* (Nawa) (Blattodea: Termitidae) found to infest 45 trees, followed by *Coptotermes gestroi* (Wasmann) (Blattodea: Rhinotermitidae) (2 trees), *Microcerotermes crassus* Snyder (Blattodea: Termitidae) (2 trees), and an additional unidentified *Microcerotermes* species (1 tree) with no close sequence match to identified species in NCBI GenBank. However, taxonomic revisions and broader representation of DNA markers of well-curated specimen in public databases are clearly needed, especially for the *N. takasagoensis* species complex.

Keywords: DNA barcoding, ironwood tree decline, wood-feeding pest, Termitidae, Rhinotermitidae

Issue Section: MOLECULAR ENTOMOLOGY, Research

PCR multiplex to differentiate Ralstonia *solanacearum* species complex, including R. *solanacearum*, R. *pseudosolanacearum* and Select Agent R3bv2 strains

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Bacterial wilt strains in the Ralstonia *solanacearum* species complex (RSSC) pose serious threats worldwide to economically important crops. In 2014, Safni and co-workers proposed that the four phylotypes of RSSC be reclassified into three genospecies: R. *pseudosolanacearum* (Rps), R.*solanacearum* (Rs) and R. *syzygii* (Rsy). The revision of RSSC into three genospecies necessitates the proper identification and differentiation of strains for characterization, diagnostics, and epidemiological studies. Therefore, this study aimed to develop an endpoint PCR multiplex for detection and differentiation of Rps, Rs, Rsy, the Rs Select Agent R3b2 subgroup and RSSC strains with an undetermined phylotype. Genomes representing different phylotypes and hosts were retrieved from the NCBI GenBank database and utilized to search for unique gene regions using OrthoMCL. Designed primers for each group were validated in silico for specificity. AT-rich flap sequences were added at the 5' position of each primer to optimize the reaction thermodynamics. The in silico specificity of the assay was tested in vitro with representative strains of each group and other genera. Neither false positives nor false negatives were detected. The detection limit for each of the primers was 10 pg (Rps), 100 pg (Rs), 100 pg (R3b2), 100pg (Rsy) and 10 pg (RSSC) of genomic DNA. The use of GoTaq green in this multiplex PCR provides an easy and inexpensive option for routine diagnostics. The tool is highly specific, reliable, and economical for potential use in culture characterization, diagnostics, surveys, and quarantine decisions.
Keywords: Ralstonia *solanacearum* species complex, characterization, diagnostics, quarantine, Multiplex PCR

Field deployable recombinase polymerase amplification assay for rapid and accurate detection of Ralstonia *solanacearum* species complex

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The Ralstonia *solanacearum* species complex (RSSC)—recently separated into three genospecies (R. *solanacearum*, R. *pseudosolanacearum* and R. *sygygii*)—is associated with bacterial wilt of numerous plant species and has high economic consequences worldwide. Prevention of pathogen dissemination in symptomless planting stocks necessitates rapid and sensitive point-of-need detection for all three species. Recombinase polymerase amplification (RPA), a relatively new isothermal technique, is becoming popular among diagnosticians due to its speed, sensitivity and ability to overcome reaction inhibitors. A rapid point-of-need Exo RPA assay was developed to detect multiple RSSC strains in field settings. A unique conserved genomic region was identified through a comparative genomics approach using OrthoMCL and Geneious to design robust primers and probe. The specificity of the assay was validated with representative strains from each of three genospecies and non-target genera. No false positives or false negatives were detected. The detection limit was assessed with 10-fold serially diluted genomic DNA and determined to be 10 pg. Sensitivity in spiked assays—1 µl sap from 100 mg host tissue macerated in 500 µl of TE buffer—was also 10 pg. The potato host tissue showed no adverse effects on the detection limit. The developed assay is useful in field settings with high accuracy and minimum instrument sophistication. The assay has wide-range applications in biosecurity, quarantine, routine diagnostics and the formulation of effective disease management strategies.

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Ecology of Guam's *Casuarina equisetifolia* and research into its decline

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Abstract

On Guam, the estimated lifespan of *Casuarina equisetifolia* subsp. *equisetifolia* is 35 to 90 years and trees may reach a height of 24 m and a diameter at breast height of 92 cm. The majority of its trees are monecious. In 2002, it was noticed that many trees were in a slow decline and when felled, droplets often formed on their fresh stumps. In 2009, this condition was coined ironwood tree decline (IWTD). The droplets which consisted of various mixtures of plant exudates and bacterial ooze were found to contain *Ralstonia solanacearum* and various wetwood bacteria. Reported in 2015 and confirmed in 2020, Guam's *R. solanacearum* strains are of the phylotype 1 (Asia). Cross-sections of IWTD trees from Guam are very similar in appearance with those of bacterial wilt from China. In 2012, a project was initiated to diversify the gene pool and identify resistance of *C. equisetifolia* in Guam trees. Using the 1991-1993 international provenance trials of *C. equisetifolia*, 11 geographically paired seedlots were planted at a farm where IWTD was present. Over the succeeding years, none of these trees have developed IWTD nor tested positive for *R. solanacearum*. Over the years, the following pests were investigated and ruled out as contributors to IWTD: *Protaetia orientalis*, *Protaetia pryeri*, *Selitrichodes casuarinae*, *Helicotylenchus* sp. and *Phellinus* sp. Between 2010-2019, data analyses identified three prominent variables as predictors of IWTD: bacterial wilt pathogen *R. solanacearum*, butt and root rot fungus *Ganoderma australe* and termites. In 2019, *Nasutitermes takasagoensis* complex was found to be the dominant termite species infesting Guam's *C. equisetifolia*.

Keywords: *Casuarina equisetifolia*, *Ralstonia solanacearum*, *Ganoderma australe*, wetwood, ironwood tree decline on Guam, bacterial wilt China, bacterial wilt India

1 Introduction

Much of the information presented in this article is contained in a general review of Guam's *C. equisetifolia* (Schlub, 2019).

1.1 History

Casuarina equisetifolia, subsp. *equisetifolia*, is tightly integrated into the local culture and the Guam's environment, where it is locally known as ironwood (in English) and "gago" (in the native Chamorro language). It has been continually propagated on Guam since the 1600s. It is a hardy, pioneer, salt-resistant tree that occurs on the island's main soil types: limestone, volcanic, and coral sand. It is propagated for windbreaks, erosion control, and urban landscapes. Because *C. equisetifolia* is the dominant tree species on many of the sandy beaches of the Mariana Islands, it has become an important perching tree for the white-collared kingfisher (*Halcyon chloris*), the Mariana fruit-dove (*Ptilinopus roseicapilla*), and the white fairy tern (*Gygis alba*), which commonly lays eggs in ironwood trees. Since the 1980s and prior to appear of tree decline in the 2000s, the Guam Department of Agriculture provided approximately 250,000 seedlings to farmers, the public, and government agencies for various tree planting projects.

1.2 Ecology

On Guam, estimated lifespan of *C. equisetifolia* is 35 to 90 years, and it may reach a height of 24 m and DBH of 92 cm. Its population on Guam is estimated to be 80% monecious, 3% male, and 10% female. Ironwood thickets are a component of Guam's forestland, where *C. equisetifolia* is considered a secondary forest species. In the Mariana Islands, it grows in the clay volcanic soils of savanna grasslands and in the calcareous and loamy sands of coastal strands. On Guam, *C. equisetifolia* is only one of eight tree species larger than 28 cm in diameter at breast height (DBH). In addition, *C. equisetifolia* is a prominent member of the halophytic (sea-salt adapted) vegetation type. This vegetation type is found along beaches in northern and southern Guam, where it may be composed solely of *C. equisetifolia* or a mixture of other species, including *Cocos nucifera, Guettarda speciosa, Hernandia sonora, Pandanus tectorius, Scaevola taccada, Thespesia populnea*, and *Tournefortia argentea*.

1.3 Ironwood tree decline (IWTD)

Symptomatic *C. equisetifolia* began appearing in tree stands across Guam in 2002. In one farm location, five 10-year-old trees planted as part of a windbreak exhibited symptoms of rapid yellowing (chlorosis) and mortality. At the same time, trees at this location and elsewhere on Guam were exhibiting symptoms of thinning foliage and a lethal progressive dieback. Age of affected trees ranged from 10 years to several decades. A study was commissioned in 2004, after Natural Resources personnel with Commander Navy Region Marianas (COMNAVMAR) observed high mortality among trees at the Naval Station. The study failed to identify a cause for the mortality but did rule out two invasive beetles, *Protaetia pryeri* (Janson) and *Protaetia orientalis* (Gory and Percheron). By 2005, one third of all trees at the Naval Station were dead. In 2008-2009, the condition of foliage thinning and dieback on *C. equisetifolia* was referred to as ironwood tree decline (IWTD) (Schlub et al., 2011). At that time a visual tree scoring system was developed based on five ordinal categories of branch fullness and dieback: 0= symptomless, 1=slight damage, 2=distinctly damaged, 3=heavily damaged, and 4=nearly dead) (Schlub et al., 2011). As of today, trees continue to die, although the rate of loss appears less than that in 2005.

2 Materials, methods and results

2.1 International provenance trial

To identify potential resistance to IWTD, 11 geographically paired seedlots obtained from the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Australian Tree Seed Centre were planted on Guam in 2012. The majority of these seedlots were used in the 1991-1993 international provenance trials of *C. equisetifolia*. The fastest growing pairs were those from Solomon (CSIRO 18402), Vanuatu (CSIRO 18312) and Papua New Guinea (CSIRO 20586 and CSIRO 18153), while the slowest pairs were from Australia (CSIRO 19821 and CSIRO 18378), Thailand (CSIRO 18297 and CSIRO 18299), and Guam. Though these trees were planted in the immediate area where IWTD occurs, today these trees remain healthy and free of infection by *R. solanacearum* (bacterial wilt pathogen) or *G. australe* (wood-rot fungus).

2.2 Gall-inducing wasp

After discussions among attendees of Guam's IWTD conference in 2009 (Schlub et al., 2011) and subsequent surveys, the gall-inducing wasp (*Selitrichodes casuarinae*) was discovered. Subsequently, this gall-inducing was found to be widespread across Guam (Fisher et al., 2014). On healthy trees, its impact is likely negligible, but may be significant on trees in decline.

2.3 Nematodes

In 2011, over a 5-day period, nematodes were extracted from *C. equisetifolia* roots and associated soils from five sites with healthy trees, and nine sites with declining trees. Soil samples from sites with decline contained a higher number of nematodes and nematode species than samples from sites with healthy trees. *Tylenchus* and *Helicotylenchus* were the most common genera isolated, with both genera found on 50% of samples from healthy sites, and 44% and 78%, respectively, of samples sites with declining trees. The highest average nematode counts from 10 g samples from both sites (healthy and declining trees) were those of *Helicotylenchus*, with 10 (healthy tree sites) and 39 (declining tree sites). It was concluded that *Helicotylenchus* sp. was likely the only nematode isolated with a potential to negatively impact *C. equisetifolia.*

2.4 Termites

In 2015, termites from 48 infested *C. equisetifolia* trees across Guam were collected. *Nasutitermes takasagoensis* or a closely related species from the *Nasutitermes takasagoensis* complex was found to have infested 45 trees. *Coptotermes gestroi* and *Microcerotermes crassus* were found to infested two trees (Park et al., 2019).

2.5 Conk forming basidiomycetes

Tree surveys during 2008-2009 found that 65% of *C. equisetifolia* were nearly dead (DS=4) and conks (fruiting bodies/basidiocarps of wood-decay fungi) were visible on most of these trees. In 2010, five conk-forming basidiomycete genera of the class Agaricomycetes, belonging to the orders Polyporales (*Ganoderma, Favolus, Pycnoporus*), Hymenochaetales (*Phellinus*) and Thelephorales (*Sarcodon*), were identified based on macro- and micro-morphology and DNA sequencing (e.g. ITS rDNA). A short survey for conk-forming basidiomycete was conducted in 2012 in healthy and IWTD sites on Guam and the nearby island of Saipan where IWTD does not occur.

Conks found growing on live trees belonged primarily to two species: *Ganoderma* sp. (*austral* group), which fruits on the tree roots, butt, and less commonly bole, and *Phellinus* spp., which primarily fruited on the butt. Both species were commonly found on Guam and infrequently found on Saipan. *Phellinus* does not appear to be a primary contributor to IWTD by itself. In contrast, *Ganoderma* appears more likely as a factor that contributes to IWTD on Guam, because it is a consistent indicator of IWTD (or a tree in decline) and its occurrence is irrespective of tree size. The *G. australe* complex identification was confirmed from samples collected at three sites on Guam and one site on Saipan in 2013. ITS sequences of a sample (*G. austral* complex) from Guam showed a 99% identity with sequences of *Ganoderma* sp. from China (GenBank GU213473), and a sample from Saipan showed a 99% identity with other sequences of *Ganoderma* sp. from China (GenBank FJ392286). Appropriate taxonomy within the *G. australe* complex remains unclear.

2.6 Detection and isolation of *R. solanacearum* on Guam

Within minutes of felling a tree in decline, droplets often formed on the cut surface. The droplets, hereafter referred to as ooze, consist of various mixtures of plant exudates and bacterial ooze. In 2011, it was discovered that ooze and tissue samples from decline trees tested positive using *R. solanacearum* specific Immunostrips (Agdia, Inc.) (Ayin et al., 2015; Ayin et al., 2019).

Three forms of ooze were observed: white to off-white viscous ooze (VO), watery amber ooze (WO), and a mixture of the two (MO). Drops of VO commonly appear in sapwood and sapwood-transition zones, may occur in unstained tissue, and frequently tested positive for *R. solanacearum*. Drops of WO commonly appear in the sapwood-transition and heartwood zones, always appear in stained tissue, and frequently tests negative for *R. solanacearum*. Drops of VO and MO were not randomly distributed, but appeared to coincide with growth rings (Fig. 1).

Though *R. solanacearum* could be detected from wood chips and drill shaving from roots, stems and branches of trees, attempts to isolate these bacteria from these same drill shavings failed. The only means

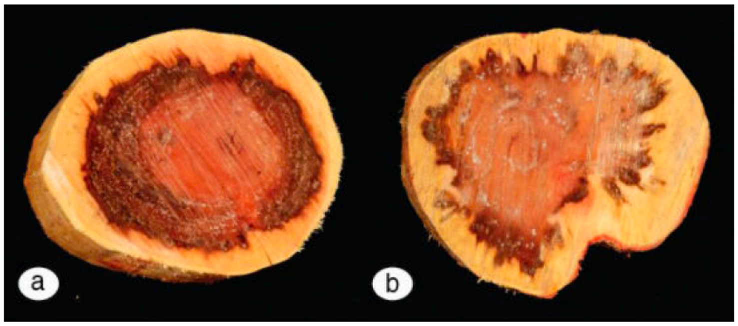

Fig. 1. Cross-sections of *Casuarina equisetifolia* trees on Guam with decline symptoms: (a) decline level DS=1 (slight damage), 63% wetwood symptomatic tissue present in sapwood and sapwood-heartwood transition zones, extensive bacterial ooze formed within minutes of felling, the majority of which is viscous, positive for *R. solanacearum*, positive for *Klebsiella oxytoca*, and negative for *Ganoderma australe*; (b) decline level DS=3 (heavily damaged), 58% wetwood symptomatic tissue present in sapwood and sapwood-heartwood transition zones, extensive bacterial ooze, of which the majority is viscous, positive for *R. solanacearum*, and negative for *Ganoderm australe*.

by which *R. solanacearum* could be isolated was by streaking ooze that formed on slices of stems, roots, or large branches from infected trees onto selective medium. To enhance the production of ooze, slices were placed on saturated paper-towel in a moisture chamber for 24 hrs. Once formed, the ooze was streaked on Engelbrecht's semi-selective medium (SMSA) (Ayin et al., 2015; Ayin et al., 2019). Colonies were re-streaked on to SMSA, which was followed by streaking onto modified Kelman's tetrozolium chloride medium (TZC) before subculturing on TZC.

2.7 Statistical links to decline

Various statistical methods have been applied to variables in search of predictors for IWTD. These variables were related to tree growth (e.g. diameter), abiotic factors (e.g. management practices and site density), or biotic organisms (e.g. termites and bacteria). In 2008-2009, 1,427 trees were surveyed for decline severity (DS) using an ordinal scale consisting of five categories (0=no damage, 1=slight damage, 2=moderately damaged, 3=heavily damaged, and 4=nearly dead). In addition 13 predictive variables were measured: latitude, longitude, altitude, number of tree stems, tree stand density, tree site location, typhoon damage, fire damage, CBH, intensity of management practices (none, moderate, or high), whether the tree was naturally planted or not, and whether a tree had conks or termites (Schlub et al., 2011). Through the application of multinomial modeling, three variables were determined to be significant (Schlub, 2010) (Table1).

In 2012, variables derived from 16 GIS map characteristics were added to the survey data. These GIS-derived variables included: cemetery buffer, fire risk, fires per year, proximity to golf courses, land cover, management areas, school buffer, soil available water at 150 cm, available water at 25 cm soil depth, soil depth to restrictive layer, soil series, vegetation, and the 2002 USDA Forest Service Inventory Analysis (FIA) map of trees with conks. The soil series was dropped from the analysis because of correlations with regressors. Nine GIS-derived variables were determined to be significant (Table 1).

After establishing in 2013 that *R. solanacearum* (bacterial pathogen) and wetwood bacteria were present in declining trees (Ayin et al., 2015), trees were surveyed to determine the relationship of bacteria and *G. australe* (wood-rot fungus) with IWTD. Using data collected in 2015 from a set of 77 whole trees, a proportional odds logistic regression model was fit with the following covariates: DBH, height, and presence/absence of *R. solanacearum* and *G. australe*. In addition, nine covariates (percent wetwood area, ooze initiation, ooze quantity, ooze type, and presence/absence of *Klebsiella* colony types, *K. oxytoca*, *R. solanacearum*, and *G. australe*) were applied univariately to data from a 30-tree subset of the original 77. From the two studies, four explanatory variables or covariates were determined to be significant (Ayin et al., 2019)

Table 1 Explanatory variables that were found to be significant positive (+) or negative (-) predictors of ironwood tree decline (*Casuarina equisetifolia*) (IWTD) from three statistical studies, variables are listed in order of significance.

Study*	Variable	Significance
2010	When conks were present	+
	When termites were present	+
	With increases in landscape management practices	+
2012	When conks were present	+
	When trees were intentionally planted	+
	When located where soil water is available at 25 cm	-
	With increases in altitude of location	+
	When located on a golf course	-
	When located in a forested areas	-
	With increases in tree circumference	+
	When termites were present	+
	When located in an urban landscape area	+
2019	When *Ralstonia solanacearum* was detected	+
	When *Ganoderma australe* was present	+
	With increases in percent wetwood in tree cross-sections	+
	When ooze bacterial ooze forms within 24 hrs of tree felling	+

*Analyses (Schlub 2010; 2019 and Ayin et al., 2019).

(Table 1).

2.8 Comparison between IWTD in Guam and bacterial wilt in China and India

Though IWTD in Guam and bacterial wilt in China and India have the pathogen *R. solanacearum* in common, IWTD differs with respect to symptomatology and abiotic and biotic contributors (Table 2).

3 Discussion

Based on our current information, it appears that IWTD is unique to Guam and it has no single cause. Though *R. solanacearum* occurs in Guam, China and India, it only accounts for 65% of the trees with IWTD symptoms in Guam, whereas in China and India is accounts for 100% of trees with bacterial wilt symptoms. Another stark difference between Guam and China and India is that the symptoms are nondistinctive and gradual with IWTD in Guam, whereas in China and India thedisease symptoms are distinct and sudden.

The identification of several significant explanatory variables strengthens the concept that IWTD is not solely caused by *R. solanacearum*, but it is instead the result of a disease complex comprising multiple biotic and abiotic factors in which biotic factors play a dominant role. The presence of *R. solanacearum* and *Klebsiella* colony types in symptomless trees suggests that at least some trees could possibly remain symptomless when infected with *R. solanacearum* and wetwood bacteria, provided its sapwood is not compromised through the formation of wetwood or ooze (Ayin et al., 2019).

4 Conclusion

On Guam, where IWTD is prevalent 20% of the outwardly asymptomatic trees test positive for *R. solanacearum* and 50% have some degree of wetwood. Only a few trees exhibit no internal discoloration or bacterial ooze, and test negative for *R. solanacearum* (Fig. 9).

Table 2. Comparison between ironwood tree (*Casuarina equisetifolia*) decline (IWTD) in Guam and bacterial wilt caused by *Ralstonia solanacearum* in China and India.

	Guam	China	India
Age at onset	10 years and older	Several months to 10 years (Fig. 4)	Sapling to one year
Foliage symptoms	Gradual thinning and die-back of branches (Fig. 3a)	Rapid wilt and death of branchlets (Fig. 4, 5)	Rapid yellowing of lowest branchlets and progressing upward
Onset to tree death	Months to years	Weeks to months but heavy rain can extend the period	Weeks
Appearance of freshly cut stomp or stem cross-sectional disc/slice	Those in severe decline usually produce ooze (Fig. 1), others may (Fig. 2) but often do not (Fig. 3b), wetwood may occur in the sapwood, sapwood heartwood transition zone (Fig. 1) or heartwood (Fig. 2), asymptomatic trees may (Fig. 2) or may not (Fig. 3b) have wetwood	Usually produce ooze (Fig. 6, 7) and may have evidence of wetwood in the sapwood or the sapwood-heartwood transition zones (Fig 6.)	Often show ooze and discoloration
Occurrence	Roughly 5% of the tree population, dense tree cohorts often <0.5 ha or as a few scattered trees across several hectares	Roughly 5,000 hectares mainly in Guangdong province, occurring dense tree cohorts close to agricultural lands (Fig. 4)	Dense plantations
Maximum Incidence	Approximately 85%	>90% within 1-2 months following a typhoon	40%
Phylogenetic Analysis	phylotype 1 (Ayin et al., 2015)	phylotypes 1, biovar 3 or 4 and race 1 (Jiang et al., 2017)	Phyloptype I, biovar 3 & 4, race 1 (Ramesh et al. 2014; Singh et al., 2018)
Contributing factors	In sites with decline approx. 65% of the symptomatic trees are infected with *Ralstonia solanacearum*, other factors linked to IWTD included site conditions, management practices, bacterial wetwood, the termite *Nasutitermes takasagoensis*, and the butt and root rot fungus *Ganoderma australe*	100% of the symptomatic trees are infected with *R. solanacearum*, appears sporadically at first and gradually reaches >90%, sudden death often follows typhoon (Fig. 4,5), occurs in plantations, disease level increases with repeated plantings in infected sites, disease is greatest near crops infected with bacterial wilt	Usually occurs under wet conditions, most of the plantation are adjacent to tomato fields, biocontrol organisms such as *Trichoderma* and *Micromonospora* controlled the disease and reversed the symptoms if applied during early stages of infection, revived trees showed no discoloration of stem or oozing when tested at the age of 18 to 24 months (Fig. 8)

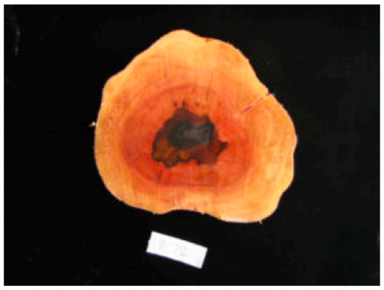

Fig. 2. Cross-section of a *Casuarina equisetifolia* tree on Guam with no outward symptoms of decline: DS=0 (symptomless), 14% wetwood symptomatic tissue present in heartwood, slight viscous and watery bacterial ooze, negative for *Ralstonia solanacearum* and *Ganoderma australe*

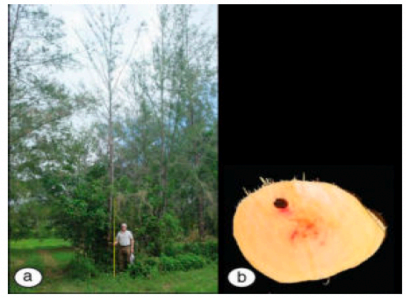

Fig. 3. *Casuarina equisetifolia* tree on Guam with decline symptoms: (a) level DS=2 (distinctly damaged) (b) tree 'a' cross-section, 0% wetwood symptomatic tissue, no bacterial ooze, positive for *Ralstonia solanacearum*, and negative for *Ganodera australe*.

Fig. 4 Mortality of various aged clonal *Casuarina equisetifolia* trees in China, following a strong typhoon in 2015.

Fig. 5. Mortality of bacterial wilt-infected *Casuarina equisetifolia* trees in a clonal trial in Wuchuan, China, 2 months after a strong typhoon in 2015.

Fig. 6. Cross-section of bacterial wilt-infected *Casuarina equisetifolia* tree in Xuwen, China in 2019: severe bacterial wilt, 38% wetwood symptomatic tissue present in sapwood-heartwood transition zone, extensive viscous bacterial ooze is formed within minutes of felling, *Ralstonia solanacearum* positive.

Fig. 7. Cross-section of bacterial wilt infected *Casuarina equisetifolia* tree in Xuwen, China in 2019: severe wilt, 0 % wetwood symptomatic tissue, slight viscous ooze bacterial exudate is formed within minutes of felling, *Ralstonia solanacearum* positive.

Fig. 8. Cross-section of an 18-month-old tree in India that recovered from bacterial wilt with no signs of wood discoloration or bacterial ooze, after showing symptoms at 6 months and following treatment with *Micromonospora maritima.*

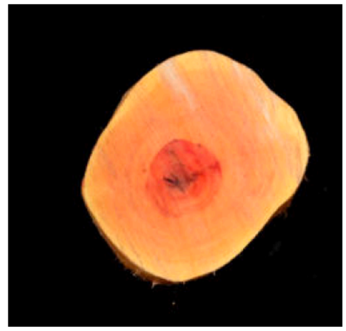

Fig. 9. Cross-section of a *Casuarina equisetifolia* tree on Guam with no outward symptoms of decline: IWTD level DS=0, symptomatic tissue 3%, no wetwood or bacterial ooze, negative for *Ralstonia solanacearum,* negative for *Klebsiella oxytoca,* and negative for *Ganoderma australe.*

These healthy trees tend to occur in natural stands, at low altitude or areas not prone to drought. Due to the slow progression of IWTD and its general sporadic nature, it is likely that IWTD could be reduced through the following measures: (1) increasing the genetic diversity and species diversity of *C. equisetifolia* in Guam, (2) removal of *R. solanacearum / G. australe* infected trees, (3) prevention of root-grafts, and (4) the application of cultural practices that promote healthy growth.

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This article is the columniation of 17 years of research led by Dr. Robert Schlub. Though it is impossible to acknowledge all of the nearly 50 individuals and various professional organizations, universities, and other institutions that have contributed to these studies over the years, Dr. Schlub would like to especially note the contributions of Roger Brown, Joe Afaisen, Victoria Santos, Kevin Eckert, Phil Cannon, Ananda Persad, Khongsak Pinyopusarerk, Bal Rao, and Pauline Spaine. This work is supported by McIntire- Stennis project accession no. 1017908 from the USDA National Institute of Food and Agriculture. Other major financial contributors over the years include the University of Guam, University of Guam Cooperative Extension & Outreach, Western Pacific Tropical Research Center, and various United States Government Programs (USDA, NIFA, NSF, EIPM, WIPM, RREA, WPDN, and USDA Forest Service).

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Part III.
2022 Update

Appendix B

Forward: This appendix serves as an update to my last revision of the 2013 guide: Gago, Guam Ironwood Tree, *Casuarina equisetifolia* Past, Present, Future. Contributors and funding sources not previously mentioned in past revisions are indicated here.

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Other contributors: There are dozens of individuals on Guam and off island that have been instrumental in making things happen and keeping ironwood related projects going. Off island contributors include Saipan Forestry, Northern Marianas CREES, and Rota Forestry.

Without the teamwork of my current employees (Julia Hudson, Elizabeth Hahn, Ethan So, Alexander Chingyan, and Thomas Fies) much of the field work that is supporting the research efforts at the University of Hawaii and at Louisiana State University would not have been possible. Their team effort ensured that the University of Guam was able to put on a world class conference in January 2022 ironwood decline. I am most grateful to Julia for her willingness to take on Roger W. Brown's tasks upon his untimely death May 17, 2021.

Funding sources: The advances made in the last three years were funded by many institutions and organizations that funded the early research including University of Guam, University of Guam Cooperative Extension & Outreach, Western Pacific Tropical Research Center, and various

United States Government Programs (USDA, NIFA, EIPM, WIPM, RREA, WPDN, US Forest Service, and McIntire-Stennis). Noteworthy was the $304,263, 2019-2022, WSARE Research and Education grant: Restoring *Casuarina equisetifolia* as an agroforestry species in Guam through replacement of bacterial wilt infected trees and research into bacterial microbiomes and associated termites. An important survey for hosts of bacterial wilt was funded by a $70,000 2021-2022 Hatch project: Collection, isolation and characterization of the bacterial wilt pathogen, *Ralstonia solanacearum* species complex on the island of Guam

Findings and Discussion

Genetic enhancement of Guam's Ironwood tree population

The lack of genetic diversity within Guam's tree population is believed to be part of the reason ironwood tree decline is so widespread and severe. In 2012, the US Forestry Service funded a small ironwood provenance trial to evaluate trees from various parts of the world for suitability to Guam's environment and to establish a tree nursery from which seeds could be collected **(Figure 24, 25).** Two hundred and forty trees from 11 geographic regions were out-planted in the provenance trial. Over the course of the study, provenance trial trees were lost to storms and tree stand thinning **Figure 39.** At roughly the same time, a set of provenance trees were planted on Anderson Air Force Base golf course; fortunately, none of these were lost due to storms or other causes.

In March 2021, cones were collected from the Anderson trees and an older established natural tree from Hagatna (**Table 6**). Seeds served as a seed source for ironwood trees that were used in workshops, research, and various out-planting projects. In July and November 2021, 661 seeds from 9 different origins were planted. Origins included Australia, India, Egypt, Vietnam, Papua New Guinea, Kenya, Sri Lanka, Malaysia, and Guam. This resulted in the production of several hundred seedlings which were used in 7 out planting projects (**Table 7)**. One of the projects was the out planting of 27 trees in a demonstration garden behind the University of Guam College of Natural and Applied Sciences Building. Three trees of each variety for a total of twenty-seven trees were planted. The planting of these trees will aid in the stabilization of the cliffside soil and will contribute to improving the genetic diversity of Guam's ironwood tree population.

Deteriorating agroforestry projects across Guam were chosen for replacement of Ironwood trees **(Table 8**). Seeds sourced from different geographical locations were planted into seed cones. Saplings were raised for 4-6 months then transplanted to different locations across Guam in February 2020 Fifteen new windrows were created consisting of 10 trees each and four deteriorated windrows were refurbished with 15 trees each. (**Figure 40**)

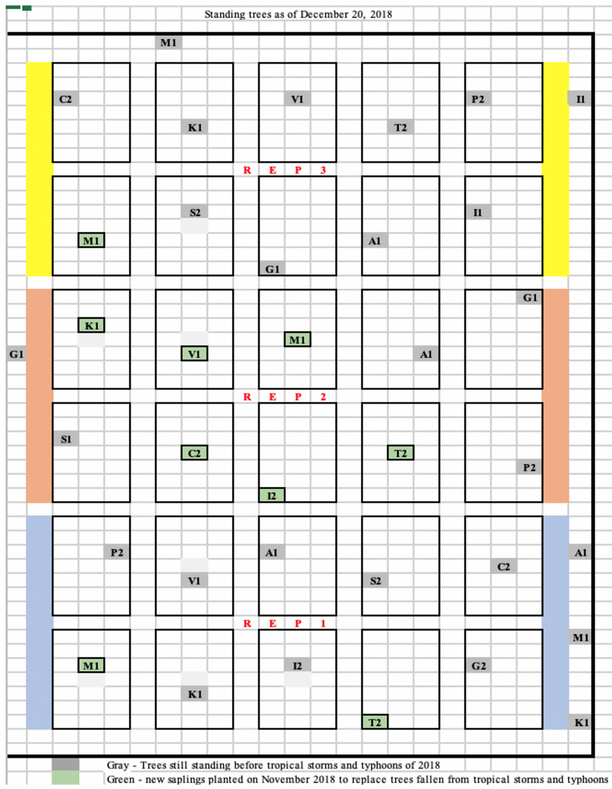

Figure 39: A schematic of the *Casuarina equisetifolia* seed nursery at Bernard Watson's farm

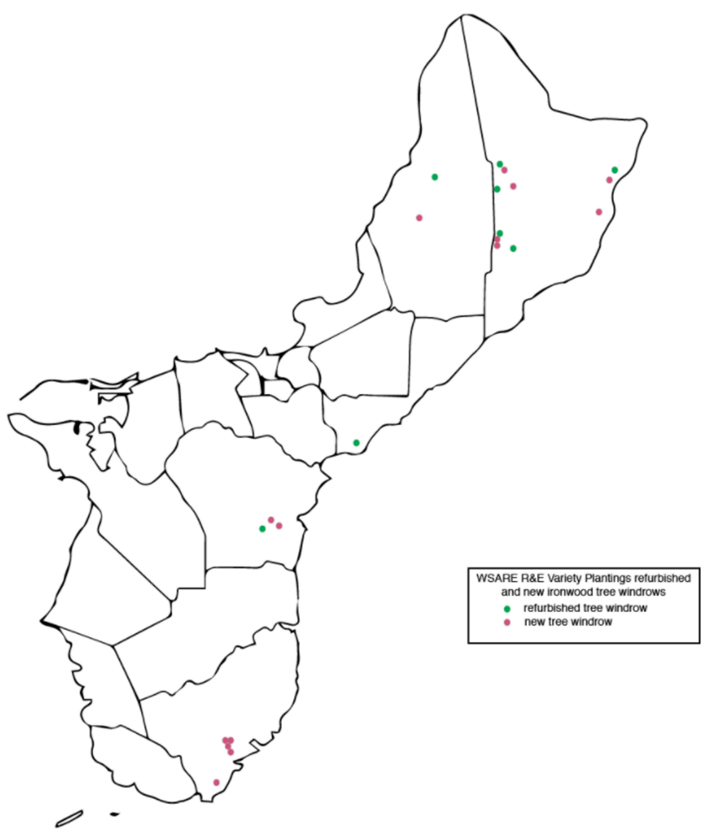

Figure 40. Locations of windrows planted across Guam

Table 6. Information on *Casuarina equisetifolia* trees from which cones were harvested and seeds collected for preservation

UOG Clinic No.	Tree Tag no.	Origin	GPS(N/E)	Location	Date Cones collected
15-216	84	Malaysia	13.56895/144.93149	AAFB, Guam	3/23/21
15-219	81	Papua New Guinea	13.56907/144.93166	AAFB, Guam	3/23/21
15-225	75	India	13.57127/144.93329	AAFB, Guam	3/23/21
15-228	72	Kenya	13.57219/144.93384	AAFB, Guam	3/23/21
15-232	68	Vietnam	13.57271/144.93423	AAFB, Guam	3/23/21
15-233	67	Australia	13.57439/144.93724	AAFB, Guam	3/23/21
15-234	66	Egypt	13.57454/144.93755	AAFB, Guam	3/23/21
15-235	65	Sri Lanka	13.57490/144.93835	AAFB, Guam	3/23/21
21-141	n/a	Guam	13.47786/144.75767	Hagatna, Guam	7/7/21

Table 7. Ironwood seedlings given away between October 2021-January 2022

Received by	**# of Seedlings**	**Date**	**Project**
Agriculture and Life Sciences Building, UOG Campus	27	10/29/2021	Demonstration Garden
Russel Young, AAFB	108	11/29/2021	Andersen Golf Course windrow
Dr. Robert Schlub, UOG	36	12/30/2021	Rs inoculation experiment
Russel Young, AAFB	140	1/13/2022	Andersen Golf Course windrow
Ren Chau	50	1/14/2022	Farm Windrow
Mangilao Golf Course	120	1/14/2022	Mangilao Golf Course windrow
Dr. Bob Bevacqua, UOG	10	1/14/2022	Prizes for UOG-sponsored public festival

Table 8. Deteriorating windrows refurbished across Guam

Location	Date planted	Number of trees planted	Tree origins used	GPS- beginning of windrow	GPS- end of windrow
Palm Tree Golf Course, AAFB	June 2019	12	Guam (9), Vanuatu (3)	13.57476, 144.93803	13.56912, 144.93175
Palm Tree Golf Course, AAFB	June 2019	6	Guam (2), Vanuatu (2), China (2)	13.56127, 144.93317	13.56187, 144.93282
Bernard Watson Farm, Yigo	June 2019	15	Vanuatu (3), Malaysia (3), Egypt (2), PNG (1), China (3), Vietnam (1), Sri Lanka (2)	13.56637, 144.87721	13.56702, 144.87700
Bernard Watson Farm, Yigo	June 2019	15	Vanuatu (3), Malaysia (3), Egypt (2), PNG (1), China (3), Vietnam (1), Sri Lanka (2)	13.56587 144.87730	13.56583, 144.87688

Distribution of *Ralstonia solanacearum* in ironwood trees in the islands of Guam, Saipan, and Rota and the potential threat posed to the islands

Background: Whether indigenous or native, the ironwood trees (*Casuarina equisetifolia*) are tightly integrated into the local culture and the island's environment. Ironwood is a hardy, pioneer, salt-resistant tree that occurs on the island's main soil types: limestone, volcanic and coral sand. Its ability to fix free nitrogen allows it to thrive in low nutrient soils. On the sandy beaches of the Mariana Islands, it has become an important perching tree for the white-collared kingfisher, and the Mariana fruit-dove. The white tern, *Gygis alba*, commonly lays eggs in it. As global warming accelerates sea level rise (estimated to be 20–60 cm by 2100) and storm intensity, it is important to have healthy stands of ironwood trees as part of the shelterbelt to protect the sandy shoreline of the Mariana Islands. Shelterbelts are strips of vegetation composed of trees and shrubs grown along the coasts to protect coastal areas from high winds. They also serve the purpose of sand binders and prevent sand erosion. Some of the important species suitable as for coastal bioshields are *Casuarina equisetifolia*, *Manilkara littoralis*, *Ficus hispida*, *Thespesia populneoides*, *Hibiscus tiliaceous*, *Baringtonia asiatica*, *Pongamia pinnata*, *Azadirachta indica*, *Morinda citrifoia*, *Cassia fistula* L., *Cocos nucifera*, *Anacardium occidentale*, *Syzygium* spp. and *Pandanus tectorius.*

In 2010, *Ralstonia solanacearum* (Rs) was detected in Guam's ironwood trees (*Casuarina equisetifolia*). This led to a survey of ironwood trees in 2015, where it was discovered that Rs is widespread in Guam's ironwood tree population. In 2019, based on the analysis of a set of 77 medium and large trees, the bacterium *Ralstonia solanacearum* and the fungus *Ganoderma australe* species complex were found to be significant predictors, with p values of <0.001 and < 0.008, respectively. Characterization of samples from Guam in 2020 by the University of Hawaii, confirmed the presence of two species in Guam's ironwood trees – *R. pseudosolanacearum,* phylotype I (origin Asia and Africa continent) (Rps) and *R. solanacearum,* phylotype II (America continent) (Rs). The Rps strain accounts for 95% of Guam's ironwood isolates. *R. solanacearum* is a highly heterogeneous bacterial species and is now considered a member of the *R. solanacearum* species complex (RSSC).

Survey for method for *Ralstonia solanacearum* (Rs) in the Mariana Islands: Drill shavings were collected from the main trunk of the tree from beneath the bark layer, using a sterilized 1/4 inch drill bit which was drilled approximately 1.5 inches into the trunk of the tree. Approximately 0.2 grams of drill shavings were collected into Agdia test buffer extraction pouch and crushed with hammer or other means. Pouch was allowed to settle for 2-3 minutes before an Agdia test strip was placed into the pouch. Results were read once the buffer solution reached the top of the Agdia test strip. Two pink lines = positive for *R. solanacearum*; one line (top line only) = negative for *R. solanacearum*; one line (bottom line only) = invalid test strip.

Survey for *Ralstonia solanacearum* (Rs) on the island of Rota: In June on the island of Rota, 89 Agdia Rs-specific ImmunoStrip tests were performed on 47 ironwood trees. During the 6-day survey, 22 ironwood trees tested strongly positive for *R. solanacearum* (**Table 9**). These trees were widespread across Rota in 13 different locations (**Figure 41**). This is extremely significant for forest health in Rota, and it is clear that this disease is not a new occurrence. The importance of healthy stands of ironwood on the coastline around the towns of Sinapalo in east central Rota and Songsong on the far western portion of the island is imperative. The disease was frequently found in small trees, thinning and die-back of branches was often accompanied by the presence of dead foliage. The symptoms observed in the ironwood trees are less like what is seen on Guam and more like the rapid death of trees found in China. Another similarity with China, is the near 100% association between Rs positive tree and trees in poor health. Also few of these unhealthy trees had conks or termites which is the opposite of what is found on Guam.

To reduce the impact of bacterial wilt on Rota's susceptible trees ironwood (Casuarina equisetifolia) soursop (*Annona* spp.), mango (*Mangifera* spp.) and guava (*Psidium* spp.), I recommended that all tools that may move the sap from one tree to another (such as chainsaws, machetes, or drills) should be disinfected between trees. Any product suitable for killing bacteria on hard surfaces will work such as 70% alcohol or Clorox (1 part bleach to 9 part water) or similar product. Also identify areas on Rota where trees are showing symptoms of bacterial wilt and confirm the presence of the disease through the use of the Agdia Ralstonia specific immunostrip. If practical, cut down infected trees and do not replant or allow the establishment of new seedlings for at least one year.

Survey for *Ralstonia solanacearum* (Rs) on the island of Saipan: In 2017, a smaller ironwood Rs survey found no positive trees. This included the Capitol Hill Baseball where 8 trees were found to be positive in the current survey (**Table 10**). In April of 2022, on the island of Saipan, ironwood and other hosts of RSSC were surveyed over a 5-day period (**Figure 42**) Of these, 41 were collected from ironwood trees and 46 were from non-ironwood trees and various crop species. A total of 9 ironwood trees in two separate locations tested positive (**Table 10**). All trees which tested positive were tested multiple times to confirm the results. The symptoms observed in the ironwood trees are very similar to those associated with ironwood decline in Guam (Gradual thinning and die-back of branches remaining attached foliage is green, occurring on medium to large trees).
To keep Saipan's ironwood trees as healthy as possible, I suggest you limit the impact of RSSC in your ironwood trees. I suggest the following:
1) Continue surveying ironwood trees for RSSC using Agdia immunostrips and recording test results. RSSC infected trees do not occur randomly across the landscape but in cohorts; therefore, give priority to those areas where trees are in poor health and or areas more prone to disease such as areas of higher elevations, areas where tools and lawn care practices may spread the bacterium, and areas prone to poor tree growth. 2) If practical, remove all trees that test positive. Dispose of such trees in a non-ironwood area. Allow grass to grow for a couple of years and replant with anything other than ironwood. If you plant another ironwood in that location, monitor that tree for RSSC every few years. 3) Disinfect all tools that may move the sap from one tree to another (such as chainsaws, machetes, or drills) with Clorox or alcohol.

Survey for *Ralstonia solanacearum* (Rs) on the island of Guam: Over the course of three years (2019-2021), 63 trees at 20 sites, scattered across Guam, were tested for Rs, of which 26 were positive (**Figure 43**). Those trees with greater degrees of decline were more likely to test positive to Rs; though, there were three healthy trees at two locations that also tested positive (**Table 11**). The decline and eventual death of many of Guam's ironwood trees is in large part believed to be due to RSSC; but instead of acting like a pathogen it acts like an opportunist. Trees on Guam are dying to a disease complex which I refer to as ironwood tree decline (IWTD). It is my belief that disease and death associated with ironwood tree decline is closely linked to stressful conditions arising from both biotic (RSSC, wet wood bacteria and the fungus Ganoderma) and abiotic factors (poorly drained soils, and the movement of bacteria from lawn and forestry tools). Though RSSC occurs in Guam, China and India, it only accounts for 65% of the trees with IWTD symptoms in Guam, whereas in China and India RSSC accounts for 100% of unhealthy trees with bacterial wilt symptoms. In China and India where RSSC acts as a pathogen, trees appear healthy one day and dead within weeks to a few months. In Guam, trees infected with RSSC gradually lose their foliage, with death occurring in a matter of years.

Due to the slow progression and general sporadic nature of IWTD on Guam, it is likely IWTD could be reduced substantially through cultivar selection and adoption of cultural practices that promote healthy growth and preclude conditions favorable for pests (termites) and pathogens (wood-rots, root-rots and bacteria).

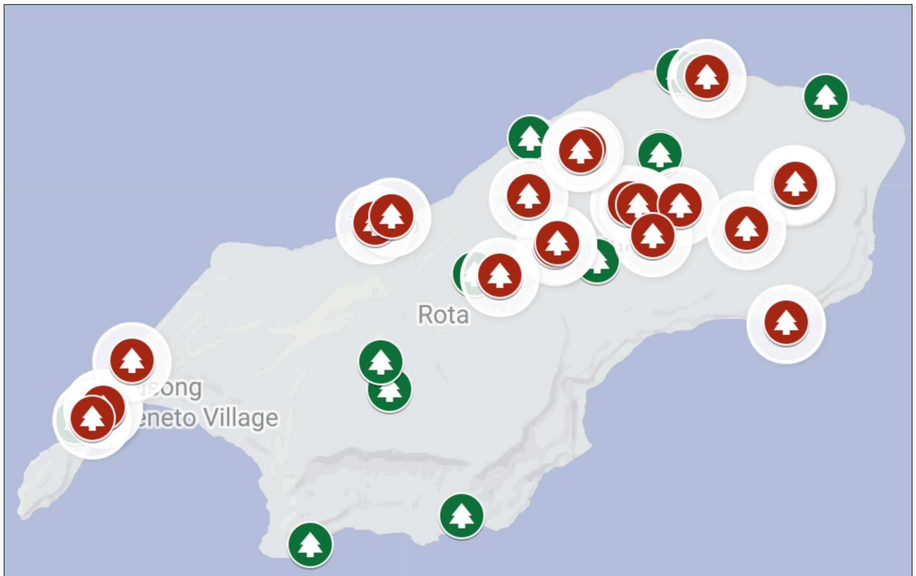

Figure 41. Locations of trees tested on Rota. Positive trees in red. Negative trees in green

Table 9. *Ralstonia solanacearum* survey of ironwood trees on Rota July 2022, clinic numbers 22-338 through 22-421

Location	Disease severity of individual trees	Respective Rs test results of individual trees (+ or -)
Mayor Prudencio Taisacan Manglona Harbor Island Park	2	-
Ace Hardware	4,0	+, -
Beach behind Ace Hardware	2	+
Sinapalu 3	3,1,0,3	+,-,-,+
Teteto Beach	4,4	+,+
Gampapa	3,1,4	+,+,+
Mount Sabana	2,1	-,-
Pona Point	1	-
Taiapu	1	-
Funta	1,4,1	-,+,-
Rota Resort	4,3,4	+,+,+
Mochong Beach	1,1,3	-,-,+
Rota Airport (back)	3,1,2,3,3	-,-,+,+,+
Mua	2,3	-,-
As Matmos Cliff	1	-
Taga Latte Stone Quarry	4	+
l'Chenchon Park Bird Sanctuary	1,3,2	+,-,-
Baseball Park	0	-
Rota Airport (front)	4,4	-,+
Pinatang Park	3,2,0	+,-,-
Behind Rota Resort Golf Course	1,1	-,-
Nanyo Kohatsu Kabushiki Kaisha Sugar Mill	2	+

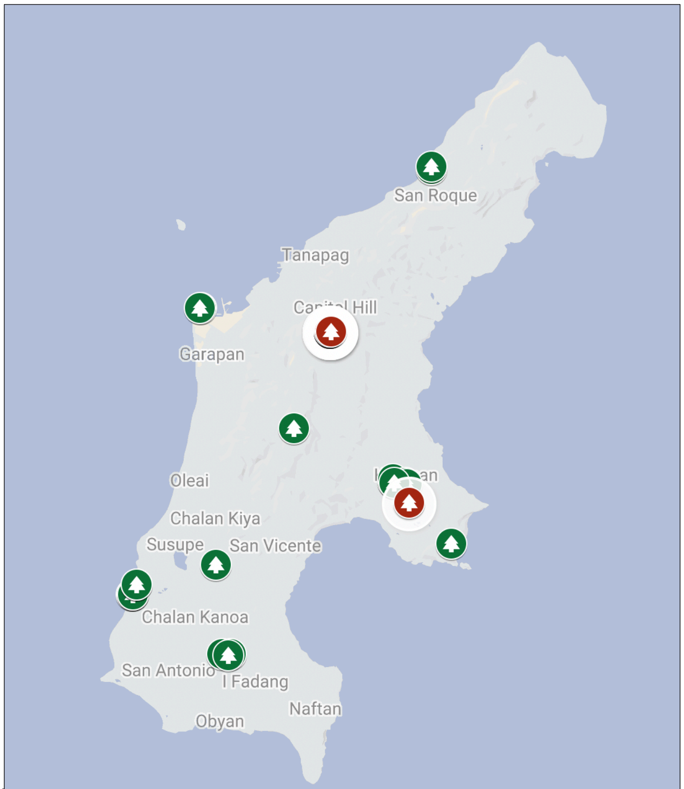

Figure 42. Locations of trees tested on Saipan. Positive trees in red. Negative trees in green

Table 10. *Ralstonia solanacearum* survey of ironwood trees on Saipan April 2022, clinic numbers 22-259 through 22-337

Location	Disease severity of individual trees	Respective Rs test results of individual trees (+ or -)
American Memorial Park, Saipan	3,4	-,-
Department of Agriculture, Kagman	2,3	-,-
Route 34	4,1,4	+,-,-
Laly 4	0,0,3,2	-,-,-,-
Pau Pau Beach	0,1	-,-
San Isidro Beach Park	3,2	-,-
Jack Ogumoro Farm	1,1	-,-
Forbidden Island	4	-
Mt. Tapocha	0	-
Capitol Hill Baseball Park	3,3,1,3,3,2,3,2,1,2,2,2	-,+,-,+,+,+,+,+,+,+,-,-
Rt 35/Flame Tree Rd (Airport)	3	-
United Cargo Building, Airport	3,3	-,-
Northern Marianas College	2	-

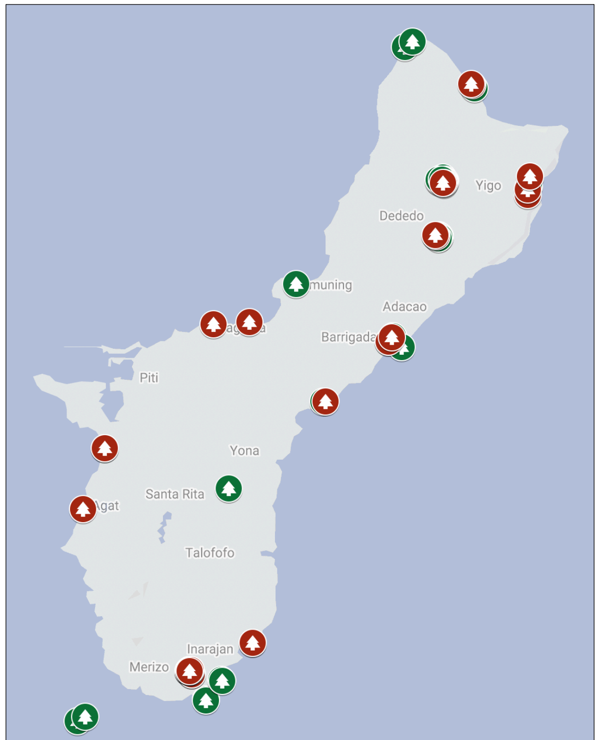

Figure 43. Locations of trees tested on Guam. Positive trees in red. Negative trees in green

Table 11. *Ralstonia solanacearum* survey of ironwood trees on Guam 2019-2021, clinic number from 19-76 to 19-109, 20-122 to 20-135, and 21-150 to 21-174

Location	**Disease severity of individual trees**	**Respective Rs test results of individual trees (+ or -)**
UOG Yigo Station	3,0,1,4,4,4	3-,-,-,+,+,+ 6
Watson's Farm, Yigo	3,4,2,0,1,1,0,1,1,0,4,3,3,4,3	2-,-,-,-,-,-,-,-,-,-,-,-,-,+,+15
UOG Ija Station	3,4,4,4,4,4,-,-,4	7-,-,+,+,+,+,+,+,+9
Mangilao Golf Course	0,4,1,1,-	2-,-,-,+,+5
Windward Golf Course	4,1	-,-2
Ritidian	1,0	-,-2
Cocos Island	2,1	-,-2
Thousand Steps	0	-1
Ysrael Beach	4,1	-,- 2
Sagan Kotturan Chamoru	1	- 1
AAFB	3,1,4,1	2-,-,+,+4
Duenas Beach	0,1	-,-2
Tarague Beach	0	-1
UOG, Mangilao	4,3	1-,+2
UOG Inarajan Station	-,-	2+,+2
Nimitz Park	0	1+1
Paseo Park, Hagatna	0	1+1
Apaca Point, Agat	0,0	2+,+2
Governor's Complex, Aniquia	0	1+1
AAFB, tarague beach	4,3	26 2+,+2 63

Risk of infection by RSSC to new tree plantings: To determine the likelihood of seedling of mixed varieties becoming infected after outplanting, (irrespective of the presence or absence of ironwood trees and whether or not infected) 113 seedlings were planted in 9 windrows and later tested for Rs (**Table 12**). Based on the fact that only one tree became infected, the risk of infection by RSSC under normal circumstances is very low.

A small experiment was conducted in 2020-2022. It consisted of planting 24 Kenya and 24 Malaysia seedlings in four locations under different pressures of RSSC: in soil where an infected tree once stood, around an infected tree, and around a healthy non-ironwood tree. Based on this experiment, there appears to be little or no risk of infection if trees are planted in soil that is not within the root zone of an infected ironwood tree, even though an infected tree may have stood there at one time. The chance of infection is 20% when trees (irrespective of variety) are planted in the root zone on an infected tree. However, when the tree variety is considered (susceptible Malaysia vs resistance Kenya) the chance of infection can be as high as 40% and as low as 0.

Table 12. Presence of *R. solanacearum* in tree stands planted in 2021 and 2022 on the island of Guam

Location	**Number of trees**	**Number of varieties**	**Alive or dead**	**Test results of live trees**	**Date tested**
Windward Hills Golf Course A	15	5	14 alive 1 dead	- (14)	7.19.2022
Windward Hills Golf Course B	15	5	11 alive 4 dead	+ (1) - (10)	7.19.2022
Yigo Experiment Station A	15	5	14 alive 1 dead	- (14)	7.20.2022
Yigo Experiment Station B	15	5	14 alive 1 dead	- (14)	7.19.2022
Bernard Watson's Farm A	15	7	5 alive 10 dead	- (5)	7.27.22
Bernard Watson's Farm B	15	7	8 alive 7 dead	- (8)	7.25.2022
Bernard Watson's Farm C	15	7	11 alive 4 dead	- (11)	7.25.2022 7.27.2022
Bernard Watson's Farm D	10	8	9 alive 1 dead	- (9)	7.27.2022
UOG Demo Garden	27	9	27 alive	- (27)	7.15.2022

Termite related research

Summary: Based on a collection of termites from 48 ironwood trees, the 'higher' termite, *Nasutitermes takasagoensis* was the most common accounting for 93% of the samples. The lack of *Ralstonia* spp. and the low presence and abundance of wetwood bacteria in the gut of these termites suggest that they are not a vector for these pathogens of IWTD. At taxonomic level two in SILVA (Phylum level), Spirochaetes (48.22 %) and Fibrobacteres (41.43 %) were found to be the most dominant phyla followed by Bacteroidetes (3.61%), Proteobacteria (3.35%), Margulisbacteria (0.84%), Acidobacteria (0.77%), Planctomycetes (0.65%), and others (1.61%). It was determined that the presence or absence of *Ralstonia* in trees from which termites were collected showed no significant effect on the total bacteria community; however, the communities of termites collected from sick trees showed significantly higher bacterial richness compared to those from healthy trees. Plot of the first two dimensions of the NMDS ordination of the weighted Unifrac distance matrix showing the similarity of bacteria communities of termite samples within and between the Ralstonia negative and Ralstonia positive groups. In conclusion, the lack of Ralstonia and the low presence and abundance of wetwood bacteria suggest that termites are not a vector for these pathogens of IWTD. For all the bioassays of *N. takasagoensis* workers consumption of wood pieces with different levels of Ralstonia and wet wood bacteria, the average mortality rate of 32.9 ± 5.69% was observed. One-Way Analysis of Variance showed a significant effect ($p = 0.0007$, R2 =0.2604) of the food source on net consumption by *N. takasogoensis* workers. "High Rs and High WW" had the lowest consumption and "No Rs and No WW" had the highest consumption.

Materials and methods, Guam: At the University of Guam, from 2019-2020 termites were collected from infested ironwood trees across Guam **(Figure 44).**

In 2019, a total of 64 termite samples from 32 ironwood trees were collected and sent to Louisiana State University for species identification and analysis of gut contents. In 2021, the above methods were repeated to collect an additional 37 termite samples from 34 ironwood trees which were also sent to Louisiana State University for species identification and analysis of gut contents (**Figure 44).**

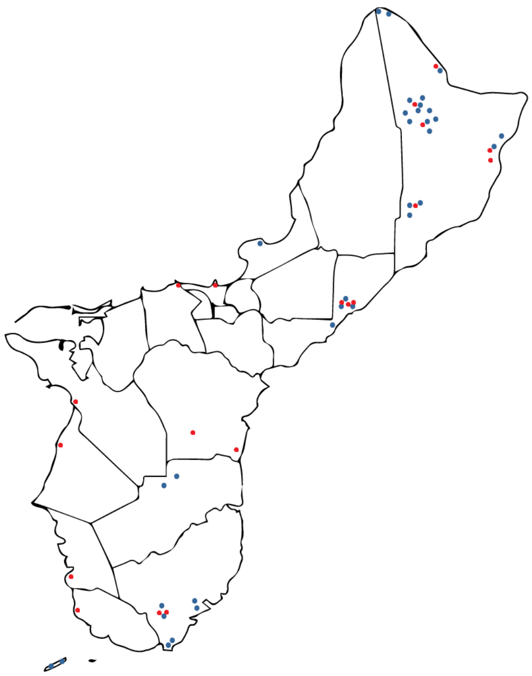

Figure 44. Ironwood tree from which termites were collected on Guam; blue dot (2019), red dot (2021)

Table 13. Site and tree variables and their descriptions from which termites were collected and sent to Louisiana State University (LSU) along with a short statement (< 50 words) about LSU findings regarding gut content of said termites.

Location related	
Factor: Description	**LSU findings**
Location: Area where the tree was located	Had no significant influence on alpha diversity in any of the three data sets (Full, Only SF and without SF)
Parent material: The type of parent material (Lime, Tuff or Sand) at that location.	
Site management: Sites were classified into three categories based on the extent of maintenance: No maintenance, Moderately managed, Highly managed	Did not have significant influence on bacterial richness
Altitude: Location of tree in relation to mean sea level (in meters), taken at ground level at the base of the tree.	The altitude of the tree location showed a weak but significant correlation to the phylogenetic diversity of the bacteria.
Altitude classification: Altitude was classified as "low" for less than 100 meters and as "high" for greater than 100 meters.	(High> 100 m vs Low< 100m): The bacterial communities in the phyla Spirochaetes and Fibrobacteres of termites collected from ironwood trees at high altitudes (n=25) showed greater phylogenetic distance within samples than those of termites from trees located at low (n=17) altitude.
Tree related	
Presence or absence of *Ralstonia*	Ralstonia: The presence or absence of Ralstonia in trees

***solanacearum* species complex:** Drill shaving of trees were tested for presence (+) or absence (-) of *Ralstonia* using the Agdia Strip Test.	from which termites were collected showed no significant effect on the total bacteria community (Full dataset). However, Ralstonia presence had a significant influence on ASV richness and Shannon diversity of the Spirochaetes and Fibrobacteres (Only SF) community and marginal influence on environmental bacteria (Without SF).
Decline Severity: The level of damage to the tree due to disease determined by visual inspection based on fullness of branches and dieback (0= symptomless, 1=slight damage, 2=distinctly damaged, 3=heavily damaged, and 4=nearly dead)	Decline severity of the tree marginally influenced phylogenetic diversity of the termites' bacteria community.
Health: Using tree Decline Severity, the individual trees were classified as "healthy" (DS= 0) or "sick" (DS= 1, 2, 3, or 4)	The bacterial communities of termites collected from sick trees (n=33) showed significantly higher richness compared to those from healthy trees (n=9). The phylogenetic distances were also greater between the bacterial communities of termites collected from sick trees than those of healthy trees.
Plot related	
Plot Average DS: Only live trees within the plot were counted and the average disease severity of the plot was determined.	The average decline severity (Plot Average DS) of the plots influenced some aspects of the bacterial diversity in the termites. The bacterial composition within termite samples was more even in less damaged tree plots.
Plot Average Health: Using Decline Severity, each tree within the plot was classified as "healthy" (DS= 0) or "sick" (DS= 1, 2, 3, or 4). An average health of the plot was determined by the percentage of sick trees to the total number of live trees in the plot.	
Percentage of dead trees in plot: Percentage (%) of dead trees within the sample tree's 30m radius	
Percentage of trees with termites in plot: Percentage (%) of live trees with existing or previous termite activity within a given plot	Increasing number of termite infested trees in a plot was significantly associated with less bacterial diversity.
Stand Maturity Estimate: It indicates the age/ maturity of trees in a stand. It was calculated using basal area per acre divided by number of trees per acre	The age of the tree stand showed a marginal correlation to the phylogenetic diversity of the bacteria in the Full dataset and significant correlation in Without SF data set.
Full data set: contains all ASVs with taxonomic assignment. Only SF data set: contains only the major phyla Spirochaetes and Fibrobacteres. Without SF data set: contains the minor phyla after excluding Spirochaetes and Fibrobacteres from the full data set.	

Abstract submitted by Garima Setia for inclusion in the 2022 Entomological Society of America-Joint Southeastern Branch and American Phytopathological Society-Caribbean Division meeting:

Investigation of potential role of termites as pathogen vectors in the decline of ironwood trees (Casuarina equisetifolia) in Guam

The numbers of an indigenous agroforestry species, Ironwood (*Casuarina equisetifolia*), in Guam are declining. This mysterious ironwood tree decline (IWTD) started in 2002 and is still prevalent to date. A previous study identified bacterial pathogens from the *Ralstonia solanacearum* species complex and wet wood bacteria species (Klebsiella oxytoca, and Klebsiella variicola) as causal organisms of IWTD. Various additional factors were statistically associated with IWTD, including termite infestation. Termites are known to carry a diverse microbiome, which enables them to thrive on a lignocellulose diet. Therefore, we hypothesized that termites could be vectors of plant pathogens causing IWTD. To investigate the potential role of termites as pathogen vectors, we employed next-generation 16S rRNA gene sequencing to describe bacteria diversity and differential abundance present in termites collected from 48 ironwood trees of different disease stages in Guam in association with location- plot-, and tree-related factors. *Nasutitermes takasagoensis* (Family Termitidae) was present in 93% of the termite infested trees. Bacterial phyla composition of *N. takasagoensis* workers was typical for wood-feeding higher termites consisting of Fibrobacteres, Spirochaetes as well as Margulisbacteria. However, *Ralstonia* species were not detected and Klebsiella species were rare even in termites collected from trees infected with *Ralstonia* and wetwood bacteria. This suggests that *N. takasagonesis* workers are not a vector for pathogens causing IWTD in Guam. Factors such as site management, tree and plot health, percentage of trees with termite damage in the plot, *Ralstonia* presence in trees, and altitude had a significant influence on bacteria diversity of termite samples.

Bacterial related research

From the analysis of 26 water cultures of the bacterial wilt pathogen by the University of Hawaii, it was determined that the bacterial wilt pathogen in Guam's trees is best described as *Ralstonia solanacearum* species complex (RSSC). Their analysis revealed two strains and thus two potential origins of Guam's pathogen. The most common was Phylotype I which is present in North and Eastern Asia, whereas less common was Phylotype II which occurs in Central America.

Materials & Methods: Though *R. solanacearum* could be detected from wood chips, drill shavings, water from root drill shavings, stems and branches of trees, attempts to isolate from these same samples failed. The only means by which Rs could be isolated was by streaking ooze that formed on disks taken from stems, roots, or large branches of infected trees onto selective medium. To enhance the production of ooze, slices were placed on a saturated paper-towel in a moisture chamber for 24 hrs. Once formed, the ooze was streaked on Engelbrecht's semi-selective medium (mSMSA) (Engelbrech 1994). Colonies were re-streaked onto SMSA, which was followed by streaking onto modified Kelman's tetrazolium chloride medium (TZC) before grow-out on TZC (Norman and Alvarez 1989). A single colony from modified SMSA media was picked and mixed in 50 microliters of nuclease-free water. The colony was denatured for 10 minutes at 95^0C and centrifuged for two minutes. The colony was used as a template to do endpoint PCR with *Ralstonia solanacearum* species complex specific primers. DNA extraction was performed with cultures found to be positive with PCR. The DNA extracted was used as a template to perform dnaA specific PCR for sending the samples for sequencing.

Results: All the strains isolated from ironwood were able to grow on modified Kelman Tetrazolium Chloride (TZC) media and modified SMSA media. The growth was observed within 48 hours with light to dark pink pigmentation at 28^0C. The identity of strains was first confirmed using colony PCR using *Ralstonia solanacearum* species complex (*RSSC*) specific end point PCR primers. The PCR products were electrophoresed at 100V for 40 minutes to visualize the amplicons. All the 25 strains isolated from ironwood were found to be positive with *RSSC* specific primers. Genomic DNA was extracted from all the strains using DNeasy Blood and Tissue kit (Qiagen, Valencia, CA). The extracted DNA was amplified using *RSSC* specific *dnaA* primers. All the strains were found to be positive. The BLASTn results showed all the strains from ironwood to be *Ralstonia pseudosolanacearum*

Ironwood Conference

The ironwood was founded by a USDA, WSARE research and education grant (SW19-906) as part of the grant's education and outreach mission. The purpose of the conference was to bring researchers together for discussion and presentations that may be helpful in unraveling the roles played by *Ralstonia solanacearum*, *Ganoderma australe*, wetwood bacteria, and termites in the decline of Guam's ironwood trees. This was accomplished by presentations from past and current researchers of ironwood tree decline (IWTD), the sharing of relevant literature, by presentations on research that have a direct bearing to the subject, and by field trips to ironwood decline sites and agroforestry outplants.

Ironwood (*Casuarina equisetifolia*) decline conference

Exchange of knowledge and research discovery to ameliorate
the impact of bacterial wilt in *Casuarina equisetifolia*

Unraveling the roles of *Ralstonia solanacearum* species complex, *Ganoderma australe*, wetwood bacteria, and termites in the decline of Guam's ironwood

Sponsors
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United States NIFA and Western Region SARE Program (WSARE)

Proceedings
Micronesia: Journal of the University of Guam

In-person and virtual conference (no charge)
January 4-7, 2022

Contacts:
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Location:
University of Guam, Agriculture and Life Sciences Building (ALS) Room 127
Mangilao, GU

Tentative Program

Location-Date-Time	**Activity**	**Presenter**
Tuesday Jan 4		
	Breakfast on your own	
8:30 am	Pickup from hotel Island tour and visit decline sites • Guam National Wildlife Refuge: Ritidian Unit (Natural stand of healthy ironwood trees)	Davis, Dr. Schlub
12:00 noon	Lunch	
1:00 pm	Continuation of tour • Ija Experiment Station (decline & exp. site) • Windward Hills golf course (decline & exp. site)	Dr. Schlub, Alex
4:30 pm	Return to UOG or hotel	

Wednesday Jan. 5		
8:00 am Room #125A (ALS)	Donuts, fruit, coffee, juice	
8:30-8:45 am Room #127 (ALS)	Start of conference	Dr. Yudin
8:45-9:15 am Room #127 (ALS)	"Overview: Guam's Ironwood (*C. equisetifolia*) and its decline"	Robert Schlub (in person and virtual) University of Guam
9:15-9:45 am Room #127 (ALS)	"Statistical applications into Ironwood trees (*C. equisetifolia*) decline on Guam and varietal selection"	Karl Schlub (virtual) Louisiana State University (Thesis)
9:45-10:15 am Room #127 (ALS)	"Overview of *Ralstonia solanacearum* species complex (RSSC) and population biology"	Mohammad Arif University of Hawaii
10:15 am	Break	
10:30-11:00 am Room #127 (ALS)	"Phylogenetic characterization and genealogy of strains in *Ralstonia solanacearum* species complex (RSSC) associated with ironwood decline in Guam"	Sujan Paudel (in person and virtual) University of Hawaii (Thesis)
11:00-11:30 am Room #127 (ALS)	"Genome evolution of *Ralstonia solanacearum* species complex (RSSC) associated with ironwood decline in Guam"	Dario Arizala (virtual) University of Hawaii (Thesis)
11:30-12:00 noon Room #127 (ALS)	"Field deployable recombinase polymerase amplification assay and other methods used at the University of Hawaii to differentiate Ralstonia solanacearum species complex and related bacteria from Ironwood samples"	Shefali Dobhal (virtual) University of Hawaii (Thesis)
12:00 noon	Lunch	
1:00-1:30 pm Room #127 (ALS)	"Comparisons between Ironwood tree decline and bacterial wilt in China and India"-	Robert Schlub (in person and virtual) University of Guam
1:30-2:00 pm Room #127 (ALS)	"Occurrence and control of bacterial wilt in *C. equisetifolia* coastal shelterbelts in Southern China"	Zhang Yong (virtual) Chinese Academy of Forestry
2:00-2:30 pm Room #127 (ALS)	"Casuarina research in China"	Zhong Chonglu (virtual) Ret. Chinese Academy of Forestry
2:30 pm	Break	
2:45-3:15pm Room #127 (ALS)	"Disease management in high density clonal plantations of Casuarina in India"	Abel Nicodemus (virtual) Indian Council of Forestry Res. & Ed.
3:15-3:45 pm Room #127 (ALS)	"Possible biocontrol agents for bacterial wilt disease in Casuarina"	Arumugam Karthikeyan (virtual) Indian Council of Forestry Res. & Ed.
3:45-4:15 pm Room #127 (ALS)	Bacterial wilt of Eucalyptus	Teresa Coutinho (virtual) University of Pretoria
6:00 pm	Dinner at Meskla Chamoru Fusion Bistro	

Thursday Jan. 6		
8:00 am Room #125A (ALS)	Donuts, fruit, coffee, juice	
8:30-9:00 am Room #127 (ALS)	"Bacterial wilt in Florida and diagnostic procedures used at the University of Florida"	Carrie Harmon (virtual) University of Florida
9:00-9:30 am Room #127 (ALS)	"Introduction into termite biology, feeding and symbiosis and termites of Guam's Ironwood trees (*C. equisetifolia*)"	Dr. Claudia Husseneder (in person and virtual) Louisiana State University
9:30-10:15 am Room #127 (ALS)	"Investigation of potential role of termites as pathogen vectors in the decline of Ironwood trees (*C. equisetifolia*) in Guam"	Garima Setia (in person and virtual) Louisiana State University (project)
10:15 am	Break	
10:30-11:00 am Room #127 (ALS)	"Ganoderma associated with tree death and decline in southern United States"	Jason Smith
11:00-11:30 am Room #127 (ALS)	"Tree varieties in place of dying Casuarina"	Anand Persad (in person and virtual) ACRT Services
11:30-12:00 noon Room #127 (ALS)	"Climate and Island States"	Anand Persad (in person and virtual) ACRT Services
12:00 noon	Lunch	
1:00-2:30 pm	Departure for Bernard Watsons Farm	
3:00-4:30pm	Yigo Experiment Station	
5:00-7:00 pm	Evening- Chamorro Village or other venue	
Friday Jan. 7		
8:00 am Room #125A (ALS)	Donuts, fruit, coffee, juice	
8:15-10:30 am Room #127 (ALS)	Conference summary of discussions and recommendations	Dr. Schlub, chair (in person and virtual)
10:30 am	Adjournment of conference	
10:30-11:30 am	WSARE Research Project team members	Dr. Schlub, research team
11:30 am	Adjournment of research team	
Saturday Jan. 8		
	Departure of research project team members and other invited researchers	

Robert Schlub (rlschlub@triton.uog.edu), Karl Schlub (schlubk@gmail.com), Mohammad Arif (arif@hawaii.edu), Sujan Paudel (sujanpau@hawaii.edu), Dario Arizala (arizala@hawaii.edu), Shefali Dobhal (shefali@hawaii.edu), Zhang Yong (zhangyongritf@caf.ac.cn), Zhong Chonglu (zhongchl@caf.ac.cn), Abel Nicodemus (nicodemus_a@rediffmail.com), Arumugam Karthikeyan (karthikarumugam13@gmail.com), Teresa Coutinho (teresa.coutinho@up.ac.za), Carrie Harmon (clharmon@ufl.edu), Claudia Husseneder (chusseneder@agcenter.lsu.edu), Garima Setia (gsetia1@lsu.edu), Anand Persad (apersad@acrtinc.com)

PCR multiplex to differentiate *Ralstonia solanacearum* species complex, including *R. solanacearum*, *R. pseudosolanacearum* and Select Agent R3bv2 strains

Paudel S, Dobhal S, Lowe-Power T, Schlub RL, Allen C, Alvarez AM, Arif M (2020) (Abstract; APS Annual Meeting, Virtual).

Ralstonia solanacearum R3bv2 strains, listed as a Select Agent in the United States, is a highly destructive pathogen causing bacterial wilt on many crops worldwide. A sensitive, accurate and discriminative tool is critical for monitoring, surveillance, and seed certification for domestic and international regulatory agencies. Unique genes in *R. solanacearum* R3bv2 and other strains in the *R. solanacearum* species complex (RSSC) were identified through comparative genomics. Primers and probes showed 100% specificity with the target strains based on *in-silico* analysis. The assay was validated using 3 sets of primers/probes specific for RSSC, RS R3bv2 and UIC (universal internal control). The RSSC primers were designed to amplify all *Ralstonia* genospecies. The 2nd primer set was specific for RS R3bv2, and UIC was used to monitor potential inhibition during amplification. The specificity of the developed assay was confirmed with 94 bacterial strains (n=42 RS R3bv2, n=43 other RSSC and n=9 other species and endophytes). No false positives or negatives were obtained with any strain in the exclusivity or inclusivity panels. The sensitivity of the developed assay was 1 pg. for both pure bacterial genomic DNA and bacterial DNA spiked into plant DNA assays (potato and geranium). The RSSC assay detected and discriminated the target pathogens from plant samples with 100% accuracy with no false positives or negatives (both naturally and artificially infected). The developed assay has potential applications in routine diagnostics, disease management, and agricultural biosecurity.

Phylogenetic characterization and genealogy of strains in the *Ralstonia solanacearum* species complex associated with ironwood decline in Guam

Paudel S, Dobhal S, Hu J, Schlub R, Alvarez AM, Arif M (2021). (Abstract for presentation: APS Annual Meeting 2021, virtual).

The bacterial wilt pathogen, *Ralstonia solanacearum* (Rs) was first detected in wood drill shavings from declining ironwood trees of Guam in 2010. Extensive surveys in 2015 and 2019 confirmed the island-wide presence of *Rs* associated with ironwood. Whereas a majority of ironwood *Rs* strains were later reclassified as Phylotype I, *R. pseudosolanacearum* (*Rps*), two strains were Phylotype II and later confirmed to be Rs through *dnaA* gene sequences. Carbon source utilization and chemical sensitivity patterns for all ironwood *Rps* strains were similar. The genetic relatedness between the Guam ironwood strains and other *Ralstonia* strains worldwide was examined in the effort to trace their ancestral genotypes. Three housekeeping and two virulence-related genes were used to infer the diversity, evolutionary relationships, and genealogy of representative ironwood strains. The *Rps* population in Guam was considered to be highly clonal based on low nucleotide diversity and lack of any significant evidence of recombination in individual gene sequences. Our analysis showed that the potential origin of ironwood Phylotype I *Rps* strains was in the geographical region of North and Eastern Asia, Indonesia, or Northern

Australian, whereas the geographical region around Central America, South-Eastern USA, Northern Latin America, and Caribbean may be the potential origin of Phylotype II *Rs* strains. The predominant ironwood *Rps* population in Guam recently diverged from a common ancestor as shown by a Timetree analysis.
Thesis title: Evolutionary relationships and molecular diagnostics of *Ralstonia solanacearum* species complex associated with declining ironwood trees in Guam. Sujan Paudel graduate research involved the analysis of Guam's ironwood tree samples. His research was conducted under the supervision of Dr. Mohammad Arif. Thesis submitted to: University of Hawaii at Manoa, Honolulu, HI

Thesis research

One Student, Sujan Paudel, graduated from this project under the supervision of Dr. Mohammad Arif.
Thesis title: Evolutionary relationships and molecular diagnostics of *Ralstonia solanacearum* species complex associated with declining ironwood trees in Guam.
Thesis submitted to: University of Hawaii at Manoa, Honolulu, HI

Paudel thesis abstract

Ironwood (*Casuarina equisetifolia*) is an important component of the island of Guam's culture and ecology. Different biotic and abiotic factors have been associated with the declining ironwood trees on Guam. The bacterial wilt disease caused by *Ralstonia solanacearum* species complex (*RSSC*) was considered an associated factor after the positive test results of *RSSC* specific immunostrips with ooze from declining trees. The long-range spread, broad host range along with emerging new hosts of the bacterium necessitated the characterization of *RSSC* ironwood decline strains to understand the diversity, evolutionary relationships among the strains and predict the future movement pattern. In this study, we developed an efficient protocol for the isolation and characterization of *RSSC* strains from the declining trees. We also used the MLST approach to study the diversity, evolutionary relationships and predict the genealogies of the isolated strains. The presence of fast-growing saprophytes in the declining trees necessitated the development of field deployable and rapid Exo-RPA assay in our study. Furthermore, we also developed a five-plex multiplex PCR assay to detect and differentiate the genospecies of *RSSC* strains associated with ironwood and all other hosts including the Select Agent (SA) group. The isolation of *RSSC* from the declining ironwood tree was found to be highly effective with the use of modified SMSA media from the root slices. Both *R. pseudosolanacearum* (Rps) and *R. solanacearum* (Rs) were found to be associated with decline although the later species was found to be much lesser in number (3) compared to the former species (35). The phenotypic characterization assays showed the similar utilization pattern for the Rps strains and for the Rs strains. The ironwood Rps population in Guam was found to be highly clonal with least nucleotide diversity and contracting population structure. Our analysis showed North and Eastern Asia and Indonesia and Northern Australia region as the potential origin of ironwood Phylotype I strains whereas Central America and South-Eastern USA and Northern Latin America and Caribbean clade may be the potential origin of Phylotype II ironwood strains. The unique target genomic region for the molecular diagnostics of *RSSC* ironwood strains was determined using whole genome based comparative genomics approach and ORTHOMCL. The developed RPA assay was found to be highly specific, rapid, field- deployable, and efficient in detection of targets directly from the infected plant material with least or no effect of plant inhibitors. When tested with a total of 110 *RSSC* strains from all the genospecies and SA group, the developed multiplex assay gave accurate results with no cross- amplification. The multiplex PCR assay also successfully differentiated the genospecies

from the artificially inoculated plant DNA proving its high usefulness in culture characterization, routine diagnostics, and surveys. Microscopy, labeling studies and whole-genome based studies will provide better understanding about the role of secondary saprophytes in ironwood decline and unlock the questions about role of the bacterium as a primary or an opportunistic pathogen.
Key words: Ironwood decline, characterization, molecular diagnostics, diversity, genealogies

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Setia, G., Chen, J., Schlub, R., Husseneder, C., (2022, March 26-30). Investigation of potential role of termites as pathogen vectors in the decline of ironwood trees (*Casuarina equisetifolia*) in Guam [Conference presentation abstract]. 2022 Joint Southeastern Branch & APS-CD Meeting, San Juan, Puerto Rico. https://esa.confex.com/esa/2022seb/meetingapp.cgi/Paper/156060

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