Population Genetics, Endosymbionts and Bunchy Top Virus Studies of Banana Aphid (*Pentalonia sp.*) in the Philippines

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Population Genetics, Endosymbionts and Bunchy Top Virus Studies of Banana Aphid (*Pentalonia sp.*) in the Philippines

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ABSTRACT

Managing and controlling perennial plant diseases to increase agricultural production while protecting the environment is an important strategy in integrated pest management and sustainable agriculture. Using molecular ecology tools, I study the banana aphid (*Pentalonia nigronervosa* Coquerel), an insect vector which play an important role in the acquisition and spread of bunchy top disease caused by *abaca bunchy top virus* (ABTV) and *banana bunchy top virus* (BBTV) affecting abaca (*Musa textilis* Nee) plants across three abaca germplasm collections in the Leyte, Sorsogon and Davao regions of the Philippines. First, I study the reproductive mode, genetic diversity and population genetic structure of the banana aphid using microsatellite markers. I found that they reproduce asexually with low genetic diversity and that two *Pentalonia* species are infecting abaca plants in the Philippines. Second, I identify the infection, composition, and distribution of facultative bacterial endosymbionts in these two aphid species, determine if aphids hosted the same symbionts within aphid colony, and evaluate if they were influenced by geographic location, aphid species and the plants susceptibility to bunchy top disease. I found that four endosymbiotic bacteria infected *Pentalonia* aphids and infections were not consistent within aphid colony. Geographic location influenced endosymbiont communities but did not vary with aphid species and across abaca accessions. Lastly, I identify the bunchy top virus infecting banana aphid and quantify the viral load present in a single aphid. I found that BBTV was present and seemed to be causing bunchy top disease in abaca. I then determine the distribution of the virus across germplasm collections and evaluate if the presence or viral copy number was influenced by collection location, *Pentalonia* species, the endosymbionts present, whether ants were present on the plants the aphids were collected from and plant susceptibility to the disease. I found that the presence of BBTV depended on collection sites and aphid species, and that infection was highest in Davao but lowest in Sorsogon. Meanwhile in Leyte, I found that *Pentalonia* species and *Wolbachia* were observed as the main factors that influenced the presence of BBTV. With

these findings on the population genetics, endosymbionts, and the bunchy top virus in banana aphids, I discuss the implications for bunchy top disease management on abaca and make recommendations for future sustainable integrated pest management strategies of *abaca bunchy top virus* in the Philippines.

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DEDICATION

I humbly dedicate this thesis to my beloved parents, Tatay Lalio and Nanay Beling, whose faith in God and unselfish service to the community have inspired me to do the same.

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LIST OF ABBREVIATIONS

AbMV	*Abaca mosaic virus*
ABTD	Abaca bunchy top disease
ABTV	*Abaca bunchy top virus*
AFP	Acquisition feeding period
BBrMV	*banana bract mosaic virus*
BLAST	Basic Local Alignment Sequencing Tool
bp	base pair
BTD	bunchy top disease
CFC	Common Fund Commodity
CMV	*cauliflower mosaic virus*
CO1	cytochrome c oxidase I
ct	cycle threshold
DNA	deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DOST	Department of Science and Technology
EtOH	Ethyl alcohol
FIDA	Fibre Industry Development Authority
glm	generalized linear model
GHGs	Greenhouse gases
H_E	expected heterozygosity
H_O	observed heterozygosity
HWE	Hardy-Weinberg Equilibrium
IPM	Integrated Pest Management
IPTG	Isopropyl-β-D-thiogalactopyranoside
LB	Luria Bertani
LD	Linkage Disequilibrium
LE	Linkage Equilibrium
LGUs	Local Government Units
lme	linear mixed model
MiMi	Multi-Individual Microsatellite Identification
MLGs	multilocus genotypes
NA	null allele
NaCl	Sodium chloride

NARC	National Abaca Research Centre
NCBI	National Centre for Biotechnology Information
ng	nanogram
NTC	no template control
ORF	open reading frame
PCR	Polymerase Chain Reaction
PhilFIDA	Philippine Fibre Industry and Development Authority
qPCR	quantitative Polymerase Chain Reaction
R&D	Research and Development
RiRkSsW	*Regialla-Rickettsia-Serrattia-Wolbachia*
RkSsW	*Rickettsia-Serrattia-Wolbacia*
RiRkW	*Regialla-Rickettsia-Wolbachia*
RiSs	*Regialla-Serrattia*
RkW	*Rickettsia-Wolbachia*
rcf	relative centrifugal force
RT-PCR	Real-time Polymerase Chain Reaction
SCMV-Ab	*sugarcane mosaic virus in abaca*
SDGs	Sustainable Development Goals
SSRs	Simple sequence repeats
Ta	annealing temperature
UNIDO	United Nations Industrial Development Organization
UPLB	University of the Philippines, Los Baños, Laguna
VSU	Visayas State University
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
µg	microgram
µL	microliter
µM	micromolar

DECLARATION

I hereby declare that the work has been done by myself and no portion of the work contained in this thesis has been submitted in support of any application for any other degree or qualification on this or any other university or institution of learning.

Signed: Marciana B. Galambao

Date: November 30th, 2020

CHAPTER 1: GENERAL INTRODUCTION

Agriculture is the backbone of the global economy and the source of food, fibre and fuel (Schmidhuber and Tubiello, 2007; Gillespie et al., 2017; Sishodia et al., 2020; Taylor and Amidy, 2020). Now more than ever, this industry faces a huge challenge in achieving global food security as the current global population of 7.8 billion people is projected by the United Nations to increase to 8.5 billion in 2030 (UN, 2019) and the required agricultural products are expected to increase by 70% - 100% by 2050 (Kastner et al., 2012; Tilman et al., 2011; Zabel et al., 2019). However, it has been reported that agriculture accounted for 80% global deforestation, 70% freshwater usage, 50% freshwater biodiversity loss, 29% global GHGs (Greenhouse gas), 70% terrestrial biodiversity loss and 52% agricultural land degradation (CBD, 2014; ELD Initiative, 2015; GSDR, 2019; WWF, 2020). In addition, the global problem of macroplastic (Patricio Silva et al., 2021; Shen et al., 2020) and microplastic pollution (Campanale et al., 2020; Schmid et al., 2021) has shifted our focus to produce and use environmental-friendly, renewable, and biodegradable products. Hence, like food, the demand for biofuel and fibre production is expected to increase. Specifically, the high demand for sustainable materials and eco-friendly fiber-based composites in industrial and manufacturing sectors has led to the use of natural fibres over synthetic fibres (Oliveira Duarte et al., 2019; Kumar et al., 2019; Thyavihalli Girijappaman et al., 2019; Lee et al., 2021). For example, Abaca (*Musa textilis* Nee) has been used in the automotive, aviation, maritime, furniture and textile industries for its tensile strength and resistance to corrosion and saltwater degradation (Armecin et al., 2014; Simbaña et al, 2020). With this, the production of plant-based fibres which are sustainable, renewable, and biodegradable is expected to increase to alleviate the environmental damage caused by using synthetic fibers (Thyavihalli Girijappaman et al., 2019; Asim et al., 2020).

Safeguarding the biotic and abiotic environment in the face of agricultural intensification to increase agricultural production has become even more

challenging with the effects of climate change. Moreover, agricultural expansion coupled with agricultural land conversion have hastened the loss of biodiversity and ecosystem services (Diaz et al., 2019; Pe'er et al., 2020). Hence, food security and climate change are two interconnecting forces that need immediate action to address world food hunger and biodiversity loss. Under the United Nations 2030 Agenda for Sustainable Development, number two of the 17 Sustainable Development Goals (SDGs) states that we should 'end hunger, achieve food security and improved nutrition and promote sustainable agriculture' (UN, 2015). This urgent need to practice sustainability in agriculture for food security and ecosystem services protection is a worldwide challenge (Garibaldi et al., 2017; Pe'er et al., 2020).

Sustainable agriculture and integrated pest management

Sustainable agriculture is a concept that considers the long-term stability in the economic, environment and social aspects of farming while promoting the resilience and persistence of production (Garibaldi et al., 2017). One of the threats to agricultural production and the greatest challenge to achieving global food security is the presence of plant diseases (Bentham et al., 2020). The emergence of insect pests, diseases and pathogens is expected to increase with the advent of climate change (Renoz et al., 2019; Garrett et al., 2006). Globally, plant diseases have devastating effects economically, socially, and ecologically (Velasquez et al., 2018). In 2019, for example, the global estimates of yield losses due to occurring and re-occurring pests and diseases accounted for 21.5% in wheat, 30% in rice, 22.5% in maize, 17.2% in potato and 21.4% in soybean (Savary et al., 2019).

To manage and control insect pests responsible for the transmission of plant diseases and pathogens is a huge challenge. One of the farming practices that reduces the environmental impact of agriculture and improves social and economic outcomes is the use of integrated pest management (IPM) (Alwang et al., 2019). It is defined as the 'farmers best use of a mix of control tactics that are biologically, environmentally, economically, socially and culturally

acceptable' (Kenmore et al., 1985). The basic concept is to use the best option in controlling plant pests and diseases using all available methods while reducing the use of chemical pesticides (Stenberg, 2017). However, a recent study showed that beneficial insects were harmed by contaminated honeydew when IPM-recommended insecticides were used (Calvo-Agudo et al., 2020). Nevertheless, different IPM management practices had been conducted already on pea aphid (reviewed in Sandhi and Reddy, 2020).

In 2015, eight principles of IPM were proposed which includes the prevention of pest occurrence and suppression of pest populations; monitoring of harmful pests; informed decision-making; priority to non-chemical methods; multi-criteria selection of pesticides; pesticides use reduction; avoidance of pest resistance to pesticides and evaluation (Barzman et al., 2015; Rossi et al., 2019). The focus of these principles is geared to the ecology, environment, and economic aspects of pest management. However, the real challenge encountered by plant scientists worldwide is how to expedite the basic aspect of understanding plant diseases (molecular, ecology and epidemiology) and develop long-term solutions for the prevention and management of these diseases confronting modern agriculture and beyond (Velasquez et al., 2018). In 2019, Dara presented a new paradigm approach to IPM which emphasize not only the ecology and economic aspects but also the management, business, and sustainability aspects of pest management, highlighting the significance of research and outreach (Dara, 2019).

Abaca and its economic importance

As an agricultural country, agriculture in the Philippines continues to provide not only food subsistence but also employment to more than 9.70 million Filipinos (PSA, 2020a). In 2019, agricultural export earnings accounted for approximately $7 billion (PSA, 2020b). Major crops include coconut, rice, corn, fruits crops (banana, pineapple, mango), legumes, abaca, plantation crops (cacao, coffee, oil palm, rubber, and sugarcane), root crops (sweet potato, cassava, gabi) and vegetables (PSA, 2016). Out of the total land area of 30

million hectares (ha), 13 million ha are devoted to agriculture, 180,302 hectares of which are cultivated for abaca (*Musa textilis* Nee) production, managed by 122,758 abaca farmers (Barbosa et al., 2020; PhilFIDA, 2018). Abaca is one of the high-value and commercially grown crops and one of the top export commodities of the country, with a revenue ranging from $93.2 million in 2019 to $97.7 million in 2020 (Arcalas, 2020). These values are high but represent a decline in export revenue from the previous revenue of $111 M in 2016, $130 M in 2017 and $111.5 M in 2018. Abaca revenue is expected further to decline this year due to the damage caused by Super Typhoon Goni that hit recently in Catanduanes, the abaca capital of the Philippines.

Abaca is an endemic plant to the Philippines and northern Borneo (Spencer, 1953) and was first formally described in 1801 by Don Luis Nee (Göltenboth, 2005). It is believed to be the first wild plant domesticated by the Filipinos (native of the Philippines) (Spencer, 1953) and is now cultivated in Indonesia, Ecuador, Central America, Equatorial Guinea and Kenya (Lalusin and Villavicencio, 2015). Grown primarily as fibre crop used in textile, paper, maritime and automotive industries (DaimlerChrysler, 2004, Milan and Göltenboth, 2005; Bledzki et al, 2006; Armecin et al., 2014; Lalusin & Villavicencio, 2015), abaca is a major source of strong natural fibres for domestic and international markets, supplying 85% of the world fibre requirements and provides direct and indirect employment to nearly 1.5 million Filipinos (Milan & Göltenboth, 2005; PhilFIDA, 2018; Barbosa et al., 2020).

To cater for demands both domestically and internationally, the industry needs to increase abaca production and expand the area used for abaca plantations. However, issues such as low productivity, unavailability of high-quality planting materials, lack of capital for farm production inputs (i.e. fertilizers, pesticides) and poor road infrastructure hinder abaca production and expansion (Milan and Goltenboth, 2005; Armecin et al., 2014). In addition, agricultural land conversion, use of traditional and susceptible varieties, over-harvesting, limited research on varietal improvement and natural calamities also limits crop production (Lalusin and Villavicencio, 2015). Moreover, the industry is

threatened with a number of viral diseases that are highly destructive and are rapidly spreading throughout the country (Gonzal et al., 2005; Lalusin and Villavicencio, 2015). The industry is threatened by bunchy top disease (BTD) (Raymundo et al., 2001; Halos, 2008); affecting crop production and quality of planting materials (Lalusin and Villavicencio, 2015; Galvez et al., 2020). The BTD disease is caused by *abaca bunchy top virus* (ABTV) and *banana bunchy top virus* (BBTV) (Raymundo et al., 2001; Bajet and Magnaye, 2002; Sharman et al., 2008) and is transmitted by the banana aphid (*Pentalonia* sp.).

Abaca diseases and control measures

The most devastating diseases affecting the abaca industry are the *abaca bunchy top virus* (ABTV), *abaca mosaic virus* (AMV) and *abaca bract mosaic virus* (ABMV) (Lalusin and Villavicencio, 2015). Of the three, ABTV is the most destructive as it suppresses plant growth making the plant stunted and unproductive (Magnaye, 1989, Raymundo et al., 2001, Lalusin and Villavicencio, 2015). The ABTV, which is ranked as the number one viral diseases of abaca, has been reported to be of economic importance since 1923 (Ocfemia, 1931). It has wiped out thousands of hectares of abaca plantations in the country to date (Calinisan, 1939; Raymundo and Bajet, 2000). Although the origin of ABTV in the Philippines is not clear, some researchers suggest that it may have been in the country since the 1910s (Bajet and Magnaye, 2002). Despite ongoing efforts, a century since the first observation of the disease, comprehensive control measures do not yet exist for ABTV.

From 1925 to the present, research on ABTV has been undertaken on the aphid vector (Ocfemia, 1926); disease epidemiology and diagnosis (Ocfemia, 1930; Raymundo and Bajet, 2000; Raymundo et al., 2001; Borines et al., 2008; Purwati et al., 2008; Raymundo and Pangga, 2011); production of high yielding varieties resistant to bunchy-top disease through breeding (Diaz, 1997; Lalusin and Villavicencio, 2015); virology (Tiongco and Celino, 1972; Thomas et al., 1997; Pietersen and Thomas, 2001; Sharman et al., 2000;

Gambley et al., 2004; Furuya et al., 2006; Natsuaki and Furuya, 2007; Sharman et al., 2008; Stainton et al., 2012; Pinili et al., 2013; Kumar et al., 2015), development of virus-free planting through tissue culture (Gonzal et al., 2005) and mutation breeding (Pinili et al., 2011; Dizon et al., 2012). New technological approaches include the identification of resistant and susceptible abaca accessions using microsatellites (Descalsota et al., 2015; Boguero et al., 2016); dipstick development to detect ABTV (Espino et al., 2007; Tulin et al., 2012), and serological and molecular detection of abaca virus through polymerase chain reaction (PCR) (Sta. Cruz et al., 2016). Genetic engineering was also explored to develop bunchy top virus resistant abaca, a project in collaboration between Fiber Industry Development Authority (FIDA) and senior scientists from University of the Philippines, Diliman (UPD) and UP-Los Baños (UPLB) (Halos, 2008).

Although control measures are strongly recommended and implemented such as the use of virus-free planting materials, quarantine of infected areas, early disease detection and immediate eradication of infected plants through roguing, control of the vector using insecticides and removal of alternate hosts and clean culture (Bajet and Magnaye, 2002; Borines et al., 2008; PhilFIDA, 2016), disease incidence is still present. Notwithstanding past and current Research and Development programs, disease eradication and management practices for effective control remain elusive. The control of disease by FIDA (now PhilFIDA) through eradication (roquing) of infected plants in 1995 was not successful, primarily due to insufficient knowledge of the abaca-virus-vector interactions (Raymundo et al., 2001). In the case of ABTV management in the Philippines, high impact research has been focused on the development of high yielding and resistant varieties to ABTV, characterization of the virus, and virus detection using symptomatic and asymptomatic abaca plants, but little effort has been put into understanding the causal agent of the disease.

Aphid biology and ecology

Aphids (Hemiptera: Aphididae) are small, soft-bodied insects belonging to the Aphidoidea superfamily (Blackman and Eastop, 2017). They have an oval shape body composed of a head, thorax, and abdomen (Podsiadlowshi, 2016) with a length between 1.5 to 3.5 millimetres (Blackman and Eastop, 2000). The head comprises of a pair of antennae, a pair of compound eyes, and rostrum (use to access plant phloem); the thorax consists of three pairs of legs and two pairs of wings (absent in apterae); and the abdomen which can possess (or not) a pair of cornicles (Turpeau et al., 2010) used for pheromone secretion and a cauda (tail) used for 'flicking' honeydew droplets (Blackman and Eastop, 2017). The mouthpart stylets of the aphid are composed of mandibles and maxillae stylets (Forbes, 1977) responsible for plant cell wall penetration and host plant selection, respectively (Powell et al., 2006; Brozek et al., 2015). The aphid body colour (green, yellow, brown, red, pink, or black) varies between and even within aphid species, which is influence by biotic (host plants, predators, endosymbionts) and abiotic (temperature, light) factors (reviewed in Tsuchida, 2016).

Aphids were distributed in temperate regions dating back to the Triassic period (Dixon, 1995; Grimaldi and Engel, 2005; Wegierek et al., 2017; Loxdale and Balog, 2018). It is in temperate regions that they are capable to alternate between sexual and asexual reproduction, known as cyclical parthenogenesis or holocyclic life cycle (Blackman and Eastop, 2000; 2017) whereas in tropical regions, they tend to reproduce by obligate parthenogenesis (anholocyclic life cycle) with asexual reproduction all year round (Figure 1). Hence, the majority of all aphid species can be found in northern hemisphere temperate zone (Blackman and Eastop, 2008) with a few aphid species in the tropical region (Dixon et al., 1987; Blackman and Eastop, 2006; Clarke et al., 2020).

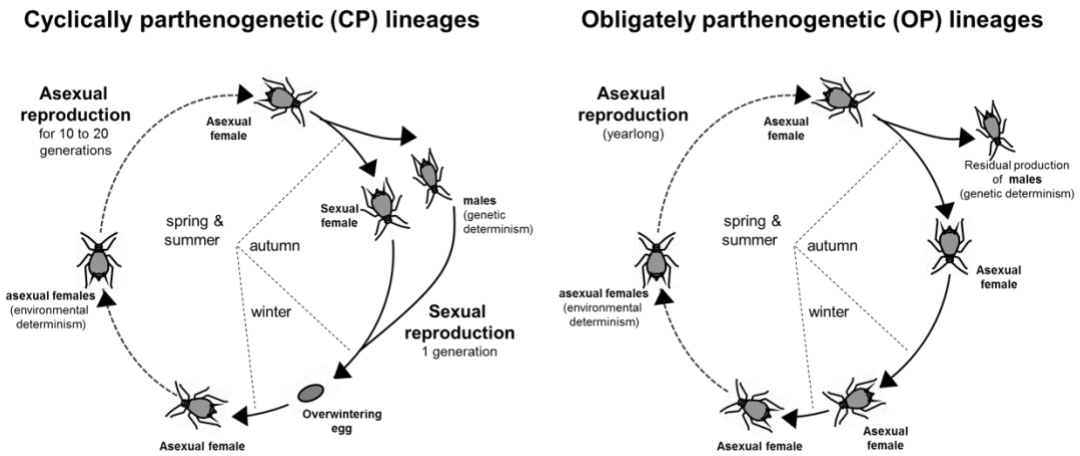

Figure 1. The life cycle of aphids showing cyclically and obligately parthenogenetic lineages typical of temperate and tropical regions respectively. Image adapted from https://www6.rennes.inrae.fr/igepp_eng/Research-teams/Ecology-and-Genetics-of-Insects/Projects/ANR-SexAphid. Copyright, Julie Jaquiery, INRAE.

Globally, more than 5000 aphid species have been identified, belonging to more than 700 genera and representing 30 subfamilies (Favret, 2018; Mille et al., 2020). They are known as major crop pests that cause damage to the agricultural, horticultural and forestry sectors worldwide through direct feeding and indirect transmission of plant viruses (Blackman and Eastop, 2000; van Emden and Harrington, 2007; 2017). Aphids transmit about 50% of all plant viruses (Nault, 1997; Gaafar and Ziebell, 2020), however, from the 450 species documented on agricultural crops, only 100 species are considered to have devastating effects economically (Blackman and Eastop, 2000; van Emden and Harrington, 2017).

Considered as the ‘insects for all seasons’, aphids have a range of adaptations with regards to their life cycle, reproduction, polyphenism and host plants (Loxdale et al., 2017; Loxdale and Balog, 2018). The life cycle of an aphid is composed of two types with regards to host plant utilization (Hardie, 2017). As sap-sucking insects, aphids host plant preference is vital for their nutrition and reproduction. The majority of aphids are known to be specialists (99%) but

some species (10%) alternate between primary (woody plants during summer, winter, and spring) and secondary host plants (herbaceous plant during summer (Eastop, 1986; Podsiadlowski, 2016). They live on primary host plants during winter, migrate (winged aphid) to herbaceous plants during spring or summer and then migrate back to their primary hosts during autumn (Dixon, 1998; Hardie, 2017). It is on the primary host plants that sexual reproduction (the mating of male and female aphids) and egg-laying takes place. The second type of life cycle is the non-host-alternating aphids where the majority of the aphids live on the same host species or other closely related host species the entire year (Hardie, 2017).

The mode of reproduction in aphids can be sexual and asexual and both forms of reproduction may co-occur within a single aphid species (Wang et al., 2007), depending on the type of environment they are in (Dixon, 1998). In the family Aphididae, the annual cycle includes a sexual generation with males and mating females (oviparae), which lay fertilized eggs that are all females. Upon hatching, each egg will give rise to a wingless “foundress” (fundatrix) that gives birth parthenogenetically to further parthenogenetic females (viviparae) (Hales et al., 1997; Ogawa and Miura, 2014). These parthenogenetic females may be winged (alate) or wingless (apterae). They tend to develop wings to fly to new plants for feeding or refuge in response to crowding, food scarcity, or in the presence of natural enemies (Hardie, 2017). The generation time of an asexual aphid ranges between 7-10 days or 7-12 days and may produce 10-100 offspring (Blackman, 1971; Loxdale et al., 2020). About 14 asexual generations are produced per growing season, or up to 20 generations annually when climatic conditions are favourable (Dixon, 1998; Simon et al., 2010; Loxdale and Balog, 2018). In addition, asexual reproduction is often associated with viviparity (live birth) and the telescoping of generations (Dixon, 1998), and this phenomenon enables them to reproduce exponentially.

Ants

The mutualistic relationship between ants and aphids called trophobiosis has been investigated for over a century and is thought to have started during the tertiary period (Stadler and Dixon, 1998; 2005; Yao, 2012; Blanchard et al., 2019). Like any mutualistic relationship, the balance between the costs and benefits is very important for a relationship to function. For the ant-aphid association, this can take the form of services such as reproduction, nutrition, protection, hygiene, and dispersal (Way, 1963; Blanchard et al., 2019). The honeydew produced by aphids, which contains carbohydrates, is an essential component of ant diets (Delabie, 2001; Oliver et al., 2008; Novgorodova, 2020). In return, aphids gain a protector against predators (Way, 1963; Nielsen et al., 2010), parasites (Brodeur et al., 2017) and sooty mold fungi (Stadler and Dixon, 2008; Novgorodova, 2020). Ants from the genus *Formica* (Nielsen et al., 2010; Novgorodova and Kryukov, 2017; Novgorodova, 2020), prevent fungal infection in aphids with their 'quarantining behaviour', which detect and eliminate fungal-infected aphids from the host plant (Novgorodova, 2020). Aside from protection, ants also help aphids to be more fecund throughout their lifetime and improve offspring production (Stadler and Dixon, 1999; Flatt and Weisser, 2000; Yao, 2014). However, their presence also limits the dispersal capacity of aphids, resulting in low genetic diversity caused by fragmented aphid populations due to limited migration (Yao, 2014).

Recent studies have shown that bacterial endosymbionts are involved in ant-aphid interactions (Ivens et al., 2018; Depa et al., 2020). The presence of endosymbionts seems to affect the protein component of honeydew in aphids, which in turn may affect ants' attendance (Sabri et al., 2013; Depa et al., 2020). Since both ants and endosymbionts confer protection in aphids, it has been hypothesized that the infection of protective endosymbionts would be reduced in the presence of ants due to duplication of function (Erickson et al., 2012). A survey conducted in 2015, showed high infection of *Serratia* in ant-tended aphids but the absence of two facultative endosymbionts (*Hamiltonella* and

Regiella) whose protective function against parasitoids is being provided by ants (Henry et al., 2015). Aside from influencing symbionts, ants also affect the aphid life cycle (Depa et al., 2020). It has been shown that the presence of ants may hamper the seasonal migrations of host-alternating aphids (Kindlmann et al., 2007; Depa et al., 2020) and may influence them to switch their mode of reproduction to asexual on the secondary hosts (Moran, 1992; Simon et al., 2002; Ivens, 2014; Depa et al., 2020).

The banana aphid and bunchy top disease

The banana aphid (*Pentalonia nigronervosa* Coquerel, 1859) is the vector that transmits bunchy top virus to abaca (Bajet and Magnaye, 2002) and banana (Kumar et al., 2015) (Figure 2). They are also called *Pentalonia nigronervosa* van der Goot and *Pentalonia caladii* van der Goot (van der Goot, 1917; Foottit et al., 2010). Another two species have been reported to be under the genus *Pentalonia*, the *P. gavarri* (Eastop, 1967; Martin, 1987) which was discovered in southeast Asia and Australia on Poaceae, and *P. kalimpongensis* Basu (Basu, 1968) which was found in northwest India on Zingiberaceae (Foottit and Maw, 2019). Aside from abaca and banana, banana aphids also infest ornamental gabi (*Caladium bicolor*), camia (*Hedychium coronarium*) and Butuan (*M. balibisian* Colla) (Lomerio, 1984; Bajet and Magnaye, 2002).

There are 100 aphid species from the Aphididae family that significantly affect agriculture and 15 species of these are economically important (Blackman and Eastop, 2017). Although banana aphid is not included on the lists, it is one of the most important agricultural insect pests commonly distributed in tropical and subtropical regions (Africa, Asia, and greenhouses in Europe, North America (Blackman and Eastop, 1984; Robson et al., 2007), Oceana and South America. It was first discovered during the 19th century on banana plant on the island of Reunion (Coquerel, 1859), recorded to be found on ferns in 1922 on Oahu (Honolulu, Hawaii) (Timberlake, 1924; Zimmermann, 1948) and first reported in the Philippines in the 1930s (Ocfemia, 1930). Previous studies

conducted on banana aphids have explored the biology (Robson et al., 2007; Anhalt et al., 2008), ecology (Bhadra and Agarwala, 2010), molecular and morphometric analysis (Foottit et al., 2010), endosymbionts (De Clerck et al., 2014; De Clerck et al., 2015), population genetics (Galambao, 2011), host plant preferences (Foottit et al., 2019), virus transmission (Hu et al., 1996; Anhalt et al., 2008; Watanabe and Bressan, 2013) and currently on genomics of the species (Mathers et al., 2020).

Figure 2. Banana aphids infesting an abaca plant (Left) and abaca with bunchy top disease caused by ABTV and BBTV (Right).

Banana aphids can be found on the pseudostem, youngest unfurled leaves and on the underside of the old leaves of Abaca (Lalusin and Villavecenccio, 2016). They are mostly found on the petiole of abaca leaves and outside of the furled young leaf of the plant. Based on vector transmission of the virus to the plants, it has been reported that ABTV and BBTV can be acquired by the plants within two hours of an ‘acquisition feeding period’ (Bajet and Magnaye 2002) with a single aphid, although infection is more pronounced if a greater density of aphids infest the plant. A previous study found that aphids can harbor BBTV for 15 to 20 days (Hu et al., 1996), although Bajet and Magnaye (2002) reported that ABTV and BBTV were only retained in the aphids for 5 to

12 days. Vertical transmission of the virus from the parent to offspring does not seem to be possible (Hu et al., 1996).

Population genetics

Population genetics is crucial for understanding the ecological and evolutionary aspects of species. This is because the genetic variation of a population is a determinant of its survival and adaptation. For insect pests, studying the population genetic structure and genetic diversity are basic tools to enhance our understanding on their biology, movement, and pest forecasting (Morales-Hojas et al., 2020a), tracking their origin (Davies et al., 1999) and deciphering genetic variants that contributed to their success (Lee, 2002; Pelissie et al., 2018). Gene flow or migration information is also vital to understand insecticide resistance management, pest management and eradication programs (Kim and Sappington, 2013). All this information is important for the refinement of pest control strategies in agriculture; however, these are also influenced by different agricultural production practices and climate change (Morales-Hojas et al., 2020b). In addition, the distribution of genetic variation in a population depends on natural selection, genetic drift, gene flow, and mutation events (Roderick, 1996; Hartl and Clark, 1997; Jessen et al., 2008).

Microsatellite markers have been used previously in population genetic studies in aphids. These markers, also known as simple sequence repeats (SSRs) are short tandem repeats of one to six base pairs in length, commonly distributed across the genomes of eukaryotic and prokaryotic species (Tautz, 1989; Weng et al., 2007; Bhargava and Fuentes, 2010). They have been used to study the mode of reproduction in aphids (Brévault et al. 2008; Razmjou et al. 2010); migration and genotypic variation (Llewellyn et al. 2003; Dedryver et al. 2008; Loxdale, 2008), genetic divergence (Wang et al., 2020), host-plant adaptation (Haack et al., 2000; Peccoud et al. 2008; Kanbe and Akimoto, 2009; Margaritopoulos et al., 2009), environmental and agricultural

landscapes (Morales-Hojas et al., 2020a), cyclical parthenogenesis (Morales-Hojas et al., 2020b); host and symbionts genetic determinants of nutritional phenotype (Chung et al., 2020). Recent studies on population genetics using microsatellites have been conducted on different aphid species such as *Sitobion avenae* (Wang et al., 2020; Morales-Hojas et al., 2020b), *Sitobion miscanthi* (Morales-Hojas et al., 2020a), *Acyrthosiphon pisum* (Chung et al., 2020;), *Aphis gossypii* (Ma et al., 2019; Dong et al., 2018; Gholamian et al., 2018), *Melanaphis sacchari* (Zehnter) (Paudyal et al., 2019; Harris-Shultz et al., 2017), *Rhopalosiphum padi* (Duan et al., 2017), *Myzus persicae* (Monti et al., 2016). In the case of banana aphids, the only study in population genetics was conducted in Hawaii (Galambao, 2011).

Studying population genetics requires polymorphic markers to genetically differentiate the populations in question. In the absence of available sequencing data, the use of microsatellite markers developed from other aphid species through cross-species amplification can be used. The repeatability of the markers is one of the advantages in using microsatellite markers, however, one of the drawbacks in using developed markers not specific to your target species is the potential lack of amplification or amplification with different banding patterns that then requires time to be spent in optimizing primers for PCR amplification.

Endosymbionts

The mutualistic relationship between insects and microorganisms is ubiquitous in nature. Endosymbionts, usually bacteria, are commonly present in aphids and are classified into obligate (primary) and facultative (secondary) symbionts. A maternally inherited *Buchnera aphidicola* is an obligate symbiont present in almost all aphid species that provides them with essential nutrients in order to survive (Buchner, 1965; Douglas, 1998; Baumann, 2005; Russell et al., 2017; Guo et al., 2018) and secretes chaperonin proteins that facilitate plant virus transmission (van den Heuvel et al., 1994; Ishikawa, 1982; De Clerck et al., 2014; Alkhedir et al., 2015). This primary symbiont is housed in

specialized host cells called the bacteriocytes. The facultative endosymbionts, believed to confer resistance and susceptibility to diseases are transmitted via host plants (Skaljac, 2016), natural enemies (Gehrer and Vorburger, 2012) and infected honeydew (Darby and Douglas, 2003). Although they are not essential, they can modify the phenotypes and host plant preference of insects (Tsuchida et al., 2004; Wagner et al., 2015; Sepulveda et al., 2016), provide protection against high temperatures (Chen et al., 2000; Russell and Moran, 2006), natural enemies (parasitoids) (Oliver et al., 2003; Oliver et al., 2005; Vorburger et al., 2009, Brandt et al., 2017), and fungal pathogens (Ferrari et al., 2004; Scaborough et al., 2005; Lukasik et al., 2013; Heyworth and Ferrari, 2015), induce the loss of fecundity and cytoplasmic incompatibility (Clancy and Hoffmann, 1996; Fukatsu et al., 2000; Simon et al., 2011), and affect the transmission of the plant viruses (Ishikawa, 1982; van der Heuvel et al., 1994; Hogenhout et al., 1998; De Clerck et al., 2014; Alkhedir et al., 2015).

There are nine (*Arsenophonus, Hamiltonella defensa*, *Regiella insecticola*, *Rickettsia*, *Rickettsiella*, *Serratia symbiotica*, *Spiroplasma*, *X-type* (now *Fukatsuia symbiotica*) *and Wolbachia*) well studied symbionts present in different aphid species (reviewed in Skaljac, 2016; Zytynska and Weisser, 2016; Guo et al., 2017). Most of these endosymbiont studies have focused on identification, diversity, and geographic distribution in pea aphids (*Acyrthosiphon pisum)* (Fukatsu et al., 2000; 2001; Guay et al., 2009; Tsuchida et al., 2002; 2010; Ferrari et al., 2012), cotton or melon aphids (*Aphis gossypii)* (Gallo-Franco et al., 2019), citrus aphid (*Aphis spiraecola*) (Renoz et al., 2020), corn leaf aphid (*Rhopalosiphum maidis* (Fitch) and bird cherry-oat aphid (*Rhopalosiphum padi*) (Guo et al., 2018).

In the Asian region, the identification and geographic distribution of endosymbionts has been conducted on *Acyrthosiphon pisum* (Tsuchida et al., 2002; Tsuchida et al., 2005; Fukatsu et al., 2000, 2001), *Aphis craccivora* and *Megoura crassicauda* (Tsuchida et al., 2006), and *Rhopalosiphum maidis* and *Rhopalosiphum viridis* (Guo et al., 2019) and their effect on aphid body color (Tsuchida et al., 2010). In terms of facultative bacteria, studies have focused

on *Regiella*, *Rickettsia* and *Spiroplasma* as well as *Hamiltonella* and *Serratia* specifically in *Aphis craccivora* (Tsuchida et al, 2006; Burke et al., 2009; Brady et al., 2014) and *Acyrthosiphon pisum* aphid species (Tsuchida et al., 2002; Fukatsu et al., 2001) (reviewed in Zytynska and Weisser, 2016). However, studies on endosymbionts present in banana aphids are limited, not only in the Asian region but also in other tropical and sub-tropical areas where bananas are planted. The identification of endosymbionts present in banana aphids can provide us with information on whether certain symbionts are associated with the host plant and susceptibility or resistance to the disease, potentially important information as they are the vector responsible for the transmission of bunchy top virus.

Endosymbionts are considered to mediate insect-plant interactions (Frago et al., 2012; Biere and Bennett, 2013; Sugio et al., 2015; Giron et al., 2017) and in three-way interactions between plant host, virus, and the insect vectors (Pinheiro et al., 2015). Their possible involvement in virus transmission was first reported in the 1990s when the *Potato leafroll virus* (PLRV) transmitted by *Myzus persica*e showed a high affinity with symbionin, a chaperonin protein produced by *Buchnera aphidicola* (van den Heuvel et al., 1994; Frago et al., 2020). This symbionin (now termed GroEL), a homoloque to GroEl protein in *Escherichia coli* (Morioka and Ishikawa, 1993) is also produced by endosymbionts in whitefly (*Bemisia tabaci*) (Gottlieb et al., 2010). The interactions of proteins in whitefly and endosymbionts have been shown to expedite the transmission of Begomoviruses in the *Geminiviridae* family (Rana et al., 2019). For example, the GroEL protein expressed from *Hamiltonella* was involved in virus acquisition, retention, and transmission of *Tomato yellow leaf curl virus* (TYLCV) in whitefly (Su et al., 2013; Gottlieb et al., 2010; Kliot and Ghanim, 2013). In addition, the high infection frequency of *Hamiltonella* in *Bemisia tabaci* had increased the efficiency of virus transmission not only in Begomovirus *(Bean golden mosaic virus, Tomato severe rugose virus),* but also in Crinivirus *(Tomato chlorosis virus*) and Carlavirus (*Cowpea mild mottle virus)* (Bello et al., 2019). Aside from *Hamiltonella*-GroEL, *Arsenophonus* infected Asia II population expressed GroEL in *B. tabaci* which have been

shown to interact with *Cotton leaf curl virus* (Begomovirus) in both *in vitro* and *in vitro* studies (Rana et al., 2012). However, no evidence of interactions between *Buchnera*-GroEL protein and *banana bunchy top virus* (BBTV) from the *Nanoviridae* family was found in both *in vitro* and *in vivo* assays in *Pentalonia nigronervosa* (Watanabe et al., 2013), suggesting virus specificity.

The transfer of virus particles called virions from infected to healthy plants involves four different phases: acquisition, retention, latency, and inoculation (Stevens and Lacomme, 2017), and each phase determines the mode of virus transmission in an insect vector, such as non-persistent, semi-persistent or persistent transmission (Gutierrez et al., 2013; Jones, 2014; Chuche et al., 2017; Stevens and Lacomme, 2017). In non-persistent transmission, viruses are acquired within seconds and retained for only a few minutes as aphids only require very short stylet penetration (Stevens and Lacomme, 2017) while the semi-persistent viruses are acquired within minutes to hours, retained for several hours and transmitted for up to 2 days after acquisition (Brevault et al., 2010). In persistent transmission, viruses require minutes to hours for acquisition and inoculation to occur. A latent period is present between acquisition and retention, where the aphid can retain the virus and becomes infective until death (Stevens and Lacomme, 2017). The route of virus transmission in an aphid vector can be either circulative or non-circulative. The circulative virus passes from the gut into the haemocoel and then to the accessory salivary glands (ASGs) prior to inoculation (Blanc et al., 2014), whereas the non-circulative virus resides on or in the aphid stylets and foregut (Blanc et al., 2011; Chuche et al., 2017).

The transmission of circulative and persistent viruses are directly facilitated by the interactions between the symbionts and virus in the insect vector (Rana et al., 2012; Su et al.,2013; Kliot et al., 2014 and Pinhiero et al., 2015; Angelella et al., 2018). These viruses can also manipulate the insect vector and host plants to increase transmission efficiency (Ingwell et al., 2012; Blanc and Michalakis, 2016; Angelella et al., 2018; Mauck et al., 2018; 2019; 2020). They indirectly manipulate the insect by influencing host-plant selection and feeding

behavior (Ingwell et al., 2012; Mauck et al., 2012), increasing production of alate individuals (Blua et al., 1992; Hodge and Powell, 2010), increasing lifespan and reproduction rates (Castle and Berger, 1993; Picket et al., 1992; Pinheiro et al., 2019) and increasing feeding on virus-infected plants (Angelella et al., 2018). In plants, they can manipulate plant palatability and attractiveness (Blanc et al., 2016; Mauck et al., 2019), weaken a plant's antiviral immune system (Li and Ding, 2006; Ding and Voinnet, 2007; Pinhiero et al., 2019) and change vector movement from infected to susceptible plants (Mauck et al., 2012; 2016).

In addition, virus transmission is also influenced by host plants (Pinheiro et al., 2017), aphid density (Claflin et al., 2017), community (landscape) composition (Angelella et al., 2016; Claflin et al., 2017; Mauck et al., 2018), vectorial capacity of insect (Chuche et al., 2017) and temporal differences and seasonal changes (Angelella et al., 2019). For example, the virus transmission efficiency of *Potato leafroll virus* (PLRV) in *Myzus persicae* was reduced in turnip-reared aphids compared to physalis-reared aphids (Pinheiro et al., 2017). Aphid density and community composition may also influence aphid movement and virus transmission but depends on aphid species and virus prevalence (Angelella et al., 2016; Claflin et al., 2017). van Munster et al. (2017) reported that severe water deprivation facilitates virus transmission and that virus infected plants were a good source of virus for the insect vector. In host-associated differentiation study, the authors demonstrated its application in tracking the origin and dispersal among insect vectors (Angelella et al., 2019). Results showed two genotypic groups of *Aphis craccivora* that thrive in alfalfa and locust plants whose dispersal capability differs, with the latter showing greater capacity in early summer, indicating locust plants as an important vector for the pumpkin virus.

Study sites

The Philippine government plays a major role in the production, development, and protection of the abaca industry through research and development

(R&D). The crops R&D agenda for 2017-2022 under the Harmonized National R&D Agenda focuses on germplasm evaluation, conservation, utilization, and management; varietal improvement and selection; production of good quality seeds and planting materials; cultural management practices; crop production systems research; and postharvest, processing and product development (DOST, 2017). Cultural management practices involve the development of eco-friendly pest and disease management and control strategies, and the development of crop-disease diagnostic kits/techniques and disease management protocols.

The Philippine Fiber Industry and Development Authority (PhilFIDA), an agency under the national government of the Department of Agriculture (DA), is tasked to undertake research and development of the fiber industry of the country. The R&D focuses on conservation of abaca accessions, development of high yielding and disease resistant varieties, and post-harvest technology to cater the domestic and global markets.

For abaca conservation and varietal improvement, PhilFIDA has an existing abaca germplasm collection distributed across the country, with accessions held at Casiguran, Sorsogon; Bago-oshiro, Davao City; Abuyog, Leyte; University of the Philippines (UPLB), Laguna and the National Abaca Research Center (NARC), Visayas State University (VSU) in Leyte (As per communication with Dr. Jose L. Catalla, Chief, Research Division, PhilFIDA). Aphids for the work presented in this thesis were collected across multiple accessions held in each of these three germplasm collections.

Luzon

The Sorsogon Fiber seedbank is located at Brgy. San Juan, Casiguran, Sorsogon. The Sorsogon province is in the Bicol region, the southernmost province in Luzon. The Bicol region is the major abaca producing region of the country where abaca production is most concentrated with favorable soil and climate conducive to abaca production. The climate of the area is classified as

Type II Climate (Modified Coronas' Climate Classification) with no dry season during the year and maximum rain period from November to January (PAGASA, 2014). Rainfall ranges from 2800 mm to 3500 mm annually and temperature ranges from 21 ºC to 32 ºC (Mias-Mamonong and Flores, 2008). Located within the typhoon belt, Sorsogon tends to experience typhoons every year.

The Sorsogon germplasm was established in the 1980s with an area of 32.7 hectares (ha). The area is divided into different blocks designated for germplasm maintenance, seedling production and distribution, and research. The main purpose of the seedbank is to distribute healthy abaca planting materials to different Local Government Units (LGUs) within the province of Sorsogon. It also caters as a research and development site of the national government. There are approximately 200 abaca accessions in the area which are being duplicated and distributed to the other germplasm banks. The area is surrounded with jackfruit (*Artocarpus heterophyllus*), and Madre de cacao (*Gliricidia sepcium*) trees. Cultural management activities such as weed control through ring weeding and under brushing, fertilizer application of complete fertilizer and spraying of herbicides are routinely practiced in the area. The area is well-maintained with properly labelled accessions and well-managed abaca seedlings in greenhouse nursery (Figure 3).

Figure 3. Sorsogon germplasm collection showing the Putian accession and abaca shade house nursery.

Visayas

The National Abaca Research Center, the agency based at the Visayas State University (VSU) in Baybay, Leyte is mandated to further help the abaca industry through research, development, and extension programs (Figure 4). It has the largest collections of more than 800 accessions since its establishment in 1987, but due to Typhoon Haiyan (known as Super Typhoon Yolanda in the Philippines) in 2013, some accessions were destroyed and did not recover. In addition, with the presence of viral diseases, the center now has 138 active collections that are maintained both in *ex situ* and *in vitro* conditions (data as per communication from Dr. Luz O. Moreno).

Figure 4. NARC germplasm collection and abaca tissue culture seedlings ready for planting and distribution.

The NARC germplasm has an area of 0.25 ha where abaca, a shade-loving plant are planted surrounded with coconut (*Cocos nucifera*), bamboo (*Bambusa spinosa*) and fruit trees (Santol (*Sandoricum koetjape*), siniguelas (*Spondias purpurea*), banana (*Musa spp*.), jackfruit (*Artocarpus heterophyllus*), rambutan (*Nephelium lappaceum*), guyabano (*Annona muricata*), breadfruit (*Artocarpus altilis*)) (Figure 4). Cogon grass (*Imperata cylindrica*), billy goat weed (*Ageratum conyzoides* L.), devil weed (*Chromolaena odorata* L.) and koronatas (*Lantaca camara*) were common weeds found in the area. Cultural management activities such as weeding,

fertilizer application and spraying of herbicides are routinely practiced in the area.

The climatic condition in the area falls under the Type IV of the Coronas climatic classifications where rainfall is evenly distributed throughout the year (Balzer and Margraf, 1994). The average precipitation is 2514.50 mm/year and mean annual temperature of 28.3 ºC (Armecin et al.,2008). Although it has no pronounced dry seasons, low rainfall is recorded during the months of March, April, and May (Jahn and Asio, 2006). The Leyte Island is also prone to natural disasters such as typhoons, earthquakes, and landslides.

NARC is one the agencies actively involved not only in abaca research and development but also in extension programs within the province of Leyte and Southern Leyte. Extension services include the distribution of tissue culture planting materials to abaca farmers, providing abaca tissue culture training to private and public institutions and individuals, especially for the establishment of new tissue culture laboratories and dissemination of information on cultural management practices to different LGUs and abaca farmers. Prior to abaca seedling distribution, tissue cultured plantlets are routinely checked for bunchy top disease through disease indexing at the Plant Disease and Diagnostic Laboratory based in VSU.

Mindanao

Located in Bago-Oshiro, Davao City, the regional office of PhilFIDA was established in the 1990s with an area of two hectares for seedbank and 0.20 ha for the collection. It is one of the four regional PhilFIDA centers in Mindanao that caters to Region 11 (Davao Region) and Region 12 (formerly Central Mindanao). Twenty accessions were being maintained *ex situ* and duplicated for *in vitro* conservation when the tissue culture laboratory was operational in 2008 (Figure 5). Plants such as ipil-pil (*Leucaena leucocephala*), coconut (*Cocos nucifera* L.), sisal (*Agave sisalana*), salago (*Wikstroemia sp*.), ramie

(*Boehmeria niveau*), sili (*Capsicum annum*), Mulberry (*Morus spp*.) and banana (*Musa spp*.) surrounded the germplasm area.

Figure 5. Davao abaca germplasm collections and tissue culture plantlets inside the greenhouse nursery.

The Davao region is categorized under Type II and Type IV climates. With no dry season, significant rainfall is experienced throughout the year which ranges from 1673.3 mm to 1941.8 mm. Average annual temperature ranges from 28 ºC to 29 ºC (DA-RFO, 2014). It rarely experienced typhoons and strong winds as it is located outside the typhoon belt. With this type of climate, agriculture remains the largest economic sector in Davao where banana, pineapple, coffee, and coconut plantations are based and managed by multinational companies such as Dole, Del Monte and Sumifru/Sumitomo. Among the crops, banana is the largest fruit export of the island.

The PhilFIDA-Davao seedbank distributes healthy abaca planting materials to different LGUs within Davao and the Central Mindanao region, and to other germplasm collection sites in the country. Aside from the current germplasm area, they have also duplicated their collections in Polomolok, South Cotabato in Mindanao. One month before the aphid sampling in November 2017, they re-established their collections due to a previous drought experienced in the area. Cultural management activities include weeding, fertilization, and application of cypenture granules Furadan when need arises.

Bunchy top disease history

The first occurrence of abaca bunchy top disease (ABTD) in the Philippines was recorded in 1910 in Albay (Bicol Region) and in Silang, Cavite in 1915 (Ocfemia, 1926; Ocfemia, 1927; Bajet and Magnaye, 2002). In 1923, it became a serious disease that eventually led to the abandonment of abaca plantations in the provinces of Cavite and Laguna in 1928 (Calinisan, 1939). It was then observed in Sorsogon in 1930 (Raymundo and Bajet, 2000) and become widespread in 1980 (San Juan, 1989; Espino et al., 2002). Thereafter, the percent disease incidence was reported by FIDA in Region 5 at 20% in 1997 and 40% in 1998 (Raymundo and Bajet, 2000). In 1937, the bunchy top disease was reported in abaca plantations in Davao (Bajet and Magnaye, 2002) and in 1960, the disease was observed in banana and became problematic in 1967 (Castillo and Martinez, 1961; Espino et al., 1989; Magnaye, 1989; Galvez et al., 2020).

In the Visayas, the bunchy top epidemic was reported to wipe out more than half of the abaca plantations (16737 out of 26374 hectares) in Eastern Visayas (Leyte and Samar) (Bajet and Magnaye, 2002; Manila Bulletin, 2003; Nuñez, 2013). In 1989, the severity of disease infection was observed in Leyte. The average disease incidence of bunchy top virus (ABTV and BBTV) was reported at 5.19% and 8.16%, respectively in the Bicol and Eastern Visayas regions (Leyte and Southern Leyte) (Raymundo et al., 2002; Halos, 2008; Koh et al., 2020). A recent nationwide study conducted by PhilFIDA on Abaca Disease Management Program showed that the level of disease incidence in virus-infected areas was below 5% (PhilFIDA, 2016). In Leyte and Southern Leyte, the % disease incidence before eradication were 28.88% and 13.18%, respectively (As per communication with Mr. Jose L. Catalla).

Thesis aims and chapters

The overall aim of this PhD thesis is to develop our understanding of the banana aphid as a vector of bunchy top virus in abaca. In order to do this, I focused on three specific areas: identifying the genetic diversity and the population genetic structure of banana aphids collected from abaca plants; identifying the endosymbionts of the collected aphids; and determining the viral load present in the aphid vector. The ultimate goal of this work is to inform future sustainable integrated pest management strategies of abaca bunchy top virus in the Philippines.

Objectives

1) To study the genetic diversity and population genetic structure of the banana aphid and evaluate whether previously described species of *P. nigronervosa* and *P. caladii* could be identified and differentiated using microsatellites

2) To identify the endosymbiont communities of banana aphid collected from abaca and assess how aphid genetic background, collection location and host plant accession influences symbiont distribution and composition

3) To identify the virus that infects banana aphids and evaluate if there was an influence of endosymbionts, ants, aphid species and plant susceptibility to bunchy top disease to viral load distribution across abaca germplasm collections

This thesis is composed of three data chapters:

In Chapter 2, I present the genetic diversity and mode of reproduction of banana aphids collected from abaca plants. Using microsatellite markers, I also evaluate the previously described species of *Pentalonia* in the Philippines.

In Chapter 3, I identify endosymbionts present in banana aphids and determine the composition, distribution, frequency, and consistency of infection of individual aphids in a colony. I also investigate the influence of aphid species and plant susceptibility to bunchy top disease to endosymbionts.

In Chapter 4, I identify the virus causing bunchy top disease, quantify the presence and concentration of bunchy top virus in aphids and determine its distribution across the three main regions in the Philippines.

In Chapter 5, I evaluate the interactions between the aphids, the virus and the symbionts and their implications for bunchy top virus management in the Philippines.

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CHAPTER 2: DIVERSITY AND DISTRIBUTION OF *PENTALONIA* FROM ABACA PLANTS IN THE PHILIPPINES

Abstract

The banana aphid (*Pentalonia nigronervosa* Coquerel) is an economically important insect pest responsible for the transmission of a destructive viral disease to abaca, banana and plantain, the bunchy top disease. In the Philippines, information about the population genetic structure and genetic variation of banana aphids, as well as evidence supporting their perceived asexual mode of reproduction, is very limited. This knowledge gap impairs the ability to develop targeted pest management strategies for aphid-transmitted diseases in the country. Therefore, an in-depth understanding of the population genetic structure and genetic diversity of banana aphids is important for the development of specific and effective bunchy top disease management strategies. In this study, cross-species amplification of previously developed microsatellite markers from related aphid species was performed on banana aphids. Seven nuclear microsatellite loci were used to genotype 376 individual aphids from 188 colonies collected from 36 abaca accessions across three abaca germplasm collections in the Luzon, Visayas and Mindanao regions in the Philippines. The Bayesian model cluster assignment implemented in STRUCTURE detected two genetic clusters, which were confirmed as *P. nigronervosa* and P. *caladii* from mitochondrial cytochrome oxidase gene sequences. This finding supports previous work proposing two species of *Pentalonia* on Abaca. Data suggested low levels of genetic diversity between *Pentalonia* species across collection sites, with global F_{ST} value of 0.001 and 0.023 for *P. caladii* and *P. nigronervosa*, respectively. The majority of the individuals from both *Pentalonia* species belonged to a single clone each, which is characteristic of asexually reproducing species. Negative F_{IS} values indicated that the mode of reproduction for banana aphids in the Philippines is obligate parthenogenesis.

This information on genetic diversity and mode of reproduction of *Pentalonia* species could help in the formulation of pest management strategies which can be tailored accordingly to each species for an effective and targeted integrated pest management program.

Introduction

The banana aphid, *Pentalonia nigronervosa* Coquerel (Hemiptera: Aphididae) is a black or brown aphid belonging to the Macrosiphini tribe (Figure 6) thought to be native to Southeast Asia (Muratori et al., 2009; Timberlake, 1924; Waterhouse, 1993), but to have expanded into Africa, Europe (Portugal and Spain), North America, Oceania and South America (Blackman and Eastop, 1984; Molina et al., 2009; Robson et al., 2007). As a plant-sucking insect, *P. nigronervosa* obtains nutrients from the phloem-sap of its hosts and is strongly associated with banana plants in the Musaceae family. However, it also infests other host plants belonging to the Araceae, Costaceae, Heliconiaceae, Malvaceae and Zingerberaceae families (Bajet and Magnaye, 2002; Diekmann and Putter, 1996; Foottit et al., 2010; Lomerio, 1984; Waterhouse and Norris, 1987).

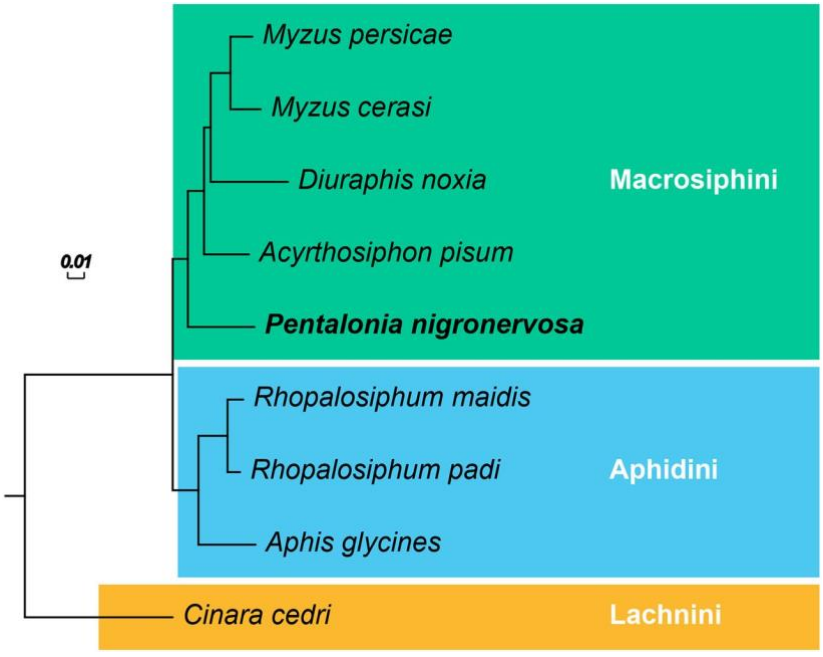

Figure 6. Maximum likelihood phylogeny of the aphid tribes showing *Pentalonia nigronervosa* under the Macrosiphini tribe. Adapted from Mathers et al., 2020.

Pentalonia nigronervosa mostly reproduces asexually (clonally) across tropical and sub-tropical regions, but sexual morphs have been recorded in

northeast India and Nepal (Bhanotar and Ghosh, 1969; Blackman and Eastop, 2000; Foottit et al., 2010). Previous studies have suggested that *P. nigronervosa* exists in two forms, the *P. nigronervosa* f. typica and *P. nigronervosa* f. caladii van der Goot (Eastop, 1966; Calilung, 1978), with the former favouring host plants in the Musaceae and Heliconiaceae while the latter has a broader host range across the Zingiberales and the Araceae (Eastop, 1966; Bajet and Magnaye, 2002; Foottit and Maw, 2019). Additional work based on molecular and morphometric analysis (Foottit et al., 2010) and on ecological and biological characters (Bhadra and Agarwala, 2010) has suggested that the two forms are distinct species (*P. nigronervosa* and *P. caladii*), a finding that is also supported by microsatellite work on populations in Hawaii (Galambao, 2011).

In the Philippines, the banana aphid has a large economic impact because it transmits the viruses causing bunchy top disease (BTD) (*abaca bunchy top virus* (ABTV) and *banana bunchy top virus* (BBTV)) to abaca (*Musa textilis* Nee) and banana (*Musa spp*.) (Raymundo et al., 2001; Bajet and Magnaye, 2002; Sharman et al., 2008; Magnaye, 1989); two of the major agricultural export commodities of the country. These viruses cause chlorotic and necrotic leaf margins, stunted growth, and bunched leaves (Ocfemia, 1926; Halos, 2008; Lalusin and Villavicencio, 2015; Qazi, 2016). They make infected plants unproductive by reducing fruit quality and quantity (Suparman et al., 2017; Kumar et al., 2015; Qazi, 2016), and reducing fibre quality or yield (Raymundo et al., 2001; Galvez et al., 2020). Few abaca accessions and hybrids have been tested for some level of resistance to the disease (Descalsota et al., 2015; Lalusin and Villavicencio, 2015; Parac et al., 2020) and no banana cultivars have been identified that are resistant to BBTV (Jekayinoluwa et al., 2020; Ferreira et al., 1997; Tripathi et al., 2016). Studies have yet to be conducted to identify markers linked to disease resistant genes or high yield in abaca and banana (Lalusin and Villavicencio, 2015). Furthermore, research is needed to better understand the virus-host interactions and the role that the vectors play in disease infection and transmission.

Population genetics is an important tool in deciphering the ecological and evolutionary processes of a target species (Hartl and Clark, 1997), and could be used to improve disease management strategies (Burdon, 1993; Powell and Powell, 2007; Margaritopoulos et al., 2009). Genetic diversity is an important factor in the emergence of insecticide resistance, and also controls vector dynamics influencing virus transmission (Hawkins et al., 2019; Jacobson and Kennedy, 2013; Monti et al., 2016; Morales-Hojas et al., 2020). For invasive insect pests such as aphids, the implementation of sustainable pest strategies could be tailored according to their population genetics (Duan et al., 2017). Information about population genetic structure coupled with assessments of population dynamics (i.e. aphids abundance) and genetically-encoded fitness traits (e.g. thermal tolerance, insecticide resistance) could facilitate forecasting of pest outbreaks and management of their populations (Wang et al., 2017; Morales-Hojas et al., 2020). Hence, a thorough knowledge of species' population structure and genetic diversity is vital to formulate an ecologically sound pest management strategy (Wang et al., 2020).

Some biotic (e.g. life cycle (Duan et al., 2017), host-plant adaptation (Via et al., 2000; Frantz et al., 2010; Ferrari et al., 2012), symbiont community (Wilson et al., 2003) and abiotic (e.g. spatial and temporal dynamics (Brunissen et al., 2009; Hlaoui et al., 2019)) factors affect the genetic diversity and population genetic structure in aphids (Loxdale et al., 2017; Loxdale et al., 2020). In addition, the intimate relationship between aphids and their host plants may lead to the development of specialized host races (Via et al., 2000) which could limit gene flow and increase differentiation across a landscape (Via, 2001; Frantz et al., 2010). Genetic diversity is higher in sexually reproducing aphids (Maynard-Smith, 1978; Simon et al., 2002; Simon et al., 2010), however, offspring from asexually reproducing aphids, although genetically identical, are commonly comprised of genotypically diverse lineages (Judson and Normark, 1996; Little and Hebert, 1996; Simon et al., 1999). These asexual lineages can exhibit heterozygote excess and also be in linkage disequilibrium (Wang et al., 2007). The genetic differentiation of asexual aphids, in the absence of recombination and hybridization could be attributed to mutation (Haack et al.,

2000), but the evolution of asexual lineages could also be influenced by bacterial endosymbionts and epigenetic effects (Wilson et al., 2003).

In studying population genetics in aphids, microsatellites (or simple sequence repeats; SSRs) are often used because they are highly polymorphic, co-dominant, reproduceable and genome specific markers (Liu, 2007; Behura, 2006; Weng et al., 2007). A good choice for within-species population genetics studies (Blackman and Eastop, 2017), microsatellites can be used to identify species, investigate genetic variation, clonality, polyphenism, molecular phylogenetics, metapopulation structure, and spatial and temporal dynamics in aphids (Loxdale and Lushai, 2007; Loxdale et al., 2017). These tools have been applied in a range of aphid species, including on life cycles in connection to climate change (Guillemaud et al., 2003; Vorburger et al., 2003), host plant adaptation (Paudyal et al., 2019; Sunnucks et al., 1997), and insecticide resistance (Jackson et al., 2020; Monti et al., 2016).

Although microsatellites tend to be most informative when they have been specifically developed for the target species in question (Wilson et al., 2004), cross-species amplification is common and has been used to good effect in aphids (Simon et al., 1999; Wilson et al., 2004; Gauffre and D'Acier, 2006; Weng et al., 2007; Michel et al., 2010; Galambao, 2011). A total of nine previously developed microsatellite loci from *Aphis gossypii*, *A. fabae fabae*, *Sitobion miscanthi* and *Myzus persicae* have been successfully shown to cross amplify in *P. nigronevosa* and *P. caladii* in Hawaii (Galambao, 2011). This panel revealed that banana aphids in Hawaii were structured according to host plant specialization and geographic isolation, with *P. nigronervosa* closely associated with banana plants (Musaceae), whereas *P. caladii* was associated with ginger plants (Zingiberaceae) (Galambao, 2011).

Previous studies on the banana aphid in the Philippines have focused on the life cycle of aphid (Ocfemia, 1926; Magnaye, 1979), feeding preferences (Facundo, 1991; Facundo and Sumalde, 1998; Lomerio, 1984; Achacoso, 2004), seasonal abundance (Gavarra, 1977) and natural enemies (predators

and parasitoids) (FIDA, 1993; Bajet and Magnaye, 2002; Calilung, 2008), but there is little information about their population genetic structure and genetic variation and little evidence supporting their perceived asexual mode of reproduction. This knowledge gap impairs the ability to develop specific pest management strategies for aphid-transmitted diseases in the Philippines. This study was conducted to 1) determine the population genetic structure of banana aphids collected from abaca plants across three islands in the Philippines and to ask if there was any population structure due to their collection site or plant accession susceptibility (resistant or susceptible) to virus; 2) evaluate whether previously described species of *P. nigronervosa typica and P. caladii* could be identified and differentiated using microsatellites, and 3) evaluate their mode of reproduction in the Philippines.

Materials and Methods

Aphid sampling and abundance

Three abaca germplasm collections, one from each of the three major islands in the Philippines (Luzon, Visayas and Mindanao) were selected for aphid sampling. Each site caters for the production, maintenance, and distribution of abaca planting materials in the region. Banana aphids were collected between November 6 - 20, 2017 from three existing abaca germplasm collections in Luzon (Casiguran, Sorsogon) (12°52′19.20″N 124°00′25.20″E), Visayas (Baybay, Leyte) (10°40′37.90″N 124°47′49.45″E) and Mindanao (Bago-Oshiro, Davao City) (7°4′38.78″N 125°29′45.60″E) in the Philippines (Figure 7, see Chapter 1 for site descriptions). Prior to collection, an intensive sampling strategy was planned, with the collection of five aphids per colony, five colonies per plant and five plants from 10 susceptible and 10 resistant abaca accessions, respectively for each site. However, plant accessions as well as aphid abundance and infestation on potentially resistant and susceptible abaca accessions differed across the three germplasm collections. Aphids were absent from some potentially resistant abaca accessions and the number of aphid colonies per accession differed per accession per site. In addition, some of the abaca accessions were missing in some sites due to disease infestation and drought. Hence, sampling of aphids was done by inspecting every plant available in the germplasm collection for the presence of aphids regardless of accession type. Where present, aphids were collected from multiple distinct colonies located from different parts of the plant and the presence of ants was also recorded.

Between one and five aphids per colony were collected from the trunk, leaf, and newly emerged or unopened shoots of the abaca plants using a sterilized paint brush dipped twice in a 15 mL Corning tube filled with absolute Ethanol (EtOH) between collections. The collected aphids were stored in sterile 1.5 mL microcentrifuge tubes filled with 200 μL 70% EtOH for air transport, then replaced with absolute EtOH and stored at -80 ºC prior to DNA extraction. A

total of 2,772 individual aphids from 679 colonies were collected from 441 abaca plants across 58 abaca accessions.

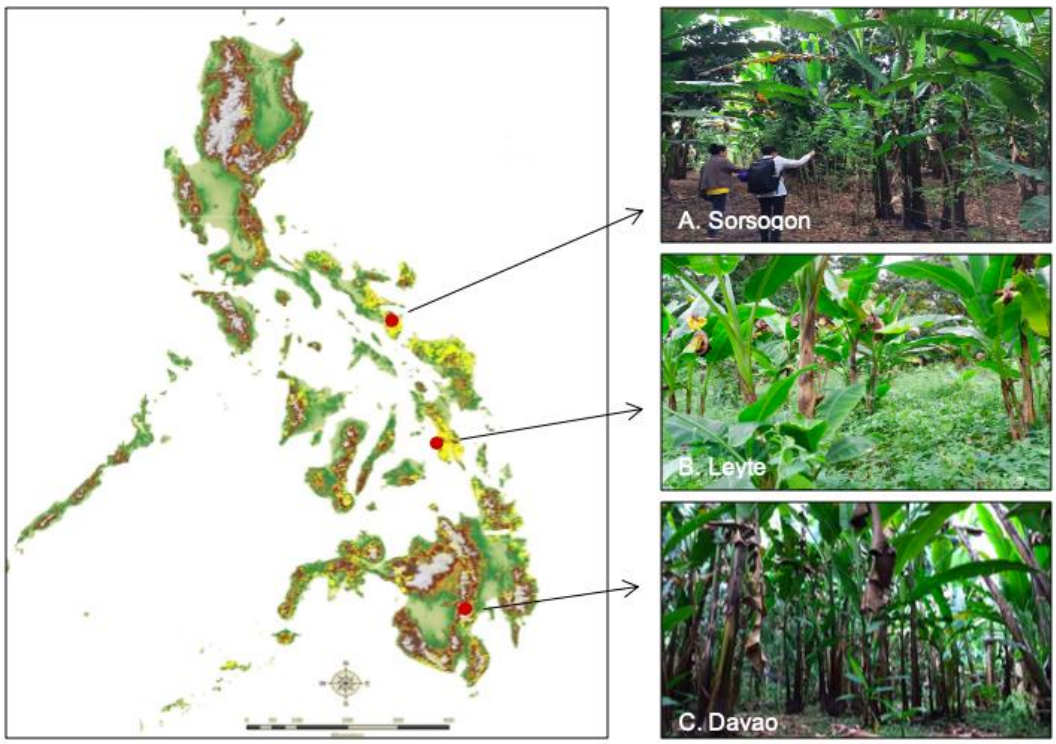

Figure 7. Map of the Philippines showing the major Abaca producing areas in Luzon (Sorsogon), Visayas (Leyte) and Mindanao (Davao) (in yellow color) (Source: PhilFIDA 2016:online) and collection sites as red circles.

Two aphids per colony were used for the analysis and colonies were distributed across two to six plants for each accession to comprehensively sample at multiple scales (Table S1). The abaca accession was categorized as resistant or susceptible (Table S2) to bunchy top disease based on the most current available data (Boguero et al., 2016; Halos, 2008; CFC/UNIDO/FIDA, 2004; Lomerio, 1991) and through personal communication with personnel at the germplasm sites (Dr. Luz O. Moreno - NARC and Ms. Analyn D. Bolivar - FIDA, Davao). The number of colonies per plant from which samples were selected ranged from one to four and depended upon field infestation rates of the aphids.

For aphid abundance, only those accessions used in this study were accounted for with regards to the total number of aphids collected. All the plants sampled per accession, the number of colonies per plant, and the number of aphids collected per site was tabulated on Table S3. Collection was subjected to one aphid to five aphids per colony and in some accessions more than five aphids were collected from large colonies. To determine the relationship between aphid abundance and plant resistance to disease, Fisher's exact test was performed to examine for statistically significant associations between aphid abundance in relation to site and plant resistance in R. software version 3.6.1 (R Core Team, 2019).

DNA extraction

Total DNA from 376 individual aphids collected from 188 colonies on 109 individual abaca plants across 36 abaca accessions from Davao, Leyte and Sorsogon (Appendix 1) was extracted following the 'salting out' protocol (Sunnucks and Hales, 1996) as follows: Individual aphids were pre-washed with 500 µL of 100% EtOH in a sterile petri dish and transferred into 1.5 mL microfuge tube. After EtOH evaporation at 37 °C for 10 mins on a heat block, the aphids were homogenised with 85 µl of TNES buffer (50 mM Tris, HCl pH 8.0, 20 mM EDTA, NAOH pH 8.0, 400 mM NaCl, 0.5% SDS) and 5 µl of Proteinase K (20 µg/µl) using a sterile pestle. A further 215 µL of TNES buffer was added before incubating the sample at 30 °C in a water bath overnight. After incubation, 1.5 µL of RNase (10 µg/µl) and 85 µL of 5 M NaCl were added to the sample, vortexed, and centrifuged at 14,000 rpm for 10 mins. The supernatant was transferred to a new 1.5 mL microfuge tube, added with 400 µL of -20°C (stored in freezer) 100% EtOH, mixed by inverting the tubes 10 times, and incubated at -20 °C in the freezer for one hour. After incubation, the samples were centrifuged at 14,000 rpm for 20 mins and the supernatant was discarded. The retained DNA pellet was added with 350 µL of -20°C 70% EtOH to wash the pellet and centrifuged at 14,000 rpm for 5 mins. The EtOH was discarded and the pellet evaporated to dryness at room temperature for

two hours. The DNA was resuspended in 30 μL of UltraPure™ DNase/RNase-Free distilled water (Invitrogen). The aphid analysis for population genetics (Chapter 2), endosymbionts (Chapter 3) and virus quantification (Chapter 4) used the same aphid samples (376 aphids from Davao, Leyte and Sorsogon). I extracted 30 μL of DNA extracts per aphid and I allocated 10 μL for each analysis. In chapter 2, aphid samples were analysed using single PCRs and then mixed into post-multiplexes for genotyping. The same aphid samples from chapter 2 were also used for the endosymbiont analysis but two multiplex-PCR (2 symbionts/PCR reaction) and one singleplex-PCR were used the analysis.

The quantity and quality of DNA were evaluated using a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific) by pipetting 1 μL of the total aphid DNA extracts onto a measurement pedestal of the NanoDrop™ machine. The nucleic acid concentration in ng/μl and the 260/280 (ratio of absorbance at 260 nm and 280 nm) and 260/230 (ratio absorbance at 260 nm and 230 nm) values were recorded to evaluate the concentration and purity of the DNA samples.

Microsatellite amplification and genotyping

Existing microsatellite primers previously developed for *A. gossypii*, *A. fabae*, *Myzus persicae* and *Sitobion miscanthi* (Wilson et al., 2004; Vanlerberghe-Masutti et al., 1998; Sloane et al., 2001; Gauffre and D'Acier, 2006) were used in this study (Table 1). These have been previously shown to cross-amplify on banana aphid species in Hawaii (Galambao, 2011). Twelve out of the 18 microsatellite markers tested successfully cross-amplified on banana aphids. However, only 10 loci were used for genotyping, as two of the loci (AF-153 and S16b) were omitted due to the absence of, or difficulty calling, allele peaks during the preliminary quality scoring.

A three-primer approach was used to fluorescently label PCR products (Neilan et al., 1997) using universal primers tagged with HEX, PET and FAM

fluorophores (Blacket et al., 2012; Culley et al., 2013). PCR reactions were performed in singleplexes using specific annealing temperatures for each locus in a final volume of 10 µl containing 5 µl MyTaq Red Mix (Bioline), 1 µl (10 ng) DNA, 0.2 µl 5 µM of the 5' modified forward primer, 0.2 µl 20 µM each of the reverse primer and universal primer and 3.40 µl water. Amplifications were performed using an Applied Biosystems™ SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific) with initial denaturation for 3 min at 94 ºC followed by 30 cycles of 15 s at 94 ºC (denaturation), 90 s of specific annealing temperature per locus (Table 2), 30 s at 72 ºC (elongation), and final extension Of 6 min at 72 ºC.

Table 1. Previously developed markers and their references tested for cross-amplification on banana aphids in this study

Locus	Primer sequence (5'-3')	Repeat Motif	Size (bp)	Reference
	F: TACGGCTAAATTGATTTGGG			
AF-I	R: TTGAATCAGGCATTGCTCTC	$(GT)_{13}$	271	Gauffre & Coeur D' Acier, 2006
	F: CTAACCCTGTCCGATCAGCC			
AF-4	R: CCACACCGCTGTATCCTTC	$(AC)_9(TC)_1(AC)_9$	75-125	Gauffre & Coeur D' Acier, 2006
	F: GTTGTAATAGCGATCGGAGG			
AF-63	R: ATGATGTGCGGTGTGAGTGT	$(AC)_{10}(GC)_1(AC)_2$	287-299	Gauffre & Coeur D' Acier, 2006
	F: CTAAAATGTTGCGCGCTTTC			
AF-93	R: TGTTGTGTTGGCTCACCTGC	$(GC)_4(AC)_{11}$	259-269	Gauffre & Coeur D' Acier, 2006
	F: GGCGAACGAATCCGACGTGT			
AF-153	R: GAACGGCGTAGACGACGAGA	$(CA)_9$	288-306	Gauffre & Coeur D' Acier, 2006
	F: CACCGATGTCATTCGAGCAA			
AF-157	R: GCACGGTAAGCATTCATGGA	$(CA)_9$	240-255	Gauffre & Coeur D' Acier, 2006
	F: CGGCCGCCAGACTTCCAAGT			
AF-169	R: ACTGATGGTGGTTGCCGAGC	$(CA)_{14}$	149-155	Gauffre & Coeur D' Acier, 2006
	F: TTTTCCCGGCACACCGAGT			
Ago24	R: GCCAAACTTTACACCCCGC	$(T\ G)_8AGT(GGGT)_2\ldots(GT)_4\ldots(TA)_4$	114-155	Vanlerberghe-Masutti et al. 1998
	F: TGACGAACGTGGTTAGTCGT			
Ago53	R: GGCATAACGTCCTAGTCACA	$(AAT)_6(GT)_3$	112-118	Vanlerberghe-Masutti et al. 1998

	F: GCGAGTGGTATTCGCTTAGT			
Ago59	R: GTTACCCTCGACGATTGCGT	$(AC)_{19}AT(AC)_5(GC)_4$	150-205	Vanlerberghe-Masutti et al. 1998
	F: TCGGTTTGGCAACGTCGGGC			
Ago66	R: GACTAGGGAGATGCCGGCGA	$(T\ G)_{11}\ldots\ (T\ G)_4(TA)_4$	97-159	Vanlerberghe-Masutti et al. 1998
	F: GGCAATAAAGATTAGCGATG			
M35	R: TGTGTGTATAGATAGGATTTGTG	$(AT)_9$~$(AC)_{22}$	178-198	Sloane et al. 2001
	F: CGCTGGGGACGAAAAACCTG			
M62	R: AACAAAAAACCGAAAACCCG	$(CA)_6(CA)_{12}$	99-121	Sloane et al. 2001
	F: AACCCATCTCACTCGTCAGCC			
myz25	R: GAATCTGGAGAGCGGTTAATGC	$(AG)_{24}$	119-126	Wilson et al. 2004
	F: ATAAAACAAAGAGCAATTCC			
S16b	R: GTAAAAGTAAAGGTTCCACG	$(CA)_{14}$	161-207	Wilson et al. 2004
	F: TTCTGGCTTCATTCCGGTCG			
S17b	R: CGTCGCGTTAGTGAACCGTG	$(CA)_{11}TA(CA)_8C(TA)_7$	127-139	Wilson et al. 2004
	F: GGTCCGAGAGCATTCATTAGG			
S23	R: CGTCGTTGTCATTGTCGTCG	$(GA)_{14}$	107-111	Wilson et al. 2004
	F: CCCGACCCCGTCCATTCAAA			
S24	R: CCTCCACCACTACTTTCACTCC	$(CA)_{20}$	151-161	Wilson et al. 2004

bp, base pair; primers in bold were used for genotyping

Table 2. Details of microsatellite loci, PCR conditions, and post-PCR multiplexes used in this study.

Locus name	Reference	Ta (ºC)	Fluorophore-universal primer combination	Post-PCR multiplex
AF 1	Gauffre & Coeur D' Acier, 2006	53	HEX – T7 term	3
AF 4	Gauffre & Coeur D' Acier, 2006	53	PET – M13 modified B	3
AF 93	Gauffre & Coeur D' Acier, 2006	59	FAM – Tail C	2
Ago 66	Vanlerberghe-Masutti et al., 1998	64	PET – M13 modified B	2
S17b	Wilson et al., 2004	55	PET – M13 modified B	1
S23	Wilson et al., 2004	55	HEX – T7 term	1
S24	Wilson et al., 2004	55	FAM – Tail C	2
M35	Wilson et al., 2004	55	FAM – Tail C	1
M62	Sloane et al., 2001	55	HEX – T7 term	3
myz25	Wilson et al., 2004	53	FAM – Tail C	3

Ta: Annealing temperature; Tail C: CAGGACCAGGCTACCGTG (Blacket et al. 2012); M13 modified B: CACTGCTTAGAGCGATGC (Culley et al., 2013); and T7 term: CTAGTTATTGCTCAGCGGT (Culley et al., 2013)

To reduce cost, three post-PCR multiplexes (Table 2) were assembled by combining 1 µl PCR prouduct from each included singlplex into a single tube; 0.5 µl from the mixture was then submitted for genotyping at the Core Genomic Facility, University of Sheffield, Sheffield, United Kingdom. The fragment length was determined using an ABI 3730 DNA Analyzer capillary sequencer (Thermo Fisher Scientific) with GeneScan 500 LIZ size standard (Thermo Fisher Scientific).

Allele scoring and binning were performed using the Fragman v1.0.9 (Covarrubias-Pazaran et al., 2016) and MsatAllele v1.05 (Alberto, 2009) packages in R version 3.5.0 (R Core Team, 2018). Three additional monomorphic and problematic loci having multiple bands (S23, AF-93, and Ago66) were removed from subsequent analysis. The final dataset consisted of seven loci for 376 individual aphids from Leyte (138), Sorsogon (178) and Davao (60). Null allele frequency was estimated using the Expectation Maximization algorithm (Dempster et al., 1977) implemented in FreeNA (Chapuis and Estoup, 2007). For genetic analysis programs that don't have their own internal conversion functions (i.e. Microsoft Excel to Genepop format

or Genepop to STRUCTURE), data were converted according to the specified format of the program using PGDSpider v2.1.1.5 (Lischer and Excoffier, 2012).

Species identification

Due to the morphological similarity between *Pentalonia* species, and the cross-amplification of the microsatellite primers used across multiple species within the genus, a preliminary analysis was conducted to determine if multiple *Pentalonia* species were present within the data. A Bayesian model-based clustering method was used to investigate for any broad-scale genetic differentiation (i.e at the species or sub-species level) prior to further analysis of population structure. The assignment of individuals (probabilistically) to populations was implemented using the software STRUCTURE 2.3.4 (Pritchard et al., 2000; Falush et al., 2003). This model identifies clusters of genetically similar individuals from multilocus genotypes without prior knowledge of their population affinities, assuming loci are at Hardy-Weinberg (HWE) and linkage equilibrium (LE) within populations. Although the markers did not meet these conditions (see Bayesian estimate of population structure section below), the test considered to still be robust because two genetic clusters were identified and confirmed as two distinct *Pentalonia* species.

For the analysis, STRUCTURE was run with a burn-in-period of 100 000 iterations followed by 200 000 iterations, with K (assumed number of clusters) ranging from 1 to 10. The model parameters selected assumed admixture of ancestry and independent allele frequencies. Three independent repetitions were run to test the consistency of the results and the ΔK quantity where it is expected to exhibit a peak for the actual K value was used to determine the most reliable number of clusters (Evanno et al., 2005). The ΔK quantity is an *ad hoc* quantity based on the rate of change in the log probability of data in relation to the real number of clusters (Evanno et al., 2005). Genotypes were assigned to a cluster if the histogram assignment probability was greater than 70%, a threshold based on the generated coefficient of matrix that would allow highest probability of membership of an individual sample to a cluster or group.

The result of the STRUCTURE analysis from the full data set showed two genetic clusters (see Results). To investigate if these constituted separate species, mitochondrial DNA barcoding was conducted to compare sequences from the two identified clusters with each other, as well as to existing sequences within reference databases.

Mitochondrial DNA amplification and sequencing

Ten randomly selected representative samples from each initial STRUCTURE inferred population were sequenced to characterize their haplotypes. DNA amplifications were performed using the primer pair LepF (5'ATT CAA CCA ATC ATA AAG ATA TTGG3') and LepR (5'TAA ACT TCT GGA TGT CCA AAA AAT CA'3) which amplify an approximately 680 bp DNA fragment of mitochondrial DNA from both regions of the mitochondrial cytochrome oxidase gene I (COI) (Foottit et al., 2008). Polymerase chain reaction was performed in a final volume of 15 µl containing 7.5 µl MyTaq Red Mix (Bioline), 0.225 µl 20 µM LepF, 0.225 µl 20 µM LepR and 1.5 µl 10ng/1 µl DNA template and 5.55 µl RNase/DNase Free Distilled Water using Applied Biosystems™ SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific) at 3 min for 94 ºC followed by 30 cycles of 15 s at 94 ºC, 30 s at 50 ºC, 30s at 72 ºC and 6 min at 72 ºC. Amplified PCR product was purified using Illustra ExoProStar (GE Life Technologies), and 14 µl of purified PCR product was sent for forward and reverse sequencing on an ABI 3730, 48-capillary DNA analyser (Thermo Scientific) at the Core Genomic Facility, University of Sheffield, Sheffield, UK.

The quality of the electropherograms for the CO1 gene were checked using 4Peaks v1.8 (Griekspoor and Groothuis, 2014) and only those with a quality of >25 Phred score (percentage quality score of the electropherogram where the peaks for each nucleotide were properly called) were chosen as the baseline to edit the sequence. The >25 Phred equates between 99% (20 Phred score) to 99.9% (30 Phred score) in terms of % base call of accuracy (Illumina, 2001; Ewing et al., 1998). Quality checked electrograms were edited and aligned using the automated ClustalW multiple alignment method (Hall,

2005) using the BioEdit software version 7.0.5.3 (Hall, 1999). The identity of the sequences was sought using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) from the National Center for Biotechnology Information (NCBI). Each sequence was uploaded individually into the NCBI BLAST program for identification of the aphid species. The percent identity of the sequence matches in the database for each species was determined by creating a consensus file (from 10 individual sequences) per species using the BioEdit software and uploading it into the BLAST program.

Population genetics analysis

The number of alleles (Na) observed (*Ho*) and expected heterozygosities (*He*), fixation index and departure from Hardy-Weinberg equilibrium (HWE) between (full data set) and within (per species) *Pentalonia* species were calculated using the program GenAlEx 6.5 for each locus (Peakall and Smouse, 2006; Peakall and Smouse, 2012). Further analyses on linkage disequilibrium, allelic richness, F-statistics and MLGs were performed for each species. Linkage disequilibrium (LD) among all pairwise sets of loci was assessed using Genepop v4.5.7 on the web (Rousset, 2008; Raymond and Rousset, 1995). Significance values were corrected for multiple tests using the p.adjust function base in R (R Core Team, 2019). Allelic richness and F-statistics (F_{IT}, F_{ST} and F_{IS}) (Wier and Cockerham, 1984) were assessed using Fstat software 2.9.4 (Goudet, 1995; Goudet, 2003). For each species, separate STRUCTURE analyses were conducted to examine population structure within species, using the same parameters as detailed previously. Multilocus genotypes (MLGs) and expected MLGs were calculated using 'poppr' package v2.8.3 (Kamvar et al., 2014; Kamvar et al., 2015) in R software version 3.6.1 (R Core Team, 2019).

MLG association with inferred species, collection location and host plant susceptibility

Fisher's exact tests were used to test for an association between MLG and collection location, between MLG and aphid species, and between MLG and the susceptibility of the host plants to BTD in R software version 3.6.1 (R Core Team, 2019). The full data set was used for MLG and location association test, while only data from Leyte was used to test the association between MLG and host plant susceptibility because it was the only location with sufficient numbers of both susceptible and resistant hosts. Prior to the analysis, any plants in Leyte that has no information about the level of resistance to BTD were removed.

Inferred species association with location and host plant susceptibility

Fisher's exact tests were used to test for an association between inferred population (i.e. based on the Bayesian model assignment to population) and collection location and between inferred population and the susceptibility of the host plants to BTD in R software version 3.6.1 (R Core Team, 2019). As previously, the full data set was used for the location association test, while only data from Leyte was used to test the association with host plant susceptibility. Prior to the analysis, any plants in Leyte that has no information about the level of resistance to BTD were removed.

Genotype consistency of aphid colonies

Colonies were given a score for consistency of the MLGs per aphid in a colony = i.e., if the same genotype was present for the two individual aphids per colony. Colonies were scored from 0 to 1, where 0 indicates the MLG of two individual aphids in that colony were not the same and 1 indicates the two aphids in that colony have the same MLG. Missing data were not counted. Total counts of 0 and 1 were tabulated in a contingency table for association test of species assignment to location and host plant's susceptibility to the disease using Pearson's Chi-squared test in R (R Core Team, 2019).

Results

Bayesian estimates of population structure

STRUCTURE analysis revealed two genetic clusters of banana aphids (Fig. 8A) as indicated by an inferred population value of K = 2 (Fig. 8B). The majority of individuals (89%) belonged to a single genetic grouping that was distributed across collection sites [Leyte (101/334), Sorsogon (175/334) and Davao (58/334)]. Most of the individuals assigned to the other genetic grouping (11%) were from Leyte (37/42) with a few isolated individuals from Sorsogon (3/42) and Davao (2/42). The inferred genetic clusters showed affinity to *Pentalonia nigronervosa* and *Pentalonia caladii* (see mitochondrial DNA analysis below).

Within-species population structure

Further analysis of assigning individuals to population based on genetic groupings has revealed two subpopulations for *P. caladii* (K=2) and no population clustering for *P. nigronervosa* species (K=1) (Fig. 8C).

Mitochondrial DNA

Mitochondrial DNA sequences from 10 individual aphids from each inferred genetic cluster suggests two different species of *Pentalonia*: *P. caladii* and *P. nigronervosa*. This was evident in the multiple sequence alignment that showed two separate groups with differences in one or two nucleotides. Ten individuals from the first population group (representing 11% of the individuals tested) showed similarity to *P. caladii* while the second group (representing 89% of the individuals tested) strongly affiliated with *P. nigronervosa*, having percentage identity of 95.95% and 99.70%, respectively as identified and confirmed through the BLAST program using the NBCI GenBank dat

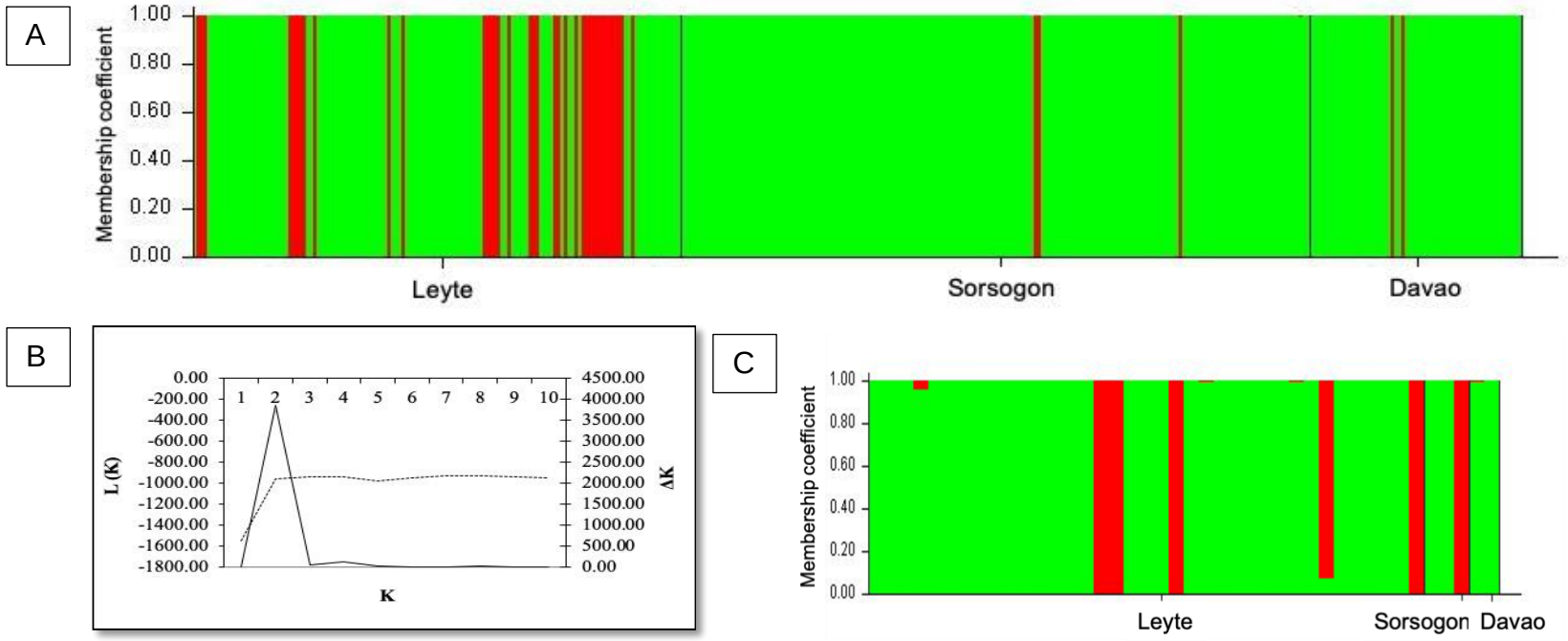

Figure 8. (A) Bayesian inference of population genetic structure of *Pentalonia* (K=2) with one genetic cluster in red and the other in green. Collection sites are delineated by black lines and indicated on the x-axis. Mitochondrial sequences revealed individuals from the red grouping to show affinity to *P. caladii* and individuals from the green grouping to show affinity to *P. nigronervosa*, (B) The likelihood of the data plotted in L (K) and ΔK against K showing the inferred number of genetic clusters and (C) Bayesian inference of population genetic structure of *P. caladii* (K=2) species and *P. nigronervosa* (K=1) (plot not shown).

Genetic diversity

Genetic diversity analysis within *P. caladii* (42 aphids) and *P. nigronervosa* (334 aphids) using seven microsatellite loci detected a total of 19 and 18 alleles across all loci, and 1 to 5 and 1 to 4 alleles per locus, respectively (Table 3). The mean number of alleles was 2.714 for *P. caladii* and 2.571 for *P. nigronervosa*. All loci were polymorphic between two *Pentalonia* species, except for loci S17b, S24 and AF1, which were found to be monomorphic for *P. caladii*, and myz25 locus was monomorphic for *P. nigronervosa*. Observed heterozygosity per locus ranged from 0 to 0.833 and 0 to 0.994 while expected heterozygosity ranged from 0 to 0.589 and 0 to 0.507, for *P. caladii* and *P. nigronervosa*, respectively. The overall allelic richness per locus ranged from 1 to 4.952 with a mean richness of 2.850 for *P. caladii* and 1.231 to 2.909 with a mean richness of 2.159 for *P. nigronervosa*. The null allele frequency for each locus which ranged from 0.001 to 0.104 was low to mild (Table S4), thus all loci were retained for the analysis. Four loci departed significantly from HW equilibrium for *P. caladii* and five loci departed significantly from HW equilibrium except M35 for *P. nigronervosa* ($P<0.001$). A total of 6 (*P. caladii*) and 2 (*P. nigronervosa)* pairs of loci out of 21 possible pairs of loci were observed to be under linkage disequilibria.

Low genetic differentiation between populations was evident within both *P. caladii* and *P. nigronervosa* according to Weir and Cockerham (1984) estimates with a global F_{ST} value of 0.001 and 0.023, and an average estimated inbreeding coefficient of 0.093 and -0.441, respectively (Table 4). The overall F_{ST} and F_{IS} values of *Pentalonia* aphids (full data set) were 0.452 and -0.288, respectively.

Table 3. Summary genetic information for *P. caladii* and *P. nigronervosa* using 7 microsatellite markers.

Locus	Number of alleles		Observed heterozygosity		Expected heterozygosity		HWE (p-value)		Allelic Richness	
	P. caladii	*P. nigronervosa*	*P. caladii*	*P. nigronervosa*	*P. caladii*	*P. nigronervosa*	*P. caladii*	*P. nigronervosa*	*P. caladii*	*P. nigronervosa*
M35	4	2	0.833	0.003	0.544	0.003	***	ns	3	2.042
S17b	1	2	0.000	0.000	0.000	0.006	0.000	***	1	1.231
S24	1	3	0.000	0.000	0.000	0.012	0.000	***	1	1.461
AF1	1	4	0.000	0.003	0.000	0.041	0.000	***	1	2.119
AF4	3	3	0.786	0.003	0.625	0.009	***	***	3	2.909
M62	4	3	0.024	0.994	0.217	0.507	***	***	4.952	2.716
myz25	5	1	0.927	0.000	0.589	0.000	***	0.000	6	2.632
Mean	2.714	2.571	0.367	0.143	0.282	0.083			2.850	2.159
SE	0.644	0.369	0.171	0.142	0.112	0.071			0.766	0.242

ns = not significant; 0.000 =monomorphic; SE = Standard error; *** = significant departures from HW equilibrium, p<0.001

Table 4. Number of individuals, fixation index and inbreeding coefficient based on the inferred population of overall and two *Pentalonia* species in the Philippines using 7 microsatellite loci.

Species	*P. caladii*		*P. nigronervosa*
K	1	2	1
N	6	36	334*
F-statistics	F_{ST} = 0.001, F_{IS} = 0.023		F_{ST} = 0.093, F_{IS} = - 0.441
Pentalonia overall F-statistics: F_{ST} = 0.452, F_{IS} = -0.228			

K = inferred population, N = number of samples, * Leyte= 101, Davao = 58, Sorsogon = 175

With regards to aphid abundance, Leyte had the highest aphid infestations followed by Sorsogon and Davao (Table 5), but there was no significant association between the number of aphids to location (p-value = 0.2949) and between the number of aphids to plant susceptibility (p-value = 0.093). However, there was a significant association between the total number of colonies to location (p-value = 0.0440) with highest aphid infestations on Inosa (63 colonies), Samoro (48 colonies) and Putomag (40 colonies) in Leyte (Table S3).

MLG determination showed 31 MLGs across species (Appendix 1), with expected MLGs ranging from 4.32 to 14 per species (Table 6). *P. caladii* had 14 MLGs dominated by MLG.7 (23 aphids) while *P. nigronervosa* had 17 MLGs dominated by MLG.25 (299 aphids). Across collection sites, Leyte had the highest number of MLGs followed by Sorsogon and Davao. Only three MLGs (MLG.7, MLG.25 and MLG.30) out of 31 MLGs were present across locations (Appendix 1) but one MLG (MLG.25) dominated across all collection sites and comprised 299 individuals (89 aphids from Leyte, 160 aphids from Sorsogon and 50 aphids from Davao) that belonged to *P. nigronervosa*.

Table 5. Summary on the number of accessions, plants sampled, and aphids collected on resistant and susceptible plants per location.

		No. of plants sampled		No. of colonies		No. of aphids	
Location	No. of accessions	R	S	R	S	R	S
Davao	6	0	34	0	60	0	323
Leyte	15	35	144	53	237	240	1065
Sorsogon	21	28	88	39	135	128	485
Total	36	63	266	92	432	368	1873

Resistant = R, Susceptible = S

Table 6. Summary of MLGs across banana aphid species

Pentalonia species	No. of Abaca accessions	No. of individual plants	Total aphids	No. of colonies*	No. of single aphids**	No. of alleles	MLGs	Expected MLGs	MLG/N
P. caladii	13	25	42	14	14	19	14	14	0.30
P. nigronervosa	35	102	334	160	14	18	17	4.32	0.05
Total	36	109	376	188	14	30	31	6.99	0.08

* two individual aphids in a colony; ** two individual aphids in a colony were mixed species, One aphid belongs to *P. caladii* and the other belongs to *P. nigronervosa*

Fisher's exact test (full data set) revealed a significant association between MLGs and location ($p<0.0005$), between MLGs and *Pentalonia* species ($p<0.0001$), and between *Pentalonia* species and location ($p<0.0001$). However, there was no significant association between MLGs and plant susceptibility to bunchy top disease (p-value = 0.4653) and between *Pentalonia* species and plant susceptibility to bunchy top in Leyte (p-value = 0.3468).

Genotype consistency of aphid colonies

Genotype consistency within a colony accounted for 80% of the banana aphid samples (i.e. both aphids sampled from a colony exhibited the same MLG; Table S5). This means that 20% of colonies contained aphids with different genotypes. Fourteen colonies were mixed species (1 aphid belonged *P. nigronervosa* and 1 aphid belonged to *P. caladii*). There was significant association between the level of genotype consistency to location site (X^2 = 17.364, df = 2, $P<0.0001$) and *Pentalonia* species (X^2 = 17.902, df = 2, p-value = 0.0001). Davao (88%) and Sorsogon (89%) had the most consistent genotype within aphid colonies and Leyte had 35% inconsistent colonies. Among the species, *P. nigronervosa* (92%) had more consistent genotypes compared to *P. caladii* (43%).

Discussion

The present study is the first analysis on banana aphid's genetic diversity collected from abaca plants in the Philippines. The STRUTURE analysis revealed two genetic clusters that were likely two species of *Pentalonia*: *P. caladii* and *P. nigronervosa* which were confirmed as separate species using the mitochondrial DNA sequences. Summary data analysis supported the suggestions that aphids primarily reproduce asexually in the Philippines as there is an observed excess in heterozygosity and negative F_{IS} values. Both *Pentalonia* species was dominated with one MLGs (MLG.25 for *P. nigronervosa* and MLG.7 for *P. caladii*) out of 31 MLGs, which further supports the idea of predominant asexual reproduction. Location seemed to influence the distribution of MLGs, and within a colony around 20% of the aphids were collected from mixed genotype colonies. There was no association of host plant susceptibility to BTD with aphid species or location. The cross-species amplification of primers from related aphid species to banana aphids were successful, but with a reduced number of loci compared to previous studies.

Genetic structure of banana aphids

The genetic structure and genetic diversity of banana aphids has not been extensively studied globally. So far, the only reported genetic information was in Hawaii that revealed two main genetics groups, constituting the species the *P. nigronervosa* and *P. caladii* (Galambao, 2011). This current study supports the existence of two main genetic groupings and that these form two distinct species (Foottit et al., 2010; Galambao, 2011). The Hawaiian study found that banana aphids were structured according to host plant specialization and geographic location, with *P. nigronervosa* was closely associated to banana, while *P. caladii* was closely associated to ginger. In Philippine banana aphids, however, aphid species seem to be partitioned by island as there was a significant association between aphid species to location. This was more prominent for *P. caladii*, where the majority of the aphid individuals came from

the Leyte collections. Host-plant specialization is common in aphid species and thus normally dictates the genetic structuring in a population, but geographic distance could also influence genetic partitioning among aphid species (Nibouche et al., 2014). This study considered only within-species diversity rather than among species diversity of host plants and showed no evidence of genetic partitioning across genetic strains of abaca.

Host plant quality is a very important factor influencing the survival and reproduction rate of aphids (Dixon, 1997). The pholem sap, which contains simple sugars, nutrients, plant secondary metabolites and essential amino acid (Douglas, 1993; Zust and Agrawal, 2016) differs with respect to the age of the plant and between parts of the plant (McLung, 2006; Petterson et al., 2017). Such was the case in *Macrosiphoniella tanacetaria* and *Uroleucon tanaceti* aphids that fed on different chemotypes from stems, young and old leaves of *Tanacetarum vulgare* where the former preferred the stems (high reproduction) while the latter favoured old leaves where it both survived and reproduced (Jakobs and Müller, 2018). In addition, the host plant resistance mechanisms such as plant toxins and antibiosis (Alvarez et al., 2006), antixenosis, tolerance and R-genes also vary between varieties and changes as the plant matures (van Emden, 2017).

It has been reported that within host-plant species genetic variation can greatly influence the growth performance and feeding preference of phloem-sap feeding aphids and ultimately indirectly affect their survival (Zytynska and Weisser, 2016; Zytynska et al., 2019). In this study, aphids were collected from different accessions of abaca across the three collection sites which were classified as susceptible or resistant to BTD. In Leyte, aphids were collected from reasonable numbers of both susceptible and resistant accessions and were identified as either *P. nigronervosa* or *P. calladii*. The two species showed no association to resistant or susceptible abaca accessions. This finding is contrary to previous studies especially for *P. nigronervosa* which showed different feeding preferences when tested on eight (Inosa, Putian, Laguis, Linawaan, Laylay, Linawaan, Meninonga and Linlib) accessions in

Leyte (Achacoso, 2004). They favored more on Inosa and preferred less on Laylay, suggesting that some accessions might contain chemical compounds harmful to the aphid. Hence, aphid feeding preferences and the chemical composition of different abaca accessions need further study.

Site-specific variation is another factor that influences the absence of an accession aside from the occurrence of BTD or drought in the area. In the screening conducted in the Bicol, Leyte, and Davao regions of the country, eight out of 40 accessions were identified as high yielding varieties which also showed resistance to bunchy top and mosaic virus (CFC/UNIDO/FIDA Report, 2004). However, adaptability study of the eight accessions varied across three regions. This site-specificity showed that abaca may have specific soil and climatic requirements for them to be more productive. With this, the PhilFIDA have recommended plant high yielding accessions specific for Bicol (Abuab, Musa Tex 51, Tinawagan Puti), Leyte (Inosa, Linawaan and Laylay) and Mindanao (Bangolanon, Maguindanao, Tangongon).

Although no associations were found among either the aphid species or the MLGs to host plant susceptibility to BTD, during collection, aphid abundance was observed to be more concentrated on certain susceptible abaca accessions (Inosa, Samoro and Putomag in Leyte). No aphids (Agutay, Pacol and Gomez Hybrid in Leyte) and few aphids (less than 2 aphids) were found on some resistant accessions in Davao (Daratex) and Leyte (five potentially resistant plants), respectively. For phytophagous insects such as aphids, the differences in plant quality and suitability can strongly govern the feeding preferences of an insect population (Ma et al., 2019).

Aphid's mode of reproduction and genetic diversity

Four (*P. caladii*) and five (*P. nigronervosa*) of the seven microsatellite markers used in this study significantly departed from Hardy-Weinberg Equilibrium (HWE) except for one marker (M35), specifically in *P. nigronervosa* samples. Six and two pairs of loci were observed to be under linkage disequilibria for *P.*

caladii and *P. nigronervosa*, respectively. This could be attributed to the presence of null alleles, occurrence of inbreeding and/or Wahlund effects (within site population structure or group sampled) as a result of secondary population subdivision (Pinheiro et al., 2009). Null alleles, which are common in insects (Chapuis and Estoup, 2007) are caused by mutations in the primer binding regions that prevented the primers from binding which is responsible for amplification failure in PCR that can results to either missing data (non-amplification in both alleles) or false homozygotes (non-amplification of one allele only). Most of the genetic analyses that have been conducted on aphid species tend to show deviation from HWE and linkage disequilibrium (LD), which indicates that assumption of HWE and LD have not been met, a common scenario for asexual or partially asexual organisms (Delmotte et al., 2008; Razmjou et al., 2010; Halkett et al., 2005a). In *Rhopalosiphum padi* for example, reproduction can be both cyclical and via obligate parthenogenesis and populations exhibiting obligate parthenogenesis showed significant departures from HWE whereas populations with cyclical parthenogenesis do not (Duan et al., 2017).

In this study, one MLG was found to dominate across the three regions for *P. nigronervosa* population. This is indicative of clonal reproduction (Halkett et al., 2005b) and suggestive that the mode of reproduction of this aphid species in the Philippines is obligate parthenogenesis (asexual reproduction all year round). In addition, the negative F_{IS} values, suggesting an excess in heterozygotes (Holsinger and Weir, 2009), also supports an asexual mode of reproduction and is an indication of clonal diploid populations (Halkett et al., 2005a; De Meeus et al., 2006). Moreover, the absence of population genetic structure within *P. nigronervosa* samples provides further evidence that they reproduce asexually. With respect to *P. caladii*, more than half of the population was dominated by one MLG that came from Leyte where most of the aphids came from. With low representation of this aphid species from two other regions, it can be inferred that their mode of reproduction could be asexual owing to their very low genetic diversity. Extensive sampling of this species is needed to validate this finding.

Notwithstanding their lower genetic diversity and population differentiation compared to their sexual counterparts, asexual aphids have high levels of heterozygosity (Balloux et al., 2003). Excess in heterozygosity is a common occurrence in a population undergoing a long-term asexual reproduction and can lead to the Meselson-effect phenomenon (Balloux et al., 2003; Halkett et al., 2005a). This occurs when, as a result of longstanding parthenogenesis, there is sequence divergence between alleles. In the absence of sex, diploid asexual organisms tend to accumulate extensive diversity within individuals through a series of mutations over time (Butlin, 2002; Simon et al., 1999; Sunnucks et al., 1996; Welch and Meselson, 2000). Moreover, in the absence of appropriate stimuli required to develop sexual morphs, (i.e. large temperature shifts), aphids in the tropical regions such as the Philippines are believed to reproduce exclusively asexually (Clarke et al., 2020). The findings in this study supports this theory that aphids in the Philippines reproduce exclusively asexually.

Genotype and species consistency of aphids within colony

Two individual aphids per colony were sampled in this study. To test for colony consistency, which is not usually carried out in aphid genetic studies, the MLGs of these two individual aphids in a colony was classified as consistent or not consistent. A colony was consistent when two individual aphids had the same MLG, while a colony was not consistent when two aphids had different MLGs. Most population genetics studies of aphids usually use only one individual aphid as a representative of the entire aphids within a colony. This is because aphids within a colony are believed to constitute as one genotype only (Stern and Foster, 1997). However, this study showed that 20% of the aphids sampled from the same colony had different MLGs, and that two individual aphids sampled from the same colony were sometimes a different species. This suggests that multiple genotypes and species of banana aphids colonize and coexist on the abaca plants at the same time. Aphids tend to develop winged morph in the event of crowding and in the presence of natural enemies (Loxdale and Lushia, 1999) and they also have some ability to walk

between host plants (Zytynska et al., 2019). Studies working on very closely related aphid species should be cautious in assuming that a single aphid represents the genotype of a colony. Inaccurate genetic diversity information from making such assumptions could affect decision making in terms of targeted insect pest management strategies.

Cross-species amplification

The use of existing loci from related aphid species is a cost-effective strategy when species-specific markers are not yet developed such as in banana aphid species. However, the occurrence of cross-amplification failure due to primer mismatch (Weng et al., 2007) and low level of allelic diversity (Wilson et al., 2004) can be an issue when using markers from non-target species. Both of these issues were evident in this study, where only seven loci were usable, and these were less variable than in other work on banana aphids (Galambao, 2011). Although cross-species amplification technique has been successful for population genetics studies in other aphid species such as *Acyrthosiphon pisum*, *Aphis gossypii*, *Aphis glycines*, *Myzus persicae*, *Rhopalosiphum padi*, *Schizaphis graminum* and *Sitobion avenae*, most of the tested loci did not show 100% successful amplification (Weng et al., 2007; Weng et al., 2010; Wang et al., 2020; Chung et al., 2020; Gholamian et al., 2018; Michel et al., 2010; Michel et al., 2009). This could be due to biological specificity and technical aspects during the PCR amplification process; the success of cross species amplification is only effective if primer sequences are conserved between species. This is more likely when both the source of loci and the target species are of the same genus (Wilson et al., 2004). Species-specific primers should be used, if possible, as these are likely to exhibit higher polymorphism and be able to provide a more comprehensive population genetics analysis. With the newly sequenced genome of *P. nigronervosa* (Mathers et al., 2020), this available information could be a valuable tool for the development of species-specific markers for future studies. The development of markers can be facilitated with the use of newly developed microsatellite design protocols such as Galaxy-based bioinformatics pipelines

(Griffiths et al., 2016) and the multi-individual microsatellite identification (MiMi) platform (Fox et al., 2019) both of which can offer a cheaper, simpler, and more efficient method of development that has high recovery rates of functional loci.

Conclusion

This study has supported the presence of two genetic clusters of banana aphids, the *P. nigronervosa* and *P. caladii* which had been detected previously and described as two distinct species. Multilocus genotypes and genetic groupings were structured according to geographic location and there was no evidence of assortative association by host plant susceptibility. The low levels of genetic differentiation, inbreeding coefficient and excess in heterozygosity supports the idea that obligate parthenogenesis is the primary mode of reproduction in banana aphids in the Philippines. It can be inferred that both species infect individual plants simultaneously and that they co-exist on plants and within colonies, which could have implications for control strategies. Aphid forecasting and monitoring should be done and implemented in every abaca germplasm collection and at the abaca farmer's field to fully monitor the occurrence and identity of aphids in the area and correlate it with the presence or onset of the bunchy top disease symptoms. Further studies are needed to elucidate the population genetic structure and genetic diversity of the banana aphids in the Philippines at a finer scale.

Supplementary Materials:

Table S1. Summary on the number of aphids, accessions, colonies, plants sampled, and the presence of ants per site.

Location	No. of aphids	No. of colonies	No. of accessions	No. of resistant and susceptible accessions*		No. of plants	No. of resistant and susceptible plants		Aphids with ants	Aphids without ants
				Resistant	Susceptible		Resistant	Susceptible		
Davao	60	30	6	0	6	20	0	20	52	8
Leyte	138	69	15	5	8	38	10	22	78	60
Sorsogon	178	89	21	6	15	51	15	36	90	88
Total	376	188	36	11	29	109	25	78	220	156

* = 2 accessions not identified as resistant or susceptible in Leyte

Table S2. Distribution of abaca accessions from three germplasm collection sites (Leyte, Sorsogon, Davao)

Abaca accessions	Bunchy top disease susceptibility	Location Site			Abaca accessions	Bunchy top disease susceptibility	Location Site		
		Leyte	Sorsogon	Davao			Leyte	Sorsogon	Davao
Bisaya	Susceptible		✓		Musa Tex 51	Resistant		✓	
Canton Forestry	Resistant	✓			Musa Tex 52	Resistant		✓	
Green Abaca	Susceptible			✓	Negro	Susceptible		✓	
Igit	Susceptible		✓		Pepita	Resistant	✓		
Inosa	Susceptible	✓	✓	✓	Putian	Susceptible		✓	
Inotang	Susceptible		✓		Putomag	Susceptible	✓		
Javaque	Resistant	✓			Samoro	Susceptible	✓	✓	
Kurukutuhan	Susceptible		✓		Sogmad	Resistant		✓	
Kutay-kutay	Resistant		✓		Suglin	Susceptible	✓		
Laguis	Resistant		✓		Tangongon	Susceptible	✓		✓
Lausigon Red	Resistant		✓		Tinawagan Pula	Susceptible		✓	
Laylay	Susceptible		✓	✓	Tinawagan Puti	Susceptible		✓	
Linawaan	Susceptible		✓		Tuod	Susceptible			✓
Linao Cultivar	Susceptible	✓			VH#23	Resistant	✓		
Lunhan	Susceptible	✓			VH#25	No data yet	✓		
Maguino	Susceptible		✓		VH#32	No data yet	✓		
Maguindanao	Susceptible		✓	✓	VH#39	Susceptible	✓		
Minenonga	Susceptible		✓		Wild#3	Resistant	✓		

Table S3. Summary on the total number of plants sampled per accession, number of aphid colonies per plant, and number of aphids collected based on the accessions (per site) used in the study.

Local Name	Plant susceptibility	Total plants Sampled	No. of plants with						Total Colonies	No. of aphids collected
			1 colony	2 colonies	3 colonies	4 colonies	5 colonies	6 colonies		
Green Abaca	Susceptible	4	4						4	16
Inosa	Susceptible	6	4				1	1	15	96
Laylay	Susceptible	7	3	3			1		14	100
Maguindanao	Susceptible	7	5	1	1				10	44
Tangongon	Susceptible	7	3	3		1			13	47
Tuod	Susceptible	3	2	1					4	20
	Total	34	21	8	1	1	2	1	60	323
Leyte:										
Canton Forestry	Resistant	3	3						3	6
Inosa	Susceptible	45	36	5	2	0	1	1	63	243
Javaque	Resistant	11	9	2					13	52
Linao Cultivar	Susceptible	14	12	2					16	64
Lunhan	Susceptible	11	10	1					12	51
Pepita	Resistant	2		1	1				5	18
Putomag	Susceptible	23	15	2	4	1	1		40	194
Samoro	Susceptible	16	5	2	3	1	4	1	48	241
Suglin	Susceptible	15	13	1	1				18	85

Tangongon	Susceptible	12	4	4	1	1	2		29	139
VH #23	Resistant	12	10	1				1	18	69
VH #25	ND	11	5	2	4				21	95
VH #32	ND	5	5						5	15
VH#39	Susceptible	8	5	3					11	48
Wild #3	Resistant	7	3	2	1	1			14	95
	Total	195	135	28	17	4	8	3	316	1415
Sorsogon										
Bisaya	Susceptible	5	2	1	2				10	30
Igit	Susceptible	7	7						7	21
Inosa	Susceptible	4	2	1				1	10	33
Inotang	Susceptible	4	4						4	11
Kurukutuhan	Susceptible	9	5	3			1		16	43
Kutay-kutay	Resistant	5	5						5	10
Laguis	Resistant	3	1	1	1				6	13
Lausigon Red	Resistant	5	3	2					7	23
Laylay	Susceptible	7	6			1			10	45
Linawaan	Susceptible	5	3	1				1	11	35
Maguindanao	Susceptible	3	2	1					4	17
Maguino	Resistant	6	4	1			1		11	45
Minenonga	Susceptible	6	5	1					7	22

Musa Tex 51	Resistant	5	5						5	20
Musa Tex 52	Resistant	6	2	2	2				12	50
Negro	Susceptible	8	8						8	28
Putian	Susceptible	7	4	3					10	18
Samoro	Susceptible	4	2	2					6	30
Sogmad	Resistant	4	4						4	12
Tinawagang Pula	Susceptible	6	4	1			1		11	50
Tinawagang Puti	Susceptible	7	5	1	1				10	57
	Total	116	83	21	6	1	3	2	174	613

Table S4. Estimates of null allele frequency for each locus

Locus	Estimate of null allele frequency		
	Leyte	Sorsogon	Davao
M35	0.03586	0.00011	0.00006
S17b	0.001	0.05222	0.001
S24	0.001	0.07403	0.001
AF1	0.0817	0.10358	0.001
AF4	0.09612	0.00031	0.0001
M62	0.01721	0	0
myz25	0.08914	0.00099	0.00098

Table S5. Genotype consistency of banana aphid's colony*

Population	No. of colonies	Consistent colony	Not consistent colony
Davao	26	23(88. 46%)	3 (11.54%)
Leyte	64	41 (64.06%)	23 (35.94%)
Sorsogon	84	76 (89.41%)	8 (9.41%)
Total	174	140 (80.46%)	34 (19.54%)

*7% of the missing data not counted

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CHAPTER 3. GEOGRAPHIC LOCATION INFLUENCES ENDOSYMBIONT COMMUNITIES IN BANANA APHID FROM ABACA PLANTS (*Musa textilis* Nee) IN THE PHILIPPINES

Abstract

Hosting bacterial endosymbionts can affect aphid development, reproduction, and resistance to natural enemies. Endosymbionts can also influence aphid adaptation to different environmental conditions and are considered as 'hidden players' in plant host, virus and vector relationships, with a potentially crucial role in plant virus transmission. The banana aphid (*Pentalonia sp.*) transmits the bunchy top virus responsible for the devastating bunchy top disease in abaca and banana. Understanding insect symbiosis, therefore, can be a powerful tool for the management of agricultural insect pests and plant health. In this study, banana aphids were collected from 36 abaca accessions across three abaca germplasm collections in the Philippines and their endosymbionts communities were identified. Alongside the primary aphid symbiont (*Buchnera aphidicola*) four secondary endosymbiotic bacteria were identified in two species of aphid: *Regiella insecticola*; *Serratia symbiotica; Rickettsia* and *Wolbachia* with individual aphids co-hosting up to four symbionts. The overall infection frequency of endosymbionts (74.5%) in banana aphids varied across location with the highest infection frequency in Davao (98.3%) and the lowest in Sorsogon (50%). Leyte had the highest number of infected aphids (132/138) which accounted for almost half of the total aphid symbiont infection (280/376). Geographic location significantly influenced endosymbiont communities, but endosymbiont communities did not vary with aphid species or across plant accessions. Multiple infections accounted for 50.8% of infections compared to single infections (23.7%), and in 25.5% of the samples no infections were detected. Often, aphids within the same colony hosted different symbionts, highlighting the highly variable composition and distribution of endosymbionts in banana aphids in this study. This high variation may be influenced by natural enemy pressure or abiotic pressures that aphids have been exposed to in a

specific location. Understanding the role of aphid symbionts in pest populations can help to improve aphid biocontrol and aid in the identification of novel methods for reducing transmission of important plant viruses.

Introduction

Symbiosis, the close association between unrelated organisms (De Bary, 1879; Heddi and Zaidman-Remy, 2018) is common among insects. In aphids (Hemiptera: Aphididae), stable and long-term mutualistic relationships with bacteria termed as 'endosymbionts', classified as obligate and facultative symbionts, have become an integral part in aphid diversification and successful invasion to wide-ranging environments (Dale and Moran, 2006; Gibson and Hunter, 2009; Brownlie and Johnson, 2009; Skaljac, 2016; Giron et al., 2017). Symbionts are vertically transmitted from parent to offspring and, in obligate relationships, provide the aphids with essential nutrients to survive (Baumann et al., 1995; Douglas, 1998; De Clerck et al., 2014; Russell et al., 2017), while facultative symbionts defend aphids against extreme temperatures, fungal pathogens and natural enemies (Tsuchida et al., 2005; Oliver et al., 2005; Vorburger et al., 2009; Brandt et al., 2017; Lukasik et al., 2013; Heyworth and Ferrari, 2015). In addition, they are coined as the 'hidden players' in insect-plant interactions (Biere et al., 2013; Frago et al., 2012; Sugio et al., 2015; Giron et al., 2017) by modifying the plant's response with regards to insect predators and natural enemies (Frago et al., 2012). Moreover, they are also considered as the potential 'fourth player' in plant host, virus and vector relationships (Pinheiro et al., 2015) as they are perceived to influence an insect's capacity to transmit and replicate the viruses, and hence may be responsible for virus persistence in the plant system (Su et al., 2013; Alkhedir et al., 2015). The occurrence of endosymbionts in aphid populations may hinder control programs by conferring host resistance to parasitoid wasps and host susceptibility to insecticides (Skaljac et al., 2018; Vorburger, 2018; Renoz et al., 2020). Understanding insect symbiosis therefore can be a powerful tool for the management of agricultural insect pests (Douglas, 2007; Ramadhar et al., 2014; Skaljac, 2016; Berasategui et al., 2016; Xie et al., 2019).

Of the known facultative symbionts, nine (*Arsenophonus, Hamiltonella defensa*, *Regiella insecticola*, *Rickettsia*, *Rickettsiella*, *Serratia symbiotica*, *Spiroplasma*, *X-type* (now *Fukatsuia symbiotica*) *and Wolbachia*) have been

widely studied in terms of their identification, functions, mode of transmission (Skaljac, 2016; Guo et al., 2017; Vorburger, 2018) and distribution across the globe (reviewed in Zytynska and Weisser, 2016). Unlike the obligate symbionts that have a high fidelity of vertical transmission (Ferrari and Vavre, 2011; Skaljac, 2016), facultative symbionts are transmitted vertically with a reduced fidelity, especially in the field and to a lower extent horizontally (Skaljac, 2016; Guo et al., 2017; Rock et al., 2017; Guyomar et al., 2018). They can be transmitted via host plants (Frago et al., 2012; Skaljac, 2016), natural enemies (ectoparasitic mites, parasitoids) (Caspi-Fluger et al., 2012; Gehrer and Vorburger, 2012; Jousselin et al., 2013), and potentially via infected honeydew (Darby and Douglas, 2003). Their presence can be beneficial under certain circumstances, but also detrimental as they can affect an aphid's development, reproduction, and adaptation under different environmental conditions. For example, *Hamiltonella*, *Regiella*, *Serratia* and *X-type* species may defend aphids against parasitoids (Oliver et al., 2005; Oliver et al., 2003; Lukasik et al., 2013; Heyworth and Ferrari, 2015; Ferrari et al., 2004); *Regiella, Rickettsia*, *Spiroplasma* and *X-type* can protect against fungal pathogens (Scarborough et al., 2005; Lukasik et al., 2013; Guay et al., 2009; Heyworth and Ferrari, 2015) while *Hamiltonella, Serratia* and *X-type* help tolerate heat stress (Montllor et al., 2002; Russell and Moran, 2006). However, *Hamiltonella, Rickettsia* and *Spiroplasma* may also reduce an aphid's lifespan and fecundity (Fukatsu et al., 2001; Simon et al., 2011), the *Arsenophonus* species can modify phenotypes and host plant preference (Lenhart and White, 2020; Wagner et al., 2015) and *Wolbachia*, *Spiroplasma, Arsenophonus and Rickettsia* can manipulate the reproduction of the host (Alkhedir et al., 2015; Amit et al., 2017; Ballinger and Perlman, 2019; Brandt et al., 2017; Crotti et al., 2012; De Clerck et al., 2014; Pinheiro et al., 2015; Vorburger et al., 2009).

As the potential function of different endosymbionts overlaps, hosting all of them would likely be costly (Zytynska et al., 2021) particularly in the absence of biotic and abiotic stress (Vorburger and Gouskov, 2011). Commonly, endosymbiont communities are generally of low diversity (Sugio et al., 2015;

and there is no assurance that multiple symbionts infections increases the defensive mechanism of the host (Weldon et al., 2020). This is because their existence in aphid populations is believed to rely on the trade-off between protective benefits and fitness costs they offer to host (Jamin and Vorburger, 2019). Of more than 150 aphid species collected in the field and assessed for endosymbionts, about 40%-60% of aphids in a population are infected with an average of 1-2 symbiont infection per aphid (reviewed in Zytynska and Weisser, 2016). However, multiple infections are normal in many of the studied aphids. For example, *Acyrthosiphon pisum* has been shown to harbour up to four symbionts (Russell et al., 2013), *Aphis fabae* also harbours four symbionts (Zytynska and Weisser, 2016*)*, while *Rhopalosiphum maidis* harbours up to six symbionts and *Rhopalosiphum padi* harbours five (Guo et al., 2019). Symbiont-symbiont interactions (and also random drift) can lead to positive and negative associations between specific symbiont species leading to some symbiont combinations occurring more often than expected at random (Mathé-Hubert et al., 2019).

The worldwide distribution of aphid symbionts is not fully known due to inadequate information for most aphid species across all regions (Zytynska and Weisser, 2016), incomplete identification of symbiotic communities (Jones et al., 2011) and the variability of the infection rate and frequency across aphid species (Sepulveda et al., 2016; Guidolin and Consoli, 2017). Nevertheless, studies have shown that endosymbiont infection would seem to depend on aphid species and genotypes (Leclair et al., 2017; Sepulveda et al., 2016; Rock et al., 2018; McLean et al., 2019) as well as host plants (Guidolin and Consoli, 2017; Russell et al., 2013). A next-generation sequencing study comparing the microbiomes of 46 aphid species revealed that the more related the species, the more similar symbiont infections are likely to be (McLean et al., 2019). Another study showed that increasing surrounding plant species richness in the field decreases and increases the diversity of aphid's symbiont community at the individual and at the population levels, respectively, which is suggested to occur through changes in natural enemy pressures (Zytynska et al., 2016). In a recent study, host plants determined the endosymbiotic

communities in Eriosomatinae aphids where different plants vary in symbiont compositions that subsequently affected the fidelity of transmitted symbionts (Xu et al., 2020). Other studies have suggested that symbiont strains (Rock et al.; 2018; Leclair et al., 2016; Brandt et al., 2017), temperature (Cayetano and Vorburger, 2013), natural enemy (parasitoids) (Vorburger, 2014; Hafer and Vorburger, 2019), geographic location (Zytynska and Weisser, 2016; Guo et al., 2019) and their associations with other symbionts (Mathé-Hubert et al., 2019; Guay et al., 2009) can also affect symbiont distribution. However, recent findings showed that symbiont distribution in aphids seemed to be not associated in terms of taxonomy and geographic separation (Xu et al., 2020). Of the above-mentioned factors affecting symbiont distribution, there is limited information with regards to the consistency of endosymbiont infection of individual aphids within an aphid colony. Consistency means that every aphid in one colony hosts the same endosymbionts. For clonally reproducing aphids, the mother aphid transmits her symbionts to the offspring and if the colony is all from the same mother, then it is assumed, they will have the same symbiont infection (De Clerck et al., 2015). Reduced success of vertical transmission, multiple different mother aphids, or horizontal transmission among different aphid clones hosting different symbionts could alter the consistency of colony infection status. However, the driving factors that influence endosymbionts' presence or absence within and among aphid populations remains elusive (McLean et al., 2019).

The banana aphid, *Pentalonia sp.* is an economically important agricultural insect pest responsible for, among other things, the spread of bunchy top viruses. These are devastating diseases with two main groups: the *banana bunchy top virus* (BBTV), affecting banana and plantain production worldwide (Harding et al., 1991; Hu et al., 1996; Thomas and Iskra-Caruana, 2000; Kumar et al., 2015); and the *abaca bunchy top virus* (ABTV), affecting abaca (*Musa textilis* Nee) production in the Philippines (Bajet and Magnaye, 2002). Studies of endosymbionts in banana aphids are very limited in terms of location, the number of individual aphids and host plants used in the study. The first recorded endosymbiont study on *Pentalonia* species was conducted

in Hawaii on *P. caladii* van der Goot, a closely related species of *P. nigronervosa* (Jones et al., 2011). In 2014, a study on endosymbionts used 20 individual aphids (reared on banana plantlets' Willian variety) from six banana aphid strains collected from Africa (Gabon, Madagascar and Burundi) and in 2015, they used five individual aphids for each strain from Africa (Madagascar, Burundi, Gabon, Rwanda), Latin America (Brazil) and Australia (Tinbeerwah and Brisbane) (De Clerck et al., 2015; De Clerck et al., 2014). Among the top three producers of banana around the world (Asia, Africa and Latin America) (FAO, 2020), no study has been conducted yet on endosymbionts on *Pentalonia* aphids in the Asian Region and in the Philippines in particular.

To expand our understanding on the diversity of endosymbionts in banana aphids and their potential role in the ecology and epidemiology of the bunchy top virus transmission, banana aphids were collected from three abaca germplasm collections in the Philippines. The aims of the study were to 1) characterize the endosymbiont community hosted by banana aphids in the Philippines; 2) assess how aphid species, collection location, host plant accession and ant presence/absence influences symbiont distribution and composition; 3) identify specific combinations of symbionts that are more often hosted by aphids across the locations and 4) determine if aphids within a colony hosted the same endosymbionts.

Materials and Methods

See Chapter 2 for sample collection and DNA extraction.

Initial screening of bacterial endosymbionts

The endosymbionts in aphids were identified by the amplification of the specific regions of the 16S ribosomal RNA (rRNA) gene from aphid genomic DNA extracts using species-specific primers (Fukatsu and Nikoh, 1998; Heddi et al., 1999; Fukatsu et al., 2000; Tsuchida et al., 2002; Sandstorm et al., 2001; Ferrari et al., 2012). The 16S rRNA gene is the preferred target because it is widespread in prokaryotes and the conserved regions, which are only weakly affected by horizontal gene transfer, can be used for designing taxon-specific primers (Head et al., 1998; Daubin et al., 2003; Větrovský and Baldrian, 2013). The symbiont specific primers produced different amplicon sizes that were distinct for each symbiont, and primer accuracy tests showed them to be highly reliable in identifying symbionts in aphids (Henry et al., 2015).

A pooling approach (Sint et al., 2016) was employed to screen the presence of endosymbionts in banana aphid samples and determine which endosymbiont species were present in the Philippines. This strategy aimed to analyse all nine potential symbionts across pooled DNA samples to reduce the number of PCR reactions required per sample. For the initial screening, representative aphid samples per collection site were chosen based on abaca accessions, the presence and absence of ants, and the total number of aphids (> 5 aphids) per colony collected. Two individual aphids per colony were extracted per abaca accession per site. Sixty-four randomly selected DNA samples from single aphids (N1-N64) from the three abaca germplasm collections were laid out in an 8 x 8 grid where aliquots (4 μL) from individual DNA are pooled together vertically (A-H) and horizontally (1-8) (Table 7).

Table 7. Pooling approach in an 8 x 8 grid

	1	2	3	4	5	6	7	8	Pooled DNA
A	N1	N9	N17	N25	N33	N41	N49	N57	PA
B	N2	N10	N18	N26	N34	N42	N50	N58	PB
C	N3	N11	N19	N27	N35	N43	N51	N59	PC
D	N4	N12	N20	N28	N36	N44	N52	N60	PD
E	N5	N13	N21	N29	N37	N45	N53	N61	PE
F	N6	N14	N22	N30	N38	N46	N54	N62	PF
G	N7	N15	N23	N31	N39	N47	N55	N63	PG
H	N8	N16	N24	N32	N40	N48	N56	N64	PH
Pooled DNA	P1	P2	P3	P4	P5	P6	P7	P8	

For the pooled DNA, nine endosymbiont primers were used to test the presence of *Hamiltonella defensa* (450 bp), *Regiella insecticola* (840 bp), *Serratia symbiotica* (1140 bp), X-type (468 bp), *Rickettsia sp*. (600 bp), *Spiroplasma* (1500 bp), *Rickettsiella* (300 bp), *Wolbachia* (450 bp), and *Arsenophonus* (~600 bp) endosymbionts in the samples (Table 8). A previous study using the above species-specific primers showed that amplified sequences with different amplicon sizes were assigned correctly to symbiont species when validated through sequencing (Zytynska et al., 2016).

The PCR reaction for each primer pair was performed using a LifeTouch Thermal Cycler (Bioer Technology, Germany) in a volume of 20 μL (4 μL 5x MyTaq Reaction Buffer which contained dNTPs, $MgCl_2$ and enhancers that eradicates an optimization requirement (Bioline), 0.4 μM primer, 0.1 μL My Taq (5U/μl) and 1 μL of DNA) under the following PCR conditions: 94 ºC for 5 min, followed by 10 cycles of 94 ºC for 15 s, 65-55 ºC for 30 s and 72 ºC for 30 s, then 25 cycles of 94 ºC for 15s, 55 ºC for 30 s and 72 ºC for 30 s and final extension of 72 ºC for 6 mins (Zytynska et al., 2016). Amplicons were separated through electrophoresis in 1.5% agarose gels and the size of each amplicon was determined using 1 KB Plus DNA Ladder. The presence and absence of corresponding bands with the expected size of the symbiont species were visualized using Ethidium bromide and scored using the Gel Documentation-Gel-Stick “Touch” Imager (Royal Biotech Gmbh, Branderburg, Germany).

Table 8. List of primers and their references used in the identification of endosymbionts in aphids.

Symbiont Species	Primer Name	Primer sequence 5' to 3'	Size	References
	10F	AGTTTGATCATGGCTCAGATTG	471	Sandström et al., 2001
Hamiltonella defensa	T419R	AAATGGTATTCGCATTTATCG		Ferrari et al., 2012
	10F	AGTTTGATCATGGCTCAGATTG	480	Sandström et al., 2001
Regiella insecticola	U443R	GGTAACGTCAATCGATAAGCA		Ferrari et al., 2012
	10F	AGTTTGATCATGGCTCAGATTG	488	Sandström et al., 2001
Serratia symbiotica	R443R	CTTCTGCGAGTAACGTCAATG		Ferrari et al., 2012
	10F	AGTTTGATCATGGCTCAGATTG	468	Sandström et al., 2001
X-type	X420R	GCAACACTCTTTGCATTGCT		Ferrari et al., 2012
	16SA1	AGAGTTTGATCMTGGCTCAG	205	Fukatsu et al., 2001
Rickettsia sp.	Rick16SR	CATCCATCAGCGATAAATCTTTC		Fukatsu et al., 2001
	10F	AGTTTGATCATGGCTCAGATTG	1500	Sandström et al., 2001
Spiroplasma sp.	TKSSsp	TAGCCGTGGCTTTCTGGTAA		Fukatsu et al., 2000
	RCL16S-211F	GGGCCTTGCGCTCTAGGT	300	Tsuchida et al., 2010
Rickettsiella	RCL16S-470R	TGGGTACCGTCACAGTAATCGA		Tsuchida et al., 2010
	W-SpecF	CATACCTATTCGAAGGGATAG	450	Werren & Windsor, 2000
Wolbachia	W-SpecR	AGCTTCGAGTGAAACCAATTC		Werren & Windsor, 2000
	Ars-23S1F	CGTTTGATGAATTCATAGTCAAA	540-560	Thao & Baumann, 2004
Arsenophonus	Ars-23S2R	GGTCCTCCAGTTAGTGTTACCCAAC		Thao & Baumann, 2004

Endosymbiont distribution analysis

A total of 376 individual aphids collected from 188 colonies on 109 individual abaca plants across 36 abaca accessions from Davao, Leyte and Sorsogon were used in the study (Appendix 1). Two aphids per colony were used for the analysis and colonies were distributed across two to six plants for each accession (Table S2). The same aphid samples were used in Chapter 2 and therefore aphid species designation information was also available. The number of colonies per plant from which endosymbiotic bacteria were identified ranged from one to four and depended upon field infestation rates of the aphids.

Optimization of multiplex-PCR for full endosymbiont screening

To screen the presence of identified endosymbionts, a multiplex-PCR was attempted using the five primer combinations that showed amplification in banana aphids. These four primers (Ric600R, PAUS16SR, PASS1140R, W2) together with universal forward primers (10F and 16SA1) were used to test the endosymbionts simultaneously in one PCR reaction (Table 9). In addition, Buch270R primer (Peccoud et al., 2014) was used as a positive control to amplify *Buchnera aphidicola*, an obligate bacterial endosymbiont presents in all aphids and UltraPure™ DNase/RNase-Free distilled water was also used as negative control.

Table 9. List of primers and their references used for the development of multiplex PCR.

Symbiont Species	Primer Name	Primer sequence 5' to 3'	Size	References
Regiella insecticola	16SA1	AGAGTTTGATCMTGGCTCAG	840	Fukatsu et al., 2001
	PAU16SR	TCGGACGCCATAACACTAGG		Peccoud et al., 2014
Serratia symbiotica	10F	AGTTTGATCATGGCTCAGATTG	1140	Sandström et al., 2001
	PASS1140R	TTTGAGTTCCCGACTTTATCG		Peccoud et al., 2014
Rickettsia sp.	16SA1	AGAGTTTGATCMTGGCTCAG	600	Fukatsu et al., 2001
	Ric600R	TTTGAAAGCAATTCCGAGGT		Peccoud et al., 2014
Wolbachia	16SA1	AGAGTTTGATCMTGGCTCAG	450	Fukatsu et al., 2001
	W2	CTTCTGTGAGTACCGTCATTATC		Heddi et al., 1999
Buchnera aphidicola	16SA1	AGAGTTTGATCMTGGCTCAG	270	Fukatsu et al., 2001
	Buch270R	TGCCTTGGTAGGCTATTACTC		

Table 10. Primers used to detect endosymbionts in singleplex and multiplex PCR.

PCR Reaction	Primer Name	Specificity	Primer sequence 5' to 3'	Size (bp)
Singleplex	16SA1	Universal F primer	AGAGTTTGATCMTGGCTCAG	
	PAUS16SR	*Regiella insecticola*	TCGGACGCCATAACACTAGG	840
Multiplex 1	16SA1	Universal F primer	AGAGTTTGATCMTGGCTCAG	
	Buch270R	*Buchnera aphidicola*	TGCCTTGGTAGGCTATTACTC	270
	10F	Forward primer	AGTTTGATCATGGCTCAGATTG	
	R443R	*Serratia symbiotica*	CTTCTGCGAGTAACGTCAATG	488
Multiplex 2	16SA1	Universal F primer	AGAGTTTGATCMTGGCTCAG	
	Rick16SR	*Rickettsia sp.*	CATCCATCAGCGATAAATCTTTC	205
	W2	*Wolbachia*	CTTCTGTGAGTACCGTCATTATC	450

The five-primer combinations did not work completely as bands representing *Serratia* and *Regiella* did not show amplification, potentially due to having lower annealing temperatures than the other primers, thus reducing the ability to multiplex these together even after optimisation. Therefore, two PCR multiplexes (Table 10) were developed to distinctly identify the endosymbionts: Multiplex 1 consisted of (*Buchnera* (0.05 μM) and *Serratia* (0.40 μM) primers and multiplex 2 consisted of *Rickettsia* (0.20 μM) and *Wolbachia* (0.05 μM) primers. Singleplex PCR reaction was used to amplify *Regiella* (0.40 μM).

The PCR reactions were performed in 10 μL, including 2 μL 5x MyTaq Reaction Buffer (Bioline), 0.1 μL My Taq and 1 μL DNA (20 μg/μL). Both simplex and multiplex-PCRs were performed using a touchdown program (at 65ºC - 55ºC) with PCR conditions (94 ºC for 5 min, followed by 10 cycles of 94 ºC for 15 s, 65-55 ºC for 30 s and 72 ºC for 30 s, then 25 cycles of 94 ºC for 15s, 55 ºC for 30 s and 72 ºC for 30 s and final extension of 72 ºC for 6 min) (Zytynska et al., 2016). Amplicons were separated through electrophoresis in 1.5% agarose gels and the size of each amplicon was determined using 1 KB Plus DNA Ladder. The presence and absence of corresponding bands with the expected size of the symbionts species were visualized using the GelGreen® nucleic acid gel stain (Biotium) on the Gel Imaging System (Life Science Research, BIO-RAD, UK) and scored manually.

Data analysis

Species richness of endosymbiont infection for the full data set (i.e. number of symbiont species) was calculated and analysed with a linear mixed effect model using the lme4 programme (Bates et al., 2015) in R version 4.0.2 (R Core Team, 2020) where location, plant accession nested within location, presence of ants and aphid species were included as fixed effects and aphid colony nested within individual plant were included as random effects. Significance values for the fixed effects were determined using the Anova

function in the car package (Fox and Weisberg, 2019). Data from Leyte only were then analysed with a linear mixed effect model where plant accession, presence of ants and aphid species were included as fixed effects and aphid colony nested within individual plant were included as random effects.

Individual aphid samples that showed amplification using the five-specific endosymbiont primers (Table 10) were scored as 1 and 0 for the presence and absence of endosymbiont, respectively. Single and multiple infections were tabulated and analysed for all aphid samples (Table S6). Food Web Designer v3.0 (Sint and Traugott, 2016) was used to visualize the infection association among endosymbionts and a chi-square test undertaken to check the association between dual and triple symbiont infections.

For the consistency of aphid infection per aphid colony, each pair of aphids was scored from 0 to 1, where 0 meant the endosymbiont community was not consistent and 1 that they were the same. Total counts of 0 and 1 were tabulated in a contingency table for association determination and analysed with a chi-square test of association.

Results

Initial screening of bacterial endosymbionts

During the initial screening of symbionts, the pooling approach successfully identified *Regiella insecticola*; *Serratia symbiotica; Rickettsia sp*. and *Wolbachia* infecting banana aphids. Identified symbionts were selected as DNA targets for multiplex-PCR containing five symbiont specific primers (including *Buchnera aphidicola* as positive control) which only showed successful amplification of *Buchnera*, *Rickettsia* and *Wolbachia* at 65-55 ºC PCR condition. The development of two multiplex PCR successfully identified symbionts in all aphid samples.

Endosymbiont species richness

For the full sample set (376 aphids), colony (nested within plant) accounted for 11% of the variation in the data and plant for 17% of the variation in the data. Of the fixed effects, only collection site significantly influenced endosymbiont species richness (X^2 = 233.40, df = 2, p <0.0001) with higher numbers of endosymbionts infecting aphids in Davao (59/60) and Leyte (132/138) compared to Sorsogon (89/178) (Figure 9, Table S6). There was no effect of plant accession nested within location (X^2 = 44.53, df = 39, p = 0.25) and no of effect of either presence of ants (X^2 = 0.0004, df = 1, p = 0.98) or the genetic grouping that the aphids were assigned to (X^2 = 0.96, df = 1, p = 0.33). For the data from Leyte only, colony nested within plant accounted for 19.8% of the variation in the data and plant for 7.4% of the variation in the data. There were no effects of ants or aphid species.

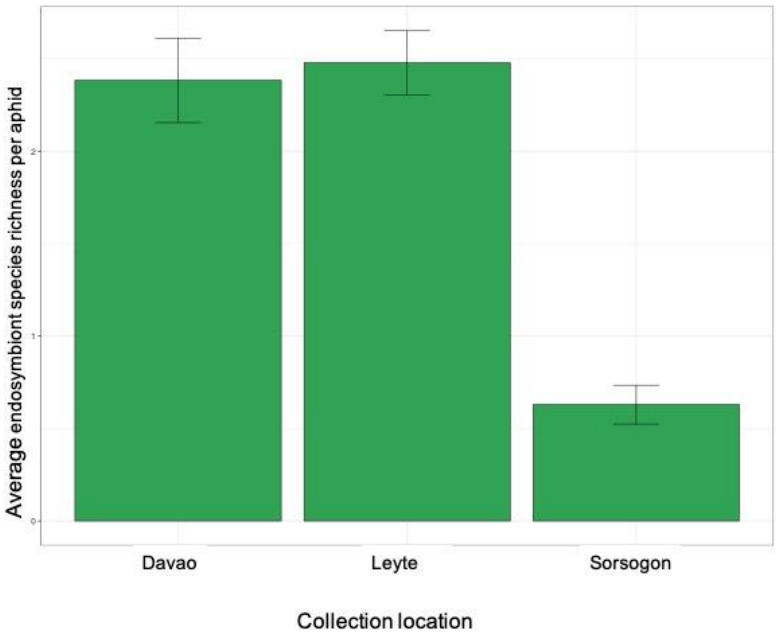

Figure 9. Average species richness of the endosymbiont community infecting banana aphids collected from three germplasm collections in the Philippines. Error bars are 95% confidence intervals.

Symbiont co-infection and distribution in P. nigronervosa

The population genetic structure analysis (see Chapter 2) showed two *Pentalonia* species: *P. caladii* (42/376) and *P. nigronervosa* (334/376). Since aphid samples that belonged to *P. caladii* were not evenly distributed across location (only two aphids from Davao, three aphids from Sorsogon and 37 aphids from Leyte), further symbiont infection analysis was focused on *P. nigronervosa*.

The overall infection frequency of endosymbionts in *P. nigronervosa* from abaca plants varied among symbiont with *Rickettsia* (45.2%) showing the highest levels of infection followed by *Serratia* (42.8%), *Wolbachia* (35.6%) and *Regiella* (23.9%). Multiple infections accounted about 46.1% of the total *P. nigronervosa* populations compared to single infections (25.7%), and in 28.1% of the samples no infections were detected. Among the aphids with a single endosymbiont, *Serratia* (14.1%) had the highest infection levels,

followed by *Rickettsia* 9.6%), *Regiella* (1.5%) and *Wolbachia* (0.6%). Overall infection was highest in Davao and lowest in Sorsogon. Co-infection with more than one symbiont was also more prevalent in Davao and Leyte than in Sorsogon (Figure 10).

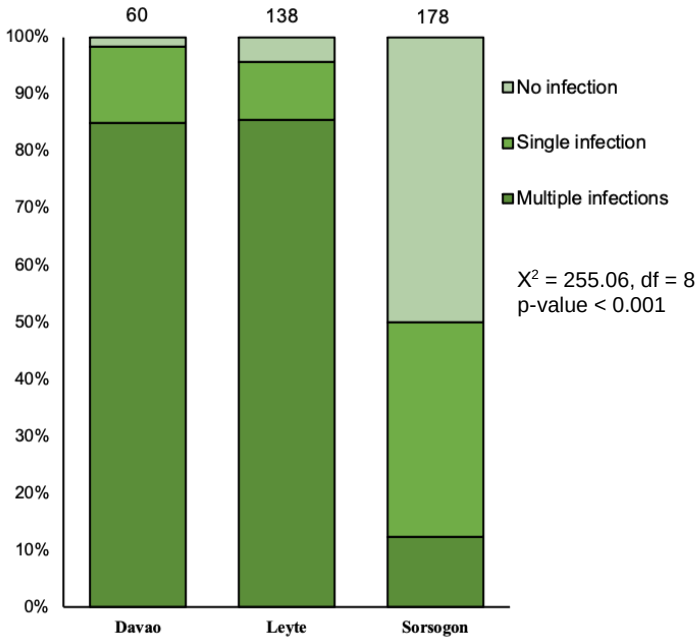

Figure 10. Single vs multiple infections of endosymbionts across collection sites.

Of the 46.1% multiple infections, 24% were dual infections, 15% for triple and 7.2% for quadruple infections. *Rickettsia-Wolbachia* (RkW) (35/80) and *Regiella-Serratia* (RiSs) (21/80) together with *Rickettsia-Serratia-Wolbachia* (RkSsW) (26/50) and *Regiella-Rickettsia-Wolbachia* (RiRkW) (18/50) associations were the most common co-infection combinations for dual and triple infection across geographic location, respectively (Figure 11).

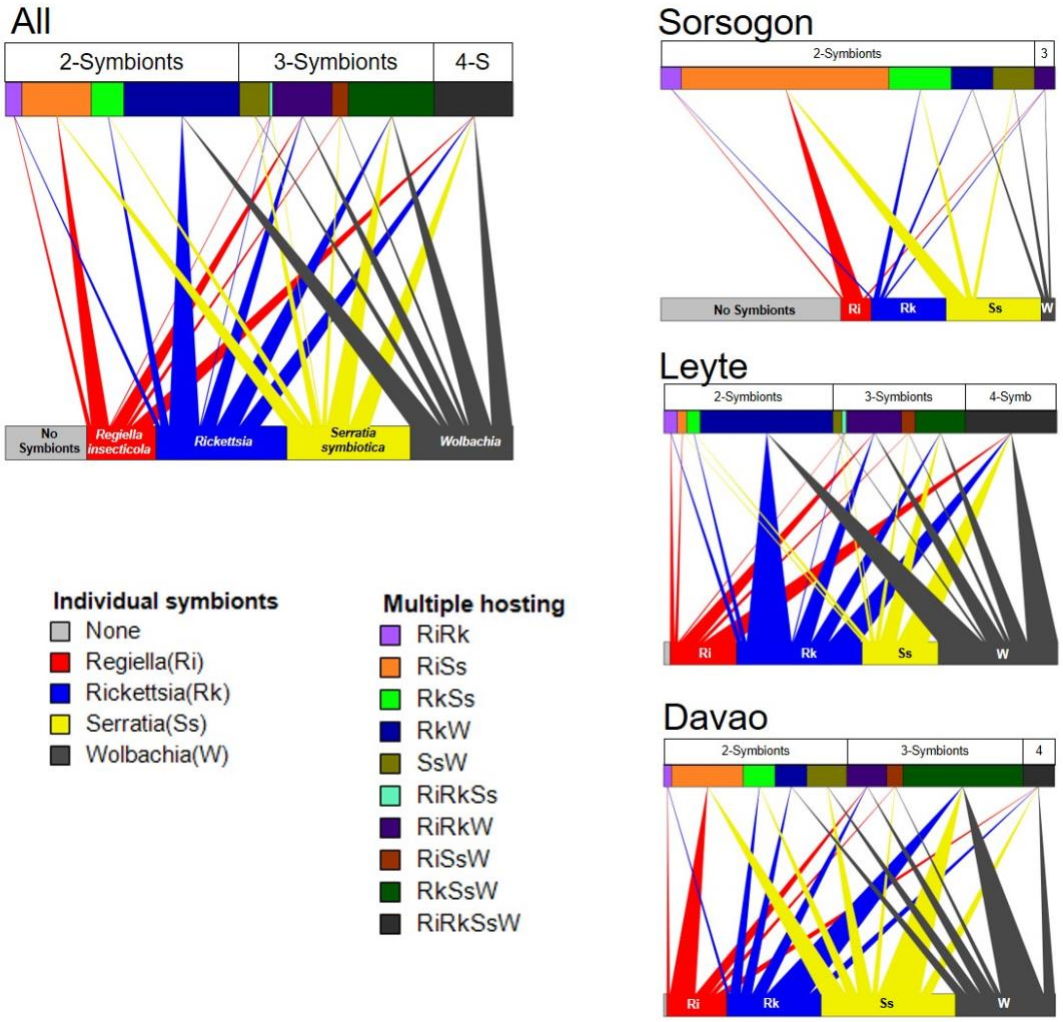

Figure 11. Multiple infections of endosymbionts across collection sites. Colours on the bottom indicates the individual symbionts and the colours at the top show the different symbiont combinations. The width of the lines represents symbionts abundance.

In Davao, RiSs (9/21) and RkSsW (15/26) were the most common endosymbiont infection combinations while only RiSs (10/21) was the most common combination in Sorsogon. Leyte collections had 20 individual aphids that hosted four endosymbionts and common dual and triple infection combinations were RkW (29/35), RiRkW (12/18) and RkSsW (11/26). Within aphids hosting two or more symbionts, 67% (103/154) hosted both RkW and this pair was found to be associated more often than expected at random with

Ss symbiont (RkWSs: X^2=8.19, df=1, P=0.004) in Davao, but similar patterns were not found in Leyte (RkWSs: X^2=3.18, df=1, P=0.075; Figure 12).

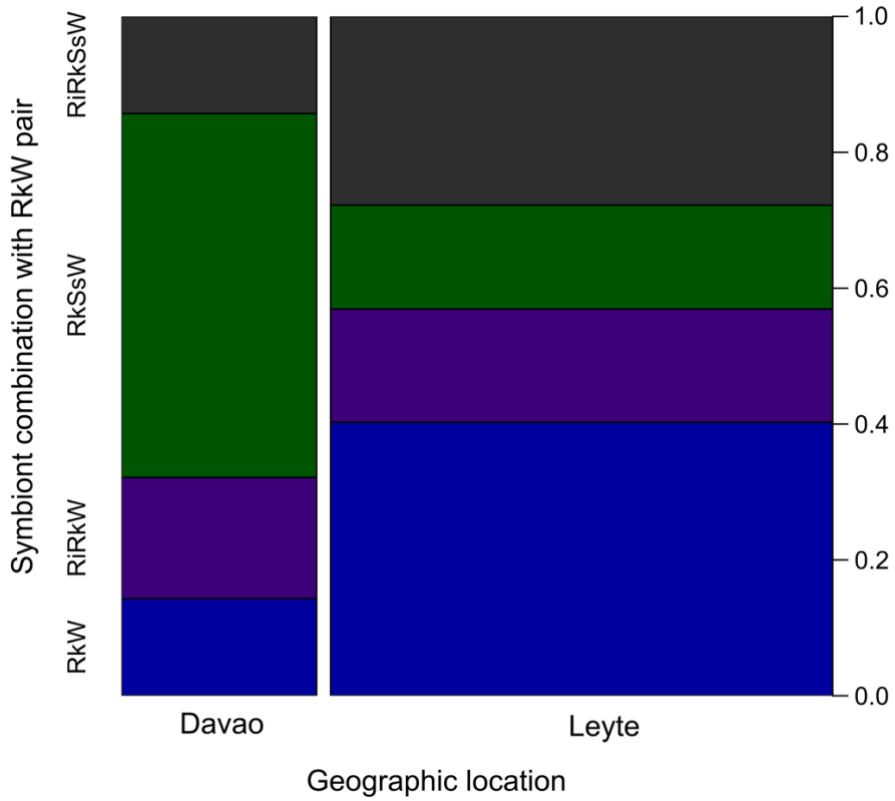

Figure 12. The distribution of different symbiont combinations with RkW pair in Davao and Leyte. Colours indicate the association of RkW to *Regiella* (RiRkW), *Serratia* (RkSsW) and *Regiella-Serratia* (RiRkSsW).

Consistency of symbiont infection per aphid colony

Across all samples there was considerable variation in the endosymbiont community detected in aphids collected from the same colony. Almost half (49.5%) of the aphid colonies across the collection sites hosted endosymbiont communities that were not consistent between individuals. Of the remaining colonies, 33% appeared to have consistent endosymbiont communities and 17.5% had no endosymbiont infection in either individual (Figure 13).

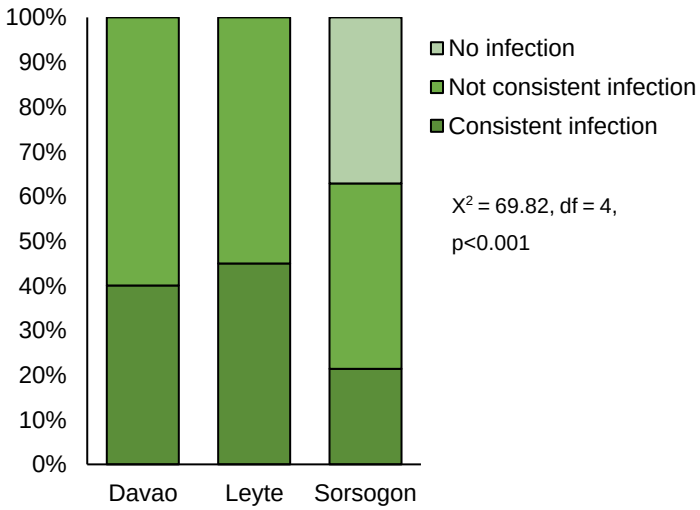

Figure 13. Consistency of endosymbiont infection within aphid colonies across collection sites.

Discussion

In this study, the presence of the common aphid secondary bacterial endosymbionts was assessed in banana aphids from different abaca accessions collected across three abaca germplasm collections in the Philippines. This is the first study conducted to identify the endosymbionts in banana aphids collected from abaca plants. Four secondary endosymbionts were identified (*Regiella*, *Rickettsia*, Serratia and *Wolbachia*) as infecting two banana aphid species: *P. nigronervosa* and *P. caladii*. Multiple hosting of secondary endosymbionts accounted for half of the aphid samples, with 7.5% of the aphids hosting all four, 18.6% hosting three and 24.7% hosting two symbionts. The number of symbionts an individual aphid hosted (symbiont species richness) was significantly influenced by the geographic collection but there was no effect of plant accession, presence of ants nor the two *Pentalonia* species. The association between *Rickettsia* and *Wolbachia* (RkW) was the most prevalent symbiont combination and was found to co-infect more often with each other as a dual infection, or with either *Regiella* or *Serattia* as triple infection. Not all aphids in a colony hosted the same combination of symbionts, indicating substantial inconsistency of colony-level symbiont status.

Symbiont identification and species richness

Total symbiont infection accounted for 74.5% of the populations while a substantial number of aphids had no infection in Davao (1.7%), Leyte (4.4%) and Sorsogon (50%). The highest number of symbiont infections were observed in Davao (59/60) and Leyte (132/138) collection areas, in contrast to Sorsogon (89/178) where only half of aphids sampled were infected. This result showed that aphids from the northern most collection site were less likely to be infected by symbionts. Previous work has shown a latitudinal gradient in symbiont frequencies for pea aphids in Japan (Tsuchida et al., 2002), and suggests some aphid-symbiont combinations may be affected by environmental or historical factors. Indeed, abiotic factors such as heat-stress

can influence symbionts (Russell and Moran, 2006) with secondary symbionts able to provide nutritional support when primary symbiont fails (Koga et al., 2003). It is also expected that infection frequencies will change over time (even within a season) as the aphid populations also respond to variable natural enemy pressures (Smith et al., 2015); however, sample collection was during a single time-point and is thus just a snapshot of the infection frequency in these populations. Interestingly, regardless of geographic location and host plants, the four identified endosymbiotic bacteria were also the same symbionts with varying infection frequencies that infect banana aphids in Africa, Australia, Brazil and Hawaii (Jones et al., 2011; De Clerck et al., 2015; De Clerck et al., 2014; Zytynska and Weisser, 2016).

Among the four endosymbiotic bacteria, *Rickettsia* (Rk) had the highest infection of almost half of the banana aphid population (45.2%:151/334) and with the highest infection in Leyte (80%:81/101). Its prevalence was commonly found in aphids having more than one facultative symbiont particularly in Leyte where it had a dual association with *Wolbachia* (RkW) and triple infection with either *Regiella* (RiRkW) or *Serratia* (RkSsW). However, this was not the case in Sorsogon where 30 out of 86 infected aphids were of single infection. This scenario indicates that certain symbionts can infect aphids either in association with other symbionts or with single infection in a particular location. *Rickettsia* has also been shown to confer protection against a fungal pathogen (*Pandora neoaphidis*) (Łukasik et al., 2013). The presence of *Rickettsia* in banana aphids could potentially be attributed to the *Fusarium oxysporum* strains that infect abaca (Halos, 2008; Borines et al., 2007; Bastasa and Baliad, 2005), since this is also one of the *Fusarium* species that harbors entomopathogenic strains (Santos et al., 2020). In other insect species, *Rickettsia* has been found to improve heat-tolerance (Brumin et al., 2011), but this has not been studied in aphids and could also explain the high infection rates in our samples.

The second highest symbiont infecting 42.8% (143/334) of the banana aphids, *Serratia symbiotica* (Ss) had the highest infection in Davao (81%:47/58)

compared to Leyte (48.5%:49/101). In Sorsogon (26.9%:47/175), 32 out of 47 aphids infected with Ss were of single infection, which was the highest single infection rate observed of Ss among geographic locations and the only symbiont with highest infection when infecting alone than when co-infecting with other symbionts. This symbiont was also found in triple infections with the common *Rickettsia*-*Wolbachia* (RkSSW) more frequently in Davao (53.6%:15/28) than in Leyte (15.3%:11/72). Reported to protect aphids against parasitoids and heat stress (Oliver et al., 2003; Russell and Moran, 2006), this symbiont was previously detected in banana aphids only from field collections in Hawaii but was absent in reared banana aphids from Africa, Australia and Brazil (De Clerck et al., 2015; De Clerck et al., 2014; Zytynska and Weisser, 2016). Field collected aphids in the Philippines could possibly experience thermal stress being a tropical country; however, this would also be expected in Africa and Australia. In *Cinara* species, *Serratia* is a permanent co-primary endosymbiont needed for survival (Meseguer et al., 2017; Augustinos et al., 2011; Leybourne et al., 2018) and their presence in Philippine banana aphids might also indicate nutrient provision to the host, with benefits to the aphid in event of reduced abundance of the *Buchnera* symbiont. We observed that *Serratia* had the highest infection among symbionts in Davao which could be an insect response to adverse abiotic conditions experienced earlier in the area prior to sampling collection.

Wolbachia (W) infected 35.6% (77/334) of the banana aphids with highest infection in Leyte (76.2%:77/101), infected more than half of aphids in Davao (60.3%:35/58) and with low infection in Sorsogon (4%:7/175). This symbiont was observed to infect more aphids when in association with other symbionts as single infection was only detected in two aphids in Sorsogon and no single infection was found in Davao or Leyte. This symbiont had strong association with *Rickettsia* (RkW) (35/80), a prominent symbiont combination which can infect either with *Regiella* (RiRkW, 18/50) or *Serratia* (RkSsW, 26/50). Considered as prominent symbiont, proposed to be vertically transmitted in *P. nigrovervosa* (De Clerck et al., 2014), this symbiont was detected earlier in *P. caladii* in Hawaii (Jones et al., 2011), *P. nigronervosa* in Africa, Australia and

Brazil (De Clerck et al., 2015; De Clerck et al., 2014; Zytynska and Weisser, 2016), and presently infected both *Pentalonia* species, *P. nigronervosa* and *P. caladii* in the Philippines. However, data have shown that although *Wolbachia* was present in both *Pentalonia* species, not all aphids were infected in Sorsogon (10/178) dominated by *P. nigronervosa* species. The role of *Wolbachia* in aphids is currently unknown but in other insect species, it has been associated with different reproductive manipulation such as parthenogenesis, feminization, cytoplasmic incompatibility, and male killing (Rousset et al., 1992; Stouthamer et al., 1993; Augustinos et al., 2011; Hoffmann et al., 1996; Werren et al., 2008). The detection in banana aphids was suggested to influence the asexual reproduction in *P. nigronervosa* (De Clerck et al., 2014); however, this *Pentalonia* species has been reproducing asexually signifying that this symbiont is more likely to have other host benefits (Jones et al., 2011). It has also been suggested that their presence in banana aphids could have evolved as co-obligate to *Buchnera* (De Cleck et al., 2015), but this claim was recently corrected as no evidence was found for *Wolbachia* to act as a co-obligate symbiont (Manzano-Marin, 2020). The low infection of *Wolbachia* in Sorsogon (4%) and the absence of any single infection in Davao and Leyte suggests little evidence that it involved as a co-obligate to *Buchnera* here. *Wolbachia* co-existence with other symbionts in banana aphids might be further enhance host fitness, as have been observed in non-aphid insects along with regards to its role in host fitness enhancement, some provision for viral defence and fecundity enrichment (Cooper et al., 2019; Gill et al., 2014; Hedges et al., 2008; Kriesner and Hoffmann, 2018; Martinez et al., 2014; Moriyama et al., 2015; Weeks et al., 2007).

The endosymbiont infecting the fewest banana aphids was *Regiella insecticola* (Ri) (24%:80/334) with higher infection frequencies in Leyte (43.6%:44/101) compared to Davao (36.2%:21/58) and Sorsogon (8.6%:15/175). This symbiont was found in combination with *Serratia* (RiSs, 21/80) and *Rickettsia-Wolbachia* (RiRkW, 18/50), but had no variation in its association with *Wolbachia* across geographic locations (as was found for *Serratia*). Although this symbiont is common in pea aphids globally (reviewed

in Zytynska and Weisser, 2016), its infection was not previously detected in banana aphids from Australia, Brazil and Hawaii but was present in Africa and, as shown here, the Philippines (De Clerck et al., 2015; De Clerck et al., 2014; Zytynska and Weisser, 2016; Jones et al., 2011). *Regiella* has been shown to provide aphids with protection against pathogenic fungi, parasitoid wasps and can be involved in host plant adaptation (Scarborough et al., 2005; Tsuchida et al., 2004; Tsutomu et al., 2011; Parker et al., 2013; Nikoh et al., 2020). However, this symbiont can also reduce heat tolerance (Russell and Moran, 2006) and lessen the production of wing and sexual morphs (Leonardo and Mondor, 2006). The high infection of *Regiella* in Leyte might be for aphid protection against parasitoid wasps and pathogenic fungi present in the area (as recent observations suggest high abundance of fungal pathogens, predators, and parasitoids in the area). Because of high infection of *Serratia* and *Rickettsia* in the population, which both gives the same protection against parasitoid and fungal pathogen, hosting *Regiella* might be negligible with their presence or provide protection from different species/strains of natural enemies. Moreover, the negative effects posed by this symbiont (reduced heat tolerance) might also hinder the aphids from hosting them in these tropical regions. Since both *Regiella* and *Wolbachia* may also have a similar effect on sexual morph production and enhancing parthenogenesis, their association is not as relevant for asexually reproducing banana aphids, hence their reduced frequency.

Symbiont co-infection frequency and distribution

The endosymbionts in banana aphids exhibited multiple co-infections with individual aphids hosting up to four symbionts. The most common symbiont combinations were RiSs and RkW for dual infections and RiRkW and RkSsW for triple infections. Across geographic locations and among symbiont combinations, RkW was the most prominent symbiont association, suggesting a strong positive association between these symbionts and most likely indicates strong positive effects for the aphids. This pair was significantly

associated more often than expected at random with Ss symbionts (RkWSs) in Davao. Among geographic locations, Leyte had the highest infection frequency for dual (38.6%), triple (26.7%) and quadruple infections (19.8%). Although Davao had 98.3% (57/58) symbiont infection, it had low to moderate infection frequencies of 39.7% for dual infection, 37.9% for triple and 6.9% for quadruple infection. In Sorsogon with only half of the aphid population (86/175) were infected, only 22.1% of the infected aphids hosted two to three symbionts. The differences in multiple infection frequencies could be in response to varying biotic and abiotic pressures across geographic locations. Co-hosting of symbionts is common across many aphid species (Fakhour et al., 2018; Guo et al., 2019) and can have negative and positive effects on the host aphids. If benefits consistently outweigh any costs of hosting symbiont combinations, we might eventually expect these to become fixed in a population (Oliver et al., 2006); however, random loss of symbionts (Rock et al., 2018) or drift (Mathé-Hubert et al., 2019) reduces the chances of this happening. For RkW infected aphids, the additive effects of hosting Ss (RkSsW) and Ri (RiRkW) or both Ri and Ss (RiRkSsW) might confer more benefits than any costs of hosting, especially if they protect the aphid from different stressors in a diverse environment. When two defensive symbionts co-infect the host system, it can increase the host defence mechanism than single infection (Łukasik et al., 2013). However, the defensive benefits of multiple infections are usually reduced when subjected to multiple stressors especially if there are substantial increases in aphid fitness in the absence of symbionts (Weldon et al., 2020). This may explain the lack of co-hosting of symbiont in Sorsogon, where there may be reduced natural enemy diversities and therefore uninfected aphids are able to survive and reproduce faster than infected ones (thus increasing the frequency on uninfected individuals in the populations).

Consistency of symbiont infection per aphid colony

This study showed that even in the same colony, individual aphids from that colony may harbour different symbionts. Of the aphid colonies (82.4%:155/188

colonies) with infection, there was a considerable variation of the symbiont infection with 60% (93/155 colonies) hosting different symbionts combinations (inconsistent colony infection) and thus only 40% with the same symbionts (consistent infection). This suggests that these aphid colonies (close group of aphids on a plant) were derived from multiple founders rather than the common assumption that a colony comes from the offspring of a single female. Yet this could also relate to how constant and fixed the transmission of symbionts in aphid is within a given area; if fidelity for vertical transmission is low, the offspring from a single mother could also host variable symbiont communities. In pea aphids, the context dependent nature of vertical transmission has influenced the endosymbiont community structure of the aphid (Rock et al., 2018). Aside from vertical transmission, regular horizontal transmission and the influence of fitness to insect host are also determining factors that could influenced the frequency and retention capacity of infection (Fukatsu et al., 2000; Fukatsu et al., 2001; Guo et al., 2019). The variation of endosymbiont infection and frequency of individual aphids in a colony presented in this study showed that infection and occurrence of endosymbionts in aphids differs not only between aphid species and geographic landscape (Vorburger and Rouchet, 2016; Guo et al., 2019) but also in aphids within colonies.

The presence of ants had no significant effect on endosymbiont species richness in banana aphid species. In this study, the infection of endosymbionts was directly proportional to the presence of ants in aphids in Davao (59/60 symbionts:52/60 ants) and Sorsogon (89/178 symbionts:90/178 ants) but inversely proportional in Leyte (132/138 symbionts:78/138 ants). Data showed a decreasing trend of symbiont infection and ant presence in aphids from Davao, Leyte and Sorsogon. Known to have evolved mutualistic relationships with many aphid species by protecting them from natural enemies in exchange for honeydew, ants can influence the interactions between aphids and their protective symbionts (Erickson et al., 2012). However, Sabri et al., (2013) demonstrated that endosymbiotic bacteria may affect the protein make-up of honeydew and Schillewaert et al., (2017) showed that ant attraction was

reduced in symbiont-infected aphids that produced honeydew with a low amino acid content. This effect may have been responsible for the observation in Leyte, where there were high levels of endosymbiotic bacterial infection but a low presence of ants. In addition, the nutritional value of honeydew depends on aphid genotype which also regulates the attractiveness of ants to aphid colonies (Mooney, 2011) as well as the abundance of predators and parasitoids (Hazel and Fellowes, 2009; Mooney, 2011; Yao, 2014). It has been hypothesized that endosymbiont infection in aphids will decrease in the presence of ants due to redundancy in protection against natural enemies (Erickson et al., 2012). However, this was not the case in Sorsogon and Davao where infection of endosymbionts and ant-tended aphids were both low and high, respectively. This scenario could be due to the absence or low level of predators and natural enemies present in Sorsogon, creating a cost of endosymbiont hosting to the aphid. Whereas in Davao, the presence of endosymbionts may be beneficial to the aphid due to enemy pressure in the area. The co-presence of ants and symbionts might have complementary benefits in aphids, but further study is needed to understand the interactions between endosymbionts and ants in aphid populations. Moreover, ants have involvement in symbiont-aphid relationships whereas, endosymbionts also take part in ant-aphid trophobiosis (Ivens et al., 2018; Depa et al., 2020), hence, a future tripartite study on aphid-ant-symbiont in the aphid system would be important.

Conclusion

The identification of endosymbionts in banana aphids expands our understanding on their composition, distribution, and frequency of infection across three abaca germplasm collections. This is the first report of facultative endosymbionts on banana aphids collected from abaca plants and in the Philippines, and the first study of endosymbionts in banana aphids in the Asian Region. Future work can help to understand the impact of these symbionts for aphid resistance to biotic and abiotic challenges, which could affect the effectiveness of biocontrol strategies. Ongoing research in other insect systems also highlights the potential of symbionts to benefit plant virus transmission, with important economic impacts for crop plants (Frago et al., 2020). Determining how aphid symbionts impact insect host and host-plant provides a novel opportunity to combine ecological knowledge with real-world impact for sustainable agriculture.

Supplementary Material:

Table S6. Number of individual aphids per aphid species infected with endosymbionts in single and multiple infections.

						Symbiont combinations											
		Single infection				2-Symbionts			3-Symbionts						4-Symbionts		
Location	No infection	Ri	Rk	Ss	W	RiRk	RiSs	RiW	RkSs	RkW	SsW	RiRkW	RiSsW	RkSsW	RiRkSs	RiRkSsW	Total
Pop 1																	
Davao	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	2
Leyte	2	1	1	1	0	1	4	0	0	5	0	12	0	7	0	3	37
Sorsogon	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	3
	2	1	1	1	0	1	4	0	0	8	0	12	0	8	0	4	42
Pop 2																	
Davao	1	0	0	8	0	1	9	0	4	4	5	5	2	15	0	4	58
Leyte	4	2	2	7	0	3	2	0	3	29	2	12	3	11	1	20	101
Sorsogon	89	3	30	32	2	1	10	0	3	2	2	1	0	0	0	0	175
	94	5	32	47	2	5	21	0	10	35	9	18	5	26	1	24	334
Grand Total	96	6	33	48	2	6	25	0	10	43	9	30	5	34	1	28	376

Ri – *Regiella insecticola*, Rk – *Rickettsia sp.*, Ss – *Serattia symbiotica*, W - *Wolbachia*

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CHAPTER 4: QUANTIFICATION OF BUNCHY TOP VIRUS IN BANANA APHIDS COLLECTED FROM ABACA PLANTS IN THE PHILIPPINES

Abstract

Abaca bunchy top disease (ABTD) caused by *banana bunchy top virus* (BBTV) and *abaca bunchy top virus* (ABTV) is the most destructive viral disease of abaca (*Musa textilis* Nee) in the Philippines. It is transmitted by banana aphids (*Pentalonia sp.*) in a persistent and circulative, non-propagative manner. A better knowledge of virus-vector interactions is important for elucidating the ecology and epidemiology of the bunchy top disease for effective insect pest management strategies. In this study, the viral copies of 376 individual aphids collected from 36 abaca accessions across three germplasm collections in the Philippines (in Davao, Leyte and Sorsogon) were quantified using SYBR green-based quantitative polymerase chain reaction. Results showed that BBT1/BBT2 specific primer was able to detect BBTV in aphids that caused ABTD in abaca plants. About 51.1% of the aphid populations across all locations were infected, with viral copies in a single aphid ranging from 65.41±33.40 to 13,583 copies/aphid. Presence of BBTV depended on the collection site and the aphid species. The highest infection frequency was recorded in Davao (100% of aphids sampled) while lowest BBTV infection frequency was found in Sorsogon (33% of aphids sampled). Infection frequency was also higher when more than one endosymbiont was present within the aphid. Viral load was influenced by location alone with the Leyte germplasm collection showing the highest mean viral copy number ranging from 4476.13 to 6997.69 copies/aphid. In Leyte, the genetic background of the aphid (which species of *Pentalonia* was sampled) and the presence of *Wolbachia* were the main factors influencing presence of BBTV. *Pentalonia caladii* had a higher infection frequency than *Pentalonia nigronervosa* as did aphids where *Wolbachia* was present as an endosymbiont. There was some suggestion that the copy number of BBTV in Leyte was higher in aphids that were not tended by ants. More targeted

research into the influence of aphid species, endosymbionts, and the presence of ants on BBTV distribution and transmission in the Philippines is required.

Introduction

Abaca bunchy top disease (ABTD) is considered the most devastating viral disease of abaca (*Musa textilis* Nee) in the Philippines (Ocfemia, 1930; Raymundo et al., 2001; Bajet and Magnaye, 2002; Furuya et al., 2006). First reported in the 1920s (Ocfemia and Buhay, 1934), ABTD is still prevalent across abaca-growing areas in the country, where it decreases abaca production by lowering the yield and quality of the abaca fibre (Ocfemia, 1930; Ocfemia and Buhay, 1934; Raymundo and Bajet, 2000; Raymundo et al., 2001; Galvez et al., 2020). Abaca plants infected with ABTD normally show a yellowing and curling of the leaves, stunted growth, chlorotic to necrotic leaf margins, and underdeveloped suckers whose leaves are small, slender, and rigid in appearance (Ocfemia, 1930; Bajet and Magnaye, 2002; Sta. Cruz et al., 2016). The most distinctive feature of the disease is the formation of a 'bunched' appearance of the leaves at the top of the infected plant, giving the disease its name (Halos, 2008; Ocfemia, 1930).

Two closely related viral agents cause bunchy top disease in abaca plants: *abaca bunchy top virus* (ABTV) and the *banana bunchy top virus* (BBTV) (Raymundo et al., 2001; Kumar et al., 2015; Galvez et al., 2020; Piamonte and Sta. Cruz, 2018). Both viruses comprise six circular single-stranded DNA components: DNA-R (Master Rep); DNA-S (capsid protein); DNA-M (movement protein); DNA-C (cell cycle link protein); DNA-N (nuclear shuttle protein) and DNA-U3 (unknown protein), of about 1 to 1.1 kb each (Harding et al., 1993; Vetten et al., 2005; Natsuaki and Furuya, 2007; Sharman et al., 2008), however, ABTV lacks an internal ORF (open reading frame) in its DNA-R and DNA-U3 components (Sharman et al., 2008). The two agents are described as distinct and separate species as they only share 79-81% of their amino acid sequence identity for the putative coat protein and 54-76% for the overall nucleotide sequence identity across all components (Sharman et al., 2008). In addition, although they both belong to the genus *Babuvirus* in the *Nanoviridae* family, they are distinct serologically and phylogenetically from each other (Sharman et al., 2008). For example, enzyme-linked

immunosorbent assays showed that only two out of ten BBTV-specific monoclonal antibodies reacted to ABTV (Sharman et al., 2008) and phylogenetic analyses using the Neighbor-Joining method showed ABTV as an outgroup from two distinct clades of BBTV isolates: the Pacific Indian Oceans group (Clade 1) and the South-East Asia group (Clade 2) (Karan et al., 1994; Stainton et al., 2012; Kumar et al., 2015).

The banana aphid (*Pentalonia sp.*) is a primary vector that persistently transmits ABTV and BBTV to abaca (Ocfemia, 1931; Magee, 1953; Calilung, 2008) and banana (Magee, 1927; Dale, 1987; Hu et al., 1996; Kumar et al., 2015) in a circulative, non-propagative manner (Sharman et al., 2008). In persistent, circulative transmission, viruses are circulated from the gut lumen into the hemolymph and into the salivary glands of the aphids where they can be inoculated to a new host, however, these viruses lack the ability to replicate in the aphid host (Hogenhout et al., 2008; Gaafar and Ziebell, 2020). For banana aphids, BBTV has been shown to translocate quickly in the aphid vector, accumulating in the anterior midgut and residing either in the haemolymph or the principal salivary glands (Watanabe et al., 2013; Watanabe and Bressan, 2013). The BBTV can be retained in an aphid's body throughout their lifespan, but without being transmitted to their progeny (Hu et al., 1996). Since both ABTV and BBTV are *nanoviruses*, their infections are confined only to the phloem of the infected host plants and cannot be transmitted via seeds or mechanical methods (Grigoras et al., 2018; Vetten et al., 2005; Gaafar and Ziebell, 2020). However, vector transmission is prevalent and is subject to the life stage of the aphid, the external temperature, and a minimum plant access period (Anhalt and Almeida, 2008).

A single aphid can acquire either virus (BBTV and ABTV) within two hours of the acquisition feeding period (AFP) and the appearance of symptoms takes between 20 days to 78 days after plant inoculation (Bajet and Magnaye, 2002). During the initial stage of infection (the latent period), preliminary symptoms are absent (Galvez et al., 2020) and plants are usually asymptomatic until about 25 days after infection (Magnaye and Valmayor, 1995). In the absence

of early symptoms of bunchy top disease, virus elimination, diagnosis and control strategies are very difficult (Barbosa et al., 2020; Galvez et al., 2020). This becomes more challenging as both viruses can affect abaca either via single or mixed infections, and they can interact with the presence of other viral diseases such as *cucumber mosaic virus* (CMV), *banana bract mosaic virus* (BBrMV), and *sugarcane mosaic virus* (SCMV) (Furuya et al., 2006; Barbosa et al., 2020; Galvez et al., 2020; Sharman et al., 2008; Sta. Cruz et al., 2016; Sta. Cruz et al., 2017). Hence, a sensitive and reliable detection method for early disease diagnoses with or without viral symptoms is very important.

Early detection methods that have been used for BBTV and ABTV in abaca plants include nucleotide sequence analysis (Furuya et al., 2006; Sharman et al., 2008) and serological and molecular virus detection (Sta. Cruz et al., 2016; Sta. Cruz et al., 2017). In 2018, Piamonte and Sta. Cruz (2018) successfully developed a sensitive and reliable detection method for BBTV in abaca using an optimized DNA extraction protocol followed by PCR with the specific BBTV primer pair, BBTT1/BBT2 (Thomson and Dietzgen, 1995). Recently, a loop-mediated isothermal amplification assay has been developed to detect the presence of ABTV and BBTV in abaca, which appears to be more sensitive, faster, and more accurate than conventional PCR, and can detect asymptomatic ABTV or BBTV infected abaca (Galvez et al., 2020). A multiplex-PCR assay has also been developed to more accurately diagnose multicomponent *Babuviruses* infecting abaca, that can simultaneously amplify all genome segments of ABTV and BBTV (Barbosa et al., 2020). Moreover, antisera against CPs of ABTV and BBTV were also developed to detect ABTV and BBTV in asymptomatic abaca and banana plants (Koh et al., 2020). Nevertheless, most of the developed and tested ABTV and BBTV detection methods have been focused on symptomatic or asymptomatic abaca leaf samples, detecting only the presence or absence of the virus. Information on the viral load concentration of the symptomatic or asymptomatic abaca plants as well as in the aphid vector is still lacking.

A better understanding of the dynamics of the abaca-virus-vector relationship is very important for successful disease eradication and to control disease spread (Raymundo et al., 2001; FIDA, 1995; Pinili et al., 2013). The host plant, virus and insect vector are three important players responsible for the spread of the virus, but in general, virus transmission is more facilitated by the interactions between the virus and the insect vector (Dietzgen et al., 2016; Urizarna España and López-Moya, 2014). Being able to quantitatively determine the viral load of a single insect vector could be very useful for the detection of the virus prior to the appearance of the symptoms in an infected plant (Chen et al., 2013). It could also be an important tool for studying virus replication and spread (Fabre et al., 2003; Olmos et al., 2005). In persistently transmitted bunchy top virus for example, the viral load in the vector and the host is critical for virus spread, as virus titre influences the rate of infection and the pace of disease formation; wherein the higher the viral copies in the vector, the higher the transmission rate and the earlier manifestation of symptoms (Jebakumar et al., 2018). The infection capacity determination of a single aphid seems to be a crucial factor in the evaluation of aphid-borne virus outbreaks and for the development of effective strategies to control the virus (Fabre et al., 2003; Marroquín et al., 2004; Jebakumar et al., 2018).

Viral load present in an insect vector has previously been quantified for BBTV in *Pentalonia nigronervosa* (Chen et al., 2013; Watanabe et al., 2013; Mware, 2016; Jebakumar et al., 2018), *Citrus tristeza virus* in *Aphis gossypii* (Bertolini et al., 2007; Saponari et al., 2008) and *Potato virus Y* in *Myzus persicae* (Berthelot et al., 2019) using the Real-time PCR (RT-PCR) or quantitative PCR (qPCR). Known to be sensitive for the detection of viral nucleic acid in aphid species (Fabre et al., 2003; Olmos et al., 2005), this RT-PCR technique uses fluorescent reporter molecules, either dyes (i.e. SYBR® Green) or probes (Molecular beacons (Laikhter, 2015), TaqMan® probes (Chen et al., 2013) that allow quantification of amplified products. The principle is based on the real-time detection of a fluorescent reporter molecule whose increase in fluorescence intensity (signal) is directly correlated with the amount of DNA present at each cycle of amplification (Nolan et al., 2006). In this study, the

viral loads of bunchy top virus from banana aphids collected from three abaca germplasm collections (Davao, Leyte and Sorsogon) in the Philippines were quantified using the SYBR Green qPCR assay to 1) identify the virus that infects banana aphids in the Philippines, 2) determine the viral load concentration in a single banana aphid, 3) determine the distribution of bunchy top virus across three abaca germplasm collection sites and 4) evaluate if there was an influence of endosymbionts, ants, aphid species and plant susceptibility to BTD to virus copy number in aphids. The goal is to understand the bunchy top disease epidemiology for the development of specific ABTV and BBTV management strategies in the Philippines.

Materials and Methods

See Chapter 2 for banana aphid collection and DNA extraction.

PCR amplification for bunchy top virus detection and sequencing

Since the quantity of the virus in an infected aphid or infected abaca plant varies, cloning was undertaken to generate a template for absolute quantification. Prior to cloning, the identity of the banana aphid from which the virus was extracted was determined using a fragment of the mitochondrial cytochrome c oxidase subunit I gene (COI). A barcoding PCR using LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') primers (Folmer et al., 1994) was performed in a final volume of 15 μL containing 2.5 μL 5x MyTaq Reaction Buffer which included dNTPs, $MgCl_2$ and enhancers that remove an optimization requirement (Bioline), 0.15 μL My Taq (5U/μL), 0.15 μL each for LCO1490 and HCO2198 primers (0.20 μM) and 1 μL DNA (10 ng/μL). After a denaturation step of 95 ºC for 3 min, PCR was carried out for 35 cycles at 95 ºC for 15s, 51 ºC for 30s, 72 ºC for 30s and final extension of 72 ºC for 3 min using the SimpliAmp Themal Cycler (Applied Biosystems). Amplicons were separated through electrophoresis of 5 μL PCR product in a 1.5% agarose gels and the size of each amplicon was determined using 1 KB Plus DNA Ladder. The remaining 10 μL of the amplified DNA products were purified using the Enzymatic PCR Clean-up Technology (GE Healthcare Life Sciences) with Illustra Exonuclease 1 (2μL) and Shrimp Alkaline Phosphatase (2 μL) and 14 μl of the purified DNA product were sent for Sanger sequencing at the Core Genomics Facility, University of Sheffield, UK.

The screening for the presence of the *abaca bunchy top virus* (ABTV) and *banana bunchy top virus* (BBTV) on representative banana aphids collected from symptomatic abaca plants was done using previously developed primers (Table 11). Only the BBTV (replicase gene) primer pair, with a fragment size

of 349 bp (Harding et al., 1993), showed amplification and therefore was cloned into the vector pGEM-T Easy® (Promega, Madison, USA) for virus titre quantification on banana aphids.

Table 11. Previously developed primers with references used for ABTV and BBTV viral detection on banana aphids.

Primer	Target Virus	Primer Sequence (5'-3')	Reference
BBT1	BBTV	F: CTC GTC ATG TGC AAG GTT ATG TCG	Thomson and
BBT2	BBTV	R: GAA GTT CTC CAG CTA TTC ATC GCC	Dietzgen, 1995
1108C3A	ABTV	F: GGATWACATATCATGTATAAAC	Sharman et al. 2008
1108C3B2	ABTV	R: TTCTTGGGATACCTCGCCAT	

Polymerase chain reaction (PCR) reaction was performed in a final volume of 15 μL, including 2.5 μL 5x MyTaq Reaction Buffer (Bioline), 0.15 μL My Taq (5U/μL), 0.15 μL each for BBT1 and BBT2 primers (0.20 μM) and 1 μL DNA (10 ng/μL). PCR conditions were set at 95 ºC for 3 min, followed by 35 cycles of 95 ºC for 15 s, 61 ºC for 30 s, 72 ºC for 30 s and final extension of 72 ºC for 3 min using the SimpliAmp Themal Cycler (Applied Biosystems). Amplicons were separated through electrophoresis of 5 μL PCR product in a 1.5% agarose gel and the size of each amplicon was determined using 1 KB Plus DNA Ladder. The remaining amplified DNA products were purified using the Enzymatic PCR Clean-up Technology (GE Healthcare Life Sciences) with Illustra Exonuclease 1 (2 μL) and Shrimp Alkaline Phosphatase (2 μL) and 14 μl of the purified DNA product were sent for Sanger sequencing at the Core Genomics Facility, University of Sheffield, UK.

The quality of the electropherograms of the sequencing results were checked using 4Peaks v1.8 (Griekspoor and Groothuis, 2014) and only those with a quality of >25 Phred score were chosen as the baseline to edit the sequence. Quality checked electrograms were edited and aligned using the automated ClustalW multiple alignment method (Hall, 2005) using the BioEdit software version 7.0.5.3 (Hall, 1999). The identity of the sequences was sought using

the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) program from the National Center for Biotechnology Information.

Preparation of bacterial growth media and selection antibiotics

Luria-Bertani (LB) liquid media was made from 1.25 g yeast extract, 2.5 g tryptone and 1.25 g NaCl and bringing to a final volume of 250 mL with UltraPure™ DNase/RNase-Free distilled water. The media was then autoclaved at 121 ºC for 15 min to sterilise it.

The ampicillin (100 mg/mL) antibiotic was prepared by mixing 100 mg with 1 ml deionized water and filter sterilized before storing at 20 ºC. The *X-Gal* (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (40mg/1mL*)* was prepared by dissolving 40 mg 5-bromo-4-chloro-3 indolyl-ß-D galactoside with 1 mL Dimethyl sulfoxide (DMSO). Prior to storage, the mixture was covered with aluminium foil and stored at -20ºC. The IPTG (Isopropyl-β-D-thiogalactopyranoside) (0.1M in 5ml) was prepared by mixing 0.12 g IPTG with 5 mL distilled water, filter-sterilized and stored at 4 ºC.

The LB plates with ampicillin/IPTG/X-Gal were prepared by adding 3.75 g LB agar to 250 mL of LB medium and autoclaved at 121 ºC for 20 mins. The medium was allowed to cool to 50 ºC and 250 μL ampicillin (final concentration of 100 μg/mL), 1.5 mL (final concentration 0.5 mM) IPTG and 500 μL (final concentration 80 μg/mL) of X-Gal added. The medium was dispensed (30-35 mL) into 85 mm petri plates inside the Laminar Hood, cooled and stored in the fridge prior to use.

Cloning using pGEM®-T Easy Vector System

The cloning of a 349 bp fragment of the BBTV-replicase gene into the pGEM-T Easy vector was done to serve as a template for absolute quantification because it is difficult to determine the standard BBTV copy number in an infected plant or the viruliferous aphids (Chen and Hu, 2013). This is because

the quantity of the virus differs in every infected aphid, or the severity of the symptoms exhibited by an infected plant. Cloning, sequencing, and quantifying virus titre of banana aphids followed the methods of Mware (2016), but with slight modifications. Instead of using *Escherichia coli* XL1-Blue cells, JM109 High Efficiency Competent Cells (Promega) were used for the transformation of bacteria with recombinant plasmids and BBTV primers were used instead of M13 primers for the sequencing of plasmid DNA.

DNA ligation using 2x rapid ligation buffer

In constructing a vector for transformation, a 3:1 ratio was used to determine the number of insert (PCR products in nanograms) to be added to the ligation mixture. Purified PCR products (3 μL) were ligated into the pGEM-T Easy Vector (Promega) according to the manufacturer's instructions. Three sets of 10 mL ligation reaction mix (standard (virus from aphid sample), positive (from the kit) and background control (negative control)) were prepared which included 5 μL of 2x rapid ligation buffer,1 μL T4 DNA ligase and 1 μL pGEM-T Easy Vector (50 ng/μL). For the positive control, 2 μL of control insert DNA, a 542 bp fragment from pGEM®-luc Vector DNA (from pGEM®-T Easy Systems kit) were added. Ligation reactions were incubated overnight at 4 ºC.

Transformation of JM109 High Efficiency Competent Cells

The JM109 High Efficiency Competent Cells (*Escherichia coli*) (Promega) were thawed for 5 min in an ice bath and mixed gently by flicking the tubes prior to transformation. After which, 50 μL competent cells were carefully transferred to a sterile 1.5 mL tube containing 2 μL aliquot of the ligation reaction mix on ice. Whereas 100 μL of cells were added a ligation reaction mix for the background control only. The mixture was immediately incubated on ice for 20 min and subjected to heat shock for 45-50 seconds in a water bath at 42 ºC without shaking. After 50 seconds, the tubes were immediately returned to ice for 2 min.

Prior to plating, 950 μL and 900 μL of LB medium were added to the ligation reaction transformations tubes (standard & positive control, and background control), respectively, and incubated while shaking (~150 rpm) at 37 ºC for 1.5 hours. Transformation cultures (100 μL) were plated onto duplicate LB/ampicillin/IPTG/X-Gal plates and incubated overnight at 37 ºC at the Microbiology incubation room, Manchester Metropolitan University.

Bacterial growth in liquid cultures

A single white colony from the standard plate was carefully isolated from the LB agar plate using a wooden toothpick under the laminar flow hood (Bassaire Limited, Hampshire, England) and placed directly on the 5 mL LB broth containing antibiotics. It was then incubated (with shaking) overnight at 37 ºC prior to extraction.

Plasmid isolation and purification using the Wizard® Plus SV Minipreps DNA Purification System

LB broth containing white colonies (1 mL) were transferred to 1.5 mL Microcentrifuge tubes and microfuged at 9447 rcf (relative centrifugal force) for 2 mins. The supernatant was discarded by gently pouring off the liquid. Plasmid DNA was isolated using the Wizard® Plus SV Minipreps DNA Purification kit (Promega) as per the manufacturer's instructions.

Confirmation of cloning

Fragments cloned into the pGEM-T Easy® vector were confirmed through restriction digestion with EcoR1 (5 μL plasmid DNA, 0.5 μL EcoR1, 1 μL 10x buffer and 3.5 μL UltraPure™ DNase/RNase-Free distilled water) for 3 hours at 37 ºC using the SimpliAmp Thermal Cycler (Applied Biosystems). Electrophoresis in 1.5% agarose gels was run to confirm the presence of cloned DNA fragments by comparing the size of the band between the insert and plasmid vector using the 1 KB Plus DNA Ladder from Bioline.

Plasmid DNA preparation and sequencing

Polymerase chain reaction (PCR) reaction was performed in a final volume of 15 μL (2.5 μL 5x MyTaq Reaction Buffer (Bioline), 0.15 μL My Taq (5U/μL), 0.15 μL each for BBT1 and BBT2 primers (0.20 μM) and 1 μL plasmid DNA (10 ng/μL)). Thermal cycling was set at 95 ºC for 3 min, followed by 35 cycles of 95 ºC for 15 s, 61 ºC for 30 s, 72 ºC for 30 s and final extension of 72 ºC for 3 min using the SimpliAmp Thermal Cycler (Applied Biosystems). Amplicons (5 μL) were separated through electrophoresis in 1.5% agarose gels and the remaining amplified DNA products were purified using the Enzymatic PCR Clean-up Technology (GE Healthcare Life Sciences) with Illustra Exonuclease 1 (2μL) and Shrimp Alkaline Phosphatase (2 μL). The purified DNA product (14 μL) were sent for Sanger sequencing at the Core Genomics Facility, University of Sheffield, UK, as previously.

Plasmid DNA quantification and plasmid copy number calculation

Purified plasmid DNA was quantified using the Qubit® 2.0 Fluorometer (Invitrogen™) as per the manufacturer's instructions. The plasmid copy number for qPCR were calculated using the formula:

$$\text{Number of copies (copies/μl)} = \frac{\text{Total mass of plasmid (plasmid concentration in g/μl)}}{\text{Mass of 1 recombinant plasmid (n*1.096E-21)}}$$

Where:
n = the total base pairs of the recombinant plasmid
1.096E-21 = the mass in grams per base pair (g/bp)

Establishment of standard curves

A 10-fold serial dilution series from $1x10^9$ to $1x10^0$ copies/μL was prepared for the establishment of standard curve. The dilutions were re-suspended and mixed well by pipetting 20 times and stored at -20 ºC until use.

The threshold cycle (ct) values from the reaction were used to establish the standard curve by plotting the ct on the Y-axis and the natural log of concentration (copies/μl) on the X-axis. Analysis of the unknown samples (376 aphid samples) was set up using the plasmid as standards for the determination of absolute and standard deviation mean copy numbers. The linear regression equation: $y = mx + b$ and the coefficient of correlation (R^2) displayed on the standard curve were used to determine the concentration of the unknown samples.

Real-time quantitative PCR (qPCR)

Each real time qPCR reaction was performed in triplicates for the plasmid (serial dilutions), water as non-template control (NTC) and positive control (aphid sample infected with BBTV virus) while each aphid sample was run in duplicate using the Mx3000P Stratagene Thermocycler (Agilent). Serial dilutions were run on each plate. A 10 μL qPCR reaction consisted of 1μL DNA (10 ng/μL working concentration), 5 μL 2x SensiFAST™ SYBR® Lo-R0x mix, 0.2 μL each of BBT1F and BBT2R primers (0.2 μM concentration) and 3.6 μL UltraPure™ distilled water was run following standard PCR reaction conditions: Initial hold at 95 ºC for 3 min, 40 cycles of 95 ºC for 15 s, 61 ºC for 30 s and 72 ºC for 30 s. A final cycle of 95 ºC for 1 min, 61 ºC for 30 s and 95 ºC for 30s was performed to provide dissociation curves (melting curves) and to determine the amplification specificity.

Statistical analysis

Presence or absence of BBTV was determined based on the presence or absence of viral copy number of each sample. These data were grouped sequentially by susceptibility of the plant host, genetic grouping of aphid, presence of ants, presence of each of the four endosymbionts and the species richness of endosymbionts (assigned as 0, 1, >1) and visually inspected for the distribution of the presence and absence of BBTV. Samples for which there was no susceptibility information on the host plants available were removed

from further analysis, leaving a total of 360 aphids. Based on this assessment, factors were then chosen to input the analytical models. Presence/absence data set were first analysed with a generalized linear model (glm) with a binomial distribution using the lme4 programme (Bates et al., 2015) in R versions 4.0.2 (R Core Team, 2020). Geographic location, plant susceptibility to BBTV (classified as susceptible or resistant), aphid species, presence of each of the four identified symbionts and endosymbiont species richness (classified as 0, 1, >1), were included as factors in the analysis. Significant effects were determined using the Anova function in the car package (Fox and Weisberg, 2019). For aphids in which the virus was detected (copy number >0), copy number was analysed using a generalized linear model containing the same factors. Data were first natural log transformed to improve normality of residuals. Both presence/absence and copy number data sets were then subset by location. The data from Leyte was the most balanced in terms of the factors measured and was therefore used for further analysis without the effect of location. These data were visually inspected as previously to determine which factors to include in the statistical models. Presence/absence data for Leyte were analysed with a generalized linear model with a binomial distribution as previously, but with aphid species, the presence of ants on the plants the aphids were collected from, the presence of each endosymbiont and the endosymbiont species richness included as factors. Copy number data were natural log transformed and then analysed with a generalized linear model with the same factors included. Significant effects were again determined using the Anova function in the car package (Fox and Weisberg, 2019).

Results

PCR amplification, sequencing and cloning

Two previously developed markers, 1108C3A/1108C3B2 and BBT1/BBT2 (Sharman et al., 2008; Thomson and Dietzgen, 1995; Harding et al., 1993) that successfully detected ABTV and BBTV, respectively in abaca and banana leaf samples were chosen to detect bunchy top virus in aphids. The BBTV specific primer only showed amplification from aphid samples collected from symptomatic abaca plants (Figure 14A). The sequencing result from DNA extracted from the banana aphid sample used for amplification was confirmed as *Pentalonia nigronervosa* using the COI gene and that the amplified PCR products using the BBT1/BBT2 specific marker were confirmed as the *banana bunchy top virus*. The sequenced analysis of the transformed BBTVrep-pGEM plasmid shared 98.53% identity with the BBTV isolate segment R which encodes a master replication initiation protein (Replicase gene).

Figure 14. A) A 1.5% Agarose gel showing the product of a conventional PCR C using BBT1/BBT2 primer for the detection of BBTV in aphids. The four BBTV amplified samples were sent for sequencing and were confirmed as the *banana bunchy top virus*. After BBTV sequencing confirmation, the first sample (lane 3) was used for the cloning. B) Agarose gel (1.5%) of the qPCR products showing 1 KB Ladder (Lane 2), the standard dilution's of BBTV replicase gene-pGEMT (349 bp) (Lane 3-12), NTC (Lane 13) and some of the amplified samples from Davao (Lane 15-24). Lane 1 and Lane 14 were empty lanes.

Plasmid DNA quantification, copy number calculation and standard curve

The average concentration of the purified plasmid DNA was 20 ng/µL and the initial plasmid copy number was 2.712E+09 which was used as template for absolute quantification.

The viral copy number from single aphids were evaluated using the standard dilutions of BBTV replicase gene-pGEMT plasmid (Figure 14B). A total of 14 qPCR plates were completed for the 376 aphid samples where the lowest average Ct value was obtained at 10.79±0.47 (2.71E+07) and highest average Ct value at 30.28±1.87 (2.71E+01) for the Standards (Figure 15A). The NTC (no template control) average Ct value was 34.40±2.90 with three out of the 14 plates having no Ct values with some of the aphid samples indicating no BBTV infection, hence the absence of viral amplification. The melt curve, a single peak of the qPCR amplicons of the replicase gene of BBTV and the samples was identified at 84 ºC (Figure 15B). The correlation coefficient (R^2) ranged from 0.9750 to 1.00 with a slope of -3.278 to -3.857 (Figure 16) and % PCR efficiency ranged from 81.7% to 101.9%.

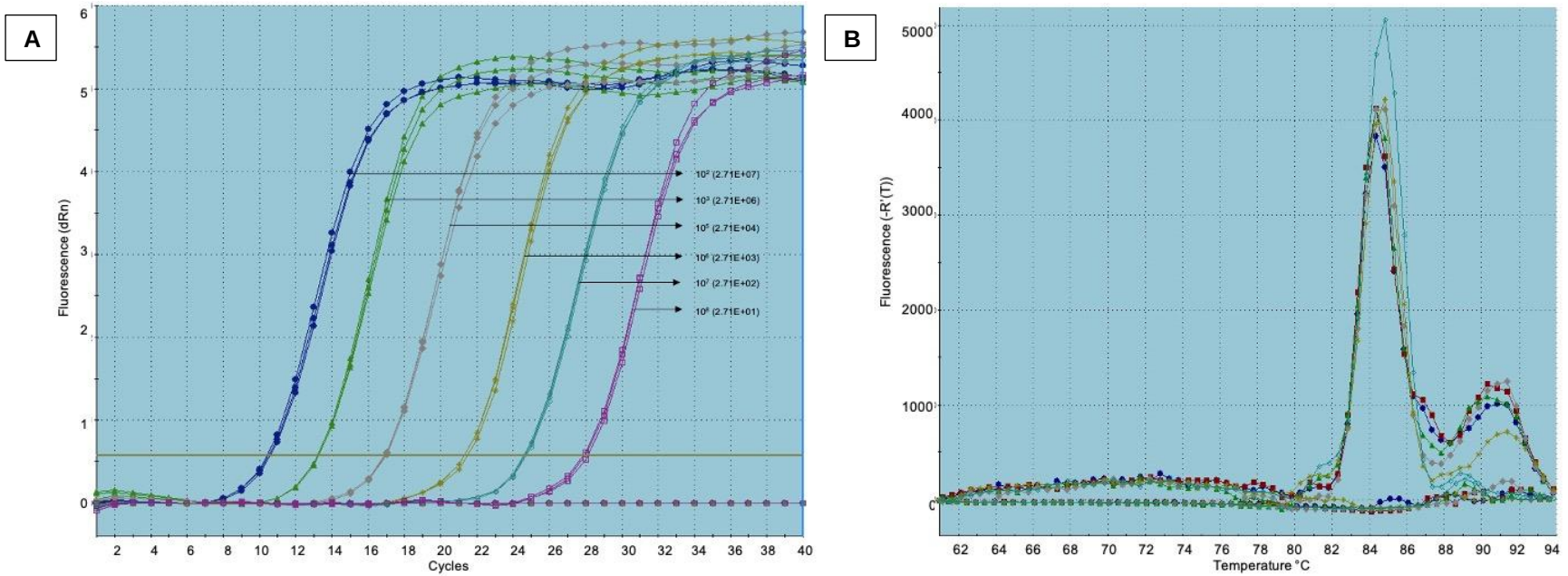

Figure 15. Example amplification plots and melt curve (from one of the plates) for BBTV detection in banana aphids using qPCR. A) SYBR green fluorescent signals generated from a dilution series of BBTV plasmid. B) Melting temperature (Mean dissociation curve or melt curve).

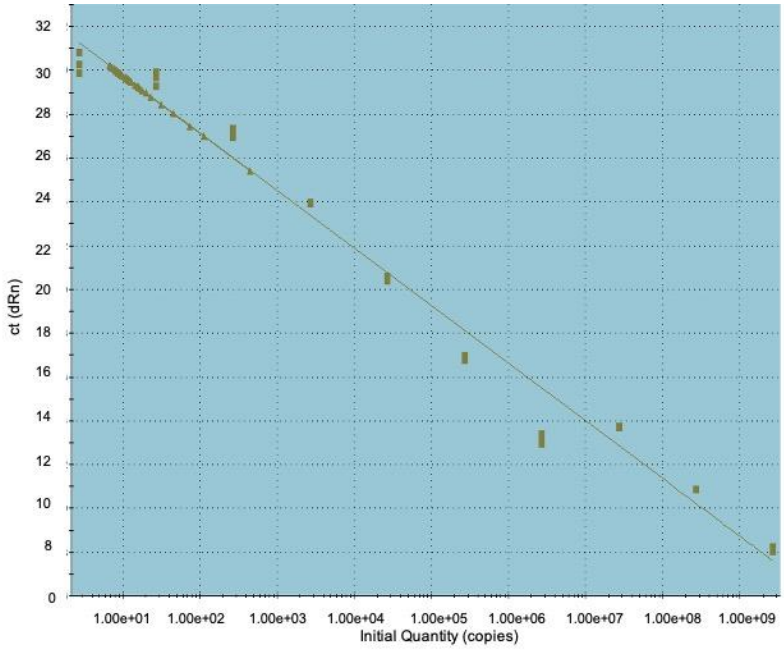

Figure 16. Example standard curve (from one of the plates) based on virus copies from BBTVRep-pGEMP plasmid and the samples versus the number of cycles.

BBTV infection and consistency across colonies

The BBTVrep-pGEM plasmid used as a standard successfully detected and quantified the presence of BBTV in a single banana aphid. Of all the banana aphids tested, 51.1% (192/376) were infected with BBTV. The BBTV titre in a single aphid ranged from 65.41±33.40 to 13,583 copies/aphid and the infection was not consistent within the aphid colony. Of the 188 colonies tested, only 67.02% (126/188) were infected and out of these infections, 52.4% (66/126) colonies had consistent infection (both individual aphids in a colony infected) while 47.6% (60/126) were not consistent (i.e. one aphid was infected while the other was not).

Factors that influenced the presence and absence of BBTV and viral copy number across all locations

The generalized linear model (glm) showed that BBTV presence was significantly influenced by geographic location (X^2=68.89, df=2, p<0.0001) and aphid species (X^2=4.09, df=1, p=0.04) (see Table 12 for full results).

Table 12. Statistical and significance values of the factors used in the generalized linear model undertaken on the presence/absence of BBTV in 360 banana aphids (residual deviance = 385).

Factor	Chi-square	Df	P value
Location	68.87	2	1.1×10^{-15}
Aphid species	4.09	1	0.04
Plant susceptibility	0.08	1	0.78
Symbiont richness	0.66	2	0.72
Serratia	0.20	1	0.65
Wolbachia	2.57	1	0.11
Rickettsia	0.03	1	0.86
Regiella	0.38	1	0.54

Infection frequencies were highest in Davao with 100% of aphids (60/60) infected. In Leyte, 57.4% (70/122) of aphids were infected and 33.1% (59/178) were infected in Sorsogon (Figure 17). Between the two banana aphid species, *P. caladii* showed higher BBTV infection of 73.3% (22/30 aphids) with viral titre ranging from 174 to 13,583 copies/aphid compared to *P. nigronervosa* with 50.6% (167/330 aphids) infection and viral titre ranged from 65 to 13,389 copies/aphid (Figure 18). However, BBTV infection frequencies were similar between the aphids without any symbionts and the aphids with a single symbiont, but they increased when aphids were infected by more than one symbiont at a time (Figure 19).

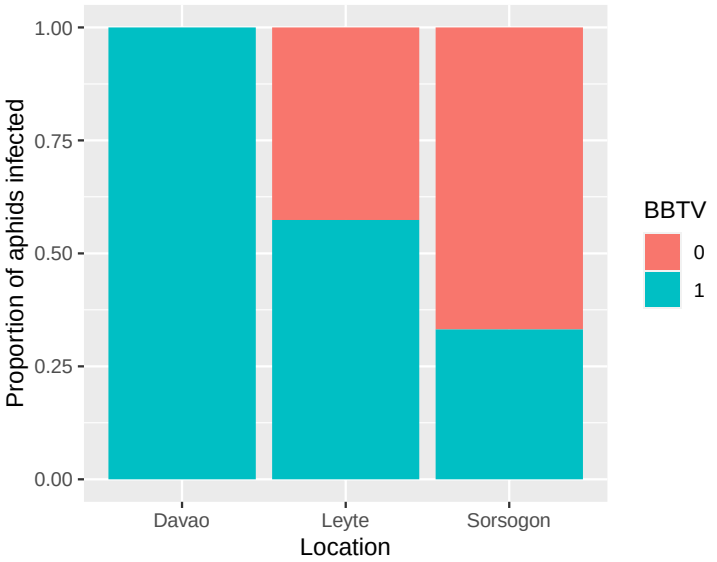

Figure 17. Proportion of aphids infected with BBTV across three abaca germplasm collections in Davao, Leyte and Sorsogon, in the Philippines.

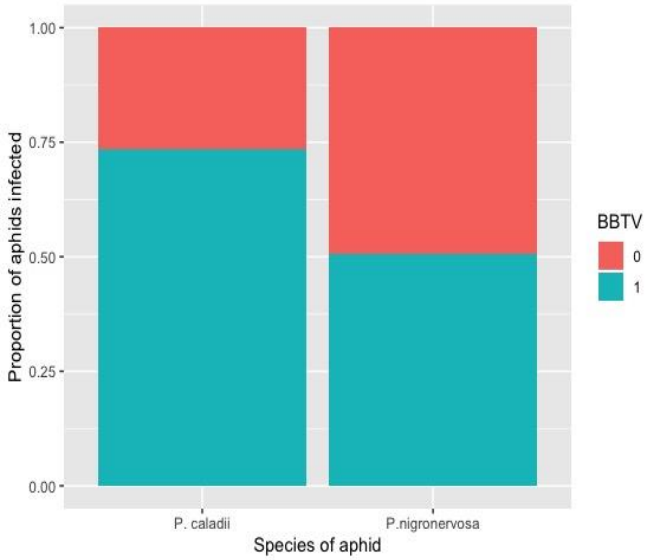

Figure 18. Influence of aphid species (across location) on the proportion of aphids infected by BBTV.

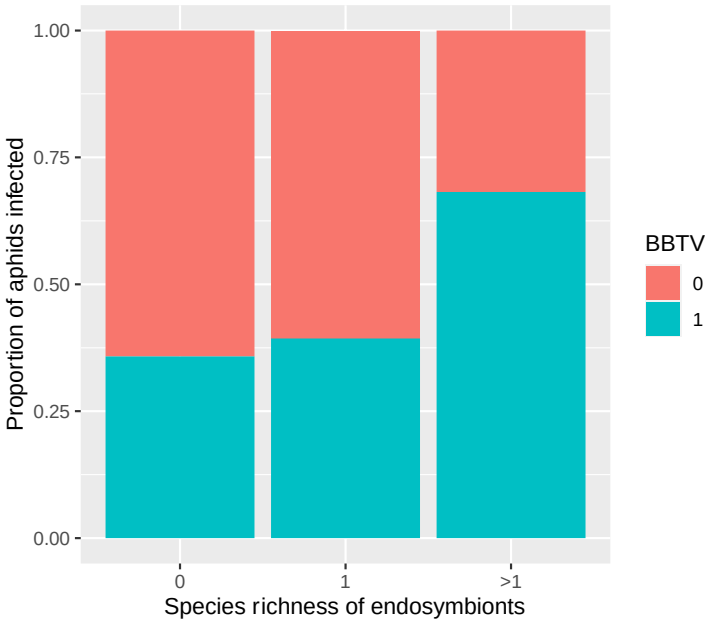

Figure 19. Proportion of aphids infected with BBTV in response to endosymbiont species richness (classified as 0, 1,>1).

Table 13. Statistical and significance values of the factors used in the general linear model undertaken on the copy number of BBTV (natural log transformed) in the 360 banana aphids (Full model R^2_{adj}=0.04, p=0.6).

Factor	Sum of squares	df	F	P value
Location	9.10	2	4.28	0.02
Aphid species	1.06	1	0.99	0.32
Plant susceptibility	2.08	1	1.96	0.16
Symbiont richness	0.78	2	0.37	0.69
Serratia	0.04	1	0.03	0.86
Wolbachia	0.14	1	0.13	0.72
Rickettsia	0.002	1	0.002	0.97
Regiella	0.76	1	0.71	0.40
Residuals	209.36	197		

The general linear model on virus copy number data was marginally significant and explained only a small amount of the variation in the data (R^2_{adj}=0.04, p=0.06). Of the factors tested, only location showed a significant effect (F=4.28, p=0.02) with aphids in Leyte containing the highest copy numbers on average (Table 13, Figure 20). Across locations, the viral titre ranged from

92.08 to 1,388.94 copies/aphid in Davao, 73.50 to 13,583.46 copies/aphid in Leyte and 65.41 to 5,115 copies/aphid in Sorsogon.

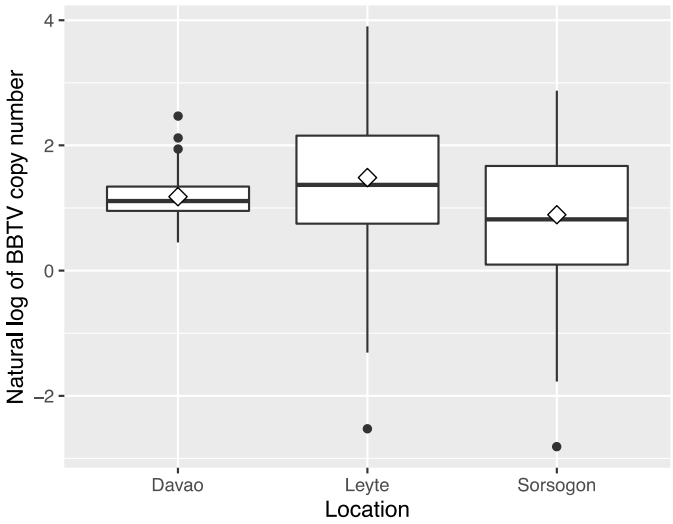

Figure 20. Natural log transformed copy number of BBTV found in aphids collected from three abaca germplasm collections in Davao, Leyte and Sorsogon, in the Philippines. Horizontal lines show the median, diamonds the mean, and boxes and whiskers represent the IQR (interquartile range) and the maximum and minimum data range.

Factors that influenced the presence and absence of BBTV and viral copy number in Leyte

The generalized linear model showed that BBTV presence in Leyte was significantly influenced by aphid species (X^2=6.16, df=1, p=0.01) and the presence of *Wolbachia* (X^2=6.50, df=1, p=0.01); see Table 14 full results). Although the absolute numbers of aphids were smaller, *Pentalonia caladii* was more likely to carry BBTV than *Pentalonia nigronervosa* (Figure 21). In addition, aphids carrying *Wolbachia* as a symbiont were more likely infected with BBTV than aphids without *Wolbachia* as a symbiont (Figure 22).

Table 14. Statistical and significance values of the factors used in the generalized linear model undertaken on the presence/absence of BBTV in 122 banana aphids in Leyte (residual deviance = 148).

Factor	Chi-square	Df	P value
Aphid species	6.16	1	0.01
Presence of ants	2.18	1	0.14
Symbiont richness	1.07	1	0.59
Serratia	0.26	1	0.61
Wolbachia	6.50	1	0.01
Rickettsia	0.37	1	0.54
Regiella	1.59	1	0.21

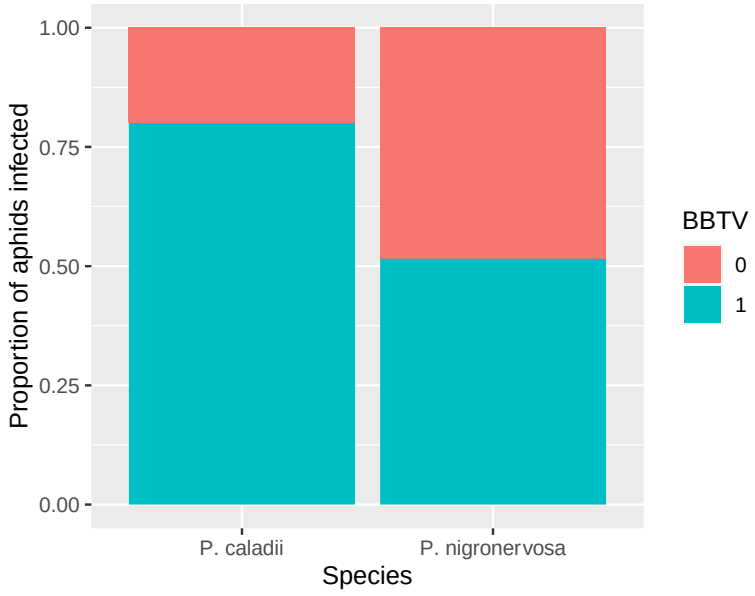

Figure 21. Influence of aphid species on the proportion aphids infected by BBTV in Leyte.

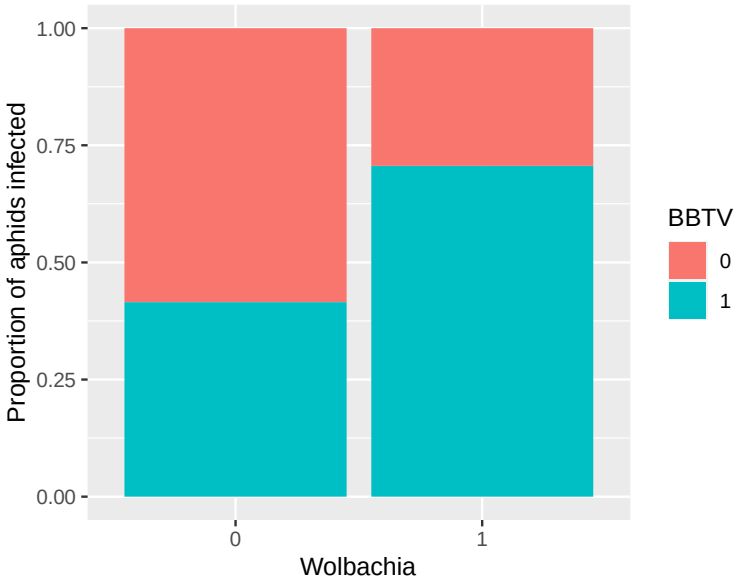

Figure 22. Influence of presence of *Wolbachia* as a symbiont on the proportion aphids infected by BBTV in Leyte.

The general linear model on virus copy number data for the aphids in Leyte, again only explained a small amount of variation in the data and was not statistically significant (R^2_{adj}=0.04, p=0.23). There was a trend that presence of ants on the plants that the aphids were collected from decreased the mean copy number of BBTV (Figure 23).

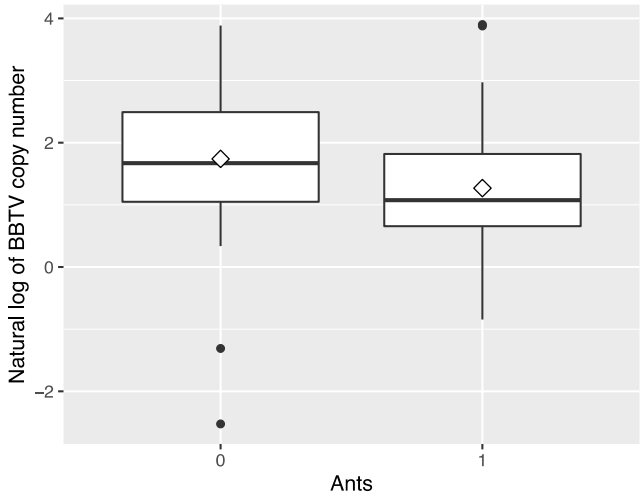

Figure 23. Natural log transformed copy number of BBTV found in aphids collected from plants with and without the presence of ants. Horizontal lines show the median, diamonds the mean and boxes and whiskers represent the IQR (Interquartile range) and the maximum and minimum data range.

Discussion

The current study is the first viral titre quantification conducted on banana aphids collected from abaca plants in the Philippines. Results showed that a putative replicase gene component of BBTV genome (DNA-R component) was detected in aphids. There were differences in the number of viral copies acquired in a single aphid in a colony. The distribution of BBTV was not consistent across collection sites with the highest proportion of infected aphids in Davao and the highest viral load in Leyte. Geographic location and aphid species significantly explained the presence and absence of BBTV across all sites. In Leyte, aphid species and *Wolbachia* significantly explained the presence of BBTV. In addition, the presence of ants is potentially influencing copy number.

Detection, quantification, and distribution of BBTV in aphids

The mode of virus transmission from one plant to another by an aphid vector governs the transferability of the viral disease. Positive detection of BBTV in an aphid vector using the qPCR technology confirms the sensitivity of the technique and that BBTV can be detected in a vector with persistent and circulative transmission (Fabre et al., 2003) such as banana aphids (Jebakumar et al., 2018). The positive detection of bunchy top virus in banana aphids using the BBT1/BBT2 primer pair is consistent with previous studies that have shown the efficacy of these primers to detect BBTV in banana aphids (Robbertse et al., 2019), in symptomatic and asymptomatic abaca (Piamonte and Cruz, 2018; Sta. Cruz et al., 2016; Sta. Cruz et al., 2017) and banana leaf samples (Thomson and Dietzgen, 1995).

The consistency and efficiency of BBTV detection using the BBT1/BBT2 primer pair which amplifies the DNA-R (also referred to as DNA-1) component of the viral genome can be attributed to the conserved nature of the replicase

gene (Piamonte and Sta. Cruz, 2018). It has been reported that the sequence analysis of this component (BBTV DNA-1), designed for the Australian isolate, can also be used to amplify BBTV isolates regardless of their origin (Thomson and Dietzgen, 1995) from the South Pacific and the Asian group, including the Philippines (Karan et al., 1994; Karan et al., 1997; Sta. Cruz et al., 2016). In this study, the sequence analysis of the amplified conventional PCR products using the DNA extracted from banana aphid and the plasmid DNA shared 98.83% identity with a BBTV isolate DNA-1 from the Philippines. The lack of amplification of ABTV in the sampled aphids could be attributed to the sensitivity and specificity of the ABTV primer being used or ABTV could be absent or in a very low concentration in the samples. It is therefore recommended to develop markers that are more sensitive and specific to the ABTV genome to detect very low concentration of the viral load in aphids.

Variation in the amount of BBTV per aphid was detected by the techniques used here, but viral copy numbers were relatively low compared to the viral load usually detected in aphids under laboratory or in an enclosed and monitored experiment set-up. In BBTV transmission efficiency studies conducted on banana aphids, a single aphid was shown to be able to acquire 861.04 copies of bunchy top virus, and 50 viruliferous aphids to be able to acquire 15,066.94 viral copies after 24 hours of acquisition access to an infected plant (Jebakumar et al., 2018). The low viral titre present in single aphids here could be due to the amount of the DNA template used in qPCR. In this study, 1 µL containing 10 ng DNA was used and this amount might contain very low copy numbers of ABTV and BBTV DNA. In abaca tissues, the concentration of viral proteins of ABTV and BBTV is low (Galvez et al., 2020). The virus infection rate in aphids could also play a role as is the case for the cross-transmission of bunchy top virus between banana and abaca plants. A 10% infection rate was recorded in aphid-transmitted BTV from abaca to banana whereas a tri-fold increase in infection rate was observed in aphid-transmitted BTV from banana to abaca (Pinili et al., 2013). This study demonstrates that BBTV ca be detected at relatively low concentrations and from a single aphid collected in the field using qPCR. The ability to do this

allows the potential detection of the virus before the appearance of symptoms in plants in the field and could be developed to provide early warnings on the risk of infection and enable the implementation of swift control measures to mitigate virus spread. Early disease forecasting can be done by monitoring the aphid's seasonal abundance coupled with the detection of BBTV in the aphid samples through deployment of suction traps in the abaca field.

Factors that influence BBTV infection and viral copy number in aphids

Location

The detection of the virus across all locations sampled has revealed the severity of BBTV in the Philippine's abaca germplasm collection. However, infection rates and viral loads were not consistent across the three germplasm sites. In fact, collection location was the driving factor that strongly influenced the presence and the copy number of BBTV. Historically, high occurrence of *abaca bunchy top virus* was concentrated in the Bicol region in the 70s and then eventually spread across other abaca-producing areas in the country (Bajet and Magnaye, 2002). In 2001, Sorsogon, a province under the Bicol region was considered a "hot spot" where bunchy top disease had wiped out abaca plantations and led to abandonment of farms (Raymundo et al., 2001). This scenario could also reduce the source of BBTV inoculum in the area as the number of banana aphids reduced and could be one reason why current disease incidence is relatively low in Sorsogon. In contrast, abaca has remained growing in the areas around Leyte and Davao, presumably supporting a population of banana aphids and retaining a persistent source of BBTV inoculum. In addition, the presence of banana in abaca-producing areas also contributed to the increase of the source of inoculum (San Juan, 1989; Magnaye 1989; Raymundo et al., 2001). Such was the case in the 60s where BBTV infected banana plantations in Davao (Halos, 2008). At present, Davao supplies the majority of banana for export from commercial plantations and small-scale growers in the area, which could serve as the source of inoculum or refugia for the aphids.

As phloem feeders, aphids prefer younger plants and favor tissue culture rather than sucker grown plants (Jebakumar et al., 2018). In addition, the rate of BBTV transmission is directly related to the number of viruliferous aphids and indirectly related to the age of the host plants (Wu and Su, 1990; Anhalt and Almeida, 2008). In the Leyte germplasm site, aphids were mostly collected from developing abaca plants derived from tissue culture, whereas in Sorsogon, most of the plants in the collection were more mature and ready to harvest. In Davao, the area had recently experienced a drought when aphid collection took place, which required some of the accessions to be replanted, resulting in the majority of the plants being in an early development stage. The different ages and provenience of the plants within the three collection areas, could contribute to the differences observed in virus prevalence across the locations.

Although no significant effect was detected between BBTV in aphids and the susceptibility to ABTD of the plants they were collected from, it was observed when collecting that aphids were more prominent on susceptible accessions and mostly absent from some resistant accessions. Therefore, it could be inferred that aphid abundance, and hence ability for the virus to spread may be related to the availability of susceptible hosts. Distribution of susceptible and resistant hosts was not even across the germplasm sites and only susceptible hosts were present at Davao. In addition, it was noted that there were a higher number of aphids per individual plant in Davao and both of these factors could have influenced the prominence of BBTV within aphids at this site.

As virus transmission depends on the interactions between the host plant, pathogen, and the insect vector (van Munster et al, 2020), environmental factors such as rainfall, humidity and temperatue significantly affect the system dynamics in the area. Wind movement facilitates aphid dispersal (Ocfemia, 1931; Halos, 2008) and rainfall inversely affects aphid abundance (Gavarra, 1977, Halos, 2008). Of the collection sites, Sorsogon is highly disturbed as it

lies in the typical annual typhoon path. Such acute weather disturbances affect not only the plants, but also the abundance of aphids and natural enemies, and hence virus inoculum vis-à-vis transmission.

Other site differences that could also affect the abundance and movement of aphids, include the situation and surrounding vegetation. The germplasm collection at Leyte is situated at the foot of Mt. Pangasugan, while at Davao and Sorsogon they are planted on flat land. The site around Leyte is planted with fruit trees, coconut, and bamboo plants and a variety of weeds, which could serve as alternative host plants for banana aphids or other insect species and could be sources of infection.

Aphid species

The *Pentalonia* species significantly influenced the presence and absence of BBTV confirming that both species were capable of carrying the *banana bunchy top virus* to abaca plants. Although viral titre quantification studies had been done on *P. nigronervosa* collected on banana plants (Chen et al., 2013; Watanabe and Bressan, 2013; Watanabe et al., 2013; Mware, 2016; Jebakumar et al., 2018), this is the first virus titre determination on *P. caladii* collected from abaca plants in the Philippines. The first and only previously recorded viral titre quantification on *P. caladii* was collected on the host plants taro, heliconia and red ginger in Hawaii (Watanabe et al., 2013).

In this study, *P. caladii* showed the highest BBTV infection frequency (60%) and viral titre (174 to 13,583 copies/aphid) compared to *P. nigronervosa* (50%) with the viral titre that ranged from 65.41 to 13,389 copies/aphid. As reported, the vectorial capacity, the ability to spread a disease (Chuche et al., 2017) of the aphid vector depends on the aphid species which differ in virus efficiency (Stevens and Lacomme, 2017). In Leyte, the presence, but not the copy number of BBTV, depended on which species of *Pentalonia* was the host vector, with *P. caladii* more likely to be infected with BBTV than *P.*

nigronervosa. In addition, *P. caladii* was also revealed to be a competent vector of BBTV in Hawaii (Watanabe et al., 2013). However, previous studies have reported *P. caladii* to be the vector of ABTV, although this is yet to be validated in the field (Bajet and Magnaye, 2002). It was not possible to detect ABTV in this study, but it could be that *P. caladii* is a more effective transmitter of both BTV species to abaca plants. Additional research is needed to ascertain the relative roles of *P. caladii* and *P. nigronervosa* in the transmission and spread of the bunchy top viruses.

Endosymbiont community

Endosymbiont species richness did not significantly influence the presence and absence of BBTV in aphids across the full data set. However, when aphids contained more than one endosymbiont, they were more likely to be carrying BBTV. Among the endosymbionts that infected banana aphids, *Wolbachia* seemed to play a role in influencing the presence of BBTV. This was most evident when examining the Leyte data alone, where presence of *Wolbachia* increased the likelihood of an aphid carrying BBTV. The role of this symbiont in aphids is not yet known. However, in Drosophila, some strains of *Wolbachia* have been reported to have anti-viral properties (Osborne et al., 2009) which might explain their presence in a parthenogenetic aphid (Jones et al. 2011). This is because *Wolbachia* inhibits virus replication in insect hosts (Lindsey et al., 2018; Pimentel et al., 2021), and this “pathogen-blocking” could also be the same strategy it exhibits when present in virus-infected aphids.

In this study, *Wolbachia* was only present at low levels in the aphids from Sorsogon in comparison to those from Davao and Leyte, mirroring infection levels with BBTV. If *Wolbachia* has antiviral properties, then increased viral pressure could lead to increased persistence of this symbiont into the aphid population. If hosting *Wolbachia* in low virus pressure Sorsogon is costly to the host, this could result in an ecological trade-off (Kwiatkowski and Vorburger, 2012) reducing the prevalence of *Wolbachia* in the area. This

interaction between *Wolbachia* and BBTV is of potential interest for disease control (Jiménez et al., 2019) and requires further investigation.

Ant presence

There was some indication that the presence of ants on the host plant influenced viral copy number of BBTV in the aphids, with aphids from plants where ants are also present showing slightly lower copy numbers on average. Ants usually tend banana aphid colonies, and their mutualistic relationship (Ocfemia, 1930) provides them with food from the aphid's honeydew in return for protection from predators (Adachi and Yano, 2017), natural enemies (Stechmann et al., 1996) and fungal pathogens (*Pandora neoaphidis*) (Nielsen et al., 2010). They have been known to move aphids from one plant to another (Billick et al., 2007), which would seem more likely to spread infections to other aphids than provide protection. Ants can also influence the infection of symbionts to aphids (Darby and Douglas, 2003), but their role with regards to the spread of the viruses in general has yet to be studied (Herwina et al., 2013).

Conclusion

In order to develop effective controls for abaca bunchy top disease, it is important to be able to quantify bunchy top viral loads within aphids in the field and determine the spread of infection across the abaca plantations in the Philippines. This study has confirmed the possibility of detecting BBTV within a single aphid and this approach could be used to develop a mass testing programme of aphids across the country to serve as an early surveillance for virus detection. Abaca bunchy top disease was confirmed to be caused by a BBTV isolate belonging to the Asian group with a high similarity to the Philippine BBTV DNA-1 isolate in these samples. Infection frequencies varied across sites and were influenced by the aphid species, as well as the presence of the endosymbiont *Wolbachia*. To our knowledge this is the first evidence of the potential role that endosymbionts might play in the transmission of BBTV. This is an important contribution to developing our understanding of the plant-virus-vector relationship for this disease and the development of specific bunchy top disease management in the Philippines.

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Chapter 5: General Discussion

Increasing agricultural production in a more sustainable way is a global challenge. With the prevalence of insect pests in a changing climate (Velasquez et al., 2018), the emergence of plant diseases will increase as insects play a crucial role in transmitting plant viruses (Wang and Blanc, 2020). One of the sustainable ways of managing insect pests is through the application of integrated pest management (IPM). Understanding the mechanisms in controlling vector transmission involves the integration of different interactions between virus-plant, virus-vector and insect-plant interactions (Blanc et al., 2011) which greatly support IPM strategies (Franco et al., 2017). In the case of aphids (Hemiptera: Aphididae), coined as the 'insects for all seasons' (Loxdale et al., 2017), the infection by endosymbiotic bacteria provides ecological benefits in terms of nutrient provision (Douglas, 2007; Baumann et al., 1995; Moran and Degnan, 2006), protection against natural enemies (Vorburger et al., 2009), and is believed to facilitate in virus transmission (Alkhedir et al., 2015; Pinheiro et al., 2015). Using molecular ecology approaches, the research presented in this thesis aimed to develop our understanding of the banana aphid as a vector of bunchy top virus in abaca (*Musa textilis* Nee). Specifically, I aimed to identify the genetic diversity of *Pentalonia* sp. collected from abaca plants across three different germplasm collections, identify the presence of endosymbionts, determine the virus that infects abaca, and quantify the viral load of individual aphids.

The first results chapter of this thesis (Chapter 2) employed the cross-species amplification of previously developed microsatellite markers from different aphid species to banana aphids to determine their mode of reproduction, genetic diversity and genetic structure. Microsatellites are species-specific genetic markers used in population genetics studies in aphids to identify aphid species, clonality, infer population genetic structure, genetic diversity, migration rates and relatedness (Loxdale et al., 2017). Genetic analysis revealed that banana aphids in the Philippines reproduce asexually, had low

genetic diversity and inconsistent genotypes within aphid colonies. The amplification of a DNA fragment from the 5' region of the mitochondrial cytochrome c oxidase 1 (COI) gene (Foottit et al., 2008) was able to differentiate *Pentalonia* species. I found that two distinct *Pentalonia* species (Foottit et al., 2010) were infesting abaca plants: *P. nigronevrosa* and *P. caladii*. Information about aphid species was then integrated into the subsequent chapters.

In the second results chapter (Chapter 3), I used previously developed markers to identify the nine most studied endosymbiotic bacteria in aphids. I identified four endosymbionts with different infection frequencies, distribution, and combinations across three abaca germplasm collections. I found that geographic location significantly influenced the endosymbiotic community in banana aphids, but there was no effect of aphid species or across plant accessions. I also found that aphids in the same colony hosted different symbionts. This high variation of symbiont infection could be an insect response to natural enemy and abiotic pressures in a germplasm area or could be due to geographic differences in founder events but needs further study. I also used these endosymbiont data in Chapter 4 to understand its influence on the bunchy top virus and the viral copy number.

In the final results chapter of this thesis, I identified the virus that infects banana aphids and quantified the viral load present in individual aphids using the SYBR-green based quantitative polymerase chain reaction (qPCR). I found that *banana bunchy top virus* (BBTV) infects both banana aphid species collected from abaca in the Philippines. No PCR amplification of *abaca bunchy top virus* (ABTV) was detected, hence, BBTV likely caused bunchy top disease (BTD) on abaca. There was variation in the prevalence of the virus among locations, with 100% of aphids in Davao infected with BBTV and the highest viral copy number infection in Leyte. The presence of BBTV depends on geographic location and the aphid species. There was no significant association between the presence of BBTV and endosymbiotic bacteria, however, I found that BBTV infection was higher when aphids hosted more

than one endosymbiont. The *Pentalonia* species showed promising information as *P. caladii* seemed to be more likely to be infected with BBTV. In the transmission study of banana aphids in Hawaii, it was found that *P. caladii* had the ability to influence BTD disease where it can vector BBTV from banana infected plants to banana (Watanabe et al., 2013). With this finding, a virus transmission efficiency study of *Pentalonia* species on abaca plants in the Philippines is highly recommended to ascertain the effectiveness of *P. caladii* or *P. nigronervosa* in virus transmission. Lastly, I found evidence in Leyte that *Pentalonia* species and *Wolbachia* infection were the main factors influencing the presence of BBTV and that there was a trend that showed a higher copy number of BBTV when not tended by ants.

Bunchy top disease management implications and recommendations on Abaca

The use of molecular ecology techniques such as polymerase reaction (PCR), quantitative PCR (q-PCR), genotyping, cloning, and sequencing in studying the population genetics, endosymbionts and virus quantification in aphids has developed our understanding of the biology of the banana aphid as well as the endosymbiotic bacteria and virus that reside inside the aphid. Understanding the population genetics, presence of endosymbionts and quantification of bunchy top virus from a single banana aphid is very important for abaca bunchy top virus (ABTV) management in the Philippines.

First, the confirmation of the asexual mode of reproduction in banana aphids, the genetic diversity determination, and the identification of two distinct *Pentalonia* species infesting abaca plants in the Philippines using population genetics approaches in Chapter 2 has provided an invaluable insight into understanding the biology of the insect vector at the molecular level. By identifying multilocus genotypes as well as the presence of two *Pentalonia* species infecting the same abaca plants I was able to show that aphids lived in mixed genotype and species colonies. Therefore, targeted pest management tailored to each aphid species, or that is successful for both species, should be considered. However, an additional factor to consider is the fact that multiple aphid vectored diseases also infect abaca plants and that these can be carried by a number of species such as *Abaca mosaic virus* (AMV) transmitted by *Aphis gossypii*, *Aphis maidis*, *Rhopalosiphum maidis* Fitch and *Myzus persicae*; *abaca bract mosaic virus* (AbMV) by *Aphis gossypii* Glover, *Rhopalosiphum maidis* Fitch and *Pentalonia nigronervosa* Coquerel; *cucumber mosaic virus* (CMV) by *Myzus persicae* and *Aphis gossypii* and *sugarcane mosaic virus* (SCMV) transmitted by *Rhopalosiphum maidis* Fitch, *Myzus persicae* Sultzer (Halos et al., 2008; Galvez et al., 2020; Sta. Cruz et al., 2017).

Another factor to consider is the host range of BBTV as this could serve as reservoir of virus inoculum and a source of infection and virus spread in the area. The *Pentalonia* aphid species have a strong association with the Musaceae plant family and infest other alternative host plant species in the Araceae, Costaceae, Heliconiaceae, Malvaceae and Zingerberaceae families (Bajet and Magnaye, 2002; Foottit et al., 2010; Foottit and Maw, 2019). However, a more limited host range of BBTV has been reported throughout Musaceae, Araceae and Zingeberaceae families (Calilung, 2008). Earlier studies showed that *P. nigronervosa* was able to transmit BBTV from banana to abaca but did not infect *Caladium bicolor* (Araceae), *Heliconia psittacorum* (Heliconiaceae) and *Hedychium coronarium* (Zingiberaceae) in the Philippines (Bajet and Magnaye, 2002). In Hawaii, *P. nigronervosa* did not transmit BBTV to *Alpinia purpurata* (Zingeberaceae) and *Colocasia* esculenta (Araceae) (Hu et al., 1996), suggesting virus specificity to Musa family. However, Dela Cueva et al. (2011) reported that *C. bicolor* and *Heliconia sp.* have shown to be infected with the BBTV but that *C. esculenta* did not host BBTV. Pinili et al. (2013) reported that aside from Musa species (banana and abaca), plants from Zingerberaceae (*Alpinia zerbet*), Cannaceae (*Canna indica*), and Araceae (*Colocasia esculenta*) families hosted BBTV isolates from banana in Japan and the Philippines. Therefore, identification of alternative host plants from cultivated and wild plant species in the Philippines that could become potential host of BBTV is highly recommended. Moreover, since BBTV isolates were grouped into Asian and South Pacific groups (Karan et al, 1994; Kumar et al., 2015), a virulence study of these isolates with different plant species is also encouraged (Pinili et al., 2013).

Second, the identification of endosymbionts in banana aphids collected in abaca plants gave us a glimpse of the potential role of symbionts in protecting the aphids against natural enemies (predators and parasitoids, virus and fungal pathogens), their importance in insect pest control (Trienens and Beukeboom, 2019) and at the same time their capacity to suppress biological control agents (Vorburger, 2018). In this study, the influence of *Wolbachia* in BBTV could guide us in the identification of innovative strategies in reducing

virus transmission. Since there was high variation in symbiont infection per location, this information could help us mitigating, if possible, the abiotic factors and encourage higher natural enemy density in the area by planting flowering plants which can help to establish natural enemy populations. As the first endosymbionts study on banana aphids collected from abaca in the Philippines, it is highly recommended to have a follow-up study to help us understand the impact of these endosymbionts for aphid resistance to biotic and abiotic challenges. Aphid samples should be collected across the country particularly from abaca and banana plantations and other alternative host plants. As endosymbionts could affect the effectiveness of biocontrol strategies, it is also recommended to update the current data on biocontrol agents and natural enemies (Calilung, 2008; BAFS, 2019) specific to the area and to test their efficacy and degree of specificity. Studies on host acceptability and suitability were previously conducted on *P. nigronervosa* using *Binodoxys communis* in the lab (Calilung, 2008) and it would be important to repeat this in the field.

Third, the determination of BBTV in aphids as the virus responsible for bunchy top disease on abaca highlighted the need to conduct virus transmission studies to determine how effective the *Pentalonia* species are in transmitting the virus, to implement a pest forecasting programme to monitor aphid abundance and to quantify virus in aphid populations across the country. This big task that needs coordination within the abaca community but having PhilFIDA offices within each region across the country, could facilitate the development of this. The influence of *P. caladii* in BBTV presence is a promising result which could help us in studying the biology of this *Pentalonia* species to know more of their role in virus transmission. Before the aphids can transmit the virus to the plants, pest forecasting and quantifying the presence of the viral load in aphids could give us an estimate of the capacity of the insect to infect and spread the virus. More studies have been conducted on *Pentalonia nigronervosa* already and hopefully they can be replicated to *P. caladii* providing a better understanding of the role of this species.

Although there was no significant effect of ants in influencing the viral copy number of BBTV, there was a suggestion from the data that their presence on plants where aphids were collected from decreased the mean copy number of BBTV. This finding is a welcome development in research as this might be suggestive of the role of ants in potentially reducing virus transmission. Aphids have a mutualistic relationship with ants as they benefit from the honeydew excreted by the aphids while protecting the aphids from natural enemies. Such activity was observed during the sampling collection in Sorsogon where large ant hills were found inside the collection area. In fact, ants can often be found on the trunk or the leaf sheet near ground. However, ants were absent on plants that are potentially resistant to the virus. This is perhaps one reason why no aphids were collected on some of the identified resistant abaca accessions or perhaps there were no ants because there were no aphids.

Most of the research and development strategies on ABTV has focused on the production of high yielding and resistant abaca varieties against bunchy top virus and the identification of the virus from asymptomatic and symptomatic abaca plants (Barbosa et al., 2020; Leny C. Galvez et al., 2020a; Galvez et al., 2020b; Piamonte and Sta. Cruz, 2018; Sta. Cruz et al., 2017). Promising developments on virus detection both for ABTV and BBTV on abaca plants is very important especially for the virus detection such as disease indexing of abaca tissue cultured plantlets. However, looking at the disease triangle concept (Scholthof, 2007; McNew, 1960) which involves the host (plant), inoculum (pathogen), and the environment, there are limited studies that have been conducted on the virus vector (the carrier of the inoculum) in terms of distribution and quantity which will likely affect the severity of viral infection on abaca plants. Hence, understanding the banana aphid as the vector of BBTV is important in elucidating IPM management strategies for BTD. Moreover, this information can also be applied not only on bunchy top virus but also to other plant viral diseases in agriculture.

The available data in this research has provided the benchmark for what needs to be done on the development of specific BTD strategies. Below are some of the recommendations based on the findings of the study.

Abaca germplasm collections

The Philippine abaca industry with an estimated value of $164 Million is projected to undergo a 20-year decline after the onslaught of Super Typhoon Rolly (International Name Goni) that devastated Catanduanes, the top producer of abaca in the country (Arcalas, 2020) recently. As a perennial crop, it will take at least a year to produce the next harvest, if at abaca rehabilitation takes place immediately. Therefore, there is an urgent need to expand the abaca germplasm collections across the country to have more duplicates of different abaca accessions. Work on this has begun already in some parts of Mindanao and hopefully will be extended to other areas in the Visayas. In addition, the priority to mass produce planting materials (corms, suckers, and tissue culture plants) should continue and expanded. Moreover, there should be a National Abaca Database Center that can be shared in every locality so that the status of abaca in the area is known. This is very helpful in deciding what accessions need immediate attention for conservation and disease monitoring in the area.

Abaca IPM management and government support

The Abaca Code of Good Agricultural Practices (BAFS, 2019) contains comprehensive information on the production of quality fibre, safety of the workers and how to reduce environmental hazards in abaca production. The IPM section of the guidance is very specific, however, research on aphids which play a crucial role in virus transmission is still limited and need to be expanded to understand the biological and ecological aspects of the insect vector and to have a holistic view in managing the viral disease. This study revealed two *Pentalonia* species on abaca that have the capacity to spread the virus. This information will help us in formulating research strategies to

ascertain which of the two species infest and can transmit the virus more. Further research on their biology and ecology of the aphids in the Philippines, monitoring their seasonal abundance, the host plants they prefer and the prevalence of disease incidence in the area are important components in IPM to prevent, suppress and monitor pest populations. This would also enable informed decision making in formulating ecologically based management practices.

Endosymbionts play a crucial role in aphid's nutrition, reproduction, and protection against biotic and abiotic factors. In IPM, strategies involve the use of biocontrol agents, reduction of pesticides application and avoidance of pest resistance. The identification of the endosymbionts presents in banana aphids is important factor that needs to be considered in IPM management as the endosymbionts affect the efficacy of the biocontrol strategies or may promote aphid resistance to pesticide.

The modified cultural practices under IPM components should also be implemented in the germplasm collections to mitigate the spread of the virus and reduce herbivore pressure in the area. As a source of abaca planting materials, germplasm should be free from bunchy top virus to produce disease-free abaca plants. The quantification of the virus titre in individual aphids in this study showed the importance of detection upon aphid's resurgence in the field for us to monitor the development of the viral disease and mitigate their spread. In IPM, field assessment of insect pest and disease monitoring are important for control measures intervention and managing disease so that it does not reach the critical levels.

Government support is still needed for research and development on abaca in mitigating the virus, increasing fibre production and quality, and in developing high-yielding abaca resistant to insect pests and diseases with the use of new technologies such as next-generation sequencing, genome editing tools, and abaca plant transformation for resistance against bunchy top virus. In addition, information dissemination and outreach programmes for proper management

of the BTD to farmers and stakeholders should continue and expand in scope across the country. Capacity building is needed to implement the different technologies at the farmer's level.

Thesis Conclusion and Future Directions

Understanding the different aspects on the banana aphid's biology is important in evaluating its capacity as a vector in spreading the disease and for viral disease management strategies. In this thesis, the mode of reproduction, genetic diversity and genetic structure of *Pentalonia* species were identified using microsatellite markers. I then used the aphid species results to verify if they have influence on endosymbiont species richness and the presence of BBTV and the viral copy number. I make some recommendations on abaca germplasm conservation, abaca cultural management practices and the research recommendation to expand our understanding of the role of aphid species, endosymbionts and ants influencing virus transmission. This information will hopefully aid in the development of targeted, specific and sustainable control strategies for bunchy top disease management on abaca in the Philippines and bananas in the tropical and sub-tropical regions.

To further understand the population genetic structure and population dynamics of the *Pentalonia* species across the country, a holistic approach for assessing their populations should be conducted. Since they reproduce asexually, it is hypothesized that they have low genetic diversity and fewer MLGs between the two *Pentalonia* species. For future population genetics studies, it is recommended to develop specific SSR markers using the available genome sequences of *P. nigronervosa* for specificity. Aphids should be collected from banana, taro, ginger and other alternative host plants present in abaca and banana germplasm collections and farmers' fields across the country. This will help us determine if aphids develop host plant specialization or show host preferences for different crop and non-crops plants. This extensive temporal and spatial aphid collection will give us an idea of how diverse and disperse they are geographically, if they are structured according to host plants and if they are regularly migrating between abaca and banana plantations or across islands. For population dynamics, the life cycle, longevity, and fecundity of two *Pentalonia* species should also be

determined to increase understanding of the impact of potential spread to new plantations.

Research to identify the chemical composition of abaca phloem sap would be important to determine the nutrient composition of the plant sap as this might affect aphid feeding behavior and preferences (across resistant and susceptible plants). Aphids determine the suitability of a host plant by probing a plant and in so doing, can already introduce the virus to the plants even if an aphid decides not to settle. Host plant preference should also be done by rearing aphids on resistant and susceptible abaca accessions to determine what planting scheme could be applied that will limit their abundance and or suppress their growth. Another aspect to consider is the identification of aphid and symbiont proteins and determine if they are involved or could facilitate virus transmission.

For cultural management, research on determining abaca accessions' susceptibility to bunchy top disease should become a priority as this could be used in planting regime where both resistant and susceptible accessions are planted side by side to act as an aphid barrier and prevent them from spreading in abaca plantations. This can be facilitated by conducting virus transmission study both in the lab and in the field using the two *Pentalonia* species with resistant and susceptible abaca accessions. Based on the results of this study, it is hypothesized that *P. caladii* could be more susceptible to BBTV infection and hence, can acquire higher virus titre loads than *P. nigronervosa*.

The identification of endosymbionts should be conducted simultaneously alongside the samples for the population genetics analysis. Results will be correlated from this study if they are location or host plant specific as this will give us an idea of the severity and or disease pressure in the area. It is hypothesized that in the absence of the virus and the abundance of natural enemies in the areas, aphids would host fewer endosymbionts compared to areas with high disease incidence. In addition, as they affect biocontrol strategies, their

performance will be tested with the identified biocontrol agents (e.g. parasitoid wasps or entomopathogenic fungi). Potential biocontrol agents will be subjected to efficacy tests if they can successfully suppress the growth or abundance of aphids. Moreover, *Wolbachia* was shown to influence virus presence in this study. To further test if they are involved in virus transmission, two *Pentalonia* species with or without *Wolbachia* will be challenged with different number of BBTV infected aphids at different time interval. In here, it is hypothesized that aphid that host *Wolbachia* has higher virus titre than *P. nigronervosa*.

In disease management, one must look at the holistic approach in managing perennial disease such as bunchy top disease. This is not only by looking the effects of one factor but the interactions between the aphids, the endosymbionts, virus, and the host plant. Therefore, it is a challenge to undergo research that will investigate the different aspects of their involvement in virus transmission, hence, collaborations with other research scientists and institutions are very important.

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APPENDIX

Appendix Table 1. Location of collections of *Pentalonia* population included in this study.

Sample ID	Region	Abaca Accession	Bunchy top Susceptibility	Plant No.	Colony No.	Aphid's location on Abaca Plant	Ants	Latitude	Longitude	Elevation (m)	MLG No.
P. caladii											
D31b	Davao	Laylay	Susceptible	9	2	newly opened leaf, front	ants	7.07395	125.4968333	115	10
D33a	Davao	Laylay	Susceptible	10	2	petiole, 1st leaf	ants	7.0739	125.4968667	117	7
N2b	Leyte	Canton Forestry	Resistant	21	1	shoot	none	11.24838333	125.3293	59	7
N3a	Leyte	Canton Forestry	Resistant	22	1	shoot	none	11.24835	125.3292667	63	7
N3b	Leyte	Canton Forestry	Resistant	22	1	shoot	none	11.24835	125.3292667	63	7
N117b	Leyte	Linao Cultivar	Susceptible	29	1	shoot	ants	11.24833333	125.3290333	70	11
N118a	Leyte	Linao Cultivar	Susceptible	29	2	petiole, 1st leaf	ants	11.24833333	125.3290333	70	7
N118b	Leyte	Linao Cultivar	Susceptible	29	2	petiole, 1st leaf	ants	11.24833333	125.3290333	70	7
N125a	Leyte	Linao Cultivar	Susceptible	30	1	petiole, 1st leaf	none	11.24848333	125.3281667	57	7
N125b	Leyte	Linao Cultivar	Susceptible	30	1	petiole, 1st leaf	none	11.24848333	125.3281667	57	5
N136a	Leyte	Lunhan	Susceptible	31	1	petiole, 1st leaf	ants	11.24876667	125.3287667	67	6
N231b	Leyte	Samoro	Susceptible	37	1	middle trunk	none	10.74763333	124.7988667	67	21
N240b	Leyte	Samoro	Susceptible	38	1	shoot, bottom	none	10.74766667	124.7983833	59	7
N323a	Leyte	Tangongon	Susceptible	44	1	unfurled leaf mid shoot	none	10.74768333	124.7982833	58	7
N323b	Leyte	Tangongon	Susceptible	44	1	unfurled leaf mid shoot	none	10.74768333	124.7982833	58	7
N325a	Leyte	Tangongon	Susceptible	44	2	petiole, top opened leaf	ants	10.74768333	124.7982833	58	7
N325b	Leyte	Tangongon	Susceptible	44	2	petiole, top opened leaf	ants	10.74768333	124.7982833	58	5
N329a	Leyte	Tangongon	Susceptible	45	1	petiole, 1st leaf	ants	10.74765	124.7983333	65	23
N330b	Leyte	Tangongon	Susceptible	45	2	petiole ,2nd leaf	ants	10.74765	124.7983333	65	23
N351b	Leyte	VH#23	Resistant	47	1	petiole, 2nd leaf	ants	10.74756667	124.7984667	68	7
N352a	Leyte	VH#23	Resistant	47	2	petiole, 1st leaf	ants	10.74756667	124.7984667	68	3
N352b	Leyte	VH#23	Resistant	47	2	petiole, 1st leaf	ants	10.74756667	124.7984667	68	4

N369a	Leyte	VH#25	No data yet	49	1	newly opened leaf, back	none	10.74758333	124.7984167	68	24
N369b	Leyte	VH#25	No data yet	49	1	newly opened leaf, back	none	10.74758333	124.7984167	68	7
N370b	Leyte	VH#25	No data yet	49	2	lower trunk	none	10.74758333	124.7984167	68	10
N377a	Leyte	VH#25	No data yet	50	2	shoot	ants	11.2484	125.3283333	55	8
N387a	Leyte	VH#32	No data yet	51	1	1st leaf	none	11.24838333	125.3294	72	9
N387b	Leyte	VH#32	No data yet	51	1	1st leaf	none	11.24838333	125.3294	72	7
N388a	Leyte	VH#32	No data yet	52	1	1st leaf	none	11.2482	125.3295333	75	7
N388b	Leyte	VH#32	No data yet	52	1	1st leaf	none	11.2482	125.3295333	75	7
N390a	Leyte	VH#32	No data yet	53	1	shoot, inside	none	11.24828333	125.32925	61	10
N390b	Leyte	VH#32	No data yet	53	1	shoot, inside	none	11.24828333	125.32925	61	7
N391a	Leyte	VH#32	No data yet	54	2	shoot, inside	none	11.2483	125.3292833	61	1
N391b	Leyte	VH#32	No data yet	54	2	shoot, inside	none	11.2483	125.3292833	61	7
N396a	Leyte	VH#39	Susceptible	55	1	petiole, 1st leaf	none	11.2483	125.32925	61	5
N396b	Leyte	VH#39	Susceptible	55	1	petiole, 1st leaf	none	11.2483	125.32925	61	6
N397a	Leyte	VH#39	Susceptible	55	2	petiole, 2nd leaf	none	11.2483	125.32925	61	7
N397b	Leyte	VH#39	Susceptible	55	2	petiole, 2nd leaf	none	11.2483	125.32925	61	7
N402a	Leyte	VH#39	Susceptible	56	2	petiole, 1st leaf lower	none	11.24823333	125.32915	65	2
S113a	Sorsogon	Maguindanao	Susceptible	85	1	petiole, 2nd leaf	none	12.83653333	124.0339	107	7
S113b	Sorsogon	Maguindanao	Susceptible	85	1	petiole, 2nd leaf	none	12.83653333	124.0339	107	7
S162b	Sorsogon	Negro	Susceptible	97	2	1st leaf	ants	12.83735	124.03305	107	7
P. nigronervosa											
D7a	Davao	Green Abaca	Susceptible	1	1	shoot	ants	7.074066667	125.4972833	122	25
D7b	Davao	Green Abaca	Susceptible	1	1	shoot	ants	7.074066667	125.4972833	122	25
D9a	Davao	Green Abaca	Susceptible	2	1	petiole, 1st leaf	ants	7.074116667	125.49725	119	25
D9b	Davao	Green Abaca	Susceptible	2	1	petiole, 1st leaf	ants	7.074116667	125.49725	119	25
D11a	Davao	Inosa	Susceptible	3	1	petiole, 2nd leaf	ants	7.074	125.4967667	107	25
D11b	Davao	Inosa	Susceptible	3	1	petiole, 2nd leaf	ants	7.074	125.4967667	107	25

D13a	Davao	Inosa	Susceptible	3	2	petiole, 7th leaf	ants	7.074	125.4967667	107	30
D13b	Davao	Inosa	Susceptible	3	2	petiole, 7th leaf	ants	7.074	125.4967667	107	27
D15a	Davao	Inosa	Susceptible	4	1	petiole, 2nd leaf	ants	7.074016667	125.49675	112	25
D15b	Davao	Inosa	Susceptible	4	1	petiole, 2nd leaf	ants	7.074016667	125.49675	112	25
D16a	Davao	Inosa	Susceptible	5	1	1st leaf, base	ants	7.074016667	125.4967333	115	25
D16b	Davao	Inosa	Susceptible	5	1	1st leaf, base	ants	7.074016667	125.4967333	115	25
D18a	Davao	Inosa	Susceptible	6	1	newly opened leaf, front lower	ants	7.074016667	125.49675	115	25
D18b	Davao	Inosa	Susceptible	6	1	newly opened leaf, front lower	ants	7.074016667	125.49675	115	25
D19a	Davao	Inosa	Susceptible	6	2	newly opened leaf, front upper	ants	7.074016667	125.49675	115	25
D19b	Davao	Inosa	Susceptible	6	2	newly opened leaf, front upper	ants	7.074016667	125.49675	115	25
D23a	Davao	Inosa	Susceptible	7	1	newly opened leaf, front	ants	7.074016667	125.4967333	119	25
D23b	Davao	Inosa	Susceptible	7	1	newly opened leaf, front	ants	7.074016667	125.4967333	119	25
D24a	Davao	Inosa	Susceptible	8	1	newly opened leaf, front	ants	7.07395	125.4967667	119	25
D24b	Davao	Inosa	Susceptible	8	1	newly opened leaf, front	ants	7.07395	125.4967667	119	25
D30a	Davao	Laylay	Susceptible	9	1	newly opened leaf, back	ants	7.07395	125.4968333	115	25
D30b	Davao	Laylay	Susceptible	9	1	newly opened leaf, back	ants	7.07395	125.4968333	115	25
D31a	Davao	Laylay	Susceptible	9	2	newly opened leaf, front	ants	7.07395	125.4968333	115	25
D32a	Davao	Laylay	Susceptible	10	1	petiole, 2nd leaf	ants	7.0739	125.4968667	117	25
D32b	Davao	Laylay	Susceptible	10	1	petiole, 2nd leaf	ants	7.0739	125.4968667	117	25
D33b	Davao	Laylay	Susceptible	10	2	petiole, 1st leaf	ants	7.0739	125.4968667	117	25
D37a	Davao	Laylay	Susceptible	11	1	petiole, 1st leaf left side	ants	7.074	125.4967833	121	25
D37b	Davao	Laylay	Susceptible	11	1	petiole, 1st leaf left side	ants	7.074	125.4967833	121	25
D38a	Davao	Laylay	Susceptible	11	2	petiole, 1st leaf right side	ants	7.074	125.4967833	121	25
D38b	Davao	Laylay	Susceptible	11	2	petiole, 1st leaf right side	ants	7.074	125.4967833	121	25
D43a	Davao	Maguindanao	Susceptible	12	1	petiole, 1st leaf, lower	none	7.074716667	125.4974667	117	25

D43b	Davao	Maguindanao	Susceptible	12	1	petiole, 1st leaf, lower	none	7.074716667	125.4974667	117	25
D45a	Davao	Maguindanao	Susceptible	13	1	petiole, 6th leaf	none	7.07465	125.4975	114	25
D45b	Davao	Maguindanao	Susceptible	13	1	petiole, 6th leaf	none	7.07465	125.4975	114	25
D46a	Davao	Maguindanao	Susceptible	14	1	1st Leaf, lower	ants	7.074683333	125.4975	114	30
D46b	Davao	Maguindanao	Susceptible	14	1	1st Leaf, lower	ants	7.074683333	125.4975	114	30
D47a	Davao	Maguindanao	Susceptible	14	2	petiole, 1st leaf	ants	7.074683333	125.4975	114	30
D47b	Davao	Maguindanao	Susceptible	14	2	petiole, 1st leaf	ants	7.074683333	125.4975	114	25
D49a	Davao	Maguindanao	Susceptible	15	1	petiole, 6th leaf	none	7.074916667	125.4969667	114	25
D49b	Davao	Maguindanao	Susceptible	15	1	petiole, 6th leaf	none	7.074916667	125.4969667	114	30
D61a	Davao	Tangongon	Susceptible	16	1	petiole, first leaf, middle	ants	7.07465	125.4975833	122	25
D61b	Davao	Tangongon	Susceptible	16	1	petiole, first leaf, middle	ants	7.07465	125.4975833	122	25
D63a	Davao	Tangongon	Susceptible	16	2	petiole, 2nd leaf	none	7.07465	125.4975833	122	25
D63b	Davao	Tangongon	Susceptible	16	2	petiole, 2nd leaf	none	7.07465	125.4975833	122	25
D64a	Davao	Tangongon	Susceptible	17	1	unopened leaf	none	7.074783333	125.4974167	122	28
D64b	Davao	Tangongon	Susceptible	17	1	unopened leaf	none	7.074783333	125.4974167	122	25
D65a	Davao	Tangongon	Susceptible	17	2	unopened leaf	none	7.074783333	125.4974167	122	25
D65b	Davao	Tangongon	Susceptible	17	2	unopened leaf	none	7.074783333	125.4974167	122	25
D68a	Davao	Tangongon	Susceptible	18	1	first leaf, base	ants	7.0749	125.4973333	117	25
D68b	Davao	Tangongon	Susceptible	18	1	first leaf, base	ants	7.0749	125.4973333	117	25
D69a	Davao	Tangongon	Susceptible	18	2	shoot	none	7.0749	125.4973333	117	25
D69b	Davao	Tangongon	Susceptible	18	2	shoot	none	7.0749	125.4973333	117	25
D70a	Davao	Tuod	Susceptible	19	1	shoot	ants	7.073883333	125.49775	112	25
D70b	Davao	Tuod	Susceptible	19	1	shoot	ants	7.073883333	125.49775	112	25
D71a	Davao	Tuod	Susceptible	20	1	petiole, 1st leaf back	ants	7.073883333	125.49775	112	30
D71b	Davao	Tuod	Susceptible	20	1	petiole, 1st leaf back	ants	7.073883333	125.49775	112	25

D72a	Davao	Tuod	Susceptible	20	2	petiole, 2nd leaf	ants	7.073883333	125.49775	112	25
D72b	Davao	Tuod	Susceptible	20	2	petiole, 2nd leaf	ants	7.073883333	125.49775	112	25
N2a	Leyte	Canton Forestry	Resistant	21	1	shoot	none	11.24838333	125.3293	59	25
N14a	Leyte	Inosa	Susceptible	23	1	1st leaf, back	none	11.24835	125.3287167	65	25
N14b	Leyte	Inosa	Susceptible	23	1	1st leaf, back	none	11.24835	125.3287167	65	25
N15a	Leyte	Inosa	Susceptible	23	2	1st leaf, front bottom	none	11.24835	125.3287167	65	25
N15b	Leyte	Inosa	Susceptible	23	2	1st leaf, front bottom	none	11.24835	125.3287167	65	25
N40a	Leyte	Inosa	Susceptible	24	1	petiole, 4th leaf	ants	11.24856667	125.32875	82	25
N40b	Leyte	Inosa	Susceptible	24	1	petiole, 4th leaf	ants	11.24856667	125.32875	82	25
N41a	Leyte	Inosa	Susceptible	24	2	petiole, 4th leaf lower	ants	11.24856667	125.32875	82	25
N41b	Leyte	Inosa	Susceptible	24	2	petiole, 4th leaf lower	ants	11.24856667	125.32875	82	25
N51a	Leyte	Inosa	Susceptible	25	1	mid trunk	ants	11.24823333	125.3291167	61	30
N51b	Leyte	Inosa	Susceptible	25	1	mid trunk	ants	11.24823333	125.3291167	61	25
N58a	Leyte	Inosa	Susceptible	26	1	petiole, 1st leaf	ants	11.24843333	125.3282667	63	25
N58b	Leyte	Inosa	Susceptible	26	1	petiole, 1st leaf	ants	11.24843333	125.3282667	63	25
N60a	Leyte	Inosa	Susceptible	26	2	petiole, 2nd leaf	none	11.24843333	125.3282667	63	25
N60b	Leyte	Inosa	Susceptible	26	2	petiole, 2nd leaf	none	11.24843333	125.3282667	63	25
N76a	Leyte	Javaque	Resistant	27	1	petiole, 1st leaf	ants	11.25018333	125.3321	59	25
N76b	Leyte	Javaque	Resistant	27	1	petiole, 1st leaf	ants	11.25018333	125.3321	59	25
N77a	Leyte	Javaque	Resistant	27	2	2nd leaf	none	11.25018333	125.3321	59	25
N77b	Leyte	Javaque	Resistant	27	2	2nd leaf	none	11.25018333	125.3321	59	25
N81a	Leyte	Javaque	Resistant	28	1	shoot, outside	ants	11.24988333	125.3329	60	25
N81b	Leyte	Javaque	Resistant	28	1	shoot, outside	ants	11.24988333	125.3329	60	25
N82a	Leyte	Javaque	Resistant	28	2	shoot, lower	ants	11.24988333	125.3329	60	29
N82b	Leyte	Javaque	Resistant	28	2	shoot, lower	ants	11.24988333	125.3329	60	25

N117a	Leyte	Linao Cultivar	Susceptible	29	1	shoot	ants	11.24833333	125.3290333	70	26
N126a	Leyte	Linao Cultivar	Susceptible	30	2	petiole, 2nd leaf	none	11.24848333	125.3281667	57	25
N126b	Leyte	Linao Cultivar	Susceptible	30	2	petiole, 2nd leaf	none	11.24848333	125.3281667	57	25
N136b	Leyte	Lunhan	Susceptible	31	1	petiole, 1st leaf	ants	11.24876667	125.3287667	67	20
N139a	Leyte	Lunhan	Susceptible	32	1	shoot	ants	11.2484	125.3293833	90	25
N139b	Leyte	Lunhan	Susceptible	32	1	shoot	ants	11.2484	125.3293833	90	25
N140a	Leyte	Lunhan	Susceptible	32	2	petiole, 2nd leaf	ants	11.2484	125.3293833	90	25
N140b	Leyte	Lunhan	Susceptible	32	2	petiole, 2nd leaf	ants	11.2484	125.3293833	90	25
N164a	Leyte	Pepita	Resistant	33	1	petiole, 3rd leaf	ants	11.24991667	125.332	53	19
N164b	Leyte	Pepita	Resistant	33	1	petiole, 3rd leaf	ants	11.24991667	125.332	53	25
N165a	Leyte	Pepita	Resistant	33	2	trunk	ants	11.24991667	125.332	53	25
N165b	Leyte	Pepita	Resistant	33	2	trunk	ants	11.24991667	125.332	53	25
N167a	Leyte	Pepita	Resistant	34	1	petiole, 1st leaf	ants	11.2501	125.3319333	55	25
N167b	Leyte	Pepita	Resistant	34	1	petiole, 1st leaf	ants	11.2501	125.3319333	55	25
N168a	Leyte	Pepita	Resistant	34	2	petiole, 3rd leaf	ants	11.2501	125.3319333	55	25
N168b	Leyte	Pepita	Resistant	34	2	petiole, 3rd leaf	ants	11.2501	125.3319333	55	31
N187a	Leyte	Putomag	Susceptible	35	1	mid trunk	ants	11.24825	125.3287667	69	17
N187b	Leyte	Putomag	Susceptible	35	1	mid trunk	ants	11.24825	125.3287667	69	25
N188a	Leyte	Putomag	Susceptible	35	2	lower trunk	ants	11.24825	125.3287667	69	25
N188b	Leyte	Putomag	Susceptible	35	2	lower trunk	ants	11.24825	125.3287667	69	25
N221a	Leyte	Putomag	Susceptible	36	1	lower trunk	ants	11.24848333	125.3284	64	25
N221b	Leyte	Putomag	Susceptible	36	1	lower trunk	ants	11.24848333	125.3284	64	30
N231a	Leyte	Samoro	Susceptible	37	1	middle trunk	none	10.74763333	124.7988667	67	12
N232a	Leyte	Samoro	Susceptible	37	2	lower leaf sheath	none	10.74763333	124.7988667	67	25
N232b	Leyte	Samoro	Susceptible	37	2	lower leaf sheath	none	10.74763333	124.7988667	67	25
N240a	Leyte	Samoro	Susceptible	38	1	shoot, bottom	none	10.74766667	124.7983833	59	25

N241a	Leyte	Samoro	Susceptible	38	2	1st leaf, back left side	ants	10.74766667	124.7983833	59	25
N241b	Leyte	Samoro	Susceptible	38	2	2nd leaf, back left side	ants	10.74766667	124.7983833	59	25
N246a	Leyte	Samoro	Susceptible	39	1	petiole, 4th leaf	ants	10.74765	124.7984	52	25
N246b	Leyte	Samoro	Susceptible	39	1	petiole, 4th leaf	ants	10.74765	124.7984	52	25
N247a	Leyte	Samoro	Susceptible	39	2	petiole, 5th leaf	ants	10.74765	124.7984	52	25
N247b	Leyte	Samoro	Susceptible	39	2	petiole, 5th leaf	ants	10.74765	124.7984	52	25
N255a	Leyte	Samoro	Susceptible	40	1	1st leaf, back left side upper leaf	none	10.74765	124.7983833	57	25
N255b	Leyte	Samoro	Susceptible	40	1	1st leaf, back left side upper leaf	none	10.74765	124.7983833	57	25
N256a	Leyte	Samoro	Susceptible	40	1	1st leaf, back left side bottom	none	10.74765	124.7983833	57	25
N256b	Leyte	Samoro	Susceptible	40	1	1st leaf, back left side bottom	none	10.74765	124.7983833	57	25
N311a	Leyte	Suglin	Susceptible	41	1	1st leaf, front	none	11.24841667	125.3287667	60	30
N311b	Leyte	Suglin	Susceptible	41	1	1st leaf, front	none	11.24841667	125.3287667	60	25
N312a	Leyte	Suglin	Susceptible	41	2	petiole, 2nd leaf	ants	11.24841667	125.3287667	60	25
N312b	Leyte	Suglin	Susceptible	41	2	petiole, 2nd leaf	ants	11.24841667	125.3287667	60	30
N316a	Leyte	Suglin	Susceptible	42	1	shoot	ants	11.24846667	125.3287333	69	17
N316b	Leyte	Suglin	Susceptible	42	1	shoot	ants	11.24846667	125.3287333	69	25
N317a	Leyte	Suglin	Susceptible	42	2	petiole, 1st leaf	ants	11.24846667	125.3287333	69	25
N317b	Leyte	Suglin	Susceptible	42	2	petiole, 1st leaf	ants	11.24846667	125.3287333	69	25
N320a	Leyte	Tangongon	Susceptible	43	1	lower back of the leaf	ants	10.74768333	124.7983167	57	25
N320b	Leyte	Tangongon	Susceptible	43	1	lower back of the leaf	ants	10.74768333	124.7983167	57	25
N321a	Leyte	Tangongon	Susceptible	43	2	fully opened leaf, upper front	none	10.74768333	124.7983167	57	25
N321b	Leyte	Tangongon	Susceptible	43	2	fully opened leaf, upper front	none	10.74768333	124.7983167	57	25
N329b	Leyte	Tangongon	Susceptible	45	1	petiole, 1st leaf	ants	10.74765	124.7983333	65	25
N330a	Leyte	Tangongon	Susceptible	45	2	petiole ,2nd leaf	ants	10.74765	124.7983333	65	25

N334a	Leyte	Tangongon	Susceptible	46	1	petiole, 1st leaf	none	10.74765	124.7983333	60	25
N334b	Leyte	Tangongon	Susceptible	46	1	petiole, 1st leaf	none	10.74765	124.7983333	60	25
N335a	Leyte	Tangongon	Susceptible	46	2	petiole, 2nd leaf	none	10.74765	124.7983333	60	25
N335b	Leyte	Tangongon	Susceptible	46	2	petiole, 2nd leaf	none	10.74765	124.7983333	60	25
N351a	Leyte	VH#23	Resistant	47	1	petiole, 2nd leaf	ants	10.74756667	124.7984667	68	25
N356a	Leyte	VH#23	Resistant	48	1	newly opened leaf upper	none	11.24843333	125.3289333	69	25
N356b	Leyte	VH#23	Resistant	48	1	newly opened leaf upper	none	11.24843333	125.3289333	69	25
N357a	Leyte	VH#23	Resistant	48	2	newly opened leaf lower	none	11.24843333	125.3289333	69	25
N357b	Leyte	VH#23	Resistant	48	2	newly opened leaf lower	none	11.24843333	125.3289333	69	25
N370a	Leyte	VH#25	No data yet	49	2	lower trunk	none	10.74758333	124.7984167	68	25
N376a	Leyte	VH#25	No data yet	50	1	1st leaf, inside	ants	11.2484	125.3283333	55	25
N376b	Leyte	VH#25	No data yet	50	1	1st leaf, inside	ants	11.2484	125.3283333	55	25
N377b	Leyte	VH#25	No data yet	50	2	shoot	ants	11.2484	125.3283333	55	25
N401a	Leyte	VH#39	Susceptible	56	1	petiole, 1st leaf upper	none	11.24823333	125.32915	65	25
N401b	Leyte	VH#39	Susceptible	56	1	petiole, 1st leaf upper	none	11.24823333	125.32915	65	25
N402b	Leyte	VH#39	Susceptible	56	2	petiole, 1st leaf lower	none	11.24823333	125.32915	65	25
N403a	Leyte	Wild#3	Resistant	57	1	base of unfurled leaf/ unopened leaf	ants	10.7476	124.7986333	67	25
N403b	Leyte	Wild#3	Resistant	57	1	base of unfurled leaf/ unopened leaf	ants	10.7476	124.7986333	67	25
N404a	Leyte	Wild#3	Resistant	57	2	back opened leaf	ants	10.7476	124.7986333	67	25
N404b	Leyte	Wild#3	Resistant	57	2	back opened leaf	ants	10.7476	124.7986333	67	25
N406a	Leyte	Wild#3	Resistant	58	1	open leaf back midrib, right	ants	10.74761667	124.7986667	67	25
N406b	Leyte	Wild#3	Resistant	58	1	open leaf back midrib, right	ants	10.74761667	124.7986667	67	25
N407a	Leyte	Wild#3	Resistant	58	2	fully opened leaf, back left	ants	10.74761667	124.7986667	67	25
N407b	Leyte	Wild#3	Resistant	58	2	fully opened leaf, back left	ants	10.74761667	124.7986667	67	25
N410a	Leyte	Wild#3	Resistant	58	3	4th leaf, inside	ants	10.74761667	124.7986667	67	25
N410b	Leyte	Wild#3	Resistant	58	3	5th leaf, inside	ants	10.74761667	124.7986667	67	25

N411a	Leyte	Wild#3	Resistant	58	4	youngest leaf, top petiole	ants	10.74765	124.7986667	67	25
N411b	Leyte	Wild#3	Resistant	58	4	youngest leaf, top petiole	ants	10.74765	124.7986667	67	25
S10a	Sorsogon	Bisaya	Susceptible	59	1	mid runk	ants	12.83668333	124.0339333	108	25
S10b	Sorsogon	Bisaya	Susceptible	59	1	Mid runk	ants	12.83668333	124.0339333	108	25
S11a	Sorsogon	Bisaya	Susceptible	59	2	lower trunk	ants	12.83668333	124.0339333	108	25
S11b	Sorsogon	Bisaya	Susceptible	59	2	lower trunk	ants	12.83668333	124.0339333	108	25
S15a	Sorsogon	Bisaya	Susceptible	60	1	mid trunk	ants	12.83673333	124.0339667	109	25
S15b	Sorsogon	Bisaya	Susceptible	60	1	mid trunk	ants	12.83673333	124.0339667	109	25
S16a	Sorsogon	Bisaya	Susceptible	60	2	lower trunk	ants	12.83673333	124.0339667	109	25
S16b	Sorsogon	Bisaya	Susceptible	60	2	lower trunk	ants	12.83673333	124.0339667	109	25
S18a	Sorsogon	Igit	Susceptible	61	1	petiole, 1st leaf	none	12.83725	124.03325	98	25
S18b	Sorsogon	Igit	Susceptible	61	1	petiole, 1st leaf	none	12.83725	124.03325	98	25
S19a	Sorsogon	Igit	Susceptible	61	2	upper trunk	none	12.83725	124.03325	98	25
S19b	Sorsogon	Igit	Susceptible	61	2	upper trunk	none	12.83725	124.03325	98	25
S21a	Sorsogon	Igit	Susceptible	62	1	shoot	none	12.83725	124.0332167	104	25
S21b	Sorsogon	Igit	Susceptible	62	1	shoot	none	12.83725	124.0332167	104	25
S22a	Sorsogon	Igit	Susceptible	62	2	petiole, 1st leaf	ants	12.83725	124.0332167	104	17
S22b	Sorsogon	Igit	Susceptible	62	2	petiole, 1st leaf	ants	12.83725	124.0332167	104	25
S28a	Sorsogon	Inosa	Susceptible	63	1	petiole, 5th leaf	none	12.8369	124.0499667	104	26
S28b	Sorsogon	Inosa	Susceptible	63	1	petiole, 5th leaf	none	12.8369	124.0499667	104	26
S29a	Sorsogon	Inosa	Susceptible	63	2	petiole, 3rd leaf	ants	12.8369	124.0499667	104	30
S29b	Sorsogon	Inosa	Susceptible	63	2	petiole, 3rd leaf	ants	12.8369	124.0499667	104	25
S30a	Sorsogon	Inosa	Susceptible	63	3	petiole, 2nd leaf	ants	12.8369	124.0499667	104	25
S30b	Sorsogon	Inosa	Susceptible	63	3	petiole, 2nd leaf	ants	12.8369	124.0499667	104	25
S31a	Sorsogon	Inosa	Susceptible	63	4	petiole, 1st leaf	none	12.8369	124.0499667	104	25
S31b	Sorsogon	Inosa	Susceptible	63	4	petiole, 1st leaf	none	12.8369	124.0499667	104	25

S34a	Sorsogon	Inosa	Susceptible	64	1	petiole, 1st leaf	none	12.83698333	124.0334	105	25
S34b	Sorsogon	Inosa	Susceptible	64	1	petiole, 1st leaf	none	12.83698333	124.0334	105	25
S35a	Sorsogon	Inosa	Susceptible	64	2	petiole, 1st leaf upper	none	12.83698333	124.0334	105	30
S35b	Sorsogon	Inosa	Susceptible	64	2	petiole, 1st leaf upper	none	12.83698333	124.0334	105	25
S38a	Sorsogon	Inotang	Susceptible	65	1	1st leaf	none	12.83765	124.0332	97	25
S38b	Sorsogon	Inotang	Susceptible	65	1	1st leaf	none	12.83765	124.0332	97	25
S39a	Sorsogon	Inotang	Susceptible	66	1	1st leaf	none	12.83765	124.0333167	98	25
S39b	Sorsogon	Inotang	Susceptible	66	1	1st leaf	none	12.83765	124.0333167	98	25
S40a	Sorsogon	Inotang	Susceptible	66	2	petiole, 1st leaf	none	12.83765	124.0333167	98	25
S40b	Sorsogon	Inotang	Susceptible	66	2	petiole, 1st leaf	none	12.83765	124.0333167	98	25
S42a	Sorsogon	Kurukutuhan	Susceptible	67	1	shoot, bottom	none	12.83745	124.0330333	97	25
S42b	Sorsogon	Kurukutuhan	Susceptible	67	1	shoot, bottom	none	12.83745	124.0330333	97	25
S43a	Sorsogon	Kurukutuhan	Susceptible	67	2	petiole, 5th leaf	none	12.83745	124.0330333	97	22
S43b	Sorsogon	Kurukutuhan	Susceptible	67	2	petiole, 5th leaf	none	12.83745	124.0330333	97	25
S55a	Sorsogon	Kurukutuhan	Susceptible	68	1	petiole, 2nd leaf	ants	12.83753333	124.03315	99	25
S55b	Sorsogon	Kurukutuhan	Susceptible	68	1	petiole, 2nd leaf	ants	12.83753333	124.03315	99	25
S56a	Sorsogon	Kurukutuhan	Susceptible	68	2	petiole, 1st leaf	none	12.83753333	124.03315	99	25
S56b	Sorsogon	Kurukutuhan	Susceptible	68	2	petiole, 1st leaf	none	12.83753333	124.03315	99	25
S59a	Sorsogon	Kutay-kutay	Resistant	69	1	shoot	none	12.8366	124.0340167	101	25
S59b	Sorsogon	Kutay-kutay	Resistant	69	1	shoot	none	12.8366	124.0340167	101	25
S60a	Sorsogon	Kutay-kutay	Resistant	70	1	shoot	none	12.8365	124.0340333	104	25
S60b	Sorsogon	Kutay-kutay	Resistant	70	1	shoot	none	12.8365	124.0340333	104	25
S61a	Sorsogon	Kutay-kutay	Resistant	71	1	1st leaf	ants	12.83726667	124.0331667	97	25
S61b	Sorsogon	Kutay-kutay	Resistant	71	1	1st leaf	ants	12.83726667	124.0331667	97	25
S62a	Sorsogon	Kutay-kutay	Resistant	72	1	1st leaf, right	ants	12.83726667	124.0332	95	25
S62b	Sorsogon	Kutay-kutay	Resistant	72	1	1st leaf, right	ants	12.83726667	124.0332	95	25

S64a	Sorsogon	Laguis	Susceptible	73	1	petiole, 1st leaf	ants	12.83783333	124.0330167	103	25
S64b	Sorsogon	Laguis	Susceptible	73	1	petiole, 1st leaf	ants	12.83783333	124.0330167	103	25
S65a	Sorsogon	Laguis	Susceptible	73	2	petiole, 2nd leaf	ants	12.83783333	124.0330167	103	25
S65b	Sorsogon	Laguis	Susceptible	73	2	petiole, 2nd leaf	ants	12.83783333	124.0330167	103	25
S66a	Sorsogon	Laguis	Susceptible	74	1	petiole, 1st leaf	none	12.83783333	124.0330167	103	25
S66b	Sorsogon	Laguis	Susceptible	74	1	petiole, 1st leaf	none	12.83783333	124.0330167	103	25
S67a	Sorsogon	Laguis	Susceptible	74	2	petiole, 2nd leaf	none	12.83773333	124.0330167	103	25
S67b	Sorsogon	Laguis	Susceptible	74	2	petiole, 2nd leaf	none	12.83773333	124.0330167	103	25
S75a	Sorsogon	Lausigon Red	Resistant	75	1	petiole, 3rd leaf	ants	12.83776667	124.0329833	89	25
S75b	Sorsogon	Lausigon Red	Resistant	75	1	petiole, 3rd leaf	ants	12.83776667	124.0329833	89	25
S76a	Sorsogon	Lausigon Red	Resistant	75	2	upper trunk	none	12.83776667	124.0329833	89	25
S76b	Sorsogon	Lausigon Red	Resistant	75	2	upper trunk	none	12.83776667	124.0329833	89	25
S77a	Sorsogon	Lausigon Red	Resistant	76	1	petiole, 1st leaf	none	12.83785	124.0330333	102	25
S77b	Sorsogon	Lausigon Red	Resistant	76	1	petiole, 1st leaf	none	12.83785	124.0330333	102	25
S78a	Sorsogon	Lausigon Red	Resistant	76	2	petiole, 3rd leaf	none	12.83785	124.0330333	102	25
S78b	Sorsogon	Lausigon Red	Resistant	76	2	petiole, 3rd leaf	none	12.83785	124.0330333	102	25
S79a	Sorsogon	Laylay	Susceptible	77	1	petiole, 1st leaf	none	12.83758333	124.03295	101	25
S79b	Sorsogon	Laylay	Susceptible	77	1	petiole, 1st leaf	none	12.83758333	124.03295	101	25
S80a	Sorsogon	Laylay	Susceptible	77	2	petiole, 3rd leaf	ants	12.83758333	124.03295	101	25
S80b	Sorsogon	Laylay	Susceptible	77	2	petiole, 3rd leaf	ants	12.83758333	124.03295	101	25
S81a	Sorsogon	Laylay	Susceptible	77	3	petiole, 2nd leaf	none	12.83758333	124.03295	101	25
S81b	Sorsogon	Laylay	Susceptible	77	3	petiole, 2nd leaf	none	12.83758333	124.03295	101	25
S82a	Sorsogon	Laylay	Susceptible	77	4	shoot	none	12.83758333	124.03295	101	25
S82b	Sorsogon	Laylay	Susceptible	77	4	shoot	none	12.83758333	124.03295	101	25
S85a	Sorsogon	Laylay	Susceptible	78	1	petiole, 1st leaf	ants	12.83623333	124.0334	100	25
S85b	Sorsogon	Laylay	Susceptible	78	1	petiole, 1st leaf	ants	12.83623333	124.0334	100	25

S86a	Sorsogon	Laylay	Susceptible	79	1	petiole, 1st leaf	none	12.83623333	124.03335	100	26
S86b	Sorsogon	Laylay	Susceptible	79	1	petiole, 1st leaf	none	12.83623333	124.03335	100	25
S89a	Sorsogon	Linawaan	Susceptible	80	1	newly opened leaf	ants	12.83763333	124.03295	97	25
S89b	Sorsogon	Linawaan	Susceptible	80	1	newly opened leaf	ants	12.83763333	124.03295	97	18
S90a	Sorsogon	Linawaan	Susceptible	80	2	upper trunk	none	12.83763333	124.03295	97	25
S90b	Sorsogon	Linawaan	Susceptible	80	2	upper trunk	none	12.83763333	124.03295	97	25
S92a	Sorsogon	Linawaan	Susceptible	81	1	petiole, 1st leaf	ants	12.83771667	124.0330833	98	30
S92b	Sorsogon	Linawaan	Susceptible	81	1	petiole, 1st leaf	ants	12.83771667	124.0330833	98	25
S93a	Sorsogon	Linawaan	Susceptible	81	2	petiole, 2nd leaf	ants	12.83771667	124.0330833	98	25
S93b	Sorsogon	Linawaan	Susceptible	81	2	petiole, 2nd leaf	ants	12.83771667	124.0330833	98	25
S104a	Sorsogon	Maguindanao	Susceptible	82	1	petiole, 2nd leaf	none	12.83668333	124.0339	108	25
S104b	Sorsogon	Maguindanao	Susceptible	82	1	petiole, 2nd leaf	none	12.83668333	124.0339	108	25
S105a	Sorsogon	Maguindanao	Susceptible	82	2	petiole, 1st leaf	none	12.83668333	124.0339	108	25
S105b	Sorsogon	Maguindanao	Susceptible	82	2	petiole, 1st leaf	none	12.83668333	124.0339	108	25
S106a	Sorsogon	Maguindanao	Susceptible	83	1	petiole, 1st leaf	none	12.8364	124.0341333	100	25
S106b	Sorsogon	Maguindanao	Susceptible	83	1	petiole, 1st leaf	none	12.8364	124.0341333	100	25
S107a	Sorsogon	Maguindanao	Susceptible	84	1	petiole, 1st back	ants	12.83663333	124.0338167	107	25
S107b	Sorsogon	Maguindanao	Susceptible	84	1	petiole, 1st back	ants	12.83663333	124.0338167	107	25
S108a	Sorsogon	Maguindanao	Susceptible	84	2	petiole, 3rd leaf	ants	12.83663333	124.0338167	107	25
S108b	Sorsogon	Maguindanao	Susceptible	84	2	petiole, 3rd leaf	ants	12.83663333	124.0338167	107	13
S110a	Sorsogon	Maguindanao	Susceptible	84	3	petiole, 4th leaf	ants	12.83663333	124.0338167	107	25
S110b	Sorsogon	Maguindanao	Susceptible	84	3	petiole, 4th leaf	ants	12.83663333	124.0338167	107	25
S111a	Sorsogon	Maguindanao	Susceptible	84	4	petiole, 5th leaf	ants	12.83663333	124.0338167	107	25
S111b	Sorsogon	Maguindanao	Susceptible	84	4	petiole, 5th leaf	ants	12.83663333	124.0338167	107	25
S114a	Sorsogon	Maguino	Resistant	86	1	mid trunk	none	12.83721667	124.0334333	106	25
S114b	Sorsogon	Maguino	Resistant	86	1	mid trunk	none	12.83721667	124.0334333	106	25

S115a	Sorsogon	Maguino	Resistant	86	2	upper trunk	ants	12.83721667	124.0334333	106	25
S115b	Sorsogon	Maguino	Resistant	86	2	upper trunk	ants	12.83721667	124.0334333	106	25
S119a	Sorsogon	Maguino	Resistant	87	1	petiole, 7th leaf	ants	12.83721667	124.0334667	105	25
S119b	Sorsogon	Maguino	Resistant	87	1	petiole, 7th leaf	ants	12.83721667	124.0334667	105	25
S120a	Sorsogon	Maguino	Resistant	87	2	petiole, 2nd leaf	none	12.83721667	124.0334667	105	25
S120b	Sorsogon	Maguino	Resistant	87	2	petiole, 2nd leaf	none	12.83721667	124.0334667	105	25
S128a	Sorsogon	Maguino	Resistant	88	1	petiole, 2nd leaf	ants	12.8372	124.0334667	102	25
S128b	Sorsogon	Maguino	Resistant	88	1	petiole, 2nd leaf	ants	12.8372	124.0334667	102	25
S129a	Sorsogon	Maguino	Resistant	88	2	petiole, 1st leaf	ants	12.8372	124.0334667	102	25
S129b	Sorsogon	Maguino	Resistant	88	2	petiole, 1st leaf	ants	12.8372	124.0334667	102	25
S135a	Sorsogon	Minenonga	Susceptible	89	1	petiole, 1st leaf	ants	12.83701667	124.0333333	101	25
S135b	Sorsogon	Minenonga	Susceptible	89	1	petiole, 1st leaf	ants	12.83701667	124.0333333	101	25
S138a	Sorsogon	Minenonga	Susceptible	90	1	1st leaf, bottom	ants	12.83701667	124.0333667	100	25
S138b	Sorsogon	Minenonga	Susceptible	90	1	1st leaf, bottom	ants	12.83701667	124.0333667	100	25
S139a	Sorsogon	Minenonga	Susceptible	90	2	1st leaf, middle	ants	12.83701667	124.0333667	100	25
S139b	Sorsogon	Minenonga	Susceptible	90	2	1st leaf, middle	ants	12.83701667	124.0333667	100	25
S140a	Sorsogon	Musa Tex 51	Resistant	91	1	petiole, 1st leaf	ants	12.83668333	124.03325	101	25
S140b	Sorsogon	Musa Tex 51	Resistant	91	1	petiole, 1st leaf	ants	12.83668333	124.03325	101	25
S141a	Sorsogon	Musa Tex 51	Resistant	92	1	petiole, 1st leaf	none	12.83668333	124.0332667	104	25
S141b	Sorsogon	Musa Tex 51	Resistant	92	1	petiole, 1st leaf	none	12.83668333	124.0332667	104	25
S142a	Sorsogon	Musa Tex 51	Resistant	93	1	petiole, 1st leaf	none	12.83666667	124.0331833	94	25
S142b	Sorsogon	Musa Tex 51	Resistant	93	1	petiole, 1st leaf	none	12.83666667	124.0331833	94	25
S146a	Sorsogon	Musa Tex 52	Resistant	94	1	2nd leaf, back	ants	12.8376	124.0332333	97	17
S146b	Sorsogon	Musa Tex 52	Resistant	94	1	2nd leaf, back	ants	12.8376	124.0332333	97	30
S147a	Sorsogon	Musa Tex 52	Resistant	94	2	1st leaf, back	ants	12.8376	124.0332333	97	25
S147b	Sorsogon	Musa Tex 52	Resistant	94	2	1st leaf, back	ants	12.8376	124.0332333	97	25

S154a	Sorsogon	Musa Tex 52	Resistant	95	1	upper trunk	ants	12.83773333	124.0333667	98	25
S154b	Sorsogon	Musa Tex 52	Resistant	95	1	upper trunk	ants	12.83773333	124.0333667	98	25
S155a	Sorsogon	Musa Tex 52	Resistant	95	2	shoot	ants	12.83773333	124.0333667	98	25
S155b	Sorsogon	Musa Tex 52	Resistant	95	2	shoot	ants	12.83773333	124.0333667	98	25
S157a	Sorsogon	Negro	Susceptible	96	1	petiole, 1st leaf	none	12.83773333	124.0329	100	25
S157b	Sorsogon	Negro	Susceptible	96	1	petiole, 1st leaf	none	12.83773333	124.0329	100	25
S158a	Sorsogon	Negro	Susceptible	96	2	petiole, 1st leaf	none	12.83773333	124.0329	100	25
S158b	Sorsogon	Negro	Susceptible	96	2	petiole, 1st leaf	none	12.83773333	124.0329	100	25
S161a	Sorsogon	Negro	Susceptible	97	1	upper trunk	ants	12.83735	124.03305	107	25
S161b	Sorsogon	Negro	Susceptible	97	1	upper trunk	ants	12.83735	124.03305	107	25
S162a	Sorsogon	Negro	Susceptible	97	2	1st leaf	ants	12.83735	124.03305	107	25
S166a	Sorsogon	Putian	Susceptible	98	1	petiole, 2nd leaf	none	12.83758333	124.0330167	106	25
S166b	Sorsogon	Putian	Susceptible	98	1	petiole, 2nd leaf	none	12.83758333	124.0330167	106	25
S168a	Sorsogon	Putian	Susceptible	98	2	3rd leaf	none	12.83758333	124.0330167	106	25
S168b	Sorsogon	Putian	Susceptible	98	2	3rd leaf	none	12.83758333	124.0330167	106	25
S169a	Sorsogon	Putian	Susceptible	99	1	newly opened leaf	ants	12.83751667	124.033	107	25
S169b	Sorsogon	Putian	Susceptible	99	1	newly opened leaf	ants	12.83751667	124.033	107	25
S170a	Sorsogon	Putian	Susceptible	100	1	petiole, 1st leaf	none	12.83756667	124.0331	99	25
S170b	Sorsogon	Putian	Susceptible	100	1	petiole, 1st leaf	none	12.83756667	124.0331	99	25
S177a	Sorsogon	Samoro	Susceptible	101	1	upper trunk	ants	12.8373	124.0332	98	25
S177b	Sorsogon	Samoro	Susceptible	101	1	upper trunk	ants	12.8373	124.0332	98	25
S178a	Sorsogon	Samoro	Susceptible	101	2	mid trunk	ants	12.8373	124.0332	98	25
S178b	Sorsogon	Samoro	Susceptible	101	2	mid trunk	ants	12.8373	124.0332	98	25
S179a	Sorsogon	Samoro	Susceptible	102	1	petiole, 4th leaf	none	12.83733333	124.0332833	99	25
S179b	Sorsogon	Samoro	Susceptible	102	1	petiole, 4th leaf	none	12.83733333	124.0332833	99	25
S180a	Sorsogon	Samoro	Susceptible	102	2	lower trunk	ants	12.83733333	124.0332833	99	25

S180b	Sorsogon	Samoro	Susceptible	102	2	lower trunk	ants	12.83733333	124.0332833	99	25
S182a	Sorsogon	Sogmad	Resistant	103	1	petiole, 1st leaf	ants	12.83773333	124.0333167	98	25
S182b	Sorsogon	Sogmad	Resistant	103	1	petiole, 1st leaf	ants	12.83773333	124.0333167	98	25
S183a	Sorsogon	Sogmad	Resistant	104	1	petiole, 1st leaf	none	12.8377	124.0331667	96	25
S183b	Sorsogon	Sogmad	Resistant	104	1	petiole, 1st leaf	none	12.8377	124.0331667	96	15
S187a	Sorsogon	Tinawagan Pula	Susceptible	105	1	petiole, 1st leaf	ants	12.83728333	124.033	100	25
S187b	Sorsogon	Tinawagan Pula	Susceptible	105	1	petiole, 1st leaf	ants	12.83728333	124.033	100	25
S190a	Sorsogon	Tinawagan Pula	Susceptible	105	2	petiole, 3rd leaf	ants	12.83728333	124.033	100	14
S190b	Sorsogon	Tinawagan Pula	Susceptible	105	2	petiole, 3rd leaf	ants	12.83728333	124.033	100	25
S192a	Sorsogon	Tinawagan Pula	Susceptible	106	1	petiole, 4th leaf	none	12.83723333	124.0329	106	25
S192b	Sorsogon	Tinawagan Pula	Susceptible	106	1	petiole, 4th leaf	none	12.83723333	124.0329	106	25
S193a	Sorsogon	Tinawagan Pula	Susceptible	106	2	3rd leaf, back	none	12.83723333	124.0329	106	25
S193b	Sorsogon	Tinawagan Pula	Susceptible	106	2	4th leaf, back	none	12.83723333	124.0329	106	25
S197a	Sorsogon	Tinawagan Puti	Susceptible	107	1	1st leaf	ants	12.83778333	124.0333167	90	25
S197b	Sorsogon	Tinawagan Puti	Susceptible	107	1	1st leaf	ants	12.83778333	124.0333167	90	25
S199a	Sorsogon	Tinawagan Puti	Susceptible	108	1	petiole, 2nd leaf	ants	12.8378	124.03315	96	25
S199b	Sorsogon	Tinawagan Puti	Susceptible	108	1	petiole, 2nd leaf	ants	12.8378	124.03315	96	25
S206a	Sorsogon	Tinawagan Puti	Susceptible	109	1	back of the leaf, right	none	12.83658333	124.0332667	99	25
S206b	Sorsogon	Tinawagan Puti	Susceptible	109	1	back of the leaf, right	none	12.83658333	124.0332667	99	16
S207a	Sorsogon	Tinawagan Puti	Susceptible	109	2	back of the leaf, left	none	12.83658333	124.0332667	99	25
S207b	Sorsogon	Tinawagan Puti	Susceptible	109	2	back of the leaf, left	none	12.83658333	124.0332667	99	25