THE ORIGIN AND SPREAD OF THE WEST INDIAN DRYWOOD TERMITE *Cryptotermes brevis* (WALKER) IN THE AZORES USING GENETIC MARKERS, AND TESTING OF COLONY FOUNDATION PREVENTATIVE MEASURES TO CONTROL ITS FURTHER SPREAD.

By

MARIA TERESA MONTEIRO DA ROCHA BRAVO FERREIRA

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2011

To my Dad, my Mom, and my brother who have always supported me

ACKNOWLEDGMENTS

I acknowledge and thank Dr. Rudolf Scheffrahn for guiding me on this scientific road, offering an environment that provided cultivation of knowledge. I also acknowledge the Portuguese Foundation for Science (SFRH/BD/29840/2006), the Azorean DRCT (Project M221-I-002-2009 TERMODISP), and the Structural School of Fumigation for providing me with the funds that allowed me to pursue this degree. I would like to thank all the teachers and scientists at the FLREC, namely Dr. Robin Giblin-Davis, Dr. Nan-Yao Su, Dr. William Kern, Dr. Hartwig Hochmair and Dr. Nigel Harrison for helping me increase my knowledge and aiding with any questions that I had. I acknowledge Dr. Paulo Borges for initiating my path into the world of termites, and for supporting me unconditionally throughout this whole process.

I would like to acknowledge my roommates, who provided a relaxing and fun environment at home, but also an intellectually stimulating environment. I would like to thank Dr. Seemanti Chakrabarti and Ericka Helmick M.Sc. for all the help with the genetics laboratory as well as interpretation of results, and for their support, and friendship. I would like to acknowledge Dr. Thomas Chouvenc, and Dr., Hou-Feng Li for their friendship and inspiration. And finally, I would like to thank my mother and my brother for being a huge support in my life even from far away, and to all my friends back home who supported me anyway that they could from a distance. To all the people that one way or another have contributed to this work, I give a most sincere thank you.

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LIST OF ABBREVIATIONS

16S rRNA	16S ribosomal RNA mitochondrial gene.
BLAST	Basic Local Alignment Search Tool.
Cytb	Cytochrome b mitochondrial gene.
DNA	Deoxyribonucleic acid.
mtDNA	Mitochondrial DNA.
PCR	Polymerase Chain reaction.
RNA	Ribonucleic acid.
UPGMA	Unweighted Pair Group Method with Arithmetic Mean

Abstract of Dissertation Presented to the Graduate School
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DETERMINING THE ORIGIN, AND SPREAD OF THE WEST INDIAN DRYWOOD TERMITE *Cryptotermes brevis* (WALKER) IN THE AZORES USING GENETIC MARKERS. AND TESTING COLONY FOUNDATION PREVENTATIVE MEASURES TO CONTROL FURTHER SPREAD OF THE SPECIES.

By

Maria Teresa Monteiro da Rocha Bravo Ferreira

December 2011

Chair: Rudolf H. Scheffrahn
Major: Entomology and Nematology

Cryptotermes brevis (Walker) is a drywood termite of the Kalotermitidae family (Isoptera). The West Indian drywood termite, *C. brevis*, is a very destructive wood pest that causes high levels of damage to wood and wood structures throughout the world in tropical and subtropical areas. This species was first identified in the Azorean archipelago in 2000, and has since then become the number one urban pest in that archipelago. The Azores is a small group of nine islands located in the Atlantic Ocean, about half way between Europe and the United States.

In order to identify the origin and spread of *C. brevis* in the Azores, genetic markers were used to elucidate if the introduction of the species to the islands was a consequence of a single introduction or multiple introductions. Mitochondrial DNA and microsatelites were used to statistically model the relationships between the populations in the Azores. Nine different locations were chosen to collect termites in four of the islands. The termite DNA was extracted, and 16SrRNA and Cytb mitochondrial genes were used to assess phylogenetic relationships between the populations. Furthermore, five loci for microsatellite DNA were used as well. The data showed that there were

multiple introductions of the species and that they still appear to be occurring, seeing that the genetic diversity is high. This is important information to have in order to advise stricter regulations for the exchange of wood products into and between the islands. To determine the origin of these introductions, samples from 11 different countries were used. The same genetic markers were used to show that the two possible places of origin might be the endemic region of Chile and Peru, and Venezuela.

Lastly, colony preventative treatments were studied in order to optimize control of the species in the Azores and possibly decrease the spread of the infestation. Different light wavelengths were studied to determine if there was a preference for a determined wavelength, and it was shown that lights in the white, blue, and green spectrum are preferred to red or no light. Also chemicals were tested against colony foundation and it was found that the two most common wood treatments used in the Azores, permethrin and cypermethrin, were effective at preventing infestations. This knowledge can now be applied to help reduce the spread of the infestation in the locations where it is already present.

CHAPTER 1
INTRODUCTION

Termites are eusocial insects that comprise an order of their own, the Isoptera. These insects were first described to the Royal Society of London in 1781 by Henry Smeathman (Howse 1970). Their name comes from the characteristic of the reproductive adults having two pairs of wings of almost equal size (iso = equal, ptera = wings). There are over 2,600 described species of termites (Kambhampati and Eggleton 2000) which have been traditionally distributed over seven families. More recently, termites have been classified into nine families with seven extant, two fossil families and one with uncertain status (Engel et al. 2009). These families are the Mastotermitidae, Termopsidae, Hodotermitidae, Archotermopsidae, Stolotermitidae, Kalotermitidae, Stylotermitidae, Rhinotermitidae, Serritermitidae, and Termitidae (Table 1-1 for details on ecology and common names of each family). Termites follow the species richness latitudinal gradient pattern, with a higher number of species in tropical areas, and a lower number towards the temperate areas (Eggleton 2000).

The isopterans are closely related to the cockroach order (Blattodea) (Inward et al. 2007). The termites are believed to have evolved from an extinct primitive type of wood feeding cockroach ancestor. It has been acknowledged that termites share several characteristics with the wood roaches of the Cryptocercidae family within the genus *Cryptocercus*. Some termites share several groups of symbiotic flagellates, housed in their hindgut, with the wood roaches. Another characteristic that shows similarity is that wood roaches have a semi-social behavior, displaying some brood care. Other cockroach hatchlings are independent at birth but woodroaches must acquire the intestinal symbionts by proctodeal feeding from their parents (Cleveland et al. 1934).

The similarity between termites and cockroaches has been phylogenetically analyzed and proven with studies on the cocladogenesis between termites, cockroaches, and their symbionts (Lo et al. 2003), and it has even been proposed that the order Isoptera be sunk into a family within the order Blattodea (Inward et al. 2007).

Table 1-1. Food and nesting habits of the extant families and subfamilies of termites.

Family	Subfamily	Food	Nesting	Common names
Mastotermitidae		wood	In tree trunks	Giant Northern termite of Australia
Hodotermitidae		grass	In soil	Harvester termites
Termopsidae		wet wood	In damp wood	Dampwood termites
Kalotermitidae		dry wood	In dry wood	Drywood termites
Rhinotermitidae		wood	In soil	Subterranean termites
Serritermitidae		nests	In decayed wood and other termite nests	
Termitidae	Apicotermitinae	soil, dung, grass	Few build nests	Soldierless termites
	Macrotermitinae	fungus	Epigeal	Fungus growing termites
	Nasutitermitinae	wood, dung, soil	Arboreal, epigeal, or subterranean	Nasutes
	Termitinae	wood, grass, dung	Arboreal, epigeal, or subterranean	Snapping or piercing termites
	Syntermitinae	soil, dung	Epigeal and other termite nests	

Termites are truly social insects. They possess the three main characteristics to being eusocial: there is cooperative brood care in their colonies, they are divided into different castes which perform different tasks within the colony, and they have overlapping generations. In termites, the larvae not only must acquire symbionts from their nestmates but must also be fed for the first couple of instars because they cannot feed directly on wood themselves (Noirot and Noirot-Timothée 1969).

Termites are often separated into two groups, “higher termites” and “lower termites”. In the “higher termites” (Termitidae), which makes up 75% of all termite species, only bacteria are present in the gut. In the “lower termites” protozoan symbionts can be found in the gut in addition to bacteria (Krishna 1969). These symbionts help with the digestion of cellulose. The lower termites are generally more primitive, having simple galleries but not well formed nests (with the exception of some Rhinotermitidae which have mounds for nests). They have colonies without true workers, and generally eat only wood. Lower termites usually occur in more temperate latitudes than higher termites. Higher termites (Termitidae) are much more diverse ecologically. While many still consume wood, others have evolved different diets of herbage, grass, dung, humus, fungus, lichens, or organic material in soil. The higher termites rely either on internal digestion with gut bacteria or external digestion in fungus combs (Edwards and Mill 1986). The higher termites often build large nests or mounds, and are common in tropical areas, but are rare or absent in temperate areas.

Termites can also be separated into groups according to their habitat types. They can be earth-dwelling termites and wood-dwelling termites. There are several types of earth-dwelling termites, such as the subterranean termites and mound building termites.

The wood-dwelling termites on the other hand, confine themselves to wood. Wood-dwelling termites can be classified as, either drywood termites, attacking dry, sound wood, or dampwood termites attacking damp usually decaying wood (Kofoid 1934).

Termite colonies are comprised of individuals that are separated into three main castes which are differentiated morphologically and behaviorally. These castes are: 1) reproductives (king, queen, and unmated winged forms called alates); 2) soldiers; and 3) workers (higher termites) and false workers or pseudergates (lower termites) (Snyder 1926, Krishna 1969) (Figure 1-1).

Alates are usually darker in color with a fully developed, pigmented chitinous exoskeleton, and with well-developed compound eyes (Light 1934a). Alates have deciduous wings that are broken off along a basal suture after a dispersal flight. After losing their wings (becoming dealates), the dealates become the primary reproductives of a colony. A colony begins with a single pair of dealates, a male and a female that cohabitate with copulation occurring at different intervals throughout their lives (Krishna1969). Besides the primary reproductives, a colony of termites may produce secondary reproductives that develop from nymphs with external signs of wing buds (brachypterous), and tertiary reproductives with no signs of wing buds (apterous) of both sexes (Snyder 1926).

Soldiers have modified heads and mandibles to protect the colony. There are several mechanisms of defense by soldiers. They can have phragmotic heads which serve to plug entry holes to the colony, they can use mandibular biting in which they will use their strong or sharp mandibles to bite enemies, and they can have mandibular snapping in which there is an elastic distortion of the mandibles that when released,

snap, striking a hard blow to the approaching enemy. Soldiers also have chemical defenses with glandular structures in their heads that exude chemical compounds. Glands can be located in the front of the head (frontal glands) which are found in Rhinotermitidae and Termitidae, in the mouth area, or the abdomen, salivary glands and cibarial glands. The chemicals exuded can have different functions like keeping a wound from healing (lipids), causing irritation (irritants), causing toxicity (contact poisons), and acting like an entangling agent (glues) (Prestwich 1984). Some species of termites however do not have soldiers. These termitids (subfamily Apicotermitinae) have lost their soldiers and developed other forms of defense, like autothysis, which is the rupturing of the abdomen when in contact with a predator to exude their sticky gut contents (Sands 1982).

The remaining castes of workers, pseudergates, and immature reproductives are usually light in color, with no specialized heads or mandibles. The immature reproductives (brachypterous nymphs) may have wing buds of different sizes. The pseudergates and the workers carry out all the work in the colony. The immature reproductives (nymphs) may also help with this (Edwards and Mill 1986). Work in the colony consists of taking care of the eggs and larvae and moving them when the nest is disrupted, foraging for food, feeding the larvae and the soldiers, building tunnels and nest structures, and excavating wood and soil.

Termites are ecologically important (Bignell and Eggleton 2000), being a key decomposer in numerous ecosystems, with their role in improving soil quality increasingly emerging (Holt and Lepage 2000). Termites, however, can be important pests to crop and timber. Of all the species of termites described (>2,600) only 183 are

known to cause damage to buildings with 83 species causing severe damage (Edwards and Mill 1986). The pest status of termites is recognized worldwide, and in the United States alone, the cost for termite control has been estimated to be between $1.02 and 1.5 billion (Edwards and Mill 1986, Su 1994). As for Europe, in France alone the estimated cost for termite treatment is about 200 million euros per year (Bagnéres et al. personal communication). Drywood termites are responsible for about 20% of the budget spent on termite control in the United States (Su and Scheffrahn 1990).

The West Indian drywood termite (Figure 1-1), *Cryptotermes brevis* (Walker) is a drywood termite of the family Kalotermitidae. It was first described in Jamaica (Walker 1853) and, with the exception of Asia, has a tropicopolitan distribution (Scheffrahn et al. 2008) (Figure 1-2). Although drywood termites are a widespread pest, they have certain temperature and moisture requirements (Edwards and Mill 1986). *Cryptotermes brevis* infests buildings and furniture, being mainly reported in the tropical and subtropical areas with some isolated occurrences in warmer temperate regions (Light 1934b; Edwards and Mill 1986). It is endemic to Chile and Peru where it occurs in nature, away from structural wood (Scheffrahn et al. 2008).

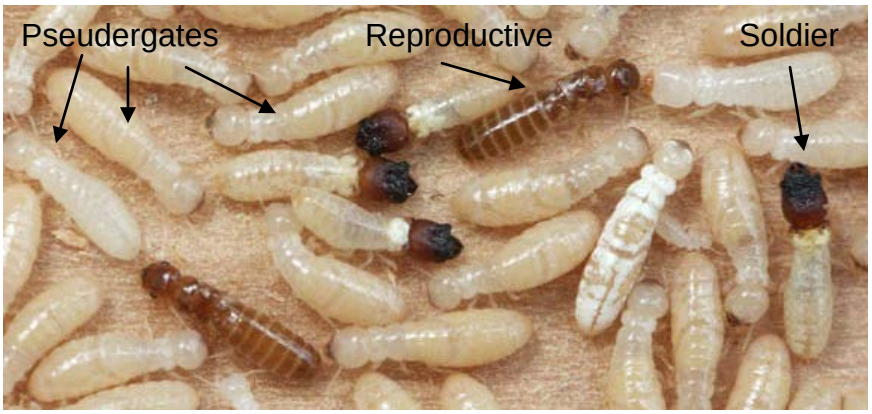

Figure 1-1. Picture of *Cryptotermes brevis* showing soldiers, reproductives and pseudergates (adapted from Scheffrahn).

Cryptotermes brevis was found infesting structures in Portugal by Mateus and Goes (1953) in the Atlantic Island of Madeira. Later it was reported in Mozambique (Carvalho 1972), which was then a Portuguese colony. For decades this species had not been reported anywhere else on Portuguese territory until it was identified as the cause of structural damage in the Azorean Island of Terceira in the year 2000 (Borges et al. 2004, Myles 2004). Since then, it has been further reported in the Islands of Faial, São Miguel, Santa Maria (Borges 2007), São Jorge (personal observation), and Pico Island (O. Guerreiro personal observation) all part of the Azorean archipelago (Figure 1-3). The Azores is an archipelago of nine main islands located in the Atlantic Ocean about 1500 km from Lisbon, Portugal, and 3900 km from the East Coast of the United States of America. The archipelago was discovered in 1427 by the Portuguese and the first settlements on this uninhabited archipelago started in 1439. Due to its strategic position, the Azores has been a stopping point for ships, yachts, and airplanes which make it vulnerable to invasive pests. The Azores is at temperate latitude but due to sea currents, enjoys a subtropical climate without extremes of either very hot or freezing temperatures. Because of the surrounding ocean the humidity is always high and therefore the climate is extremely favorable for termites, either of temperate or tropical origin. There are three more species of introduced termites know in the Azores that have been recently identified. One of these species is another Kalotermitidae that is common in the Mediterranean area of Europe, *Kalotermes flavicollis* (Fabr.). A dampwood termite, it has been found in living trees in three of the islands in the Azores (Terceira, São Miguel, and Faial), with few occurrences in wood structures (Myles et al. 2007a). The two other species that have been identified in the Azores are both

rhinotermitids of the genus *Reticulitermes*. One species has been reported in the island of Faial, where it was found infesting a coastal neighborhood in 2006 and it was identified as *R. grassei* Clément a common termite in southern Europe, and was probably brought into the island from there. The second is *R. flavipes* (Kollar) which was identified in an American Military air base in Terceira island about 20 years ago (Myles et al. 2007a), and more recently in the area surrounding the air base (personal observation). *Reticulitermes flavipes* is a common pest in the United States, and there was much traffic of goods from the U.S. into the Military base. Because of the location of the infestation it is believed that these termites may have been introduced through military shipment at the Lages Air base. The main urban pest at this moment in the Azores is *C. brevis*. The level of infestation in the city of Angra do Heroísmo on the island of Terceira is very high, with about 43% of the buildings in the historical center infested, and of these, about 50% show high levels of infestation (Borges et al. 2004, Guerreiro et al. 2009). The origin of the Azorean population of this species is unknown, but was presumed to be either from the importation of infested wood, or dispersal flights from infested boats in Azorean harbors, or both (Scheffrahn personal observation).

Cryptotermes brevis is the most destructive drywood termite pest in the world. Because of its ability to withstand wood with low moisture content it is able to attack all kinds of wood in service including structural timbers, beams, studs, flooring, molding, doors, window frames and even wooden articles such as carvings, tools, picture frames, musical instruments, etc. Like other drywood termites, *C. brevis* is a cryptic species which nests in its food source, wood, spending almost all of its life cycle within the confines of wood. A colony of drywood termites can vary in number from hundreds to a

few thousand termites (Nutting 1970) and several colonies can be found inside a single piece of wood. A *C. brevis* colony is estimated to have anywhere from 2 individuals (incipient colony) to 296 individuals with an average of 45 individuals (Myles et al 2007a). The early development of a kalotermitid colony is slow and numbers are small in the first year (Nutting1969). For *C. brevis,* the number of eggs in an incipient colony is very low with an average of 4 eggs laid per pair of female and male dealates in the first month (Ferreira 2008). In the first year there will be an average of 3.4 nymphs and no soldiers (McMahan, 1960). These low numbers indicate that many colonies of *C. brevis* can co-habit a single piece of wood. Williams (1977) suggests that a colony of *C. brevis* will gradually move from highly-infested timber into timber that is less than 30% damaged if it can do so, although it maintains activity in more damaged timbers. A majority of reports describe aggressive responses in non-kalotermitids when encounters between colonies of the same species occur. Violent agonistic responses, such as attack and dismemberment of intruders by workers have been reported for several termite species (Thorne and Haverty 1991). However, colony fusion has been observed for termite species like *Reticulitermes speratus* (Kolbe) where members of a host colony accept a lower-nymph ratio colony not showing agonistic behavior towards it (Matsuura and Nishida 2001). This is an important factor for invasive social species, because reduced genetic variability after an introduction can lower the intercolonial agonism making possible for a larger unicolonial society to become dominant (Tsutsui and Suarez 2003).

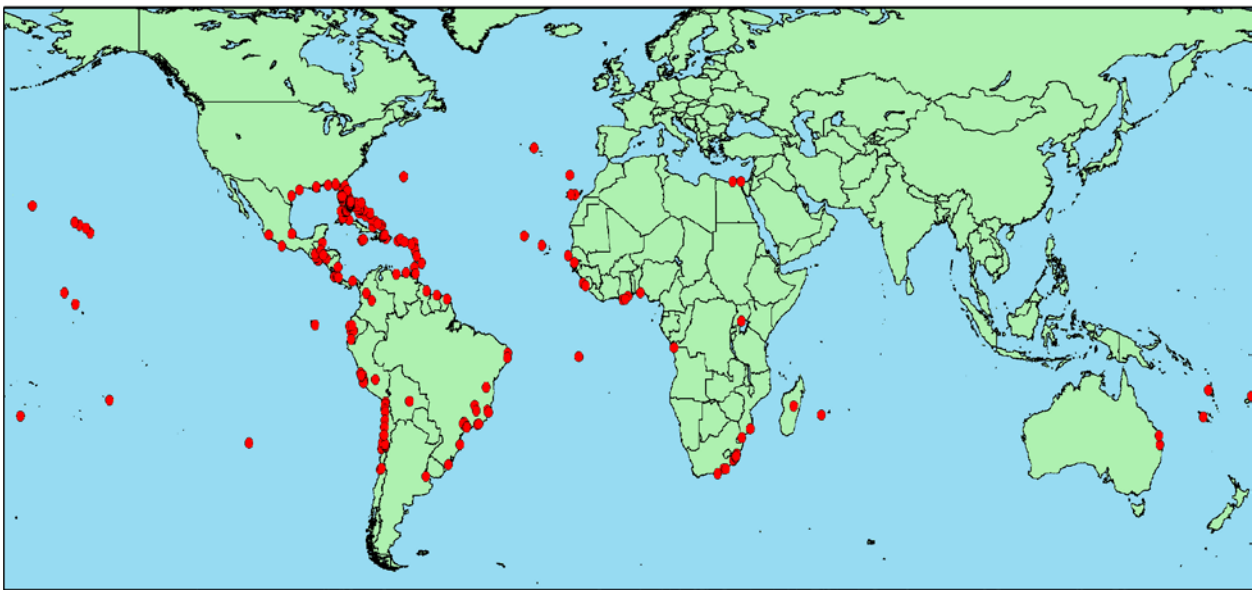

Figure 1-2. Map of the distribution of C. brevis in the world. (Adapted from Scheffrahn et al. 2008).

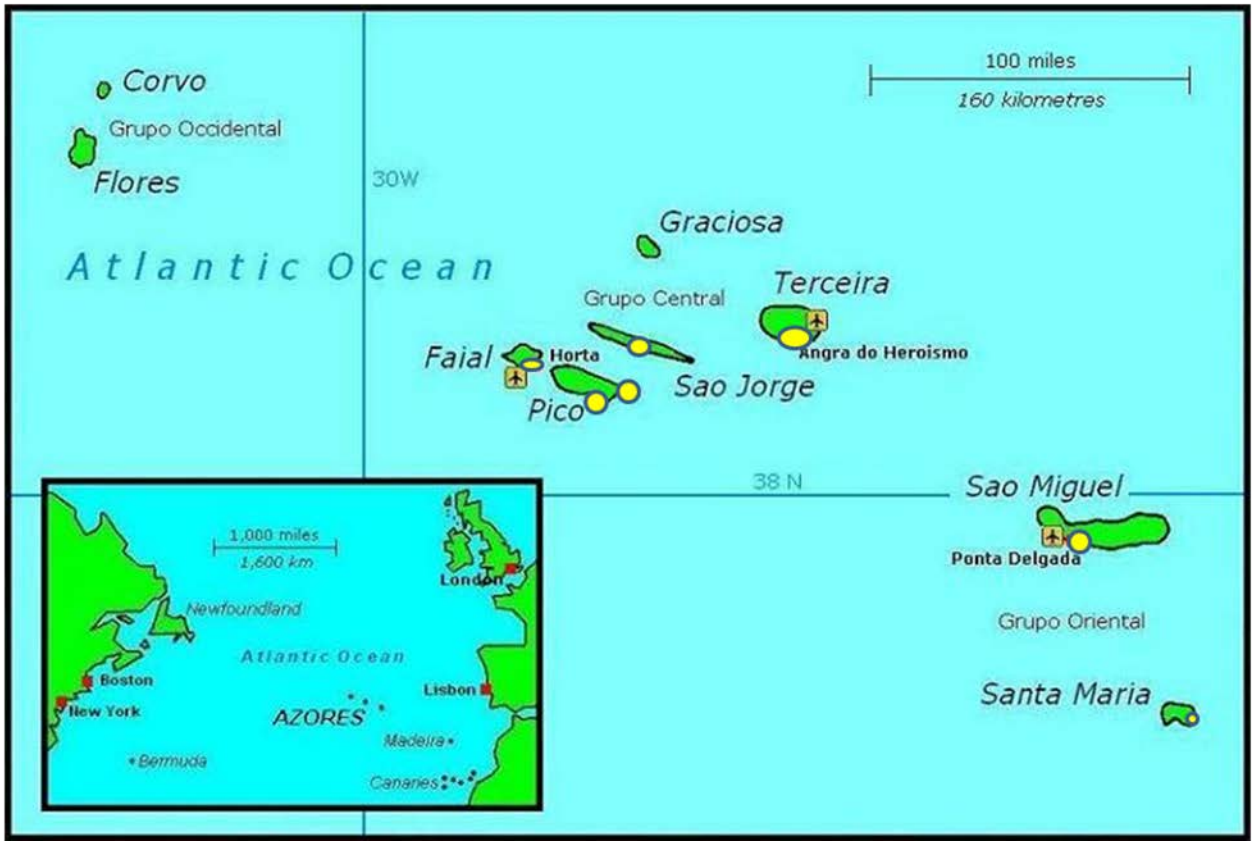

Figure 1-3. Confirmed locations of *C. brevis* infestations in the Azorean Archipelago. Yellow dots mark the locations of the known infestation sites.

The life cycle of *C. brevis* begins with a dispersal flight where the alates leave their previous gallery system in order to form new colonies. The dispersal flights of termites occur at different times of the year depending on the species. For *C. brevis* the main flight season in South Florida occurs between April and July, with a secondary smaller flight season in November (Minnick 1973). In the Azores the flight season occurs between May and September (Borges et al. 2004). The dispersal flights are the only occasion that this species is found outside of wood (Kofoid 1934). Otherwise, it never leaves the nest to exploit new food sources (Korb and Katrantzis 2004) although they will bridge small gaps between wood members. The new colony does not produce alates for about 5 years, at which point the colony is considered mature. The size of a colony and its maturity is important in determining the number of alates to be produced by a colony. It is known that a colony of *C. brevis* can survive with as few as four pseudergates and that this species can produce more neotenics than other *Cryptotermes* species (Williams and Morales 1980). The percentage of alates that leave the colony for some species has been determined, *e.g.*: *Pterotermes occidentis* (Walker), 30%; *Paraneotermes simplicicornis* (Banks), 14-16%; *Zootermopsis laticeps* (Banks), 39%; *Stolotermes victoriensis* Hill, 17.5%; and *Nasutitermes exitiosus* (Hill), 2.4% (Nutting 1969). Myles et al. (2007a) estimated that approximately 25% of *C. brevis* colony matures into alates and leave the colony, based on alates and alate nymphs present in active colonies inside wood.

One of the main methods that have been used for controlling this pest has been fumigation (Bess and Ota 1960). Sulfuryl fluoride eliminates existing colonies very effectively with no termites detected in a structure up to two years after treatment

(Minnick et al. 1972). However in places like the Azores, structural fumigation is not an option for remedial treatment of *C. brevis* due to logistical and European safety rules where there is a 10 m exclusion zone. In addition, fumigation does not prevent new infestations from occurring, and a residual termiticide should be applied for preventing new colony formation.

Detection is a very important aspect of the local control of drywood termites. Due to their cryptic nature however, detecting exact locations for drywood termite colonies is difficult. The most used type of detection is by visual inspection (Scheffrahn et al. 1993). This involves either finding alates inside structures during the dispersal flight season, which provides evidence that there is an infestation somewhere in the structure, finding frass near wood objects or structural wood, or tapping the wood surface and listening for hollow areas, or finding thin surfaces over galleries and kick-out holes. These methods are only reliable to identify an infestation when the colony is already well established. For the first years of the colony, there are few fecal pellets and this allows it to grow and consume the wood without being detected. Some of the other methods used for detection include the use of dogs to detect the scent of termites (Lewis et al. 1997). Dogs can be 97.3% accurate in detecting drywood termites in structures (Brooks et al. 2003). Studies performed on acoustic emissions detectors (AED) demonstrated that this is a reliable non-destructive method for detecting drywood termites (Fuji et al. 1990, Scheffrahn et al. 1993, Lemaster et al. 1997, and Lewis et al. 2004). An AED can detect, depending on the position, as far as 8 to 80 cm inside the wood (Scheffrahn et al. 1993). This method is effective since wood structures are composed of wood that is not very thick. Some other detection methods involve

infrared, laser, x-ray, and microwave technologies (Evans 2002, Grossman 2006, Lewis et al. 2005, Mankin 2004). These detection methods are important for remedial control in order to assess the extent of the existing infestation as well as the best methodology to apply. They are also important when using spot treatments, to help guide where the treatment should be applied.

For remedial control of termites, there are several types of treatments which can be categorized into whole structure treatments, compartmental, or localized treatments. Another method used for control for whole structure (besides fumigation) is heat treatment. Heating of structures has been shown to produce 96% mortality (Lewis and Haverty 1996). Field studies have shown that reaching a mean core temperature in the wood of a targeted 49ºC can take as little as 5h (Woodrow and Grace 1998a). Commercial heat operators use exposures of 54.4ºC for an hour as a standard for effective control (Quarles 2006).

The localized treatments used against drywood termites can be of two kinds, either chemical or non-chemical. In the chemical treatments, there are several insecticides that are currently used. The most common method for applying localized chemicals is by drill and injection. In this method, the pest control operator drills holes into the infested wood, and upon finding a gallery will inject the chosen chemical into the termite's galleries (Hickin 1971), or inject into "kickout" holes. Some of the presently used chemicals in the United States are disodium octoborate tetrahydrate (DOT), imidacloprid, and fipronil (liquid, foam, and dry formulation), and d-limonene. All of these insecticides can produce 100% mortality on contact (Scheffrahn et al. 1997a, Myles et al. 2007b). In Europe, other insecticides used include cypermethrin, permethrin, and

borates. A chemical being used currently in drywood termite control in California is d-limonene, an essential oil extract from citrus. However, this active ingredient (AI) has shown a high repellency to termites (Myles et al. 2007b), which is a non-desirable characteristic if termites are to be exposed to it. Another chemical being currently investigated against termites is chlorantraniliprole. This chemical is non-repellent to subterranean termites (Yeoh and Lee 2007) as well as having a 90% mortality rate when *R. hesperus* were exposed to it for an hour (Rust 2008, personal communication). This chemical has shown high toxicity to *R. flavipes* when applied topically (Spomer et al. 2009). Another class of chemicals that has been tested against termites is juvenile hormone analogues like hydroprene. When tested against subterranean termites, hydroprene showed a low mortality rate (50%) after 14 days, but a high production of soldiers which is a non-reproductive caste. This might affect the long term survival of a colony (Hrdý et al. 2006). Chitin synthesis inhibitors are also used against termites. Hexaflumuron was tested against *R. flavipes* and *Coptotermes formosanus* Shiraki, demonstrating non-deterrence and a 90% mortality at concentrations of 15.6-62.5 ppm, and also non-deterrent making it a good choice for baiting subterranean termites (Su and Scheffrahn 1998).

Non-chemical localized treatments against drywood termites include electrocution, microwaves, heat, cold, carbon dioxide and entomopathogenic fungi. Electrical shock treatment has been shown to produce almost 100% mortality in laboratory tests (Creffield et al. 1997) 4 weeks after the electrical shock treatment (Lewis and Haverty 1996). However it is not considered to be an effective method for infested boards in houses, because it causes scorching and the number of holes drilled into wood can be

up to 70% of a board (Lewis and Haverty 2001). Laboratory studies with microwave ovens revealed a mortality of over 84% (Lewis et al. 2000) although there is great variability in mortality across different studies (Lewis and Haverty 1996). For heat treatments, Scheffrahn et al. (1997b) showed that *C. brevis* has high mortality when exposed to temperatures above 50ºC for at least 15 min. Heat may be applied directly in infestation sites to increase the temperature of localized spots preventing an increase of temperature more than necessary and decreasing the danger of damage to structures by heat (Woodrow and Grace 1998b). The use of liquid nitrogen as a cold treatment which is injected inside wall voids in structures can produce 100% mortality (Lewis and Haverty 1996). However, close monitoring must be done because insulation materials such as fiberglass may deflect the nitrogen and prevent termites from being exposed to the freezing temperatures (Rust et al. 1997). The exposure to carbon dioxide in chambers has shown a high mortality for drywood termites (Borges et al. 2007). Finally, the use of entomopathogenic fungi as a non-chemical treatment against termites has shown high mortality, above 70%, when termites were exposed to two strains of fungi *Metarhizium anisopliae* and *Verticillium indicum* (Nars and Moein 1997) in the laboratory. Although the contact mortality of termites is high, the indirect application of fungi to termites is not as effective. Untreated termites placed in arenas with treated termites did not produce an increase in mortality (Chouvenc et al. 2008).

Preventative treatments may be used to reduce or prevent alates from flying into a structure and forming a new colony. Prevention of colonization can be of two types, either physical or chemical. Physical prevention can be of various types, from traps for alates to resistant materials used in construction, mosquito screening, caulking, and

other exclusion treatments. These resistant materials may or may not be wood. Some types of wood are more resistant to termite attack than others and are not heavily consumed by drywood termites (Wolcott 1924, Williams 1934, Minnick et al. 1973, Gonçalves and Oliveira 2006, Ferreira et al. 2007).

Alates are attracted to light when flying (positive phototaxis) and the more intense light attracts a higher number of alates (Ferreira and Scheffrahn 2011). Light traps can be optimized by using high intensity lights to capture alates before they enter wood. Insects can be attracted to different wavelengths of light and using a preferred wavelength can bring a higher efficacy to a light trap. Blacklight lamps have been effectively used for attraction of insects for many years (Nabli et al. 1999). Minnick (1973) reported differences in wavelength of light preference by *C. brevis*. He compared sunlight with U.V. light and incandescent light, and found that sunlight was most preferred followed by U.V. light, and incandescent light. However no work on exact wavelength preference has been reported until now.

Insecticides have long been used to prevent infestations by termites. Wood pressure-treated with preservatives increased the durability against termites (Randall and Doody 1934), and also preservatives can deter drywood termite infestations (Hunt 1959). Inert dust materials that have been sprayed on wood as preventatives have also had some positive results (Wagner and Ebeling 1959). As colony foundation preventatives, insecticides like disodium octoborate tetrahydrate and chromium copper arsenate when used as a surface treatment, are an unpreferred spot for colony establishment (Scheffrahn et al. 1998). Other chemicals that have been tested as colony prevention treatments were imidacloprid, silica gel, and fipronil (Scheffrahn et al

2006). These were shown to be effective and their use is recommended in wood structures, either in construction or applied to the wood surfaces that are exposed to infestation (Scheffrahn et al 2001). However these insecticides are not commercially available in Europe, and for insecticides like cypermethrin and permethrin which are available, no tests against drywood termite colonization have been performed.

Objectives

Resolving the question of the origin of *C. brevis* in the archipelago, whether there was a single or multiple introductions, can help make the case for new and stricter laws for handling import of wood, wood materials, and maritime boat regulations. Also by comparing the Azorean populations with populations from other countries and the putative countries of origin, this study can help resolve the question about the geographic origin of this species in the Azores.

In order to apply an effective method of reducing dispersal during the flight season of *C. brevis,* it is important to understand what happens during these flights. Finding the percentage of the colony that actually leaves during dispersal flights can help understand how effective a method of control for the alates can be. If a very large percentage of the colony becomes alates and leaves it, then improving control methods can help reduce the numbers of termites infesting wood. Improvement of some control methods, like the use of different chemicals as preventative treatments and the use of light traps can be viable alternatives for use in Europe where chemical usage is very restricted.

The general aim of this dissertation is to study the origin, spread, and control of the West Indian drywood termite *C. brevis* with application to the Azores Islands by testing the following hypotheses:

Hypothesis 1. The introduction of *C. brevis* to the Azorean archipelago is widely spread, with six of the nine main islands being infested. This suggests that there might have been multiple introductions of the species to the Archipelago. In order to test this hypothesis a genetic study will be performed comparing the different subpopulations of the islands.

Hypothesis 2. The origin of the introduction of *C. brevis* to the Azorean archipelago is unknown, and due to the position of the archipelago the introduction of this species might have come from various sources, including the American Continent (North, Central, and South America), Africa, or even as far as Oceania. In order to test this hypothesis a genetic study will be performed comparing the island subpopulations to subpopulations from various countries.

Hypothesis 3. Dispersal flights are the only time *C. brevis* is found outside of wood, with part of the colony leaving the wood as alates, and the remainder staying behind to continue the colony. After leaving the colony, the alates will fly in search of new places to colonize. During this time preventative treatments are important in reducing the risk of infestation. The hypothesis being tested is that some light wavelengths are more attractive to alates than others for use in light traps. And also insecticides can be used to prevent alates from establishing a new colony in wood.

CHAPTER 2
POPULATION DIVERSITY OF *Cryptotermes brevis* IN THE AZORES: SINGLE VS MULTIPLE INTRODUCTIONS

The use of genetic markers in population analysis is becoming an established method for inferring relationships amongst different populations, ancestry, and relatedness. Mendel's work showed an individual organism possesses both a heritable form of a gene (genotype), as well as individual variants (alleles). Population genetics is the study of the occurrence of alleles within and between populations. In population genetics, a population is considered a group of organisms of the same species living within a sufficiently restricted geographical area so that any member can potentially mate with any other member of the opposite sex (Hartl and Clark 1997).

There are techniques used to detect genetic variability, each with some advantages and disadvantages. The study of mitochondrial DNA is widely used and it provides sufficient variation for studies at the individual, population, and species level, depending on the region of the mtDNA that is analyzed. The reasons for the adoption of mtDNA as a marker are that experimentally, mtDNA is relatively easy to amplify because it appears in multiple copies in the cell. Mitochondrial genes are maternally inherited, and are strongly conserved across animals. Mitochondrial DNA is highly variable in natural populations because of its elevated mutation rate, which can generate conclusive data about population history over short time frames. Variable regions (e.g. the control region) are typically flanked by highly conserved ones (e.g. ribosomal DNA), in which PCR primers can be designed (Galtier et al. 2009). However, when looking at the population level, one genetic marker is usually not enough. The variability may not be sufficient to show differences at a population level. And so, mitochondrial DNA studies of populations often involve the use of another genetic

marker, microsatellites that have a high mutation rate and can show variability within a species.

Microsatellites are short segments of DNA that have repetitive sequences and are usually non-coding (Hamada et al. 1984). Over time, as animals in a subpopulation breed, they recombine their microsatellites during sexual reproduction and the subpopulation will maintain a variety of microsatellites that is characteristic for that subpopulation and distinct from other subpopulations which do not interbreed. For this reason the study of microsatellites is useful in subpopulation studies, and in the case of termites even at a colony level, to help understand the interactions between different colonies and possible interbreeding between closely located colonies (Husseneder et al. 2006).

The Azorean Islands are a small archipelago in the Atlantic Ocean, where most towns have harbors with extensive marine traffic. It is therefore highly susceptible to invasive species introduction from organisms such as *C. brevis*. Presumed to be recently introduced to the islands, the question to whether there was a single introduction or multiple introductions of this species is still unresolved.

For this study the genetic markers chosen to solve this question were two mtDNA markers, 16S rRNA and Cytb, and also microsatellites. Mitochondrial 16S rRNA was chosen because it is has clearly resolved many questions in termite phylogeny studies (Kambhampati et al. 1996, Legendre et al. 2008). Cytb gene has yielded good results in Kalotermitid phylogenetic studies (Thompson et al. 2000), especially with the genus *Cryptotermes* (Legendre et al. 2008). The microsatellite loci used were amplified by primers developed for a congeneric species *C. secundus* (Hill) (Fuchs et al. 2003). The objective of this study was to use these markers to determine if the infestation in the

Azorean islands was caused by a single introduction, or if there were several introductions. Furthermore, in case there were multiple introductions, to determine if these are still occurring and if there is any inter-island spread of the species.

Material and Methods

Termites

Termites were collected during the month of August, 2009 in four of the nine Islands of the Azorean archipelago. The islands where the termites were collected were Terceira Island, in the city of Angra do Heroísmo, São Miguel Island, in the city of Ponta Delgada, on the Island of Faial, in the city of Horta, and on the Island of Santa Maria, in the village of Maia (Figure 2-1). All termites were collected from infested structures. To collect the termites, access to private houses was received, and alates were gathered from spider webs or homeowner's light traps. In a few locations, infested wood was recovered from private houses and was later chopped open to collect termites. The termites were immediately processed into 95% non-denatured ethanol vials at room temperature (≈20ºC). Table 2-1 shows the collecting sites for the islands, where there were three collecting sites for Terceira Island (Angra), three sites for São Miguel (P.D.), two sites for Faial (Horta), and one site for Santa Maria Island (Maia). Only alates were used and the number varied between a minimum of 4 and a maximum of 20 per site (Table 2-1).

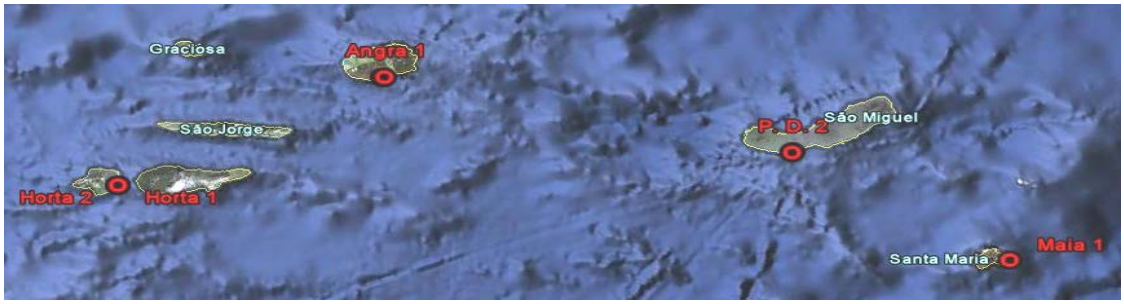

Figure 2-1. Location of the towns where *C. brevis* was collected in the four islands.

Table 2-1. Locations of collection sites in the Azores and number of individuals (N) used for analysis.

Island	Town	Sample name	N	Latitude	Longitude
Terceira	Angra do Heroísmo	AA	7	38.655475°	-27.219519°
Terceira	Angra do Heroísmo	AB	20	38.656608°	-27.228125°
Terceira	Angra do Heroísmo	AC	4	38.654694°	-27.226614°
São Miguel	Ponta Delgada	PDA	7	37.739322°	-25.669872°
São Miguel	Ponta Delgada	PDB	5	37.742244°	-25.665503°
São Miguel	Ponta Delgada	PDC	6	37.742300°	-25.662792°
Faial	Horta	HA	20	38.540844°	-28.625658°
Faial	Horta	HB	15	38.540731°	-28.625842°
Santa Maria	Maia	M	20	36.939256°	-25.014958°

DNA extraction and amplification

Mitochondrial DNA. DNA was extracted by using a DNeasy ® Blood and Tissue kit (Qiagen), following the protocol for extracting DNA from animal tissue from the DNeasy® Blood and Tissue Handbook (2006). After extraction, DNA was amplified using PCR. The primers used for mitochondrial 16S rRNA and Cytochrome b were the same primers used by Legendre et al. (2008) (Table B-1), because they previously successful for *C. brevis*. A maximum of five different specimens per collection site was used for the mtDNA amplification (with the exception of Maia where ten individuals were used because there was only one point of collection), and individually sequenced. A PCR for temperature gradient was run to determine the best temperature for annealing both 16S rRNA and Cytb primers, and the optimum PCR profile for 16S rRNA and Cytb

are described in Table 2-2. The template (3 µl of DNA) was loaded into a thermo cycler (DNA Engine® Thermal Cycler) with a PCR Master Mix formulation (Promega M7502) (7 µl), and the primers (1.5µl of each forward and reverse primer at a concentration of 10 µM) according to the gene that was to be amplified, and diluted with water (5 µl). After the DNA was amplified 5 µl of the PCR products were run on a polyacrylamide gel (8%) along with an exACTGene 50bp Mini DNA Ladder (Fisher) to ensure the products obtained were the right size (Figure A-2). The gels were then stained with Ethidium Bromide (0.5 µg/ml) and observed under U.V. light. The remainder of each of the samples was cleaned with a Montáge® PCR Centrifugal filter device (Millipore, Montáge ®) following the protocol for Millipore. After being cleaned the samples were sent to the University of Florida DNA Sequencing Laboratory, where they were sequenced using an ABI 3130 automated sequencer.

Table 2-2. PCR Cycles for the mitochondrial16S rRNA, and Cytb genes optimized for amplification in *C. brevis*.

Gene	Heat	Denaturation	Annealing	Extension	Final Extension	Cycles
16S rRNA	94ºC (2min)	94ºC (1min)	50ºC (1min)	72ºC (1min 15s)	72ºC (7min)	40
Cytb	94ºC (2min)	94ºC (1min)	56ºC (1min)	72ºC (1min 15s)	72ºC (7min)	40

Microsatellites. The same extracted DNA was used for the microsatellite data analysis. Up to 20 specimens were used per collection site (Table 2-1). The primers used for microsatellite amplification were adapted from Fuchs et al. (2003). These primers were developed for *C. secundus,* but proved to successfully amplify five loci in *C. brevis* (Table B-2). For all five loci a temperature gradient was run to assess the optimal annealing temperature. For two of the loci (Csec 5 and 6) an optimum

consistent annealing temperature was found, using the PCR profiles provided in the literature (Table 2-3). For these loci, the same PCR Master Mix and quantities were used as for the mitochondrial gene amplification. However, for the remaining loci (Csec1, 3, and 4), due to inconsistencies in results, the Terra™ PCR Direct Polymerase Mix (Clontech Industries Inc. CAT: 639270) was used with the specific PCR profiles recommended by the manufacturer. For the Terra PCR, 25 µl of buffer, 9 µl of water, 5 µl of both forward and reverse primer, 1µl of Taq, and 5 µl of DNA were loaded into the thermocycler. The PCR products for all loci were loaded into a 12% gel (Hoeffer, GE Healthcare) following the same protocol as described above, to determine if the amplified products were in the right size range (Figure A-3). All the forward primers for the microsatellite were tagged with a fluorescent tag. Loci Csec 3, 4, 5, and 6 were tagged with FAM™ (Integrated DNA Technologies, Inc.), and Csec1 was tagged with HEX™ (Integrated DNA Technologies, Inc.). Loci Csec1 and Csec 4 were multiplexed. All PCR products were sent to the University of Florida Sequencing Laboratory for genotyping.

Table 2-3. PCR profiles used for all the *C. brevis* microsatellite loci.

Locus	Heat	Denaturation	Annealing	Extension	Final extension	Cycles
Csec1	98ºC (2min)	98ºC (10s)	60ºC (15s)	68ºC (30s)	68ºC (7min)	40
Csec3	98ºC (2min)	98ºC (10s)	60ºC (15s)	68ºC (30s)	68ºC (7min)	40
Csec4	98ºC (2min)	98ºC (10s)	60ºC (15s)	68ºC (30s)	68ºC (7min)	40
Csec5	95ºC (1min30s)	95ºC (45s)	57ºC (45s)	72ºC (45s)	72ºC (7min)	35
Csec6	95ºC (1min30s)	95ºC (45s)	57ºC (45s)	72ºC (45s)	72ºC (7min)	35

Data analysis

Mitochondrial DNA. Phylogenetic and molecular evolutionary analyses for the 16S rRNA gene and Cytb gene were conducted using MEGA version 5 (Tamura et al. 2011). Sequences were aligned in MEGA version 5 (Figure A-4) using the ClustalW method set at default, as recommended by the software. The sequences from 16S rRNA and Cytb were combined in order to create more robust sequence data. After the sequences were aligned they were run through the Gblocks software (Talavera and Castresana 2007) to eliminate poorly aligned positions, divergent regions of the alignment and insertions and deletions. Distance matrixes for number of nucleotides were created for all the islands. The distance p-value was then calculated to test if the data from the sequences were good. Similar sequences were eliminated from the analysis, and a representative sequence was chosen to represent those sequences. The representative sequences were then analyzed using the UPGMA method (Sneath and Sokal 1973). The bootstrap consensus tree was inferred from 5000 replicates (Felsenstein 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are based on the number of base substitutions per site. Sequences from *Kalotermes flavicollis* for both genes were used as out-groups. These representative sequences were obtained from GenBank by using BLAST for each gene. For mitochondrial 16S rRNA gene the *K. flavicollis* sequence used was reported in Szalanski et al. (2004) and identified by accession number AY486437. For Cytb gene, the *K. flavicollis* sequence used was the one reported in Legendre et al. (2008) and identified with the accession number EU253919. Haplotypes for the individual termites were calculated using TCSv1.21 (Clement et al. 2000) and a tree was created showing the genealogical relationships between haplotypes. The program

ARLEQUIN 3.5.1.2 (Excoffier and Lischer 2010) was used to calculate the AMOVA (Analysis of Molecular Variance) between haplotypes of the different subpopulations.

Microsatellites. For the microsatellite analysis the Peak Scanner™ Software v1.0 (© Copyright 2006, Applied Biosystems) was used to score the microsatellites (Figure A-5). The EXCEL Microsatellite toolkit (Park 2001) was used to estimate the number and frequency of alleles, as well as observed and expected heterozygosity. An ANOVA was used to test if there were significant differences between expected and observed heterozygosities, and allele frequencies between populations using SAS (2003). FSTAT 2.9.3.2 (Goudet, 2001) and GENEPOPV4 (© F. Rousset) were used to calculate F_{ST}, F_{IS}, and F_{IT} to quantify the respective loss in heterozygosity, linkage disequilibrium, and the frequency of null alleles. They were also used to estimate the modified Wright's F-statistics by Weir and Cockerham's (1984) Θ_{ST}, Nei's (1987) G_{ST}, and Michalakis and Excoffier's (1996) ρ_{ST}. The gene flow was quantified in terms of migrants exchanged among subpopulations per generation (Nm), calculated using GENEPOPV4 (© F. Rousset). Genetic distance was calculated using Nei's (1972) method and a genetic distance matrix and tree was generated using Genetic Distance Analysis 1.1 (Lewis and Zaykin 2001).

To test different scenarios of introduction the program DIYABC (Cornuet et al. 2010) was used. The program uses approximate Bayesian computations to infer origins considering bottleneck effects when introduction occurs. The assumptions used for the models were that there was an ancestral population of size NA from where the introduced subpopulations originated. The size of the ancestral population was assumed to be higher than that of the introduced subpopulations N1-N4 (Figures 2-2 to 2-7). For the computations, the subpopulations of each Island were considered as one

single subpopulation per Island. The data of subpopulations AA, AB, and AC were merged into Population 1 (default nomenclature in the program), subpopulations PDA, PDB, PDC were merged into Population 2, subpopulations HA, and HB, were merged into Population 3, and the M subpopulation was represented as Population 4. A generalized mutation model (GSM) was assumed for the microsatellite loci, with a gamma distribution for the mutation rate bounded between $5x10^{-4}$ and $5x10^{-3}$. The reference table was created for 50,000 simulations. The posterior probability of each scenario was assessed by a polychotomous weighted logistic regression that estimates the difference between simulated (from the reference table) and observed data sets. The number of observed data sets used was 500 and the number of simulated data used was 5,000. Six scenarios of introduction were tested with no admixture assumed.

In scenario 1, Population 1 (Angra) and Population 2 (Ponta Delgada) were assumed to be part of an ancestral common population of size NA, and were then separately introduced to the Islands at an undetermined time (td). Population 3 (Horta) was assumed to split from Population 1 at a later time (ta), while Population 4 (Maia) split from Population 2 (Figure 2-2).

In scenario 2, Populations 1 and 2 were assumed to be part of an ancestral common population of size NA, and were then separately introduced to the Islands at an undetermined time (td). Populations 3 and 4 were assumed to have both split from Population 1 at a later time (ta) making Population 1 the common ancestral population for Populations 3 and 4 (Figure 2-3).

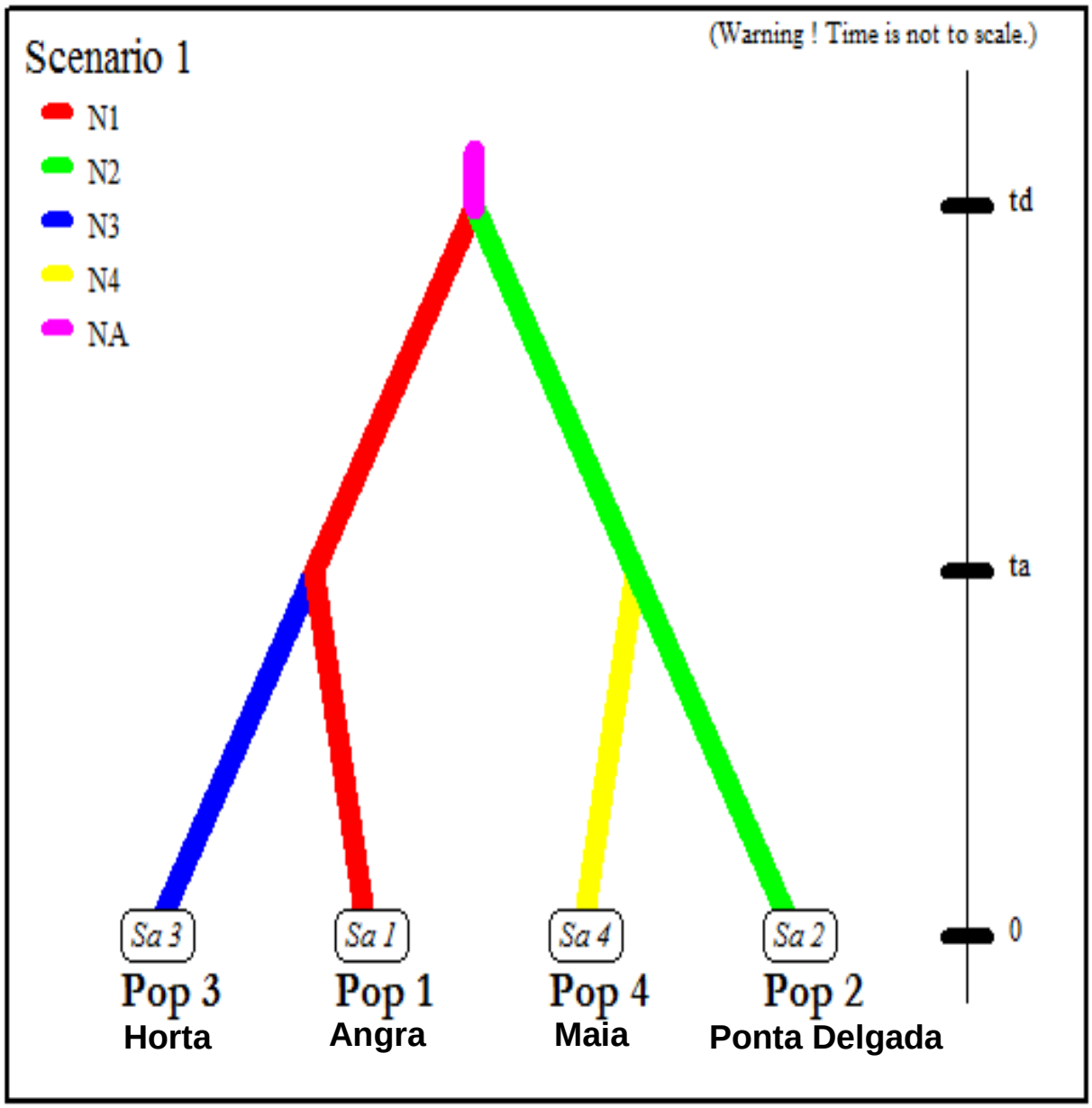

Figure 2-2. Scenario 1 of introduction to the Islands tested with the DIYABC software. The program assumed an effective random population size for each population N1, N2, N3, and N4. Computations were made based on the samples from each population (Sa 1-4). The splitting of populations was assumed to happen at a determined time for each event, td and ta, with the condition that td >ta. Each Population corresponded to a different Island with the different sites per Island merged into one single Population. Population 1 was the subpopulation of the city of Angra, Population 2 was the subpopulation of the city of Ponta Delgada, Population 3 is the subpopulation of the City of Horta, and Population 4 was the subpopulation of the city of Maia. The scenario assumed that populations 1 and 2 were part of one ancestral population of size NA with the condition that NA>N1-N4, and that subsequently population 3 split from population 1, while population 4 split from population 2.

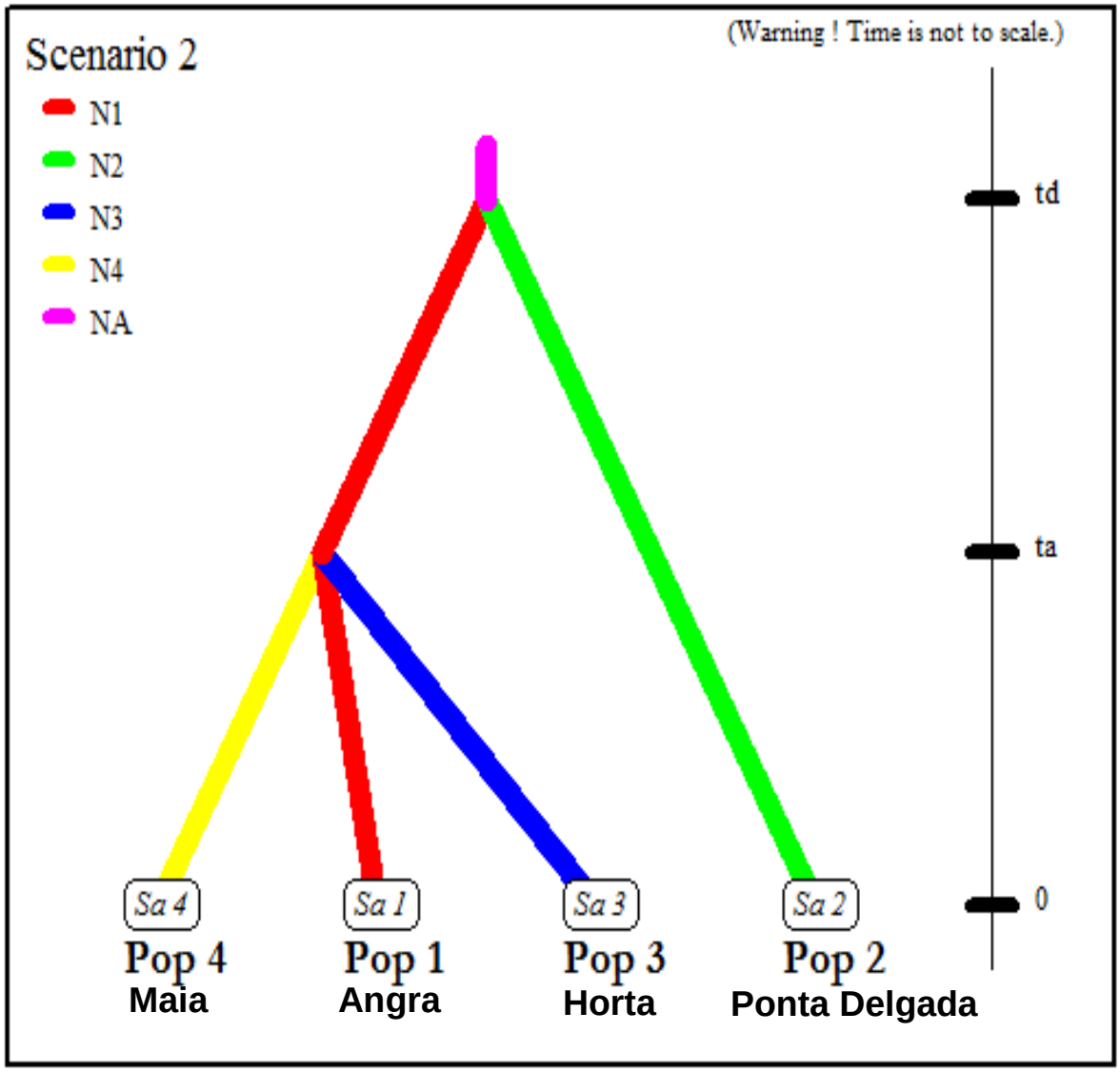

Figure 2-3. Scenario 2 of introduction to the Islands tested with the DIYABC software. The program assumed an effective random population size for each population N1, N2, N3, and N4. Computations were made based on the samples from each population (Sa 1-4). Each Population corresponded to a different Island with the different sites per Island merged into one single Population. Population 1 was the subpopulation of the city of Angra, Population 2 was the subpopulation of the city of Ponta Delgada, Population 3 was the subpopulation of the City of Horta, and Population 4 was the subpopulation of the city of Maia. The splitting of populations was assumed to happen at a determined time for each event, td and ta, with the condition that td >ta. The scenario assumed that populations 1 and 2 were part of one ancestral population of size NA with the condition that NA>N1-N4, and that subsequently Population 3 and Population 4 split from Population 1.

In scenario 3, Populations 1 and 2 were assumed to be part of an ancestral common population of size NA, and were then separately introduced to the Islands at an undetermined time (td). Populations 3 and 4 were assumed to have both split from Population 2 at a later time (ta), making Population 2 the common ancestral population for populations 3 and 4 (Figure 2-4).

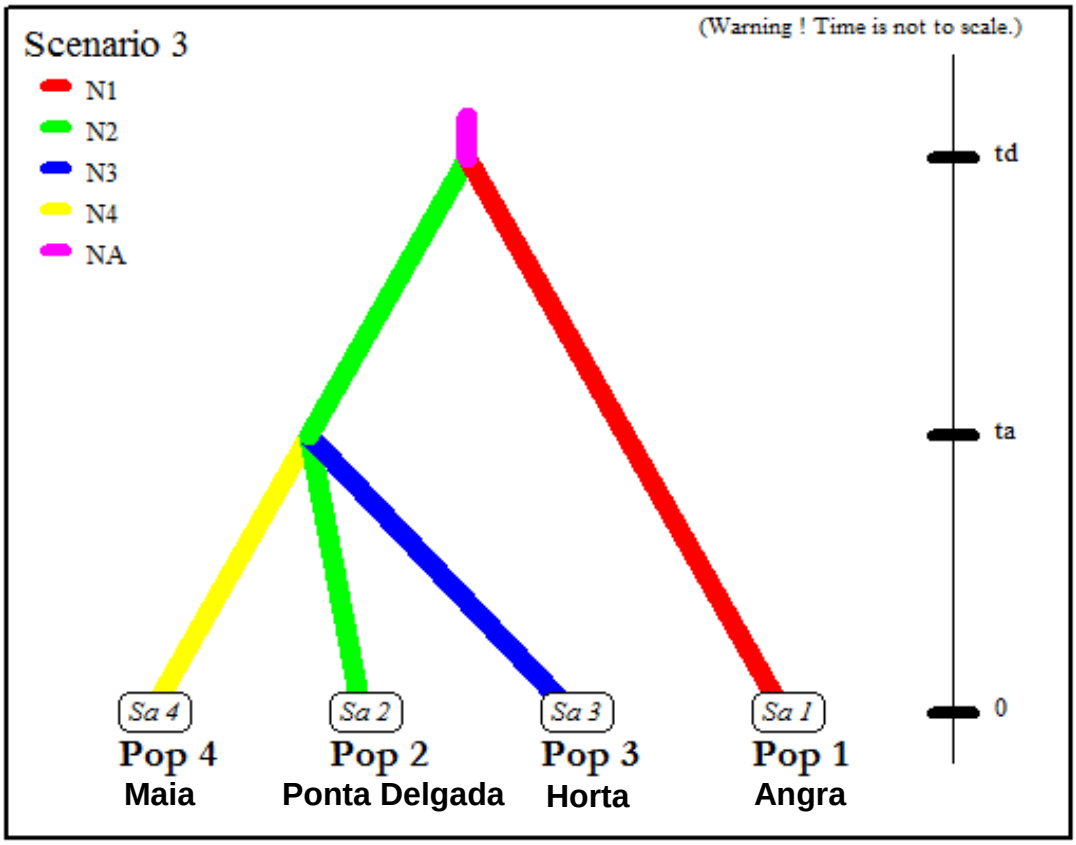

Figure 2-4. Scenario 3 of introduction to the Islands tested with the DIYABC software. The program assumed an effective random population size for each population N1, N2, N3, and N4. Computations were made based on the samples from each population (Sa 1-4). Each Population corresponded to a different Island with the different sites per Island merged into one single Population. Population 1 was the subpopulation of the city of Angra, Population 2 was the subpopulation of the city of Ponta Delgada, Population 3 was the subpopulation of the City of Horta, and Population 4 was the subpopulation of the city of Maia. The splitting of populations was assumed to happen at a determined time for each event, td and ta, with the condition that td >ta. The scenario assumed that populations 1 and 2 were part of one ancestral population of size NA with the condition that NA>N1-N4, and that subsequently Population 3 and Population 4 split from Population 2.

In scenario 4, all populations were assumed to have come from the same ancestral common population of size NA, and were subsequently introduced from that one population into the different islands at an undetermined time (td). In this scenario the different subpopulations of the islands are considered to have been introduced independently from each other (Figure 2-5).

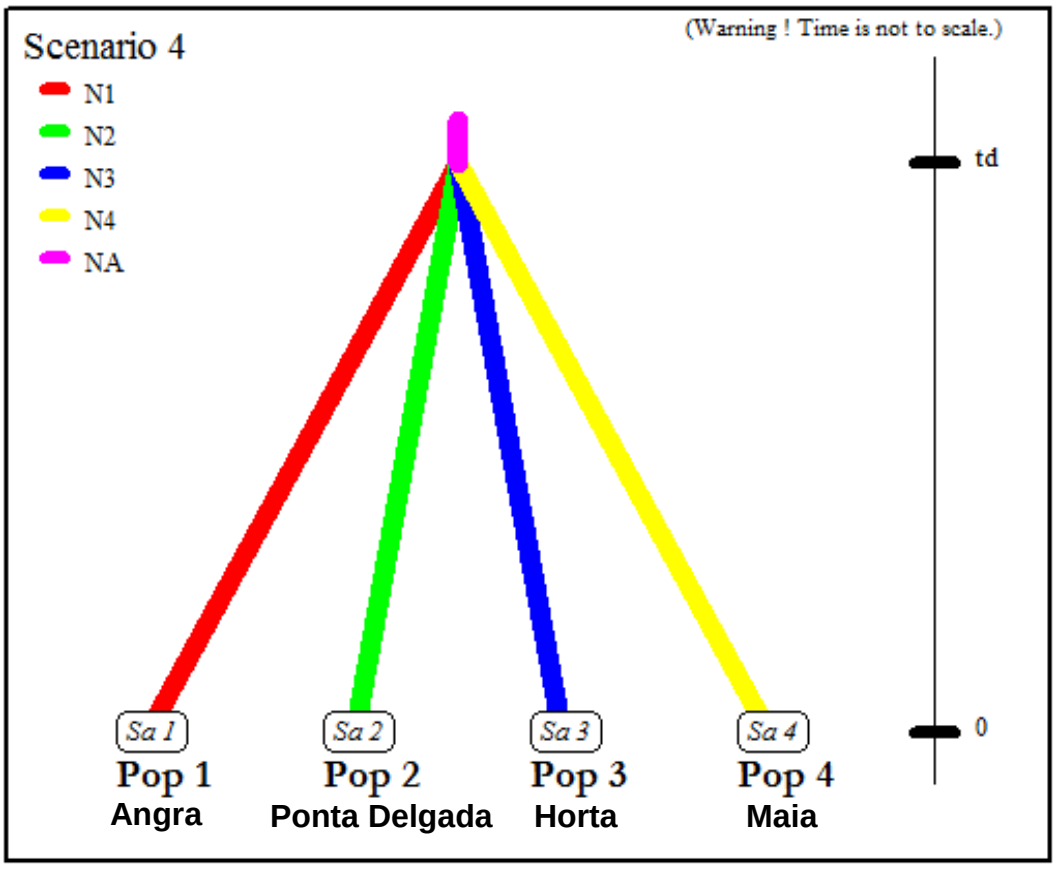

Figure 2-5. Scenario 4 of introduction to the Islands tested with the DIYABC software. The program assumed an effective random population size for each population N1, N2, N3, and N4. Computations were made based on the samples from each population (Sa 1-4). Each Population corresponded to a different Island with the different sites per Island merged into one single Population. Population 1 was the subpopulation of the city of Angra, Population 2 was the subpopulation of the city of Ponta Delgada, Population 3 was the subpopulation of the City of Horta, and Population 4 was the subpopulation of the city of Maia. The splitting of populations was assumed to happen at a determined time, td. The scenario assumed that populations 1, 2, 3, and 4 were all part of a single ancestral population of size NA with the condition that NA>N1-N4, and were all introduced, from that ancestral population, separately to the islands.

In scenario 5, Population 1 and 2 were assumed to be part of an ancestral common population of size NA, and were then separately introduced to the Islands at an undetermined time (td). Population 3 was assumed to have split from Population 2 at time (ta) and subsequently Population 4 was assumed to have split from Population 3 with Population 1 being completely independent from the other populations (Figure 2-6).

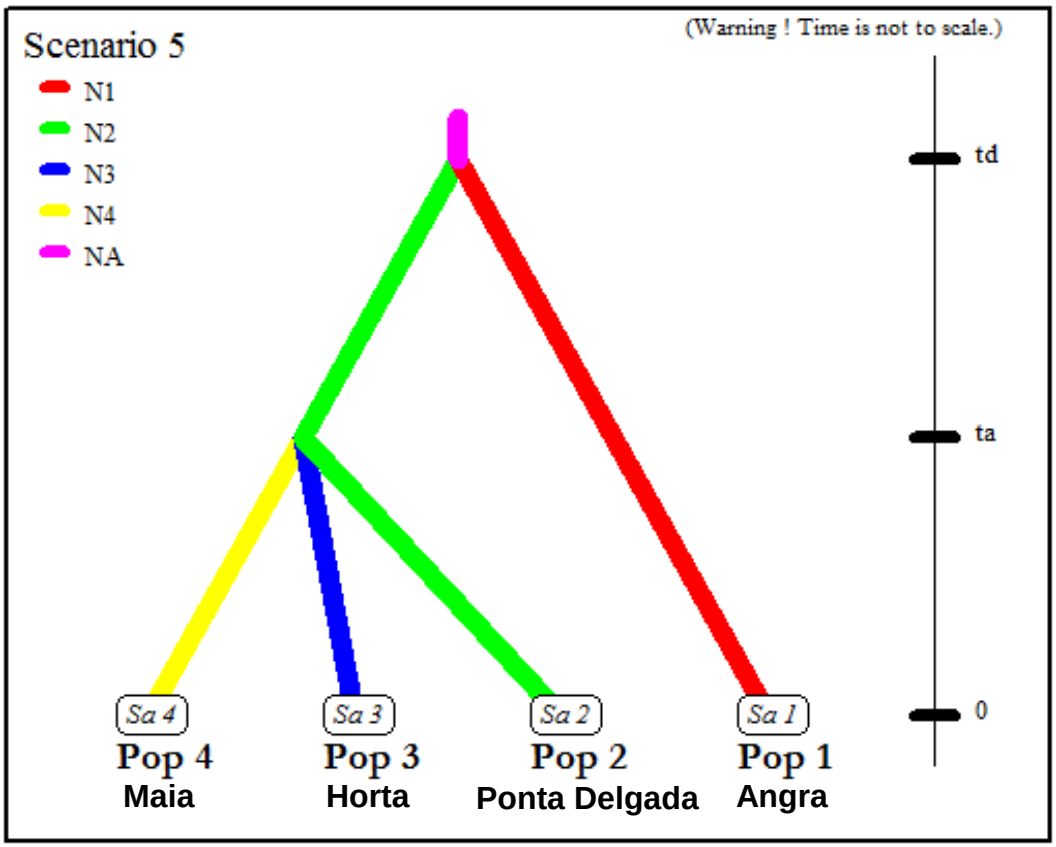

Figure 2-6. Scenario 5 of introduction to the Islands tested with the DIYABC software. The program assumed an effective random population size for each population N1, N2, N3, and N4. Computations were made based on the samples from each population (Sa 1-4). Each Population corresponded to a different Island with the different sites per Island merged into one single Population. Population 1 was the subpopulation of the city of Angra, Population 2 was the subpopulation of the city of Ponta Delgada, Population 3 was the subpopulation of the City of Horta, and Population 4 was the subpopulation of the city of Maia. The splitting of populations was assumed to happen at a determined time for each event, td and ta, with the condition that td >ta. The scenario assumed that populations 1 and 2 were part of one ancestral population of size NA with the condition that NA>N1-N4, and that subsequently population 3 split from population 2 and population 4 split from population 3.

In scenario 6, Populations 1 and 2 were assumed to be part of an ancestral common population of size NA, and were then separately introduced to the Islands at an undetermined time (td). Population 3 was assumed to have split from Population 1 at time (ta) and Population 4 was assumed to have split from Population 3 with Population 2 being completely independent from the other populations (Figure 2-7)

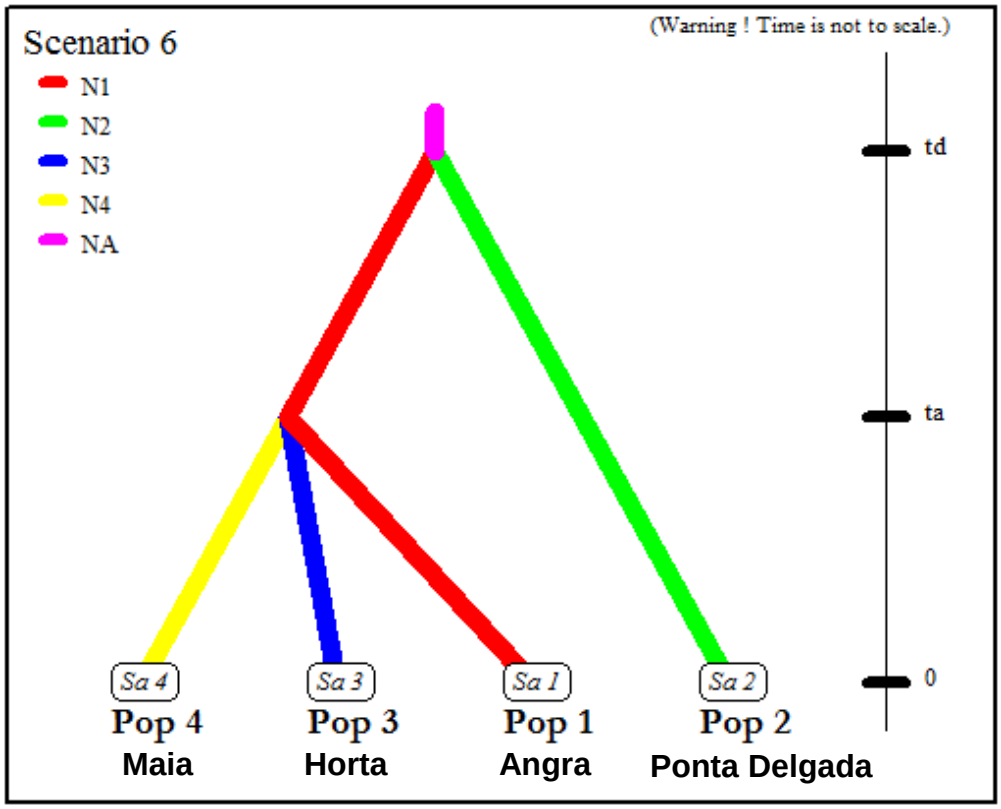

Figure 2-7. Scenario 6 of introduction to the Islands tested with the DIYABC software. The program assumed an effective random population size for each population N1, N2, N3, and N4. Computations were made based on the samples from each population (Sa 1-4). Each Population corresponded to a different Island with the different sites per Island merged into one single Population. Population 1 was the subpopulation of the city of Angra, Population 2 was the subpopulation of the city of Ponta Delgada, Population 3 was the subpopulation of the City of Horta, and Population 4 was the subpopulation of the city of Maia. The splitting of populations was assumed to happen at a determined time for each event, td and ta, with the condition that td >ta. The scenario assumed that populations 1 and 2 were part of one ancestral population of size NA with the condition that NA>N1-N4, and that subsequently population 3 split from population 1 and population 4 split from population 3.

Results

Mitochondrial DNA

A total of 49 samples of PCR product per gene were sent to the sequencing laboratory. Of these samples only 31 produced readable sequences for 16S rRNA, and 28 for the Cytb gene. Only complete sequences from both Cytb and 16S rRNA were used and combined with a total of 28 combined sequences for analysis. Of these sequences a total of 9 sequences for the combined 16S rRNA, and Cytb were not used for the phylogenetic analysis because they were not different from other sequences as determined by the calculated distance matrix (Tables 2-4 to 2-6). For the remaining 19 combined sequences, the p-distance was calculated at < 0.33, and the data were considered good for proceeding with the analysis.

MEGA 5.0 produced a phylogenetic tree for the combined mtDNA genes. For the combined sequences the UPGMA bootstrap consensus tree shows two distinct branches with 100% resolution (Figure 2-8). The subpopulations of Ponta Delgada (PD) and Maia (M) are placed together in one of the main branches. The subpopulation of Horta is placed in the other main branch. The subpopulation of Angra is placed in both of the branches, with individuals in the branch that includes Horta and individuals in the branch that includes both Ponta Delgada and Maia.

The analysis of the 28 combined sequences for haplotypes yielded a total of 22 different haplotypes (Table B-3 and 4) separated into two major groups that showed no genealogical steps between one another (Figure 2-9). Of the haplotypes, 16 had one representative sequence while the remainder six, had two sequences with the same haplotype. The haplotypes of the Angra subpopulation showed more distance from the haplotypes represented in the Ponta Delgada and Maia subpopulations than from the

Horta subpopulations (Figure 2-9). The F_{ST} value for the AMOVA was 0.77576 with a p-value of 0.000±0.000 not being significantly different from 0. The percentage of variation among and between subpopulations is shown in Table 2-7 where the variation among populations was above 77%.

Table 2-4. Distance matrix of estimates of evolutionary divergence between the combined sequences of Cytb and 16S rRNA of *C. brevis* for the Azorean subpopulations. The number of base differences between sequences is shown. The analysis involved 28 nucleotide sequences.

	AA-1	AA-3	AB-1	AB-2	AB-4	AB-5	AC-2	PDA-2	PDA-3
AA-1									
AA-3	0								
AB-1	40	39							
AB-2	40	39	0						
AB-4	6	6	39	39					
AB-5	42	41	4	4	41				
AC-2	41	40	3	3	40	1			
PDA-2	37	36	8	8	38	13	12		
PDA-3	44	43	2	2	43	7	6	9	
PDA-5	42	40	5	5	42	10	9	5	6
PDB-1	37	36	8	8	38	13	12	2	11
PDB-2	39	38	4	4	40	9	8	4	7
PDC-1	40	39	4	4	41	9	8	5	7
HA-1	2	2	39	39	6	41	40	38	43
HA-2	2	2	41	41	8	43	42	38	45
HA-3	3	3	40	40	7	42	41	39	44
HA-4	1	1	38	38	5	40	39	37	42
HA-5	0	0	40	40	6	42	41	37	44
HB-1	1	1	38	38	5	40	39	37	42
HB-2	2	2	39	39	6	41	40	38	43
HB-3	2	2	39	39	6	41	40	38	43
HB-4	2	2	42	42	8	44	43	37	44
HB-5	0	0	39	39	6	41	40	36	43
M-1	41	40	6	6	42	11	10	6	9
M-2	39	38	4	4	40	9	8	4	7
M-5	39	38	5	5	40	10	9	4	8
M-7	39	38	4	4	40	9	8	4	7
M-9	42	41	7	7	43	12	11	7	10

Table 2-5. Continuation of distance matrix of estimates of evolutionary divergence between the combined sequences of Cytb and 16S rRNA of *C. brevis* for the Azorean subpopulations. The number of base differences between sequences is shown. The analysis involved 28 nucleotide sequences.

	PDA-5	PDB-1	PDB-2	PDC-1	HA-1	HA-2	HA-3	HA-4	HA-5
PDB-1	5								
PDB-2	1	4							
PDC-1	2	5	1						
HA-1	42	38	40	41					
HA-2	42	38	40	41	4				
HA-3	43	39	41	42	3	5			
HA-4	41	37	39	40	1	3	2		
HA-5	41	37	39	40	2	2	3	1	
HB-1	41	37	39	40	1	3	2	0	1
HB-2	42	38	40	41	2	3	3	1	2
HB-3	42	38	40	41	2	3	3	1	2
HB-4	43	39	41	42	4	2	5	3	2
HB-5	40	36	38	39	2	2	3	1	0
M-1	3	6	2	3	42	42	43	41	41
M-2	1	4	0	1	40	40	41	39	39
M-5	1	4	0	1	40	40	41	39	39
M-7	1	4	0	1	40	40	41	39	39
M-9	4	7	3	4	43	41	44	42	42

Table 2-6. Continuation of distance matrix of estimates of evolutionary divergence between the combined sequences of Cytb and 16S rRNA of *C. brevis* for the Azorean subpopulations. The number of base differences per sequence from between sequences are shown. The analysis involved 28 nucleotide sequences. There were a total of 707 positions in the final dataset.

	HB-1	HB-2	HB-3	HB-4	HB-5	M-1	M-2	M-5	M-7	M-9
HB-2	1									
HB-3	1	0								
HB-4	3	4	4							
HB-5	1	2	2	2						
M-1	41	42	42	43	40					
M-2	39	40	40	41	38	2				
M-5	39	40	40	41	38	2	0			
M-7	39	40	40	41	38	2	0	0		
M-9	42	43	43	42	41	5	3	3	3	

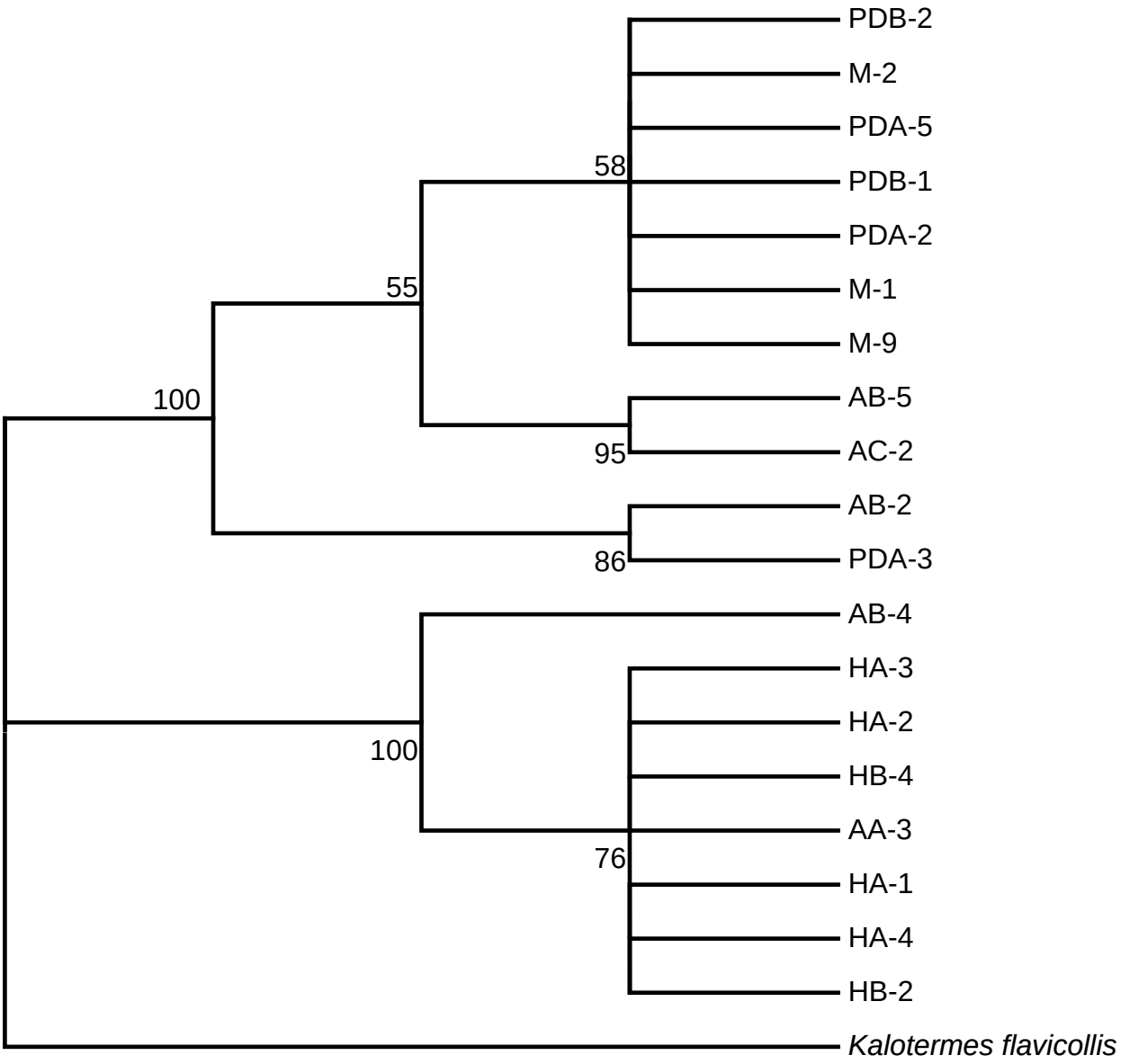

Figure 2-8. Evolutionary relationships of *C. brevis* subpopulations. The evolutionary history was inferred using the UPGMA method. The bootstrap consensus tree inferred from 5000 replicates is taken to represent the evolutionary history of the subpopulations analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated subpopulations clustered together in the bootstrap test are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 701 positions in the final dataset.

Table 2-7. Summary statistics for the AMOVA among and within subpopulations of the different Islands. Haplotype data was used to determine the percentage of variation in the Arlequin 3.5 software.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	8	246.921	9.35878	77.58
Within populations	19	51.4	2.70526	22.42
Total	27	298.321	12.06405	

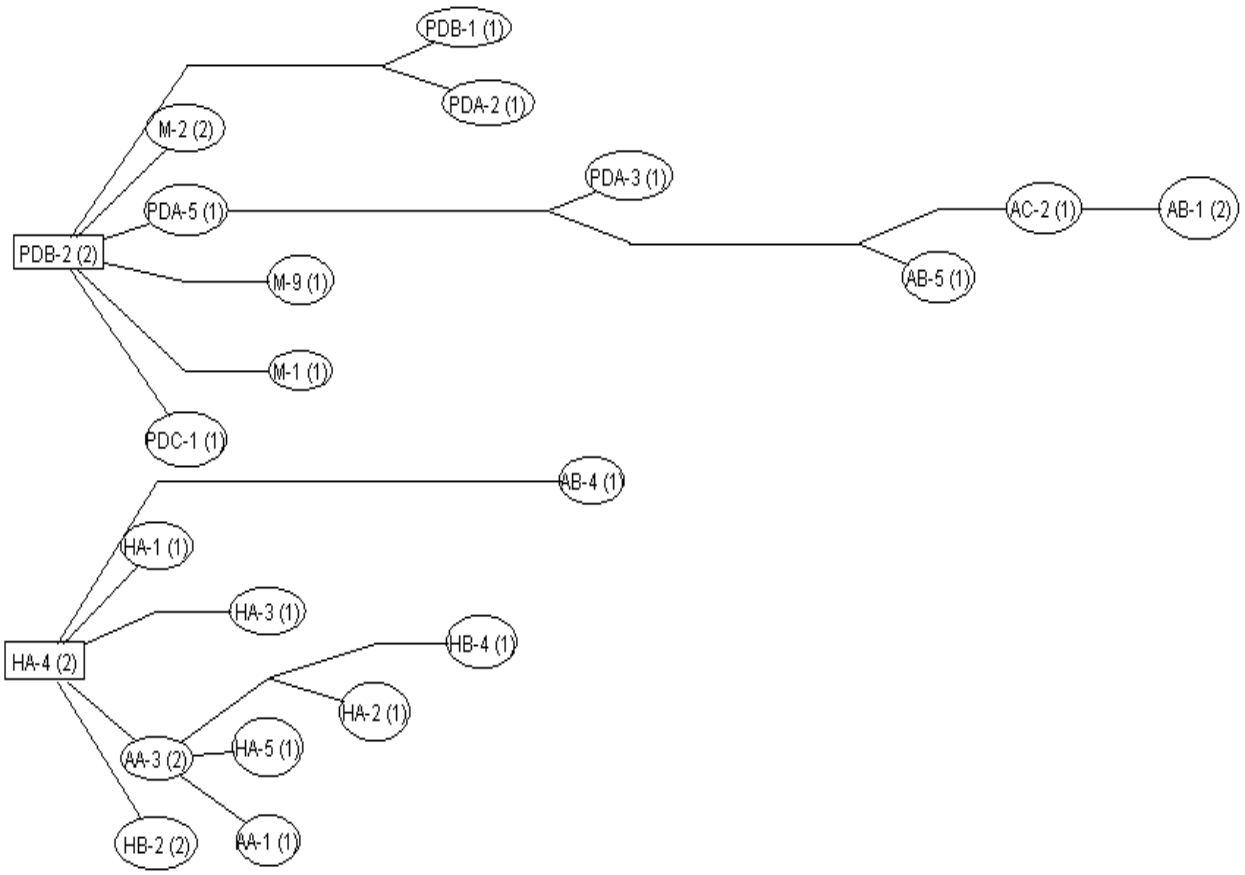

Figure 2-9. Genealogical relationships among 22 haplotypes of *C. brevis* estimated by TCS. Haplotypes in rectangular boxes were randomly chosen by software to root the tree. Length of branches indicates number of changes in haplotype. Number in parenthesis represents the number of individuals sharing the haplotype.

Microsatellites

A total of 33 alleles were detected in the populations over the five loci. Alleles per locus for all subpopulations are given in Table 2-8, and allele frequencies per locus are given in Tables 2-9 to 2-13. The allele 234 for the locus Csec1 was unique for the AA subpopulation, and for the Csec3 locus the allele 134 was unique for the subpopulation HA, while the allele 138 was unique for the subpopulation M. The loci Csec 1, 3, and 4 had the higher number of alleles for the Azorean subpopulations, with nine, ten and seven alleles, respectively. The Csec6 and Csec5 loci had three and four alleles, respectively. The mean number of alleles varied between 2.6 and 4. The frequencies of alleles varied between 0-100% for the loci (Tables 2-9 to 2-13). The allele frequencies were not significantly different between subpopulations (F=0.11, p-value=0.999).

Table 2-8. Size of alleles in number of base pairs per locus for the five microsatellite loci of *C. brevis* analyzed.

Locus	Alleles (bp)									
Csec6	143	146	149							
Csec5	122	125	128	131						
Csec4	129	144	148	154	157	166	169			
Csec1	208	214	216	220	224	226	230	232	234	
Csec3	116	118	120	122	126	128	130	132	134	138

Table 2-9. Allele frequencies in percentage for Csec6 locus for the subpopulations of *C. brevis* calculated by the Microsatellite tool kit for Excel.

Allele	AA	AB	AC	PDA	PDB	PDC	HA	HB	M
143	78.6	77.5	75.0	100.0	100.0	75.0	95.0	93.3	95.0
146	21.4	22.5	25.0	0.0	0.0	16.7	5.0	3.3	5.0
149	0.0	0.0	0.0	0.0	0.0	8.3	0.0	3.3	0.0

Table 2-10. Allele frequencies in percentage for Csec5 locus for the subpopulations of *C.brevis* calculated by the Microsatellite tool kit for Excel.

Allele	AA	AB	AC	PDA	PDB	PDC	HA	HB	M
122	7.1	30.0	12.5	71.4	50.0	33.3	40.0	20.0	12.5
125	92.9	60.0	62.5	28.6	50.0	50.0	60.0	80.0	85.0
128	0.0	7.5	25.0	0.0	0.0	8.3	0.0	0.0	2.5
131	0.0	2.5	0.0	0.0	0.0	8.3	0.0	0.0	0.0

Table 2-11. Allele frequencies in percentage for Csec4 locus for the subpopulations of *C. brevis* calculated by the Microsatellite tool kit for Excel.

Allele	AA	AB	AC	PDA	PDB	PDC	HA	HB	M
129	14.3	17.5	12.5	78.6	40.0	0.0	0.0	0.0	5.0
144	0.0	0.0	0.0	14.3	20.0	0.0	0.0	0.0	0.0
148	0.0	0.0	0.0	0.0	20.0	0.0	2.5	0.0	0.0
154	21.4	20.0	37.5	7.1	0.0	8.3	0.0	0.0	0.0
157	64.3	55.0	50.0	0.0	10.0	25.0	2.5	13.3	0.0
166	0.0	2.5	0.0	0.0	10.0	16.7	57.5	50.0	72.5
169	0.0	5.0	0.0	0.0	0.0	50.0	37.5	36.7	22.5

Table 2-12. Allele frequencies in percentage for Csec1 locus for the subpopulations of *C. brevis* calculated by the Microsatellite tool kit for Excel.

Allele	AA	AB	AC	PDA	PDB	PDC	HA	HB	M
208	50.0	87.5	87.5	50.0	40.0	75.0	62.5	66.7	62.5
214	0.0	2.5	0.0	0.0	0.0	0.0	2.5	0.0	0.0
216	0.0	7.5	0.0	0.0	10.0	0.0	0.0	0.0	0.0
220	0.0	0.0	0.0	7.1	20.0	0.0	2.5	0.0	2.5
224	0.0	0.0	0.0	42.9	30.0	0.0	0.0	0.0	2.5
226	14.3	0.0	12.5	0.0	0.0	8.3	22.5	10.0	7.5
230	7.1	0.0	0.0	0.0	0.0	8.3	10.0	16.7	15.0
232	14.3	2.5	0.0	0.0	0.0	8.3	0.0	6.7	10.0
234	14.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 2-13. Allele frequencies in percentage for Csec3 locus for the subpopulations of *C. brevis* calculated by the Microsatellite tool kit for Excel.

Allele	AA	AB	AC	PDA	PDB	PDC	HA	HB	M
116	0.0	0.0	0.0	0.0	0.0	0.0	2.5	0.0	0.0
118	0.0	0.0	25.0	57.1	60.0	41.7	62.5	66.7	57.5
120	0.0	5.0	0.0	0.0	0.0	0.0	2.5	3.3	0.0
122	64.3	75.0	50.0	21.4	20.0	41.7	12.5	16.7	17.5
126	0.0	0.0	0.0	7.1	20.0	0.0	12.5	10.0	15.0
128	14.3	7.5	0.0	0.0	0.0	0.0	0.0	3.3	0.0
130	0.0	5.0	25.0	14.3	0.0	16.7	0.0	0.0	5.0
132	21.4	7.5	0.0	0.0	0.0	0.0	5.0	0.0	2.5
134	0.0	0.0	0.0	0.0	0.0	0.0	2.5	0.0	0.0
138	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.5

Table 2-14. Estimates of genetic diversity at 5 polymorphic microsatellite loci in *C. brevis* from the Azorean Islands.

Sub-pop	Mean Expected Hz	Mean Observed Hz	ANOVA's F statistics	p-value for ANOVA	Mean No of Alleles per locus	F_{IS}	p-value*
AA	0.47 ± 0.10	0.54 ± 0.08	0.16	0.702	3 ± 1.22	-0.16	0.9613
AB	0.44 ± 0.07	0.39 ± 0.05	0.24	0.635	4 ± 1.22	0.13	0.0681
AC	0.54 ± 0.09	0.45 ± 0.11	0.26	0.627	2.6 ± 0.55	0.182	0.2132
PDA	0.42 ± 0.11	0.37 ± 0.08	0.07	0.802	2.6 ± 1.14	0.114	0.2954
PDB	0.56 ± 0.15	0.4 ± 0.10	0.72	0.421	3 ± 1.58	0.304	0.0394
PDC	0.59 ± 0.06	0.57 ± 0.09	0.04	0.849	3.6 ± 0.55	0.05	0.4262
HA	0.46 ± 0.09	0.43 ± 0.05	0.02	0.883	4 ± 2.12	0.059	0.2835
HB	0.43 ± 0.09	0.48 ± 0.06	0.14	0.715	3.4 ± 1.14	-0.124	0.9446
M	0.40 ± 0.10	0.46 ± 0.05	0.14	0.722	4 ± 1.87	-0.148	0.9915

*p-value for F larger than the observed, in randomization tests.

The mean observed heterozygosity was not significantly different from the expected heterozygosity (Table 2-14), for all the subpopulations. The values of F_{IS} were low, with some values being negative, and were not significantly different from 0 (Table 2-14). The Hardy-Weinberg test for disequilibrium was significant (p-value=0.0076 for Score U test) with linkage disequilibrium found at loci Csec4 and Csec6. The null hypothesis that there is no deficiency of heterozygosity was rejected. The p-values of F_{ST} between subpopulations are presented in Table 2-15, with the significant values indicated. The Angra subpopulations were not significantly different between one another. One of the Angra subpopulations (AC) was not different from the Ponta Delgada subpopulations. The subpopulations of Horta and Maia were not significantly different from each other and from the PDC subpopulation (Table 2-15).

The estimated frequency of null alleles for the Csec4 locus varied between 0.07 and 0.4259, with null alleles present in seven of the nine subpopulations (Table 2-16). The Csec 1 loci had two subpopulations with null alleles, while the Csec 3, 5 and 6 loci had three subpopulations with null alleles. The calculated gene flow (Nm) was 3.77626

migrants per generation. The calculated values of F_{ST}, F_{IS}, and F_{IT} for the different methods are presented in Table 2-17. The distance diagram calculated for the microsatellite data is shown in Figure 2-10. The subpopulations of Maia and Horta were shown to be closer to each other than to the other subpopulations.

Table 2-15. Matrix of F_{ST} p-values between subpopulations of *C. brevis* for all microsatellite loci. p-values obtained after 36000 permutations, Indicative adjusted nominal level (5%) for multiple comparisons is 0.001389. Significant values indicated by *.

Sub-pop	AB	AC	PDA	PDB	PDC	HA	HB	M
AA	0.033	0.084	0.001*	0.002	0.000*	0.000*	0.000*	0.000*
AB		0.236	0.000*	0.000*	0.001*	0.000*	0.000*	0.000*
AC			0.003	0.025	0.554	0.000*	0.000*	0.000*
PDA				0.67	0.001*	0.000*	0.000*	0.000*
PDB					0.002	0.000*	0.000*	0.000*
PDC						0.015	0.138	0.007
HA							0.519	0.045
HB								0.524

Table 2-16. Estimated values for null allele frequencies per subpopulation, per locus for the *C. brevis* subpopulations sequenced in this study.

Locus	AA	AB	AC	PDA	PDB	PDC	HA	HB	M
Csec1	0.000	0.000	0.000	0.000	0.188	0.000	0.000	0.051	0.000
Csec3	0.000	0.000	0.107	0.072	0.000	0.000	0.039	0.000	0.030
Csec4	0.076	0.070	0.000	0.189	0.189	0.426	0.000	0.230	0.149
Csec5	0.000	0.017	0.000	0.000	0.000	0.114	0.259	0.000	0.000
Csec6	0.000	0.167	0.290	No inf	No inf	0.251	0.000	0.000	0.000

Table 2-17. Values of F_{ST}, F_{IS}, and F_{IT} calculated using three different methods for all loci for all subpopulations

	Weir & Cockerham (1984)	Michalakis and Excoffier (1996)	Nei's (1972)
F_{ST}	0.176	0.2453	0.147
F_{IS}	0.015	-0.1081	0.049
F_{IT}	0.188	0.1637	0.162

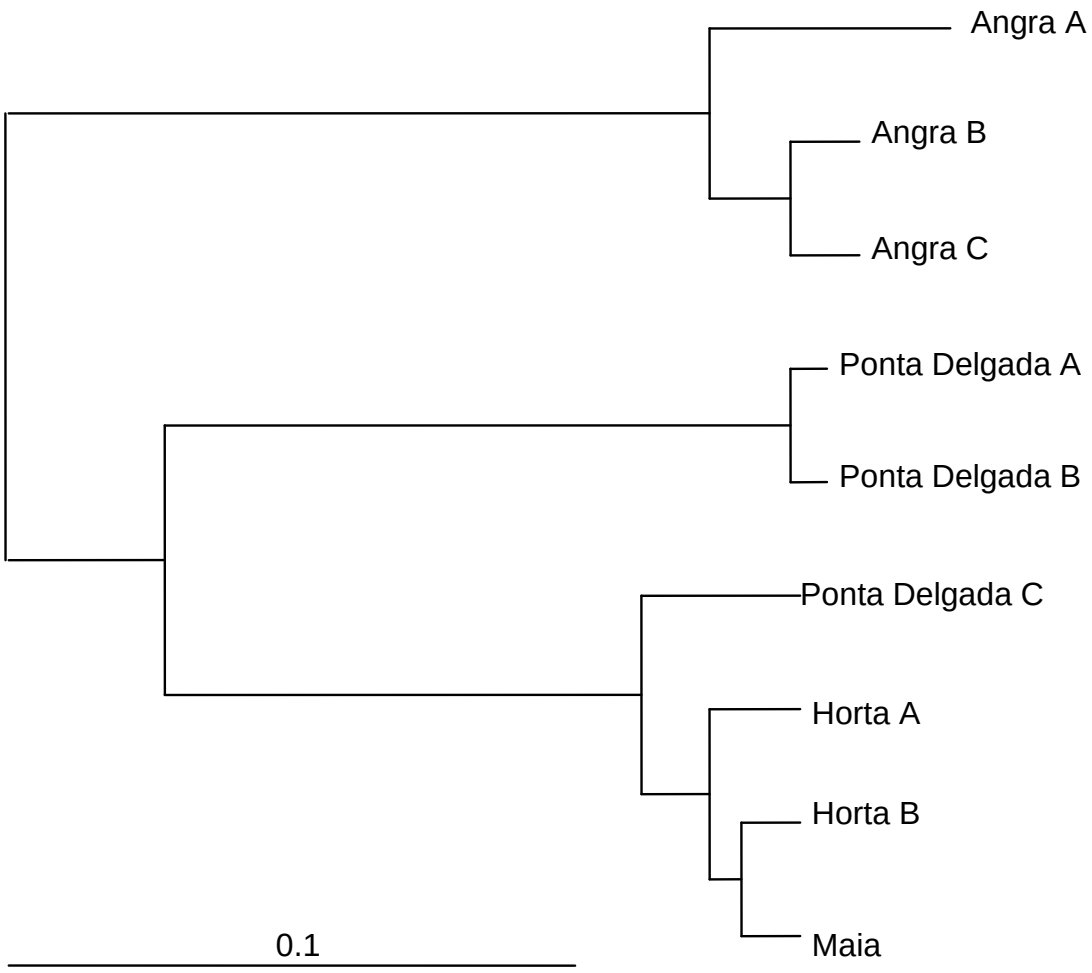

Figure 2–10. Distance diagram calculated using Genetic Distance Analysis. Length of branches represents genetic distance according to Nei's identity method. The longer the branch compared to the scale, the larger the distance between the subpopulations. All the subpopulations sampled from the Islands are represented.

The scenarios tested with DIYABC showed a low probability of being the scenario of introduction of the subpopulations to the islands for all except for scenarios 3 and 5, which showed higher probability after 5,000 simulated data were added into the logistic regression. Scenario 5 had a 50% probability, while the Scenario 3 had a 40% probability of being the introduction scenario (Figure 2-11).

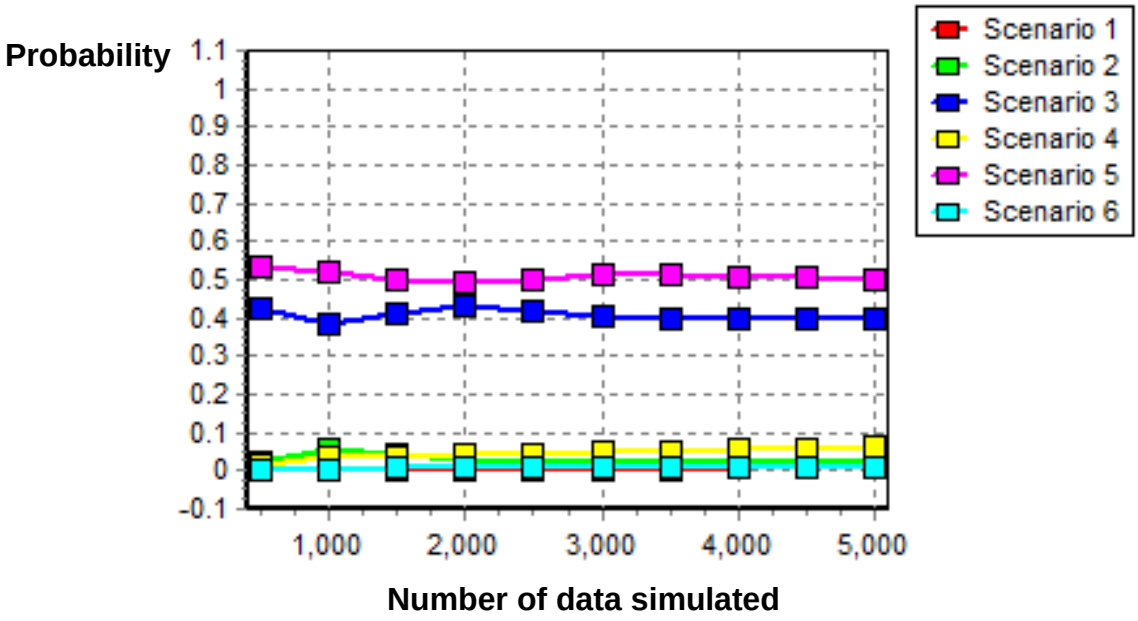

Figure 2-11. Logistic regression of the simulated data probability for the different scenarios tested in DIYABC. Probability varies between 0 and 1. All scenarios are within a 95% confidence interval for the simulations.

Discussion

The phylogenetic tree obtained from the mtDNA showed a clear pattern where the combined genes for the subpopulations were separated into two major branches. The subpopulations from Ponta Delgada and Maia were grouped in a closely related group. This is not surprising because the Islands of São Miguel and Santa Maria, where these towns are located respectively, are geographically close (Figure 2-1) and there is a lot of traffic of goods and visitors between them. The Horta subpopulation showed a distinct separation from all the other subpopulations being only closer to one of the Angra samples (AB-4). The Angra subpopulations were distributed between the two main branches showing close relationships to both the Ponta Delgada and the Horta subpopulations. Based upon the mtDNA data alone, that there is such a cleft between the two major branches would suggest that there were multiple introductions of this species to the islands with at least two major introductions. Furthermore, it seems that

the species was first introduced in the two major cities (Angra and Ponta Delgada), and from there spread out to the smaller towns in the other islands.

The genealogical relationships among the 22 haplotypes found for *C. brevis* in the Azorean subpopulations show a similar pattern to the phylogenetic tree, where two very distinct haplotype clusters were observed, the first connecting the Ponta Delgada subpopulations amongst each other and close to Maia, while the Angra subpopulations appeared as a more distant group with more changes in the haplotype, compared with the Ponta Delgada populations. Contrastingly, Horta showed a separate cluster with the haplotypes very close amongst each other but more distant from the one haplotype present in the AB population. These results confirm what was found with the phylogenetic analysis. The high number of haplotypes indicated that there is high genetic diversity in these subpopulations. This is further confirmed by the AMOVA results that showed a high value for haplotype F_{ST}, which was significantly different from 0, showing a high percentage of variance among populations. The high genetic diversity among subpopulations suggests that there was a multiple introduction scenario, because if this had been a single introduction, the diversity would be very low due to a founder effect where only a portion of the diversity of the original subpopulation is represented in the founder subpopulation, the overall diversity of the separated subpopulation is lower than that of the original subpopulation. Another common reason for loss in diversity is the bottleneck effect, which happens when a population goes through a period where the effective size (Ne) is reduced and this can result in a loss of heterozygosity, which is not the case here. A common founder effect event in nature occurs when a small group of emigrants from an established subpopulation goes on to found a new subpopulation. The mtDNA data indicates that there is high diversity

among subpopulations and that therefore the subpopulations are not under the founder effect, and this can be explained by the fact that there were multiple introductions.

The Azorean subpopulations, however, may still be exchanging genes. This is corroborated by the fact that the gene flow of Nm= 3.7 per generation is high. For isolated populations this value is usually bellow 1.6 (Hartl and Clark 2007). Over time, a high rate of gene flow can lead to maintenance of genetic similarity between subpopulations, but because this is a recent introduction the high rate of gene flow between the different islands subpopulations is not reflected in the genetic diversity, which is high. Also the mean expected heterozygosity was not significantly different from the observed heterozygosity, which suggests that there was no decrease in heterozygosity which would be expected in isolated subpopulations and shows that there is gene flow between the subpopulations.

F_{ST} values show the proportion of the total genetic variance contained in a subpopulation relative to the total genetic variance and high values mean considerable degree of difference among subpopulations (Hartl and Clark 2007). That there was great genetic diversity in the subpopulations studied herein, as shown by the high overall values of F_{ST} (≥0.15 for all methods calculated), and that there was a very low level of inbreeding for each population (F_{IS} ≈0) suggests that there is gene flow. The F_{IS} values show the proportion of the variance in the subpopulation contained in an individual as compared to its subpopulation, and the higher the value the higher the level of inbreeding (Hartl and Clark 2007). A high level of inbreeding would be expected if a subpopulation were completely isolated, so if the subpopulations of the islands were truly isolated from each other the inbreeding values would be different from 0. The F_{IT} values take into account both the F_{ST} and F_{IS} values to calculate the probability of

autozygosity of an inbred individual relative to the subpopulation as a whole (Hartl and Clark 2007). The low values obtained were again indicative that there is high diversity and low inbreeding as would be expected if there was still exchange of genes between the subpopulations, or if there were gene flow from outside subpopulations. This supports the hypothesis that there were several introductions that may still be occurring.

The distance diagram calculated using the GDA software (Figure 2-10) showed the Angra subpopulation separated from the other subpopulations. This is different from what was found with the mtDNA data, where the Angra subpopulations were close to both the Ponta Delgada and the Horta subpopulations. In the distance diagram the Horta subpopulations were close to the Maia subpopulation and the Ponta Delgada subpopulation. When comparing the F_{ST} values between the subpopulations (Table 2-15) the p-values encountered showed a similar pattern where the Horta and Maia subpopulations did not have significant differences between each other, while the Angra and Ponta Delgada subpopulations had values that were not different between some of the subpopulations. Even though the microsatellite results seem to contradict the findings of the mtDNA, the pattern of multiple introductions is still supported. In both cases the data showed that there is high diversity. Mitochondrial DNA is maternally inherited while the microsatellite loci are co-dominant markers with each allele being contributed by each parent. Over evolutionary time, microsatellites can reflect potential homoplasy. However, because the *C. brevis* introduction is recent, the microsatellites are a good marker to study the relationships between the subpopulations. This can account for the differences encountered when calculating the genetic distance between subpopulations. However, in both mtDNA and microsatellites, the data showed that

there were two distinct introductions in the two major islands and from there they may have spread to the smaller islands.

The scenarios tested with the DIYABC software were created based on what was probable given the previous results. Of all the scenarios tested, the two that showed the highest probability were scenarios 3 and 5 where the subpopulations of Horta and Maia were considered to have separated from the subpopulation of Ponta Delgada, while the Angra subpopulations were independent. The fact that scenario 4, where all of the subpopulations were assumed to have come from a common ancestral population, had near 0 probability confirms that the hypothesis of multiple introductions cannot be rejected. The data showed that there have been at least two separate introductions of *C. brevis* into the Azorean archipelago, and that the infestations are spreading through the islands. The reason why the probability of the scenarios is not higher might be due to the continuous flow of genes into the subpopulations from multiple introductions that might confound the analysis.

The present study has confirmed that multiple introductions have occurred to the Azorean islands yet further studies need to be made to resolve this issue completely. The reason why two different genetic markers were used in this study, and are normally used, is because the whole aspect of the population has to be taken into account. Using only mtDNA can induce an error if a set of populations of a species happens to have high variability for the chosen gene and because it is only maternally inherited. The opposite can also happen where there is very little variability for a particular gene that masks genetic divergence between two populations. More importantly the statistical tests used to calculate these relationships always present the caveat of sample size. When looking at studies of introductions of species, namely termites that have such a

complex life cycle, sample size is crucial. Not only in terms of actual numbers of termites, but also in terms of number of loci/genes, and number of alleles. Many population genetics studies with termites have emphasized the caveat that a large sample is necessary (Husseneder et al. 2002, Austin et al. 2006, Vargo et al 2006). However, the number of alleles found in the subpopulations of *C. brevis* from the Azores is similar to the number of alleles found by Fuchs et al. (2003) for *C. secundus*, showing that the choice to use *C. secundus* microsatellite primers for *C. brevis* is a good choice for these studies.

Cryptotermes brevis has a cryptic nature, spending most of its life cycle inside wood. Collecting such a species in urban environment, where it is mostly found, proves difficult. The permission of homeowners is usually implied and dispersal flight season happens only once a year. For this reason it is hard to gather large amounts of specimens for this species. Population genetic studies of *C. brevis* have not been performed previously, and so there were no primers specifically designed for the species, that would allow for better resolution of results. This study presents itself as the first step in studies of *C. brevis* population genetics. The results obtained from both the mtDNA and the microsatellites helped resolve a long standing question, despite the caveats. The Azorean population was introduced multiple times and it is most likely still receiving gene flow from periodic new introductions. It has also been shown by this study that the species is spreading from island to island. For these aforementioned reasons it has already been recommended and taken into action to have a more rigorous regulation over what materials enter the islands, especially wood items, like crates, or even furniture which can potentially harbor termites (Decreto Legislativo Regional n.º 22/2010/A de 30 de Junho de 2010).

Future directions call for a more detailed study with primers specifically designed for this species which would allow analysis over a large number of loci and the inclusion of a higher number of termites per sample. Also one could possibly include the recently identified introductions to other islands in the Azores over fixed periods of time to have a better grasp and better comprehension of the population dynamics within and between the islands. Recent introductions are a very interesting phenomenon to study because the evolution of the population dynamics can be followed in real time, and can help further the knowledge of a complex breeding system like the termites. Understanding the evolutionary processes that introduce and maintain genetic variability among subpopulations can help predict the dispersal pattern and therefore assist with the identification of potential spread.

CHAPTER 3
ORIGIN OF THE AZOREAN POPULATION OF THE WEST INDIAN DRYWOOD TERMITE

Cryptotermes brevis is a wood pest and is well distributed in the tropical and subtropical areas (Figure 1-2). This species is believed to be endemic to Chile and Peru, where it can be found infesting living trees, away from any structure (Scheffrahn et al. 2008). It is possible that this is the point of origin for many of the current infestations of *C. brevis* around the world, through the elevated traffic of wooden ships from the Spanish sailors that ruled the sea in the 1500's, transporting goods and possibly termites with them (Scheffrahn et al. 2008). This mode of dispersion, through maritime routes may have been the cause of the introduction of the species in the Azorean archipelago. The association between termite invasiveness through marina areas has already been shown (Hochmair and Scheffrahn 2010) for subterranean termites. Also, the import of wood for construction may be another avenue of entry for invasive drywood termite species into islands (Scheffrahn and Crowe 2011).

This study aims to resolve the possible origin of the populations in the Azores using genetic markers to compare the Azorean populations with populations from various parts of the world. For this to be achieved, microsatellite analysis along with mtDNA analysis needs to be combined.

Material and Methods

Termites

Termites from the Azores were collected as described in the previous chapter. The additional specimens used were selected from the UF termite collection at the Fort Lauderdale Research and Education Center, Davie Florida. These specimens were collected by various collaborators in recent decades from various parts of the globe, and

are preserved in vials in 85% non-denatured ethanol at room temperature. Specimens were chosen from the database to cover different parts of the world (Table 3-1).

Table 3-1. Exact locations of collection sites from the world collection and number of individuals (N) used for analysis.

Continental Area	Country	Sample name	Database Code	N	Latitude	Longitude	Collector
Africa	Republic of Congo	AFR	AFR302	5	-4.3022	15.3052	Nixon
Oceania	Australia	AUS	AUS77	5	-25.5405	152.7155	Sch*
South America	Venezuela	VZ	SA092	5	10.4980	-66.9212	Helm
South America	Chile	CLA	CL3	20	-27.8013	-70.1333	Ripa
South America	Chile	CLB	CL47	20	-33.0233	-71.6166	Paola
South America	Peru	PEA	PE101	20	-11.9790	-77.0900	Krecek
South America	Peru	PEB	PE152	20	-13.0870	-76.3970	Garcia
North America	United States	FLD	FL98	20	24.5551	-81.7795	Sch*
Central America	Honduras	HN	HN666	20	14.0087	-87.0144	Sch*
Central America	Costa Rica	CR	CTA127	15	9.9277	-84.0771	Suiter
Central America	El Salvador	ELS	CTA112	20	13.6633	-89.2580	Nixon

Sch*- Scheffrahn

DNA extraction and amplification

The procedures for DNA extraction and amplification were the same as described in the previous chapter. Five termites per population were used individually for the mtDNA work, while the microsatellite analysis used between five to twenty termites per population based on the availability of samples in the collection (Table 3-1). The PCR products for the mtDNA work were sequenced at Laragen Inc. (Culver City, CA).

Data analysis

Mitochondrial DNA. Phylogenetic and molecular evolutionary analyses for mitochondrial 16S rRNA gene and Cytb gene were conducted using MEGA version 5 (Tamura et al. 2011). Sequences were aligned in MEGA version 5 using the ClustalW method set at default, as recommended by the software. The sequences from 16S rRNA and Cytb were combined in order to create more robust sequence data. After the sequences were aligned they were run through the Gblocks software (Talavera and Castresana 2007) to eliminate poorly aligned positions and divergent regions of the alignment. Distance matrixes for number of nucleotides were created for all the islands and equal sequences were eliminated from the analysis, and a representative sequence was chosen to represent those sequences. The distance p-value was then calculated to test if the data from the sequences were good to analyze. The remaining sequences were then analyzed using the UPGMA method (Sneath and Sokal 1973). The bootstrap consensus tree was inferred from 5000 replicates (Felsenstein 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are based on the number of base substitutions per site. Sequences from *K. flavicollis* for both genes were used as an out-group. These representative sequences were obtained from GenBank by using BLAST for each gene. For mitochondrial 16S rRNA gene the *K. flavicollis* sequence used was reported in Szalanski et al. (2004) and identified by accession number AY486437. For Cytb gene, the *K. flavicollis* sequence used was the one reported in Legendre et al. (2008) and identified with the accession number EU253919.

Microsatellites. For the microsatellite analysis the Peak Scanner™ Software v1.0 (© Copyright 2006, Applied Biosystems) was used to score the microsatellites. The

EXCEL Microsatellite toolkit (Park 2001) was used to estimate the number and frequency of alleles, as well as observed and expected heterozygosities. An ANOVA was used to test if there were significant differences between expected and observed heterozygosities, and allele frequencies between populations using SAS (2003). FSTAT 2.9.3.2 (Goudet, 2001) and GENEPOPV4 (© F. Rousset) were used to calculate F_{ST}, F_{IS}, and F_{IT}, to respectively quantify the loss in heterozygosity, calculate linkage disequilibrium, and establish the frequency of null alleles. They were also used to estimate the modified Wright's F-statistics by Weir and Cockerham's (1984) Θ_{ST}, Nei's (1987) G_{ST}, and Michalakis and Excoffier's (1996) ρ_{ST}. The gene flow was quantified in terms of migrants exchanged among subpopulations per generation (Nm), calculated using GENEPOPV4 (© F. Rousset). Genetic distance was calculated using Nei's (1972) method and a genetic distance matrix and tree was generated using Genetic Distance Analysis 1.1 (Lewis and Zaykin 2001).

The program STRUCTURE (Pritchard et al. 2000) was used to evaluate the subpopulation genetic structure. This method uses the individual multilocus genotypic data to evaluate models assuming different numbers of genetic clusters (K), and each individual's genome is partitioned into fractions that represent the ancestry in each inferred cluster. The models used assumed some degree of admixture between subpopulations but considered the allele frequencies independent. The other parameters of the models were set at the default of the program. The models were run for a length of 100,000 data collection generations and 50,000 Markov chain Monte Carlo (MCMC) generations. Several subsets of data were analyzed. The number of clusters used varied between K=2 to K=10. The partitioned data evaluated was: Islands Only data; All Subpopulations data; and Islands and Endemic Only data (Table 3-2).

Table 3-2. Subpopulations included in the partitioned data used for the STRUCTURE analysis, codes of the populations are given according to the codes previously described.

Partitioned data	Subpopulations included
Islands only	AA, AB, AC, PDA, PDB, PDC, HA, HB, M
Islands and Endemic	AA, AB, AC, PDA, PDB, PDC, HA, HB, M, CLA, CLB, PEA, PEB
All Subpopulations	AA, AB, AC, PDA, PDB, PDC, HA, HB, M, CLA, CLB, PEA, PEB, HN, VZ, CR, FLD, ELS, AFR, AUS

To test different scenarios of origin the program DIYABC (Cornuet et al. 2010) was used. The assumptions used for the models were that there was an ancestral population of size N2 from where the subpopulations originated. The size of the ancestral population was assumed to be higher than that of the other subpopulations N2>N1, N3, N4, N5, and N6 (Figures 3-1 to 3-6). For the computations the subpopulations of geographical region were considered as one single subpopulation region. The data of the Azores subpopulations AA, AB, AC, PDA, PDB, PDC,HA, HB, and M were merged into Population 1 (default nomenclature in the program), the data for the South American subpopulations CLA, CLB, PEA, PEB, and VZ were merged into Population 2, Central American subpopulations HN, CR, and ELS into Population 3, the North American subpopulation FLD was represented as Population 4, the Australian subpopulation AUS was represented as Population 5, and the African subpopulation was represented as Population 6. A generalized mutation model (GSM) was assumed for the microsatellite loci, with a gamma distribution for the mutation rate bounded between $5x10^{-4}$ and $5x10^{-3}$. The reference table was created for 60,000 simulations (10,000 per scenario). The posterior probability of each scenario was assessed by a polychotomous weighted logistic regression, and the number of simulated data used for

the logistic regression was 6,000 with 600 real data used for comparison. Six scenarios of introduction were tested (scenarios 1 to 6) with no admixture assumed. In all scenarios, Population 2 was assumed to have an effective size N2 for some time (td) and eventually at a time (ta) the populations were assumed to split from this ancestral population. Population 2 is assumed to be the ancestral population because the subpopulations merged in this population include the endemic subpopulations of Chile and Peru. In scenario 1, for simplicity of the model all of the populations were assumed to split at the same unknown time (ta), with Population 2 continuing to exist after the spilt (Figure 3-1).In scenario 2, at time tb, Population 1 (Azores) splits from Population 2, with it continuing to exist after the two split. The remaining populations split form Population 2 at an earlier time (ta). Populations 1, 3, 4, 5, and 6 were assumed to have a random effective size smaller than Population 2 (N2>N1, N3-N6) (Figure 3-2). In scenario 3, at time tb Population 1 splits from Population 3, with population 3 continuing to exist after the split. The remaining populations split form Population 2 at an earlier time (ta.) Populations 1, 3, 4, 5, and 6 were assumed to have a random effective size smaller than Population 2 (N2>N1, N3-N6), and Population 1 was assumed to have a random effective size smaller or equal to N3 (Figure 3-3). In scenario 4, at time tb Population 1 splits from Population 4, with Population 4 continuing to exist after the split. The remainder populations split form Population 2 at an earlier time (ta). Populations 1, 3, 4, 5, and 6 were assumed to have a random effective size smaller than Population 2 (N2>N1, N3-N6), and Population 1 was assumed to have a random effective size smaller or equal to N4 (Figure 3-4). In scenario 5, at time tb Population 1 split from Population 5, with Population 5 continuing to exist after the split. The remaining populations split from Population 2 at an earlier time (ta). Populations 1, 3, 4, 5, and 6

were assumed to have a random effective size smaller than Population 2 (N2>N1, N3-N6), and Population 1 was assumed to have a random effective size smaller or equal to N5 (Figure 3-5). In scenario 6, at time tb Population 1 split from Population 6, with Population 6 continuing to exist after the split. The remaining populations split form Population 2 at an earlier time (ta). Populations 1, 3, 4, 5, and 6 were assumed to have a random effective size smaller than Population 2 (N2>N1, N3-N6), and Population 1 was assumed to have a random effective size smaller or equal to N6 (Figure 3-6).

An extra scenario was added to test the validity of the Population 2 as the ancestral population (scenario 2A). In scenario 2A, all the Populations were assumed to have emerged from a common ancestral population of size NA, in which NA was assumed to be higher than the effective size of the remainder populations (NA>N1-6). All the populations including Population 2 were assumed to have diverged from the ancestral population at a determined time (td), with the ancestral population not continuing to the present time (Figure 3-7). The posterior probability of each scenario was assessed by a polychotomous weighted logistic regression. The reference table created for the analysis had 20,000 simulations (10,000 per scenario), and the number of simulated data used for the logistic regression was 2,000 with 200 real data used for comparison.

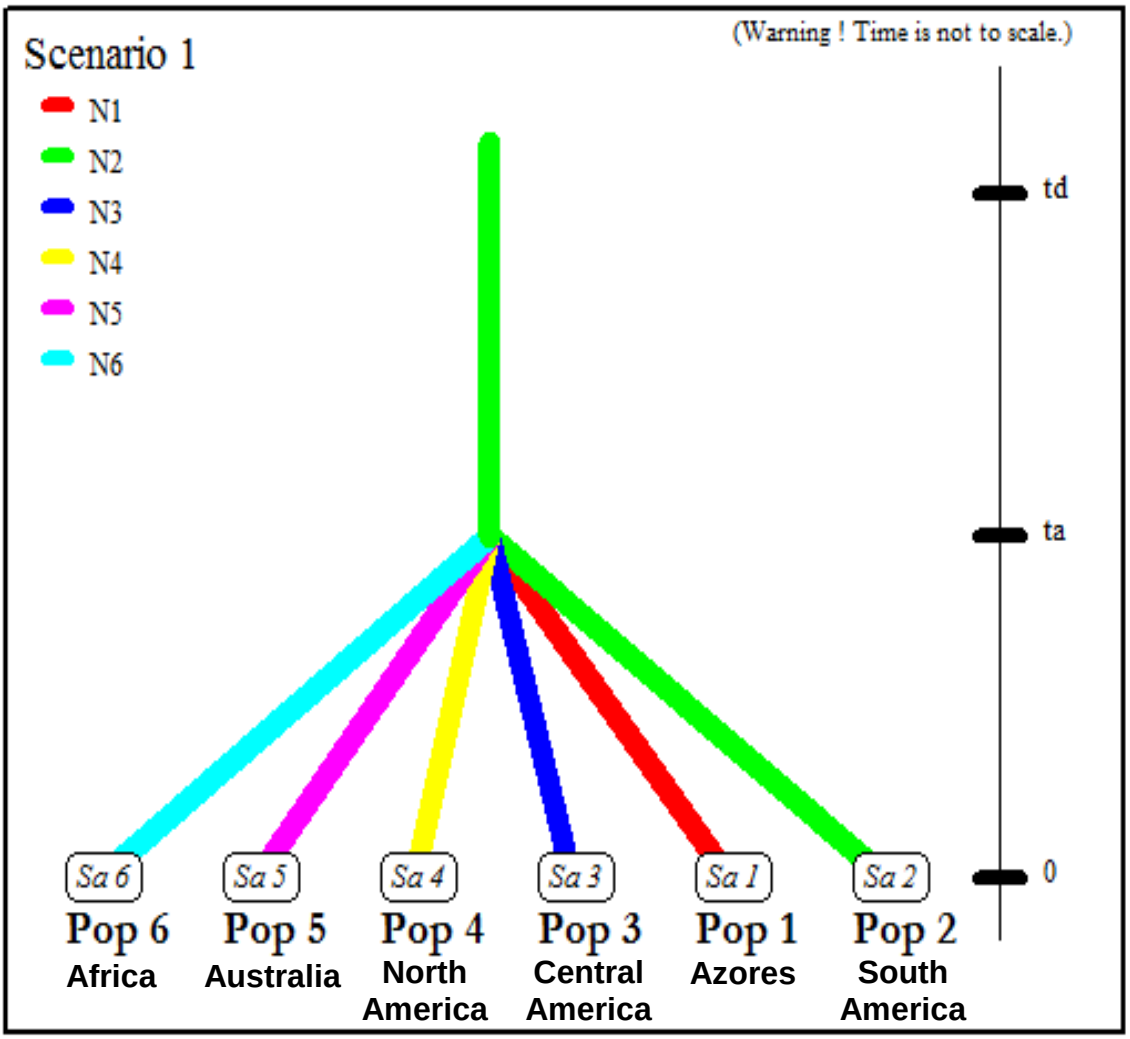

Figure 3-1. Scenario 1 of the origin of the Islands subpopulations tested with the DIYABC software. The program assumed an effective random population size for each population N1, N2, N3, N4, N5 and N6. Computations were made based on the samples from each population (Sa 1-6). The Population 2 was assumed to be the ancestral population existent for a determined time (td). The splitting of populations was assumed to happen at a determined time (ta), with the condition that td >ta. Each Population corresponds to a different Geographical Region with the different sites per region merged into one single Population. Population 1 was the subpopulation of the Azores, Population 2 was the subpopulations of the South American samples, Population 3 was the subpopulation of the Central American samples, Population 4 was the subpopulation of the North American samples, Population 5 was the subpopulation of Australia, and Population 6 was the subpopulation of Africa. The scenario assumed that populations 1, 3, 4, 5, and 6 were part of one ancestral population of size N2 with the condition that N2>N1-N6, and that subsequently they split into the different populations with Population 2 continuing its existence.

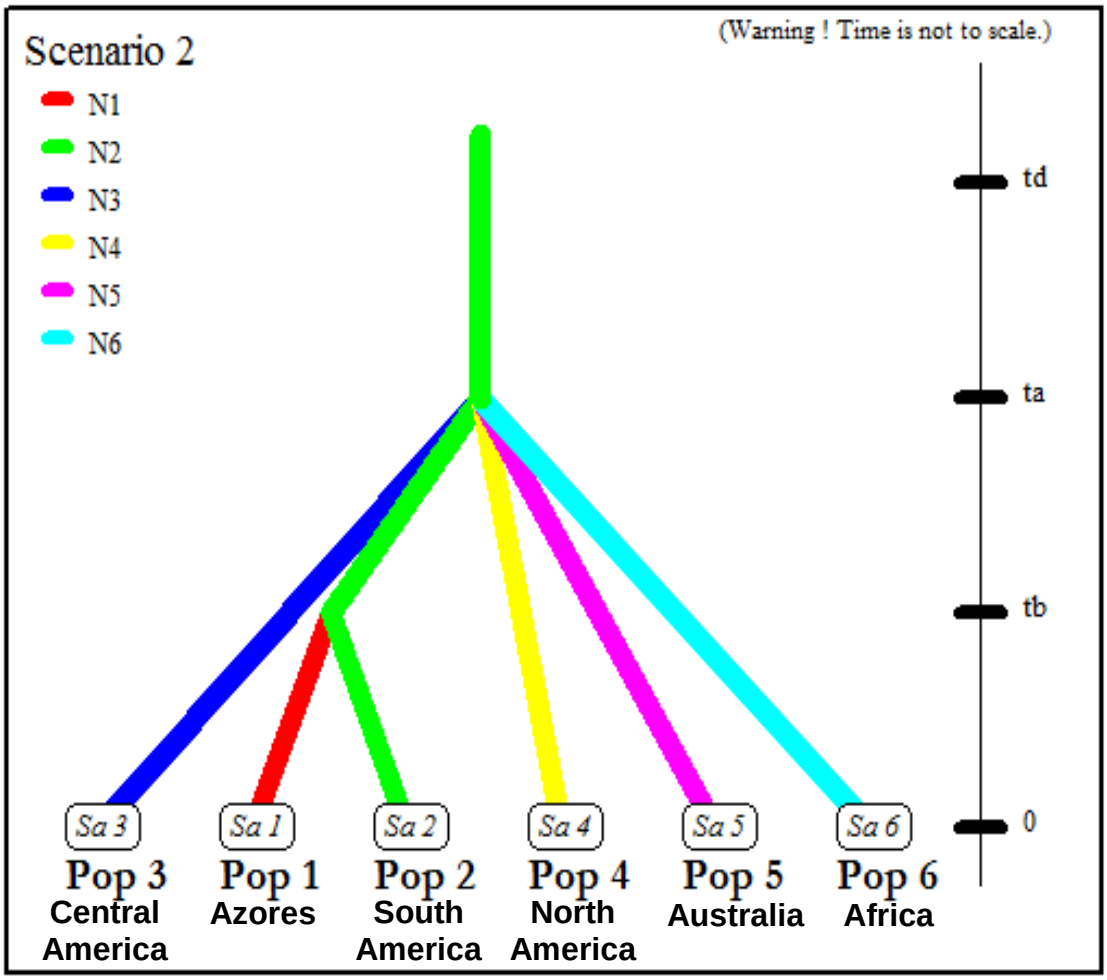

Figure 3-2. Scenario 2 of the origin of the Islands subpopulations tested with the DIYABC software. The program assumed an effective random population size for each population N1, N2, N3, N4, N5 and N6. Computations were made based on the samples from each population (Sa 1-6). The Population 2 was assumed to be the ancestral population existent for a determined time (td). The splitting of populations was assumed to happen at a determined time (ta and tb), with the condition that td >ta>tb. Each Population corresponds to a different Geographical Region with the different sites per region merged into one single Population. Population 1 was the subpopulation of the Azores, Population 2 was the subpopulations of the South American samples, Population 3 was the subpopulation of the Central American samples, Population 4 was the subpopulation of the North American samples, Population 5 was the subpopulation of Australia, and Population 6 was the subpopulation of Africa. The scenario assumed that populations 3, 4, 5, and 6 were part of one ancestral population of size N2 with the condition that N2>N3-N6, and that subsequently they split into the different populations at time ta with Population 2 continuing its existence, while Population 1 split at a later time from Population 2 at time tb with the condition that N2>N1.

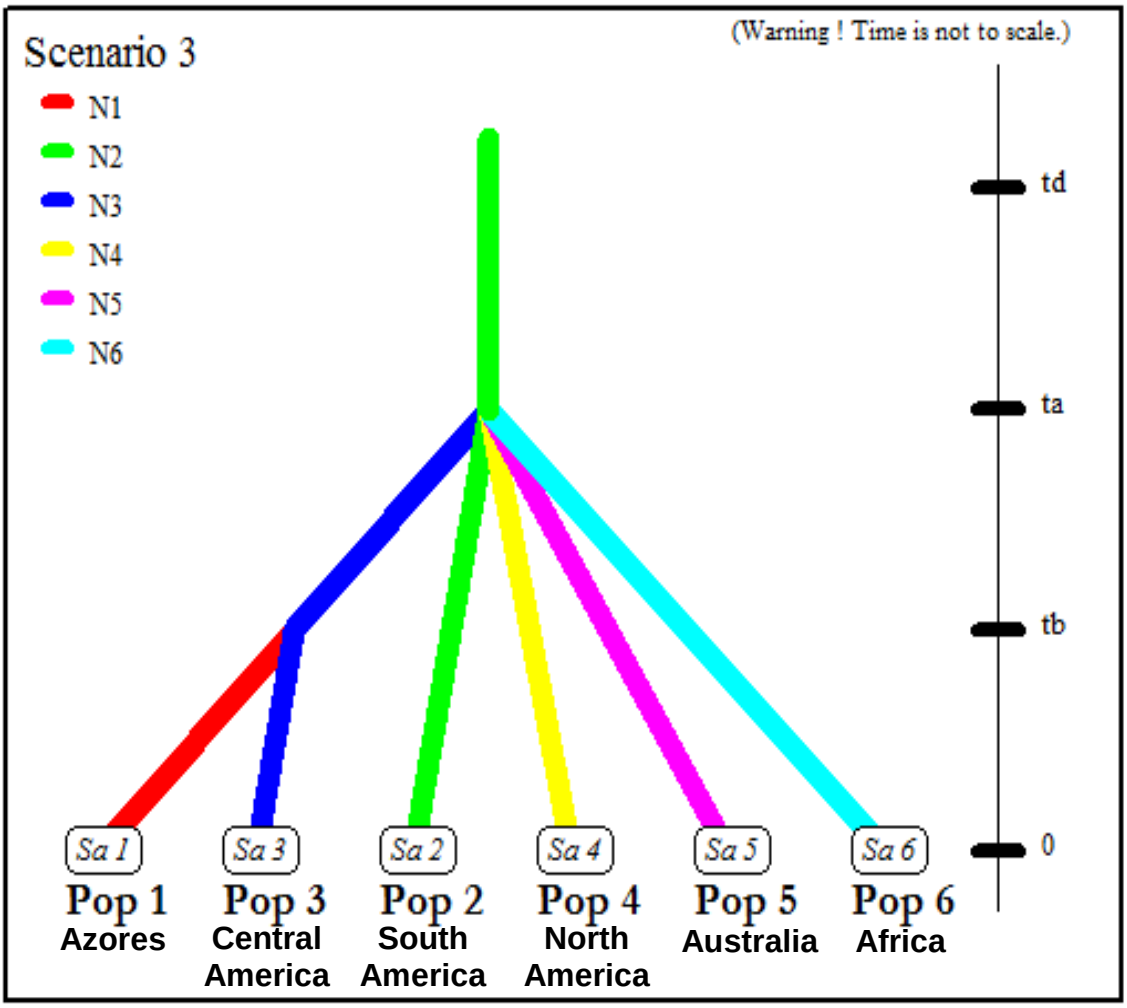

Figure 3-3. Scenario 3 of the origin of the Islands subpopulations tested with the DIYABC software. The program assumed an effective random population size for each population N1, N2, N3, N4, N5 and N6. Computations were made based on the samples from each population (Sa 1-6). The Population 2 was assumed to be the ancestral population existent for a determined time (td). The splitting of populations was assumed to happen at a determined time (ta and tb), with the condition that td >ta>tb. Each Population corresponds to a different Geographical Region with the different sites per region merged into one single Population. Population 1 was the subpopulation of the Azores, Population 2 was the subpopulations of the South American samples, Population 3 was the subpopulation of the Central American samples, Population 4 was the subpopulation of the North American samples, Population 5 was the subpopulation of Australia, and Population 6 was the subpopulation of Africa. The scenario assumes that populations 3, 4, 5, and 6 were part of one ancestral population of size N2 with the condition that N2>N3-N6, and that subsequently they split into the different populations at time ta with Population 2 continuing its existence while Population 1 split at a later time from Population 3 at time tb with the condition that N3>N1.

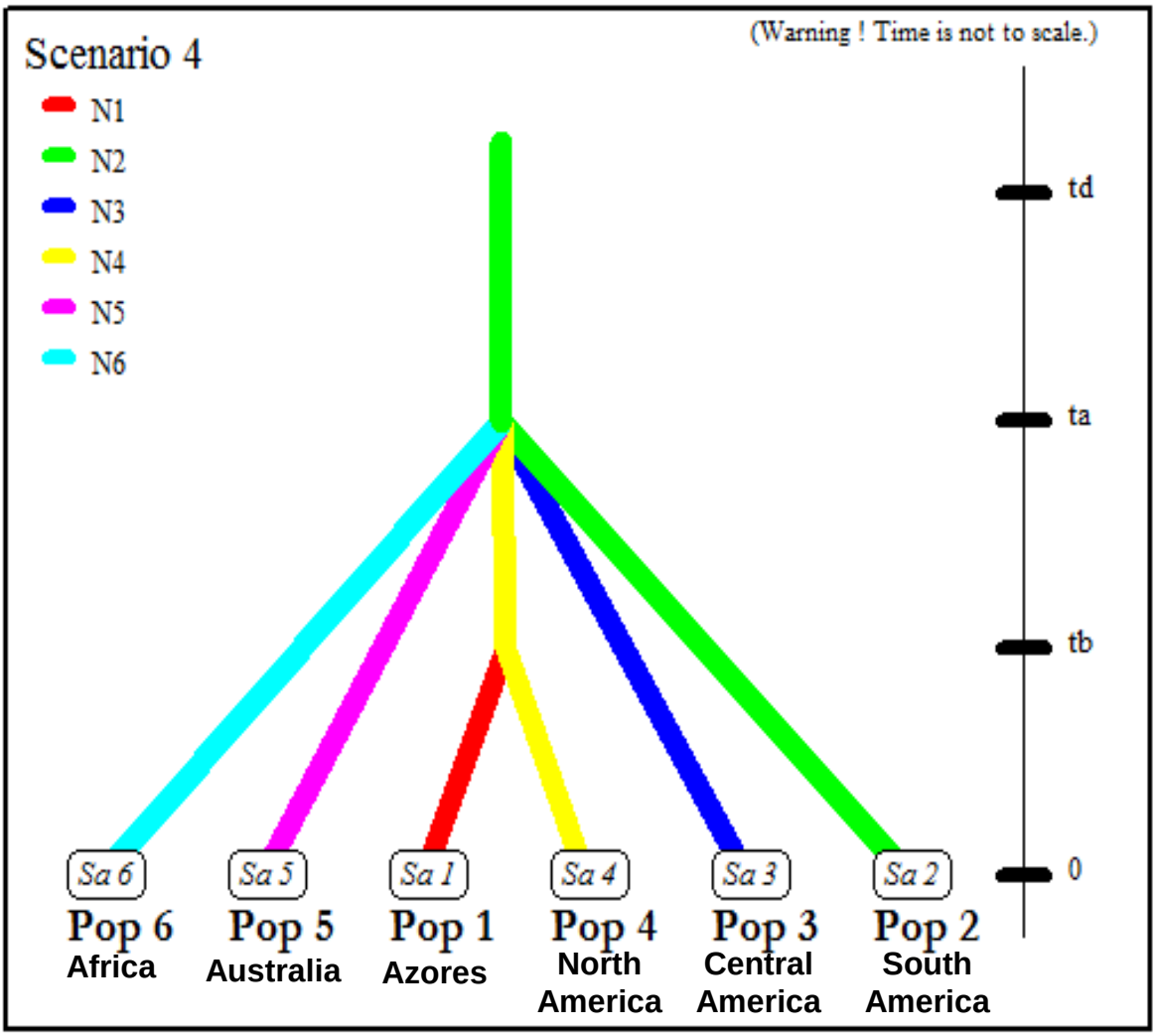

Figure 3-4. Scenario 4 of the origin of the Islands subpopulations tested with the DIYABC software. The program assumed an effective random population size for each population N1, N2, N3, N4, N5 and N6. Computations were made based on the samples from each population (Sa 1-6). The Population 2 was assumed to be the ancestral population existent for a determined time (td). The splitting of populations was assumed to happen at a determined time (ta and tb), with the condition that td >ta>tb. Each Population corresponds to a different Geographical Region with the different sites per region merged into one single Population. Population 1 was the subpopulation of the Azores, Population 2 was the subpopulations of the South American samples, Population 3 was the subpopulation of the Central American samples, Population 4 was the subpopulation of the North American samples, Population 5 was the subpopulation of Australia, and Population 6 was the subpopulation of Africa. The scenario assumes that populations 3, 4, 5, and 6 were part of one ancestral population of size N2 with the condition that N2>N3-N6, and that subsequently they split into the different populations at time ta with Population 2 continuing its existence while Population 1 split at a later time from Population 4 at time tb with the condition that N4>N1.

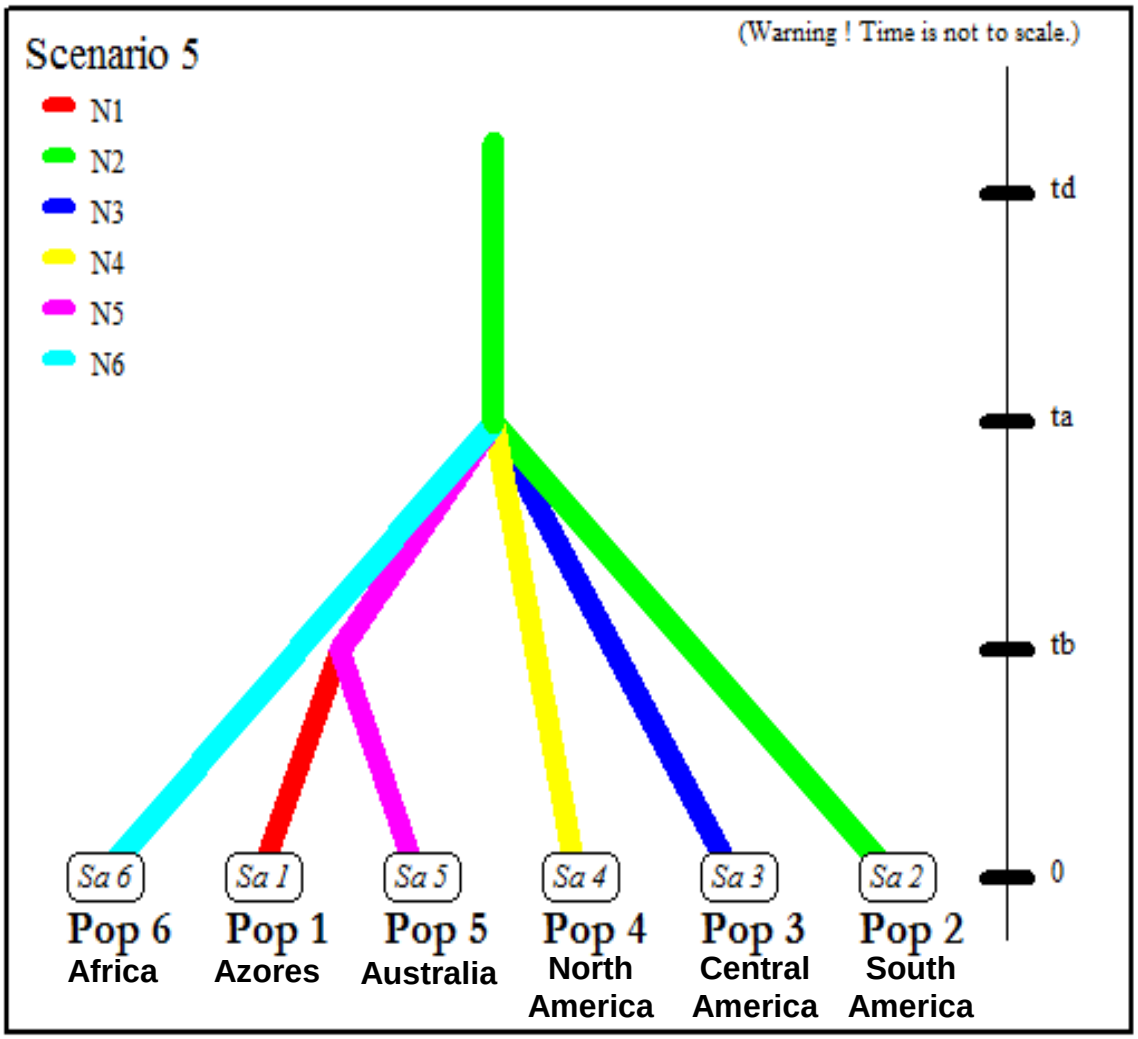

Figure 3-5. Scenario 5 of the origin of the Islands subpopulations tested with the DIYABC software. The program assumed an effective random population size for each population N1, N2, N3, N4, N5 and N6. Computations were made based on the samples from each population (Sa 1-6). The Population 2 was assumed to be the ancestral population existent for a determined time (td). The splitting of populations was assumed to happen at a determined time (ta and tb), with the condition that td >ta>tb. Each Population corresponds to a different Geographical Region with the different sites per region merged into one single Population. Population 1 was the subpopulation of the Azores, Population 2 was the subpopulations of the South American samples, Population 3 was the subpopulation of the Central American samples, Population 4 was the subpopulation of the North American samples, Population 5 was the subpopulation of Australia, and Population 6 was the subpopulation of Africa. The scenario assumes that populations 3, 4, 5, and 6 were part of one ancestral population of size N2 with the condition that N2>N3-N6, and that subsequently they split into the different populations at time ta with Population 2 continuing its existence while Population 1 split at a later time from Population 5 at time tb with the condition that N5>N1.

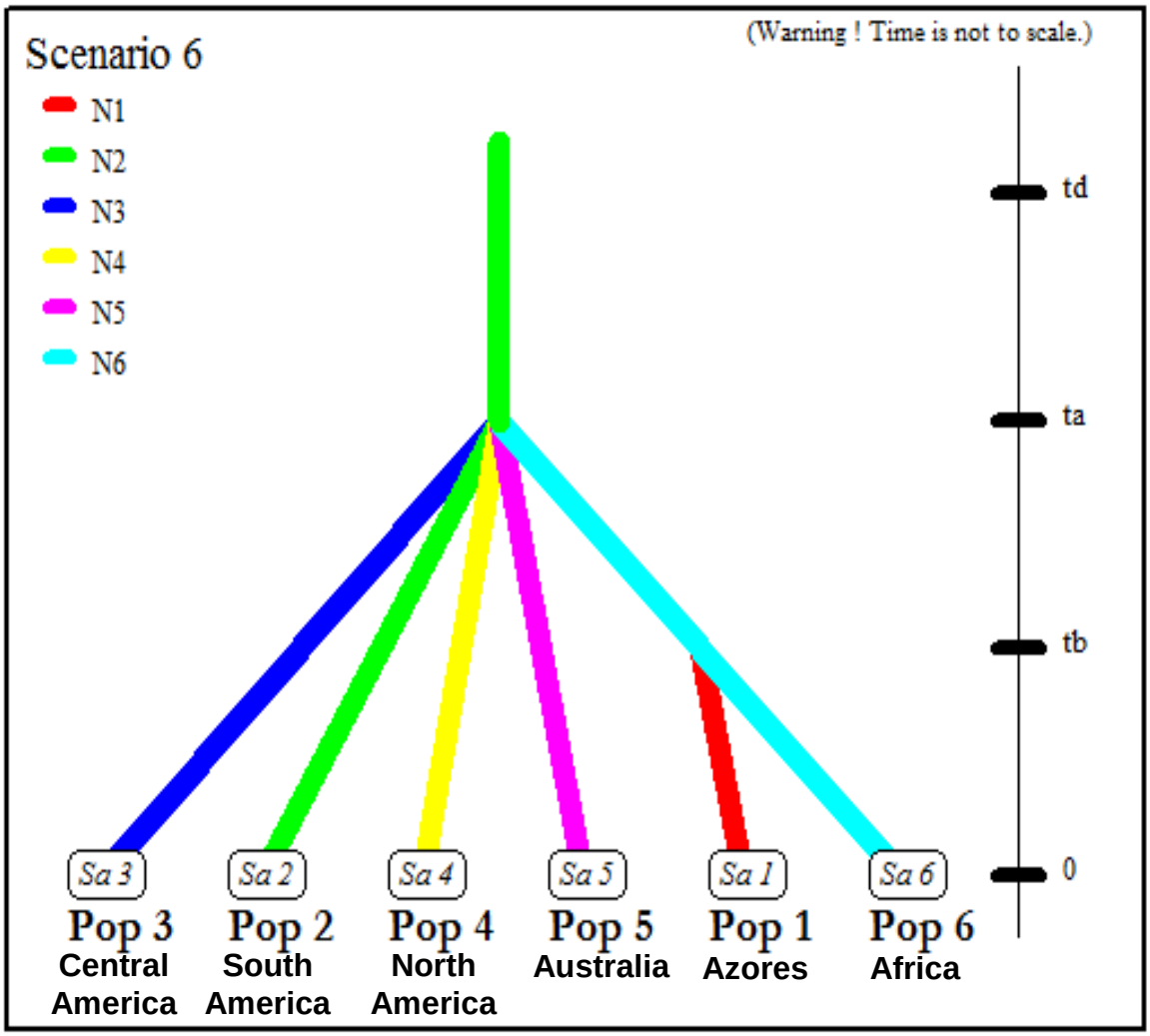

Figure 3-6. Scenario 6 of the origin of the Islands subpopulations tested with the DIYABC software. The program assumed an effective random population size for each population N1, N2, N3, N4, N5 and N6. Computations were made based on the samples from each population (Sa 1-6). The Population 2 was assumed to be the ancestral population existent for a determined time (td). The splitting of populations was assumed to happen at a determined time (ta and tb), with the condition that td >ta>tb. Each Population corresponds to a different Geographical Region with the different sites per region merged into one single Population. Population 1 was the subpopulation of the Azores, Population 2 was the subpopulations of the South American samples, Population 3 was the subpopulation of the Central American samples, Population 4 was the subpopulation of the North American samples, Population 5 was the subpopulation of Australia, and Population 6 was the subpopulation of Africa. The scenario assumes that populations 3, 4, 5, and 6 were part of one ancestral population of size N2 with the condition that N2>N3-N6, and that subsequently they split into the different populations at time ta with Population 2 continuing its existence while Population 1 split at a later time from Population 6 at time tb with the condition that N6>N1.

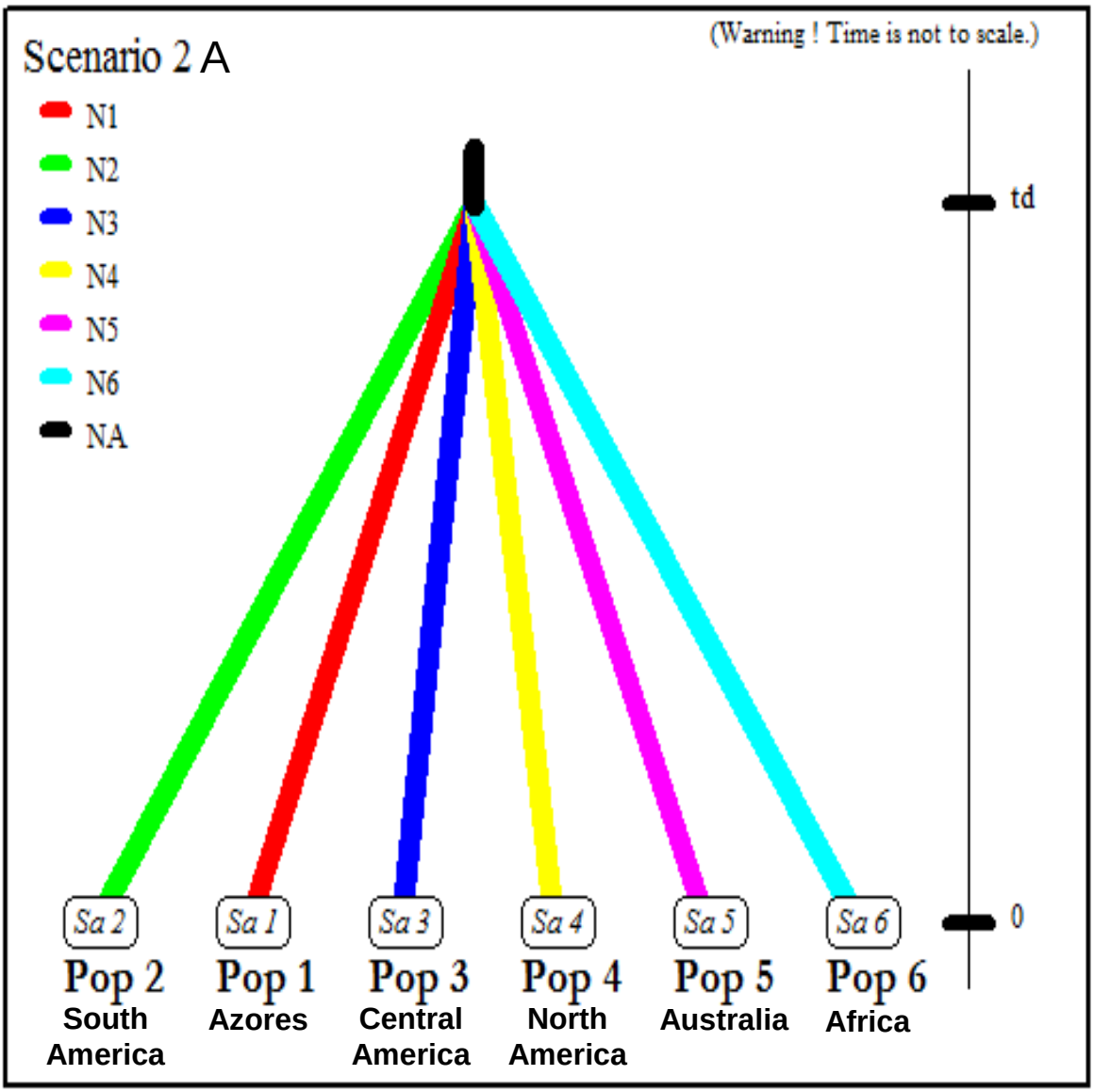

Figure 3-7. Scenario 2A of origin of the populations tested with the DIYABC software. The program assumed an effective random population size for each population N1, N2, N3, N4, N5, and N6. Computations were made based on the samples from each population (Sa 1-6). Each Population corresponds to a different Geographical Region with the different sites per region merged into one single Population. Population 1 was the subpopulation of the Azores, Population 2 was the subpopulations of the South American samples, Population 3 was the subpopulation of the Central American samples, Population 4 was the subpopulation of the North American samples, Population 5 was the subpopulation of Australia, and Population 6 was the subpopulation of Africa. The splitting of populations was assumed to happen at a determined time td. The scenario assumes that populations 1, 2, 3, 4, 5 and 6 were all part of a single ancestral population of size NA with the condition that NA>N1-N6, and were all introduced, from that ancestral population, separately to the different geographical regions.

Results

Mitochondrial DNA

A total of 55 samples were successfully amplified for each gene and PCR products were sent for sequencing. Of the 55 samples, 39 sequences were readable and analyzed with MEGA 5 for the combined 16S rRNA and Cytb. Twenty-eight of the Azorean sequences (from the previous chapter) were added to the alignment making it a total of 67 sequences. Five of the sequences were eliminated from analysis after being determined to be equal in the distance matrix (Tables B-6 to B-16) and one sequence was eliminated because it was too different and assumed to be a contaminated sequence (FLD-1 in Table B-7). The phylogenetic bootstrap consensus tree is shown in Figure 3-8. There were two main axes with a resolution below 70. The endemic subpopulations were distributed through the two main axis (highlighted in yellow), as were the Azorean subpopulations (highlighted in gray). Half of the Angra subpopulations were clustered with the Ponta Delgada and Maia subpopulations in a separate branch from the other subpopulations with poor support. The other half of the Angra subpopulations were clustered in a branch with a resolution of 90, along with subpopulations from Africa, Australia, Chile, Peru, Costa Rica, and El Salvador, as well as with the Horta subpopulations. The subpopulations from the endemic region showed a separation as far as subpopulations from the same country were concerned. The Chilean subpopulation A was clustered in the upper branch, while Chile's subpopulation B, appear clustered in the lower branch of Figure 3-8. As for Peru the B subpopulation was clustered together, while the A subpopulation was divided between the upper and lower main branch of the phylogenetic tree.

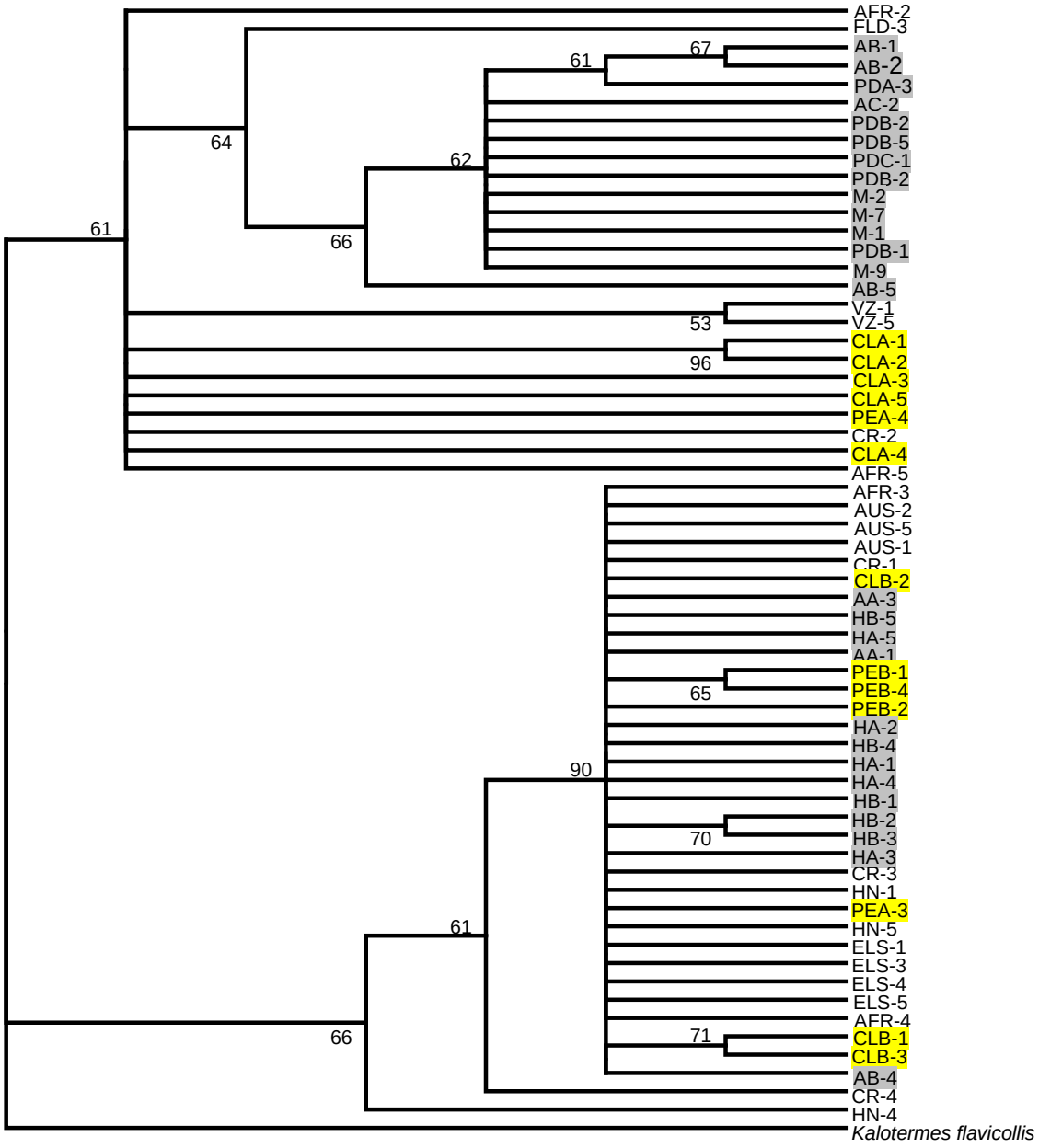

Figure 3-8. The evolutionary history was inferred using the UPGMA method. The bootstrap consensus tree was inferred from 5000 replicates and taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The analysis involved 62 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 686 positions in the final dataset. Highlighted subpopulations are Azorean (grey) and endemic (yellow).

Microsatellites

A total of 37 alleles were detected in the populations over the five loci. Alleles per locus are given in table 3-3. The loci Csec 1, 3, and 4 had the most alleles with 10, 12, and seven alleles respectively. The Csec6 and Csec5 loci had less alleles with three and five alleles, respectively. The estimated allele frequencies are given in Tables 3-4 to 3-8. No unique alleles were found for the world samples subpopulations. The frequencies varied between 0 and 100%, with no significant differences between the subpopulations (F=0.61 with a p-value=0.902 for an 0.05 value of significance).

Table 3-3. Size of alleles in number of base pairs per locus for the five microsatellite loci of *C. brevis* analyzed.

Locus	Alleles (bp)											
Csec6	143	146	149									
Csec5	122	125	128	131	134							
Csec4	129	144	148	154	157	166	169					
Csec1	208	214	216	218	220	224	226	230	232	234		
Csec3	116	118	120	122	124	126	128	130	132	134	136	138

Table 3-4. Allele frequencies, in percentage, for the Csec6 locus for the subpopulations of *C. brevis,* generated by the Microsatellite tool kit for Excel.

Allele	CLA	CLB	PEA	PEB	CR	ELS	VZ	HN	FLD	AUS	AFR
143	85.0	90.0	47.5	62.5	73.3	87.5	100.0	85.0	60.0	100.0	100.0
146	12.5	10.0	47.5	22.5	26.7	12.5	0.0	10.0	30.0	0.0	0.0
149	2.5	0.0	5.0	15.0	0.0	0.0	0.0	5.0	10.0	0.0	0.0

Table 3-5. Allele frequencies, in percentage, for the Csec5 locus for the subpopulations of *C. brevis,* generated by the Microsatellite tool kit for Excel.

Allele	CLA	CLB	PEA	PEB	CR	ELS	VZ	HN	FLD	AUS	AFR
122	7.5	2.5	12.5	20.0	26.7	0.0	0.0	2.5	32.5	50.0	0.0
125	87.5	35.0	67.5	80.0	56.7	92.5	100.0	97.5	67.5	50.0	100.0
128	5.0	40.0	17.5	0.0	6.7	7.5	0.0	0.0	0.0	0.0	0.0
131	0.0	5.0	2.5	0.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0
134	0.0	17.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 3-6. Allele frequencies, in percentage, for the Csec4 locus for the subpopulations of *C. brevis,* generated by the Microsatellite tool kit for Excel.

Allele	CLA	CLB	PEA	PEB	CR	ELS	VZ	HN	FLD	AUS	AFR
129	55.0	85.0	10.0	25.0	93.3	75.0	100.0	50.0	57.5	60.0	70.0
144	5.0	0.0	0.0	2.5	0.0	2.5	0.0	2.5	15.0	0.0	0.0
148	5.0	0.0	5.0	17.5	0.0	0.0	0.0	0.0	10.0	0.0	0.0
154	35.0	2.5	12.5	45.0	3.3	2.5	0.0	47.5	15.0	10.0	0.0
157	0.0	10.0	0.0	0.0	3.3	17.5	0.0	0.0	0.0	0.0	30.0
166	0.0	0.0	52.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
169	0.0	2.5	20.0	10.0	0.0	2.5	0.0	0.0	2.5	30.0	0.0

Table 3-7. Allele frequencies, in percentage, for the Csec1 locus for the subpopulations of *C. brevis,* generated by the Microsatellite tool kit for Excel.

Allele	CLA	CLB	PEA	PEB	CR	ELS	VZ	HN	FLD	AUS	AFR
208	42.5	67.5	55.0	67.5	70.0	85.0	50.0	57.5	67.5	80.0	90.0
214	27.5	22.5	5.0	2.5	20.0	7.5	10.0	0.0	2.5	0.0	0.0
216	0.0	0.0	0.0	0.0	0.0	2.5	0.0	22.5	0.0	0.0	0.0
218	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0	0.0	0.0
220	15.0	0.0	5.0	20.0	0.0	0.0	20.0	2.5	0.0	0.0	0.0
224	7.5	2.5	5.0	2.5	0.0	0.0	20.0	17.5	0.0	0.0	0.0
226	2.5	7.5	10.0	2.5	10.0	5.0	0.0	0.0	5.0	20.0	10.0
230	5.0	0.0	2.5	5.0	0.0	0.0	0.0	0.0	20.0	0.0	0.0
232	0.0	0.0	17.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
234	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 3-8. Allele frequencies, in percentage, for the Csec3 locus for the subpopulations of *C. brevis*, generated by the Microsatellite tool kit for Excel.

Allele	CLA	CLB	PEA	PEB	CR	ELS	VZ	HN	FLD	AUS	AFR
116	0.0	17.5	0.0	0.0	0.0	10.0	0.0	7.5	12.5	0.0	0.0
118	32.5	2.5	35.0	42.5	3.3	2.5	80.0	32.5	17.5	0.0	0.0
120	7.5	12.5	0.0	0.0	3.3	12.5	0.0	10.0	17.5	0.0	0.0
122	12.5	15.0	0.0	17.5	20.0	40.0	0.0	27.5	12.5	40.0	30.0
124	0.0	2.5	2.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
126	27.5	5.0	5.0	12.5	0.0	2.5	20.0	5.0	7.5	0.0	0.0
128	0.0	17.5	2.5	2.5	23.3	12.5	0.0	5.0	0.0	10.0	0.0
130	0.0	2.5	32.5	17.5	10.0	10.0	0.0	10.0	12.5	0.0	10.0
132	15.0	17.5	2.5	2.5	13.3	7.5	0.0	2.5	7.5	40.0	30.0
134	0.0	2.5	10.0	2.5	0.0	0.0	0.0	0.0	0.0	10.0	0.0
136	5.0	0.0	0.0	0.0	0.0	2.5	0.0	0.0	2.5	0.0	0.0
138	0.0	5.0	10.0	2.5	26.7	0.0	0.0	0.0	10.0	0.0	30.0

The mean expected and observed heterozygosities were not significantly different for all the subpopulations (Table 3-9).The mean number of alleles per subpopulation varied between 1.8 and 5.4.The F_{IS} values ranged from -0.079 to 0.302 (Table 3-9). The values were not significantly different from 0, with only Venezuela, Australia and Africa having F_{IS} values significantly different from 0.

Table 3-9. Estimates of genetic diversity at 5 polymorphic microsatellite loci in *C. brevis* from the samples from various continents.

Pop	Mean Expected Hz	Mean Observed Hz	ANOVA F stats	P-value for ANOVA	Mean No of Alleles per locus	*Fis*	p*-value
CLA	0.52 ± 0.12	0.42 ± 0.05	0.31	0.593	4.4 ± 1.52	0.199	0.0032
CLB	0.51 ± 0.13	0.45 ± 0.05	0.11	0.746	5.2 ± 3.42	0.118	0.0472
PEA	0.64 ± 0.05	0.48 ± 0.05	2.77	0.135	5.4 ± 2.07	0.249	0.0001
PEB	0.57 ± 0.08	0.44 ± 0.05	1.80	0.217	4.8 ± 2.39	0.236	0.0005
CR	0.49 ± 0.12	0.35 ± 0.06	0.70	0.428	3.8 ± 1.92	0.302	0.0006
ELS	0.37 ± 0.12	0.32 ± 0.05	0.09	0.776	4.4 ± 2.88	0.142	0.0321
VZ	0.22 ± 0.15	0.16 ± 0.07	0.11	0.751	1.8 ± 1.30	0.289	0.1676
HN	0.45 ± 0.13	0.38 ± 0.05	0.13	0.726	4 ± 2.35	0.166	0.0224
FLD	0.61 ± 0.08	0.53 ± 0.05	0.72	0.421	4.8 ± 2.68	0.13	0.0303
AUS	0.45 ± 0.13	0.48 ± 0.10	0.02	0.884	2.4 ± 1.14	-0.079	0.7564
AFR	0.29 ± 0.15	0.24 ± 0.09	0.08	0.789	2 ± 1.22	0.2	0.2784

*p-value for larger F than the observed for randomization tests

The estimated null allele frequency for all the subpopulations sampled was low for Csec 5, and 1 with values never above 0.13 (Table 3-10). The locus Csec3 showed the highest frequency of null alleles with values of 0.44 and 0.45 for CR, and AFR, respectively. The matrix of F_{ST} p-values between subpopulations had many significantly different values (Tables 3-11 to 3-14). The subpopulations of VZ and AUS were not significantly different from the Angra and Ponta Delgada subpopulations (Tables 3-12 and 3-13). The subpopulation AC was not significantly different from any of the other subpopulations. Subpopulation PEB was not different from the Ponta Delgada subpopulations (Table 3-12).

Table 3-10. Estimated null allele frequencies for all loci for the subpopulations of *C. brevis* sampled from the University of Florida Collection calculated by GENEPOP software.

Locus	CLA	CLB	PEA	PEB	CR	ELS	VZ	HN	FLD	AUS	AFR
Csec5	0.10	0.30	0.09	0.02	0.00	0.13	No inf	0.00	0.00	0.00	No inf
Csec6	0.16	0.11	0.25	0.31	0.30	0.18	No inf	0.11	0.01	No inf	No inf
Csec4	0.20	0.00	0.26	0.24	0.00	0.00	No inf	0.24	0.14	0.38	0.00
Csec1	0.06	0.00	0.02	0.04	0.00	0.00	0.16	0.00	0.00	0.00	0.00
Csec3	0.00	0.12	0.15	0.13	0.44	0.00	0.00	0.09	0.29	0.00	0.45

The Hardy-Weinberg test for disequilibrium was significant (p-value=0.000 for Score U test) with linkage disequilibrium found at all the loci. The null hypothesis that there is no deficiency of heterozygosity was rejected. The calculated gene flow was Nm=0.605876 migrants per generation. The calculated values of F_{ST}, F_{IS}, and F_{IT} for the three different methods are presented in Table 3-15. The distance diagram calculated for the microsatellite data is shown in Figure 3-9. The subpopulations of Angra are clustered in a separated branch from all the other subpopulations. Maia and Horta subpopulations are clustered together closer to the Ponta Delgada subpopulations. The Ponta Delgada subpopulations are calculated to be close to the Venezuela and Peruvian subpopulations.

Table 3-11. Matrix of F_{ST} p-values between subpopulations. Significantly different values are indicated by * after 190000 permutations. Indicative adjusted nominal level (5%) for multiple comparisons is 0.000263.

Sub pop	AB	AC	PDA	PDB	PDC	HA	HB	M
AA	0.03	0.085	0.0006	0.00118	0.00059	0.00001*	0.00001*	0.00001*
AB		0.2361	0.00001*	0.00005*	0.00055	0.00001*	0.00001*	0.00001*
AC			0.003	0.02428	0.55392	0.00014*	0.00052	0.00012*
PDA				0.66685	0.001	0.00001*	0.00002*	0.00001*
PDB					0.00209	0.00037	0.00028	0.00009*
PDC						0.01416	0.13522	0.00679
HA							0.52179	0.04845
HB								0.52604

Table 3-12. Continued matrix of F_{ST} p-values between subpopulations. Significantly different values are indicated by * after 190000 permutations. Indicative adjusted nominal level (5%) for multiple comparisons is 0.000263

Sub pop	CLA	CLB	PEA	PEB	CR	ELS	VZ
AA	0.00002*	0.00001*	0.00001*	0.00002*	0.00004*	0.0002*	0.00117
AB	0.00001*	0.00001*	0.00001*	0.00001*	0.00001*	0.00001*	0.00004*
AC	0.00354	0.00129	0.00247	0.03205	0.00776	0.01845	0.00785
PDA	0.00002*	0.00001*	0.00002*	0.00066	0.00003*	0.00001*	0.00253
PDB	0.00372	0.00003*	0.00013*	0.01324	0.00005*	0.00001*	0.07152
PDC	0.00001*	0.00002*	0.00705	0.00074	0.00002*	0.00001*	0.00219
HA	0.00001*	0.00001*	0.00001*	0.00001*	0.00001*	0.00001*	0.00003*
HB	0.00001*	0.00001*	0.00001*	0.00001*	0.00001*	0.00001*	0.0001*
M	0.00001*	0.00001*	0.00001*	0.00001*	0.00001*	0.00001*	0.00007*
CLA		0.00001*	0.00001*	0.00032	0.00001*	0.00001*	0.17167
CLB			0.00001*	0.00001*	0.00337	0.00123	0.00007*
PEA				0.00001*	0.00001*	0.00001*	0.00003*
PEB					0.00001*	0.00001*	0.00387
CR						0.00074	0.00009*
ELS							0.00001*

Table 3-13. Continued matrix of F_{ST} p-values between subpopulations. Significantly different values are indicated by * after 190000 permutations. Indicative adjusted nominal level (5%) for multiple comparisons is 0.000263

Sub pop	HN	FLD	AUS	AFR
AA	0.00001*	0.00001*	0.00127	0.00623
AB	0.00001*	0.00001*	0.00033	0.00022*
AC	0.00426	0.00149	0.00789	0.04801
PDA	0.00006*	0.00005*	0.00259	0.0013
PDB	0.00006*	0.00071	0.00797	0.00792
PDC	0.00001*	0.00004*	0.00225	0.00233
HA	0.00001*	0.00001*	0.00002*	0.00002*
HB	0.00001*	0.00001*	0.0001*	0.00008*
M	0.00001*	0.00001*	0.00002*	0.00001*
CLA	0.00002*	0.00002*	0.00008*	0.00005*
CLB	0.00001*	0.00001*	0.00262	0.01386
PEA	0.00001*	0.00001*	0.00002*	0.00002*
PEB	0.00002*	0.00009*	0.00042	0.00003*
CR	0.00001*	0.00001*	0.02756	0.08136
ELS	0.00001*	0.00001*	0.00087	0.16771
VZ	0.00408	0.00004*	0.00787	0.00789

Table 3-14. Continued matrix of F_{ST} p-values between subpopulations. Significantly different values are indicated by * after 190,000 permutations. Indicative adjusted nominal level (5%) for multiple comparisons is 0.000263

Sub pop	HN	FLD	AUS	AFR
HN		0.00001*	0.00003*	0.00002*
FLD			0.00049	0.00061
AUS				0.03228

Table 3-15. Values of F_{ST}, F_{IS}, and F_{IT} calculated using three different methods for all locus

	Weir & Cockerham (1984)	Michalakis and Excoffier (1996)	Nei's (1972)
F_{ST}	0.17	0.2528	0.165
F_{IS}	0.128	0.0062	0.123
F_{IT}	0.277	0.3015	0.172

The STRUCTURE results from the clusters tested from K=2 to K=5 showed a clear distribution of the individuals per cluster (Figures 3-10 to 3-12) for all the subsets of data tested. The K=6 to K=10 clusters did not show a clear pattern and were not considered further for the analysis (Figures A-6 to A-10). For the Islands only a subset of the number of clusters K=3 divided the subpopulations into three clear clusters with the Angra Population in one cluster (green), PDA and PDB in a distinct cluster (red) and the remaining subpopulations in the third cluster (blue) (Figure 3-10). The All Subpopulations subset STRUCTURE result (Figure 3-11) showed that for the number of clusters K=4, Angra subpopulations sharing a cluster with El Salvador and Africa (blue), while Horta and Maia shared the Peru A cluster (yellow). Chile subpopulation B and Costa Rica shared a cluster (Red), and the rest of the subpopulations (PDA-C, CL A, PEB, VZ, HN, and FLD) shared the fourth cluster (green). For Islands and Endemic only subset of data, the K=4 clusters had the subpopulation of Angra isolated from all other subpopulations (yellow), and the CLB subpopulation also isolated from the others (red).

The Horta and Maia subpopulations were clustered with the PEA subpopulation (Blue), while the PDA-C, CLA and PEB were clustered together (green) (Figure 3-12).

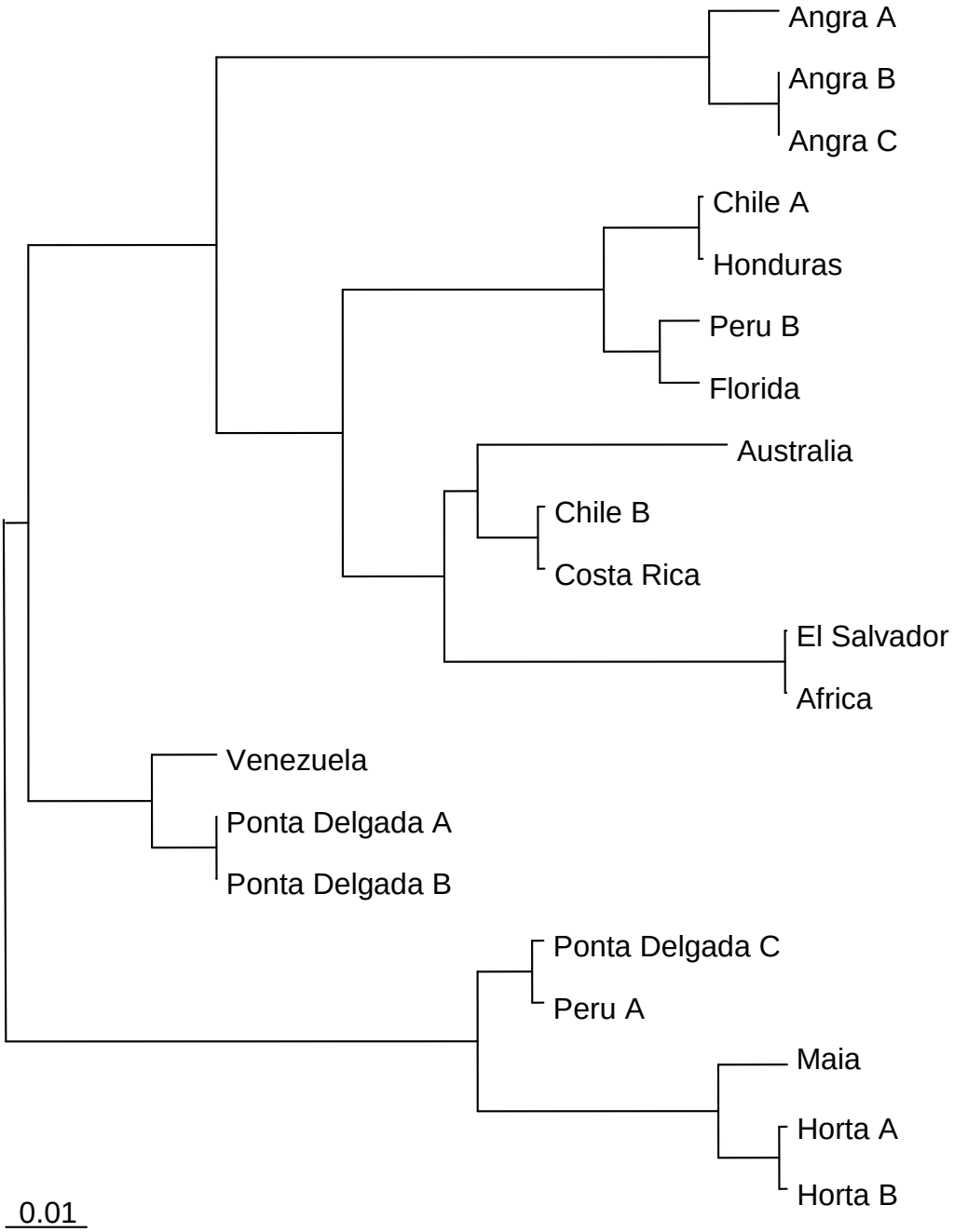

Figure 3-9. Distance diagram calculated using Genetic Distance Analysis. Length of branches represents genetic distance according to Nei's identity method. The longer the branch compared to the scale, the more distant the subpopulations. All sampled subpopulations are represented.

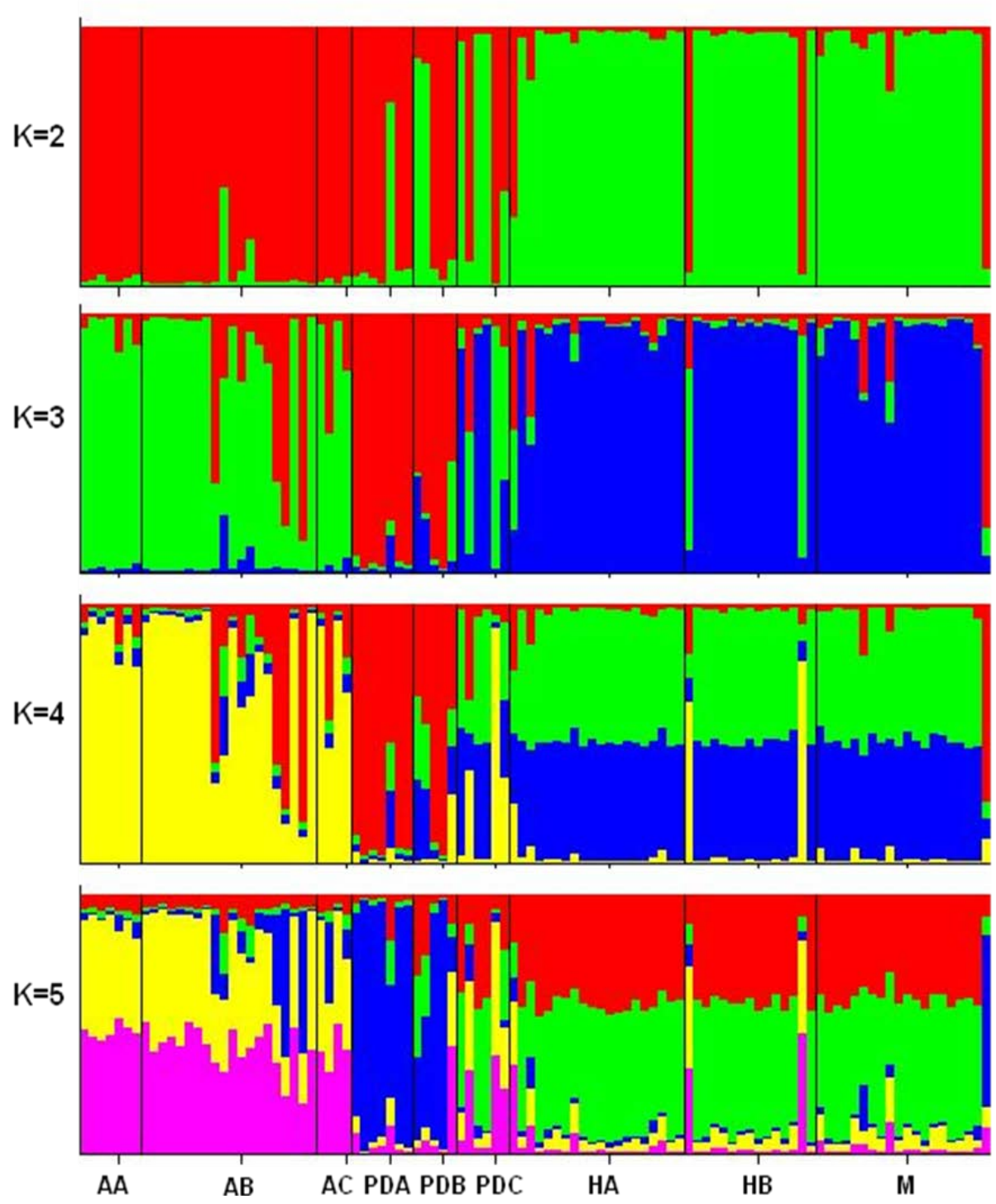

Figure 3-10. Assignments of 104 *C. brevis* individuals sampled from 9 sites to genetic clusters inferred from STRUCTURE simulations (based on average membership coefficient, Q, derived from 5 microsatellite loci). Each individual is represented by a single vertical line, with the length of each color segment representing the individual's genomic membership to that cluster. Black lines separate sample sites. Clusters K=2 to 5 are shown. Labels identify the subpopulations from each Island from where samples were collected.

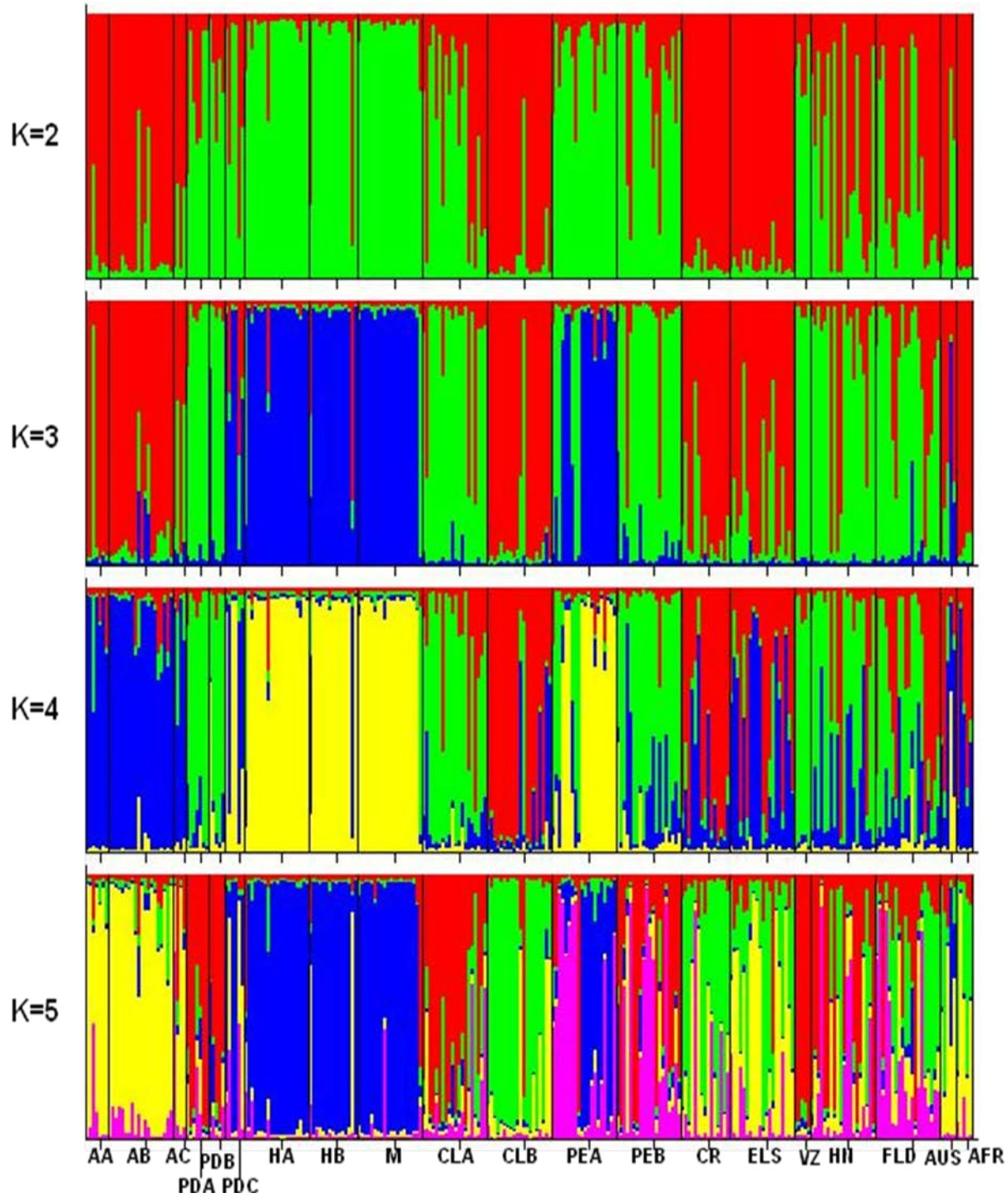

Figure 3-11. Assignments of 274 *C. brevis* individuals sampled from 20 sites to genetic clusters inferred from STRUCTURE simulations (based on average membership coefficient, Q, derived from 5 microsatellite loci). Each individual is represented by a single vertical line, with the length of each color segment representing the individual's genomic membership to that cluster. Black lines separate sample sites. Clusters K=2 to 5 are shown. Labels identify the subpopulations from each subpopulation from where samples were collected.

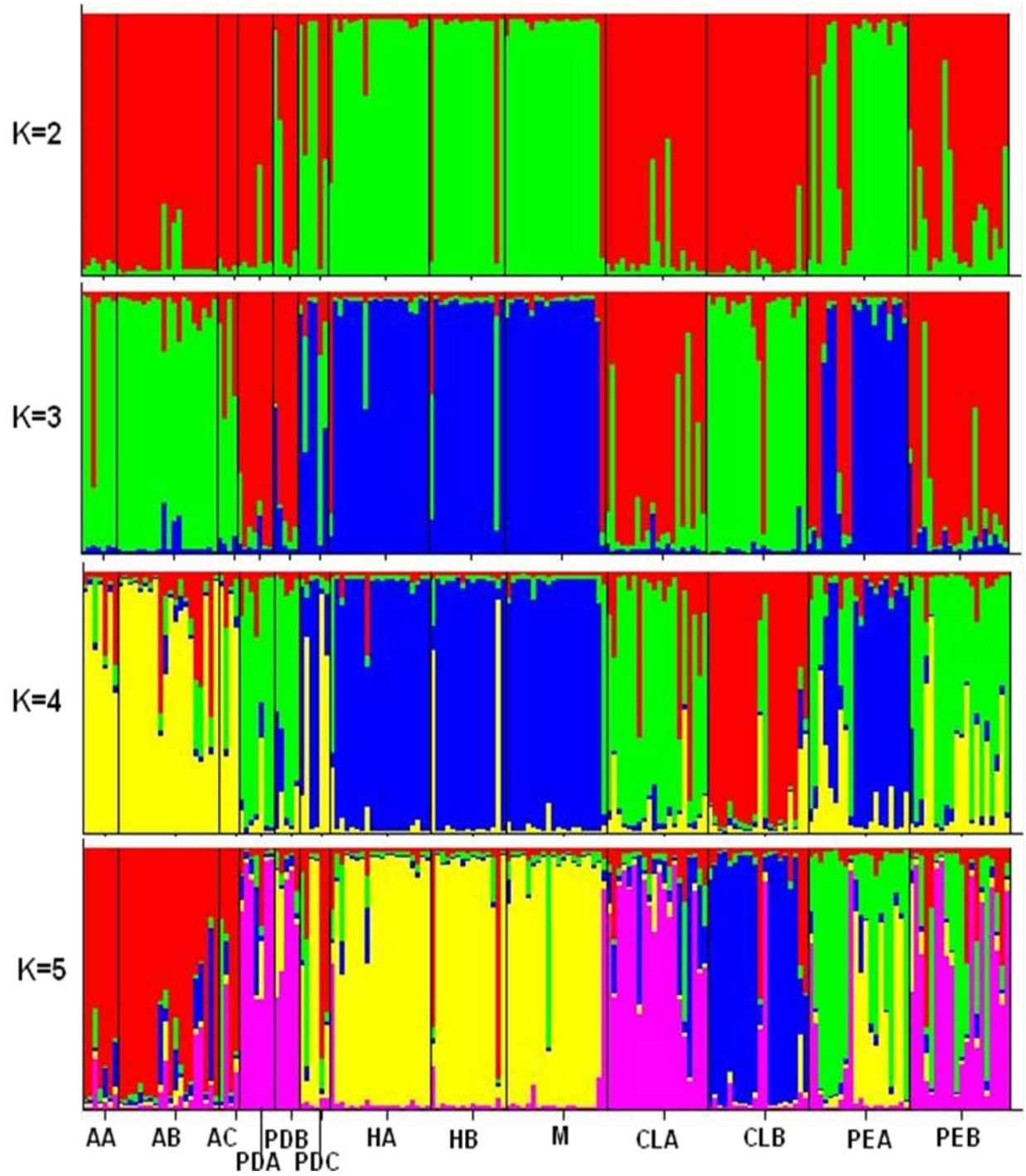

Figure 3-12. Assignments of 184 *C. brevis* individuals sampled from 13 sites to genetic clusters inferred from STRUCTURE simulations (based on average membership coefficient, Q, derived from 5 microsatellite loci). Each individual is represented by a single vertical line, with the length of each color segment representing the individual's genomic membership to that cluster. Black lines separate sample sites. Clusters K=2 to 5 are shown. Labels identify the subpopulations from the Azores and the endemic region from where samples were collected.

The DIYABC software results for the probability of the scenarios showed that for the comparison of the six scenarios of possible origin of the Azorean population, scenario 1, where all the populations were assumed to have diverted from the ancestral Population 2, was the most probable scenario with a 50% probability (Figure 3-13), while all the other scenarios had less than 20% probability of being the right scenario for the origin of the Azorean subpopulation with a 95% confidence interval. When compared with a scenario where the populations were all part of an ancestral unknown Population of size NA (scenario 2A), scenario 1 was again the most probable scenario with an 80% probability (Figure 3-14).

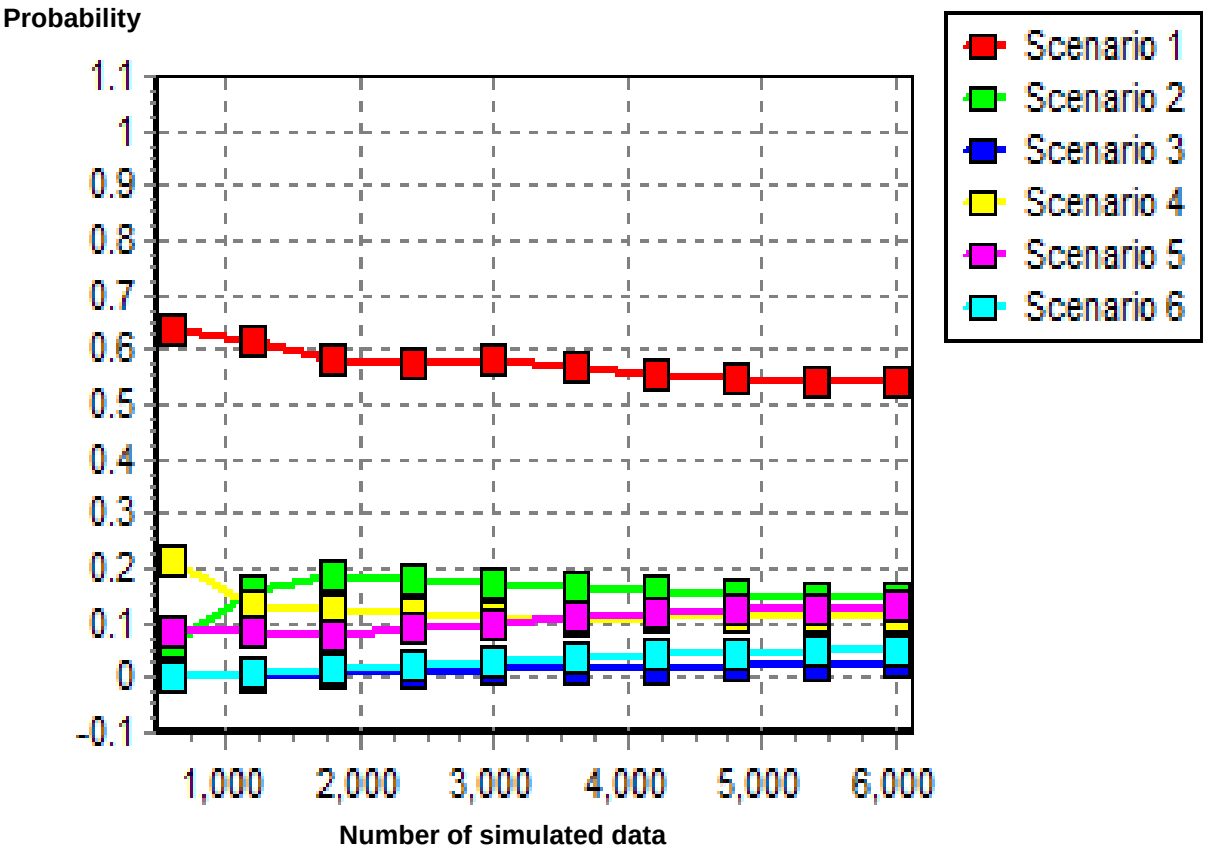

Figure 3-13. Logistic regression of the simulated data probability for the different scenarios tested in DIYABC. Probability varies between 0 and 1. All scenarios are within a 95% confidence interval for the simulations.

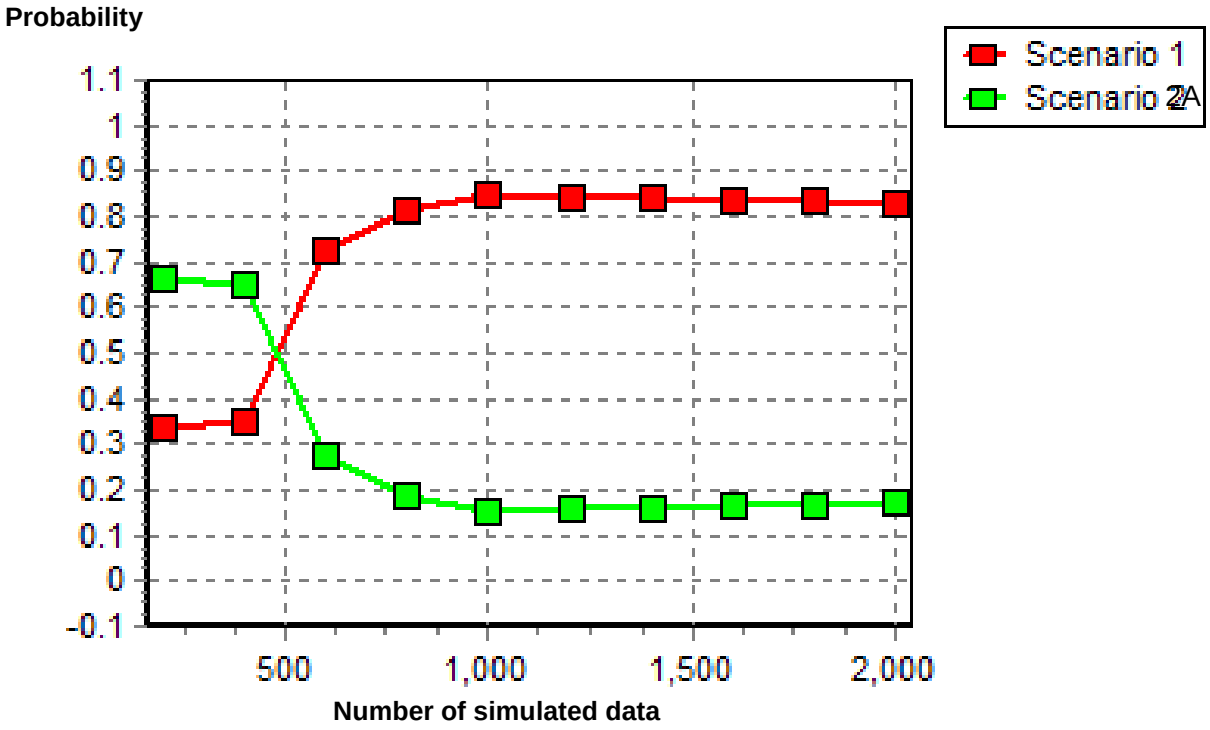

Figure 3-14. Logistic regression of the simulated data probability for the different scenarios tested in DIYABC. Probability varies between 0 and 1. All scenarios are within a 95% confidence interval for the simulations.

Discussion

The mtDNA results showed a pattern where both the Azorean subpopulations and the endemic subpopulations were divided between two main branches. Most of the Azorean subpopulations were clustered together in one branch, while some were in a completely different branch, confirming the previous findings of different introductions of the various subpopulations. The results showed that the endemic subpopulations are highly diverse, with the subpopulations spread through the two main branches. However, when focusing on some of the other subpopulations for these genetic markers the subpopulations are spread out throughout the phylogenetic tree (Figure 3-8) *e.g*. the African and Costa Rican subpopulations. This can be explained by the high variability of the 16S rRNA gene. Kambhambati et al. (1996) stated that 16S rRNA can have low resolution when the variability of the organisms being studied is very high and it will not

produce a reliable phylogenetic analysis. On a smaller scale where the populations being studied are small and closely related (as previously found) the results are reliable. However, when analyzing the relationship between very distinct populations from a great geographical range, the 16S rRNA can produce a phylogenetic tree that might have higher variability than expected for these subpopulations, even though a robust method with a high number of replications was used for analysis. The mitochondrial data does indicate that there is high diversity for both the Azorean and endemic subpopulations which would be expected given that the Azorean subpopulation is recently introduced from multiple points, and the endemic subpopulation is itself a source of genetic diversity.

The microsatellite data showed that the subpopulations do not have significant differences in the frequency of alleles between them, and that there are no unique alleles in the subpopulations of the world (Tables 3-3 to 3-7). Although the gene flow is low between these subpopulations, the alleles that are present are shared by several subpopulations, indicating their common origin. The low gene flow is indicative of subpopulation isolation (Hartl and Clark 2007) which would be expected considering that the locations where these subpopulations were sampled are geographically isolated, and these subpopulations have been well established.

The F_{IS} values were not significantly different from 0 for most subpopulations except for three of them. The African, Australian and Venezuelan subpopulations had F_{IS} values significantly different from 0, showing high levels of inbreeding for these subpopulations. That there were only five individuals sampled for the African, Australian, and Venezuelan subpopulations may have biased the results producing the appearance of higher levels of inbreeding. Even though these are long established

subpopulations, termites have been shown to have low inbreeding rates even within colonies (Husseneder et al. 1999) and have strategies to avoid inbreeding depression (Husseneder et al.2006). It would be thus expected to see these low levels of inbreeding for all the subpopulations. However for some of the subpopulations the frequency of null alleles was high and this may have caused the linkage disequilibrium to be found for all loci.

The overall F_{ST}, F_{IT} and F_{IS} values calculated with the three methods showed high diversity and low inbreeding, confirming the previous findings. When comparing the F_{ST} p-values between all the subpopulations the results showed that one of the endemic subpopulations (PEB) has values that do not differ from the Ponta Delgada subpopulations which would be expected, due to the fact that a similar value of high diversity would be expected to be found in endemic populations as well as populations recently introduced that are still getting gene flow from other sources (as previously found for the Azorean subpopulations). Also the genetic diversity found in the Venezuelan and Australian populations were not significantly different from the one found in Angra and Ponta Delgada. This could be indicative of high diversity in the subpopulations of Venezuela and Australia, even though the sample size for these subpopulations was low. The GDA results show a very clear pattern in terms of genetic distance between all the subpopulations (Figure 3-9). The Ponta Delgada, Horta, and Maia subpopulations were closer to Peru and Venezuela subpopulations, whereas the Angra subpopulations appear to be closer to the other world sampled subpopulations. This suggests that there might be a point of origin for at least the Ponta Delgada, Maia, and Horta subpopulations in Venezuela and Peru. The Angra subpopulations may just

be too diverse to determine a point of origin, or the world sample was not big enough to find a match.

The STRUCTURE analysis showed a clear pattern of ancestry for the different subsets of subpopulations analyzed. The Azorean subpopulations Islands Only subset showed that there were three distinctive ancestry clusters. The Angra subpopulations shared common ancestry, while the Ponta Delgada subpopulations were divided between two ancestries, one unique to the Ponta Delgada subpopulations and one shared with the Horta and Maia subpopulations (Figure 3-10). When compared with all the world subpopulations, the Azorean subpopulations continued to be distributed between three clusters of ancestry. The Angra subpopulations shared their ancestry with El Salvador and Africa, while the Ponta Delgada subpopulations shared their ancestry with Chile, Peru, Venezuela, Honduras and Florida. The Maia and Horta subpopulations shared their ancestry with the Peru A subpopulation. These results strongly support that there are several points of origin for the Azorean subpopulations. Most of the subpopulations appear to originate from South America. When compared with the endemic regions only, the Azorean subpopulations are divided into two distinct points of origin. The Angra subpopulations were completely separated, while the Ponta Delgada, Horta and Maia share their ancestry with the Peru and Chile subpopulations. These results substantiate what was previously observed from the GDA distance calculation, where the Angra subpopulations were isolated from the other Azorean subpopulations. Also, the origin of these other Azorean subpopulations was located in South America. This was confirmed by the scenarios tested with DYIABC software. Of all the scenarios tested the one that had the highest probability was the scenario where all the subpopulations originated from the South America continental area. Scheffrahn

et al. (2008) had found that the endemic populations of *C. brevis* were located in South America, namely in Chile and Peru. This study has confirmed this to be true. When comparing a scenario where all the subpopulations originate from an unknown ancestral population with the scenario where all the subpopulations originate from the South American group, it is shown that the endemic populations of South America were the original population for the world subpopulations. Combining the results from the STRUCTURE analysis with the DYIABC software, it was apparent that the points of origin for the subpopulations in the Azores in the towns of Ponta Delgada, Maia and Horta were from South America (Chile, Peru and Venezuela). The Angra subpopulation, however, was too diverse to account for an accurate point of origin.

That the Azorean subpopulations have a high level of genetic diversity and share ancestry with the endemic subpopulations can have two explanations. Firstly, the Azorean subpopulations may have originated from the endemic populations of Chile and Peru. Secondly, they may be showing a high level of diversity because this was a recent introduction from multiple locations that still shows a high level of diversity throughout the subpopulations. However, with the analysis performed it was shown that even though there were multiple introductions to the Islands, most of the subpopulations originated from South America. The Angra subpopulations had a completely different point of origin from the other subpopulations, which was not found in this study. Like all of these studies, further investigations and research needs be done. Working with a small number of samples and loci can only give a certain level of resolution to this story.

Future directions with this study would be surveys of infested homes in the Azores, where the origin of the lumber and furniture can be assessed. Different populations should be genotyped to further the amount of data and possibly corroborate the

hypothesis that the origin of the Azorean populations of *C. brevis* is amongst others, in countries like Venezuela, Chile, and Peru. More samples from other regions of the world would be necessary, as well as a larger number of termites collected per site. One way to circumvent the difficulty in obtaining a large number of specimens per collecting site would be to identify and create species specific primers for a large number of microsatellite loci. Analysis made with the red imported fire ant, *Solenopsis invicta* (Buren), have used as many as 67 different markers, which allowed for only one specimen to be used per colony, per location (Ascunce et al. 2011). Due to the cryptic nature of *C. brevis*, investing in augmenting the number of species-specific markers could bring an even clearer picture of the point of origin of the Azorean subpopulations, as well as the pattern of spread of this recent introduction.

CHAPTER 4
CONTROL OF THE SPREAD OF ALATES USING ATRACTION TO DIFFERENT LIGHT WAVELENGTHS AND CHEMICAL TREATMENTS

Cryptotermes brevis has become a serious pest in the Azorean islands. The levels of infestation in the major cities are elevated and some houses are at risk of structural failure. This species has also been spreading to other smaller islands of the archipelago. Measures have been taken to begin to bring this inter-island spread to a halt, with stricter laws for import and export of goods. However, the spread within cities continues with new infestation sites being discovered every year (personal observation).

The seasonal flights of *C. brevis* are the only time this species is seen outside wood, making it a good time to apply measures of control. Light traps to capture insects have been long used. *C. brevis* is known to fly towards light during its dispersal flights, and high intensity lights attract more alates of this species, than lesser intense lights, and the use of light traps has been recommended as a measure of prevention of infestation against this termite species (Ferreira and Scheffrahn 2011). However, there are no data reporting if there is any preference by *C. brevis* for a specific light wavelength. One of the objectives of this study is to determine if there is a preferred light wavelength that will attract more alates of *C. brevis* into traps, and if this preference is true in different populations.

A common preventative measure of control is the use of insecticides. Many studies have been done throughout the years with different insecticides against drywood termites in the US. However drywood termites are not a common pest in Europe so very little information is available about the efficacy of some of the commercially available products in Europe. Some of the products available in Europe are used as general wood preservatives (Wocosen, and Xylophene), others are used as

insecticides (Borowood, Gentrol, and XT-2000). With this in mind, the second objective in this study was to test these commercially available insecticides against drywood termites, and to determine how effective they are in preventing colonization by this species.

Materials and Methods

Different light wavelength preference

Experiments were conducted at the Fort Lauderdale Research and Education Center (FLREC) Davie, Florida, and in a privately owned attic in Angra do Heroísmo, Terceira, Azores. The experiments were set up in dark rooms kept at ambient temperature (T) and relative humidity (RH) (Florida: T - 25.5ºC, RH - 73.6%; Terceira: T – 20.5ºC, RH – 79.9%). In both Florida and the Azores, the rooms were filled with *C. brevis* infested wood. The Florida experiment was conducted between April and June of 2009, during *C. brevis* dispersal flight season for South Florida, and the experiment in the Azores was conducted during July and September of 2009, the dispersal flight season for *C. brevis* in the Azores.

In Florida

Twenty one transparent plastic boxes (36x23x28 cm) served as light wavelength preference chambers. Six different wavelengths were used (395 nm (ultraviolet), 460 - 555 nm (white), 470 nm (blue), 525 nm (green), 590 nm (yellow), 625 nm (red)) with three replicates per wavelength. Each box was wrapped in aluminum foil in order to isolate the light from one box to the other. Light Emitting Diodes (LED) light bulbs were used for each wavelength (Ultraviolet model# YA-UV5N30N; White model# SS5W4UAEC; Blue model# SS5B4SEEC; Green model# SS5G4UAEC; Yellow model# SS5Y4UAEC; Red model# SS5R4SDEC). Nine light bulbs were mounted in a series

circuit in clusters of three so that there would be three replicates of one wavelength with three light bulbs per replicate. Every cluster of light bulbs was five feet apart from the next one. The series circuit was mounted for each wavelength with 22-Gauge stranded hook-up wire (RadioShack technology plus ™), Full-Wave bridge rectifiers (4Amp. 50Volts RadioShack 276-1146) plug and connector (Leviton ® 15A PR-92678-10-02-0B. 125Volts), 10K Ohm resistor (¼ Watt 5% tolerance RadioShack 271-1335), 4.7K Ohm resistor (¼ Watt 5% tolerance RadioShack 271-1124), and vinyl electrical tape medium grade (Scotch ®).

The boxes were placed in a room infested with *C. brevis* with an open end facing the room. The different wavelengths were randomly distributed (Figure 4-1). Three of the boxes had unlit lights to serve as control and the remaining boxes had the different LED's hung in the middle of the box. Hefty ® EZ Foil cake pans (21.5 cm diam. and 3.8 cm deep) were placed inside the boxes underneath the light bulbs and filled up to three quarters with water (Figure 4-2). These pans served as water traps to capture the alates flying to the lights. The alates were collected and counted every day for all the boxes, throughout the flight season.

In the Azores

For the Azorean set-up the same light bulbs were used and connected with an adaptor for 240V input (standard use in Portugal). They were also randomly distributed inside 21 boxes to replicate the set-up in Florida. This set-up was assembled in an attic area filled with wood infested by *C. brevis*. The lights ran from 7 pm to 9:30 am everyday and alates in the traps were collected and counted daily.

Figure 4-1. Distribution of the different wavelengths in the boxes. The lights were distributed randomly with 3 replicates per light and 3 replicates with no light which were the controls.

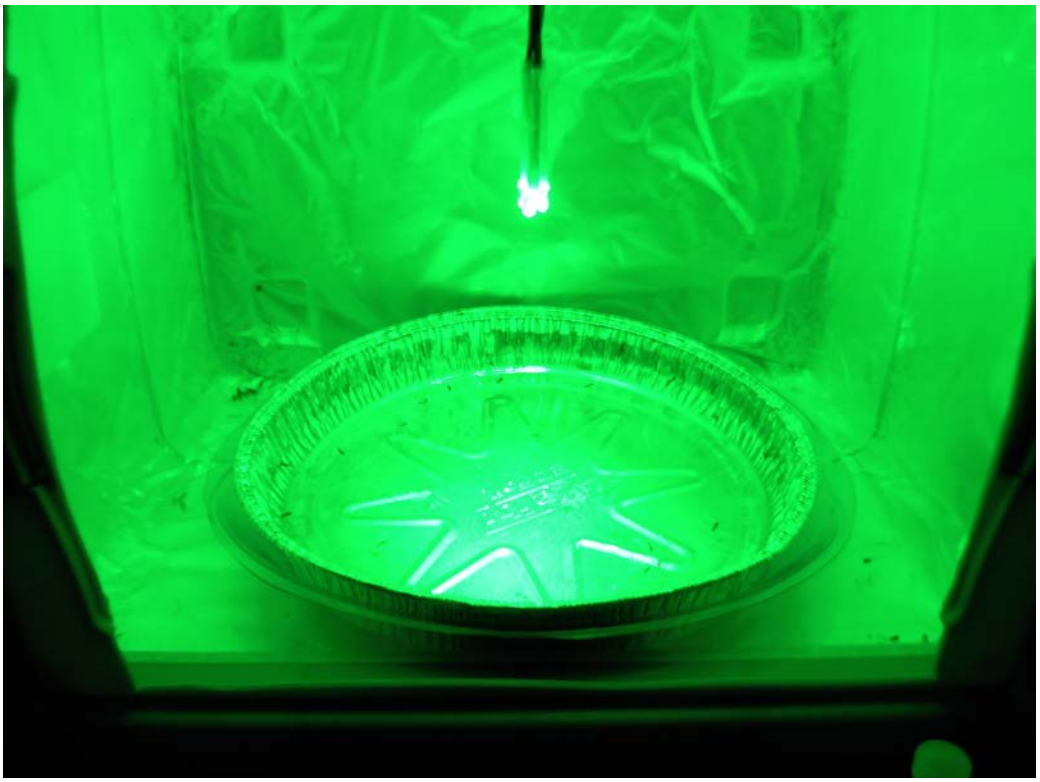

Figure 4-2. Close-up of the light chamber (green light-525nm). The bundle of three light bulbs is placed above the pan which was filled up with water. Termite alates that flew into the chamber were trapped in the water.

Data Analysis

The data collected from the experiments in the Azores and in Florida were separately analyzed using ANOVA (SAS 2003) to test if there was any difference in the variances of the number of alates per color light trap per day. Color was used as the factor for the ANOVA analysis. The data had to be transformed by ln (data+k) where k=2 in order to meet the assumptions for ANOVA (the data did not have a normal distribution). The null hypothesis being tested was that there were no differences between the variances for the number of alates caught in the light traps per day. Further analysis using Tukey's method (SAS 2003) was used to determine which wavelengths had significantly different variance of the number of alates caught in the trap for each location, separately.

Chemical prevention of colonization

Experimental set-up. Experiments were conducted in a private attic in Angra do Heroísmo, Terceira, Azores, and were conducted between July and September 2009. To test different chemicals as preventatives for colonization of this species, bioassays (attic) models that mimic the type of construction used in attics in the Azores were assembled. These attic modules were treated with the commercially available products in the Azores, Xylophene (cypermethrin 0.07%), Wocosen (permethrin 0.24%), Gentrol (hydroprene 9.0%), Borowood (disodium tetraborate decahydrate 10%), and XT-2000 (d-limonene 92%). The commonly used construction grade Criptoméria (*Cryptomeria japonica*) was used to construct the modules. Boards 39x13x13 cm and 39x13x2.5 cm were purchased from a local lumber yard, and assembled in the design and dimensions given in Figure 4-3. The sections of the boards were joined with a total of eight 3.5 cm long screws. The boards were assembled in a clapboard pattern. The attic modules

were treated on the surface with label recommended rates of the insecticides and three replicates per insecticide were used, with the three replicates for controls receiving no treatment for a total of 18 modules. After treatment, the modules were left to dry for 48h and then placed individually in a shallow plastic container, to prevent cross contamination. The modules were then placed in a *C. brevis*-infested room under a source of light for alate attraction (Figure 4-4). The source of light consisted of 70 watt power ultra violet lights commonly used in solariums. The modules were randomly distributed and left to be colonized. After one month, the modules were removed from the light source and kept for another month. At the end of the second month the modules were disassembled and survival and colony development was recorded. Although mortality was also registered, it was not used for the analysis because dead dealates found in one attic unit might have landed on other units before dealating, and so only survival was considered.

Data analysis. A one way ANOVA (SAS 2003) was used to test the difference of variances between the number of live dealates found in the units, with the treatment as the ANOVA factor. The same was used to test the difference of variance in the number of chambers, and number of eggs with the treatment as the factor. All the data was subjected to a ln (data+k) transformation, where k=2, to comply with the ANOVA assumptions (the data were not normally distributed). The null hypotheses being tested were: there were no differences in the variances of the number of live dealates in the attic units, per treatment; there were no differences in the variances of number of nuptial chambers in the attic units, per treatment; and there were no differences in variances in number of eggs in the attic units, per treatment. In order to test which treatments were

significantly different from one another a post ANOVA Tukey's analysis was performed, with significance levels set at α=0.05.

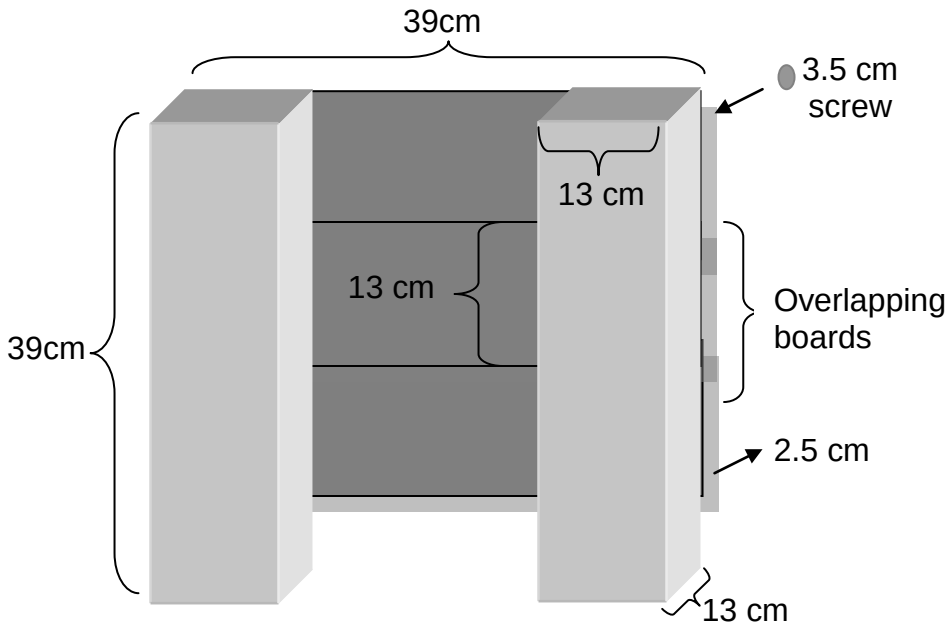

Figure 4-3. Attic module design. Mode of assembly and measures are shown Back boards are assembled in a clapboard pattern.

Figure 4-4. Attic modules randomly distributed under U.V. light.

Results

Different light wavelength preference

In Florida

A total of 991 alates were caught in the light traps in Florida. The blue light had the highest total number of alates in the traps (343) but it was not significantly different from white and green, and red and control had the lowest total number of alates with a total of 6 and 2, respectively (Table 4-1). There were significant differences between the number of alates in the traps per wavelength ($F=17.00$; $p<0.05$). Both yellow and U.V. lights had no significant differences to the lights with the lowest mean number of alates per trap per day, and the lights with the highest mean number of alates per trap per day (Table 4-1).

Table 4-1. Total and mean number of alates caught in light traps in Florida. Values with same letter were not significantly different after Tukey's analysis for $p<0.05$.

Wavelengths	Total number of alates per wavelength	Mean number of alates per trap per day (±SE)
Control	2	0.10 ± 0.02 a
625 nm (Red)	6	0.29 ± 0.06 a
590 nm (Yellow)	26	1.10 ± 0.24 a,b
395 nm (U.V.)	110	4.80 ± 1.05 a,b
460-550 nm (White)	262	11.86 ± 2.58 b
525 nm (Green)	298	13.62 ± 2.97 b
470 nm (Blue)	343	15.42 ± 3.37 b

In the Azores

A total of 1835 alates were caught in the light traps in the Azores. The blue light had the highest total number of alates in the traps (388), whereas red and control had the lowest total number of alates with a total of 79 and 97, respectively (Table 4-2). There were significant differences between the number of alates in the traps per

wavelength (F=5.4; p<0.05). Both yellow and U.V. lights had no significant differences to the lights with the lowest mean number of alates per trap per day (red and control), and the lights with the highest mean number of alates per trap per day (green, white, and blue) (Table 4-2).

Table 4-2. Total and mean number of alates caught in light traps in the Azores. Values with same letter were not significantly different after Tukey's analysis for p<0.05.

Wavelengths	Total number of alates per wavelength	Mean number of alates per trap per day (±SE)
Control	79	3.29 ± 0.63a
625 nm (Red)	97	4.04 ± 0.95a
590 nm (Yellow)	228	9.50 ± 2.37a,b
395 nm (U.V.)	304	12.67 ± 3.05a,b
525 nm (Green)	362	15.08 ± 3.78b
460-550 nm (White)	377	15.71 ± 3.17b
470 nm (Blue)	388	16.17 ± 4.017b

Chemical prevention of colonization

A total of 3,896 dead dealates were collected on all trays and modules. There were significant differences between treatments (Table 4-3) for the number of live dealates, number of chambers, and number of eggs. For both permethrin and cypermethrin no live dealates were found, and they were significantly different from all the other treatments. Dead dealates seemed to be evenly distributed throughout the treatments with no observable differences. The d-limonene treatment was not significantly different from the control (Table 4-3). No larvae were found in the chambers. Most of nuptial chambers were occupied by a single pair of dealates, as observed *in situ*. Not all of the live dealates were found in nuptial chambers. Some dealates were found crawling on the substrate with no nuptial chamber close by.

Table 4-3. Mean ± SE per attic module of *C. brevis* live dealates, number of nuptial chambers, and number of eggs. Values with the same letter were not significantly different after Tukey's analysis for $p<0.05$.

Treatment	Number of dealates	Number of chambers	Number of eggs
Control	23.33 ± 2.91a	11.67 ± 1.45 a	5.00 ± 2.00 a
D-limonene	26.00 ± 4.73 a	12.67 ± 2.33 a	4.00 ± 1.53 a
Disodium Tetraborate Decahydrate	12.67 ± 2.03 a,b	6.00 ± 1.15 a,b	1.00 ± 0.58 a,b
Hydroprene	7.67 ± 2.96 b	3.67 ± 1.45 b	1.67 ± 0.33 a,b
Permethrin	0 ± 0 c	0 ± 0 c	0 ± 0 b
Cypermethrin	0 ± 0 c	0 ± 0 c	0 ± 0 b
F	40.01	32.51	8.62
df1	5	5	5
df2	12	12	12
p	0.000	0.000	0.001

Discussion

The alates flying in both the Azores and Florida showed a similar behavior towards the wavelength of preference. When faced with choice chambers, the lights in the wavelength of the blue, green and white had a higher incidence of alates trapped compared to the remaining wavelengths. However for both Florida and the Azores these three wavelengths did not have significantly more alates caught than yellow or U.V. lights. Minnick (1973) had observed an attraction of the alates for U.V. light, preferring it to the incandescent light. Ultra violet light is commonly used in insect traps, and the common notion is that this wavelength is the most preferred by insects in general. However, more studies are looking into other wavelengths and their attraction to insects. Chang et al. (2001) looked at alate attraction of *C. formosanus* to different light

wavelengths and similarly found that blue (367-583 nm) and green (525-648 nm) lights attracted significantly more alates of this species than the red (600-733 nm) lights and the control. Previously, Yamano (1987) had also found that winged adult response to colored lights reached the maximum with the blue (400-420nm) lights. With the ease of access to simple and inexpensive LED's, these are being tested for traps for insects. Nakamoto and Kuba (2004) tested the effectiveness of green LED's in light traps against the West Indian sweet potato weevil *Euscepes postfasciatus* (Fairmaire). They found that when given the choice the weevil would choose the green light as opposed to blue, yellow, and red, and made a case for using LED's.

The experiments for light wavelength were performed with two different populations, one in Florida and one in the Azores. These two different populations were kept in different environments under different characteristics, with the population in Florida kept in a small closed, completely dark room, while the Azorean population was kept in a big open room, with some competing sunlight. However, and despite the different conditions the results were comparable, showing that this may be a preference for the species itself and not just for a particular population.

The main reason that it was important to look at the light wavelength preferences for *C. brevis* was to optimize light traps for C*. brevis,* to be used during the dispersal flight season. Using simple LED lights that are inexpensive can be a good way to get the public involved in preventative treatments for *C. brevis* in the Azores. Also there is lower fire hazard, because LED's are cold lights and do not overheat. A simple light trap composed of regular Christmas tree lights (most of these products are made up of LED's nowadays) and a sticky trap, or a container with water underneath could be

recommended for use in a household. The fact that the controls got significantly less termites flying into it shows that having a light trap is helpful in reducing infestation pressure. In the Azores the main infestation sites are in the dark often unused or cluttered attics. Promoting the use of these simple traps among homeowners can help slow the further spread of this species during the dispersal flight season.

The insecticide treatment results show that the two most commonly used insecticides for wood treatment permethrin and cypermethrin were most effective preventing colony foundation. Both the disodium tetraborate decahydrate and the hydroprene showed no significant differences in the number of eggs with permethrin and cypermerthrin, with a low number of eggs. This can be indicative that although sodium borate and hydroprene do not work in preventing colony foundation by killing the dealates, they may work on a physiological level preventing the new founded colonies from producing enough progeny to survive as a colony. Studies have shown that the average number of eggs laid for *C. brevis* in a nuptial chamber is four (McMahan 1960, Ferreira 2008), with the colonies surviving to at least the following year (McMahan 1960). The results showed an average of one to two eggs for disodium tetraborate decahydrate and hydroprene, while d-limonene showed an average of four eggs. However there were no significant differences between the average number of eggs laid in the disodium tetraborate decahydrateand hydroprene treatments, and the d-limonene and control treatments. These results may be due to the fact that a *C. brevis* incipient colony has a very low production of eggs in the first year (Ferreira 2008), and significant differences are hard to identify with these low numbers. Cypermethrin and permethrin were the most effective insecticides by causing 100% mortality with no survivors to start

a nuptial chamber or lay eggs. This is important information because it confirms that these two popular wood preservatives can be used to prevent *C. brevis* colony foundation.

Future directions can include testing the product degradation of cypermethrin and permethrin to determine what the frequency of treatments should be. The use of a combination of both light traps and an insecticide wood treatment with cypermethrin or permethrin might have a synergetic effect and could be a more effective way to reduce the spread of *C. brevis* not only within the households where there are infestations already, but as well as in the cities where this pest is already present. Also, the light traps can be used to monitor the efficacy of the pesticide treatment. Reducing the spread of *C. brevis* could help to keep the population levels lower, even if there is no complete eradication. However, more work is needed.

CHAPTER 5
CONCLUSIONS

Cryptotermes brevis in the Azores is an important urban pest problem. It is at this point well established and will only continue to spread to more islands. Since the beginning of this work in 2009, two more spots of infestation on a different island (Pico) have been identified in 2011 (Figure 1-3) (O. Guerreiro personal observation). This species has been introduced several times and it is still being introduced.

This study has shown that the multiple introductions scenario was the most probable. The points where this species might have been introduced from are Venezuela, Chile and Peru. Even though more studies are necessary, this is an early assessment of this species' population movement and dynamics. It was established in this study that the Azorean populations are still subject to high gene flow, not only from the outside but also between islands. It is therefore important to work on ways to prevent this species from spreading further, either within the islands where it is already present, or to other islands.

Studies on preventative control and ways to improve this preventative control are important for the future in order to have lower levels of infestation. Combining the use of insecticides with light traps could help decrease the spread of this species within the Islands. The knowledge of the point of origin of this species and the fact that it is still being introduced can be used to convey a higher level of regulation in the import of wood materials to the islands in order to decrease the introduction of this pest. Furthermore, this invasive species is only one of the termite species present in the Azores that is causing structural damage. This study helps to elucidate what is

happening with this particular species but more studies into the other species present on the islands are also needed for the future.

APPENDIX A
EXTRA FIGURES

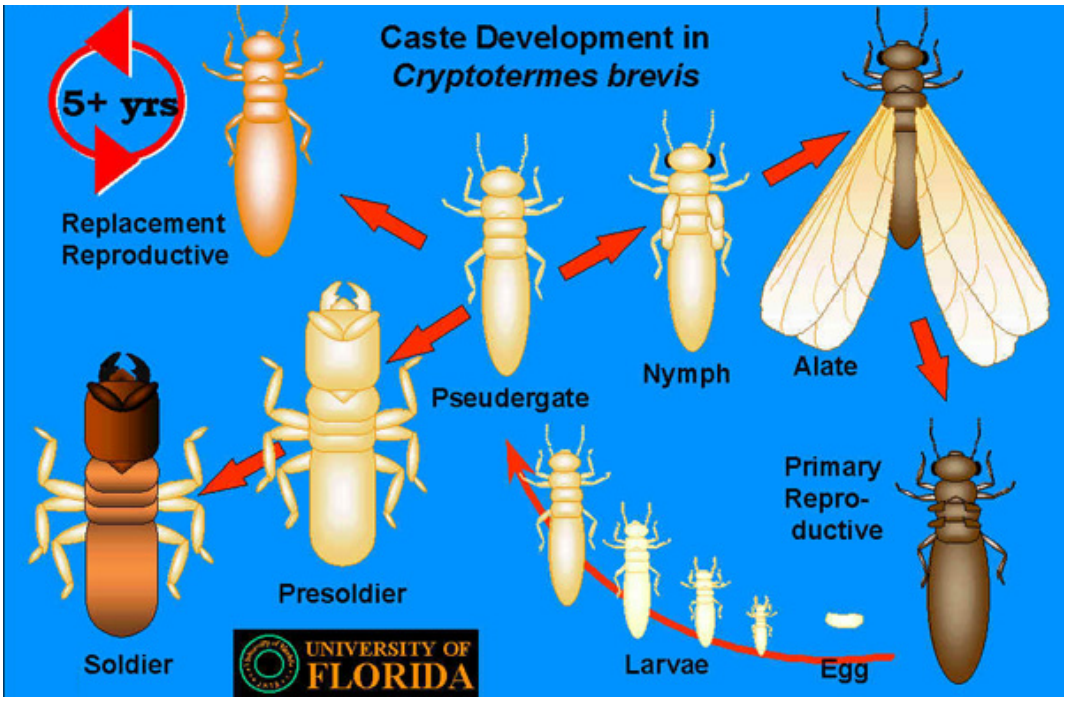

Figure A-1. Different castes of termites and life cycle (Scheffrahn).

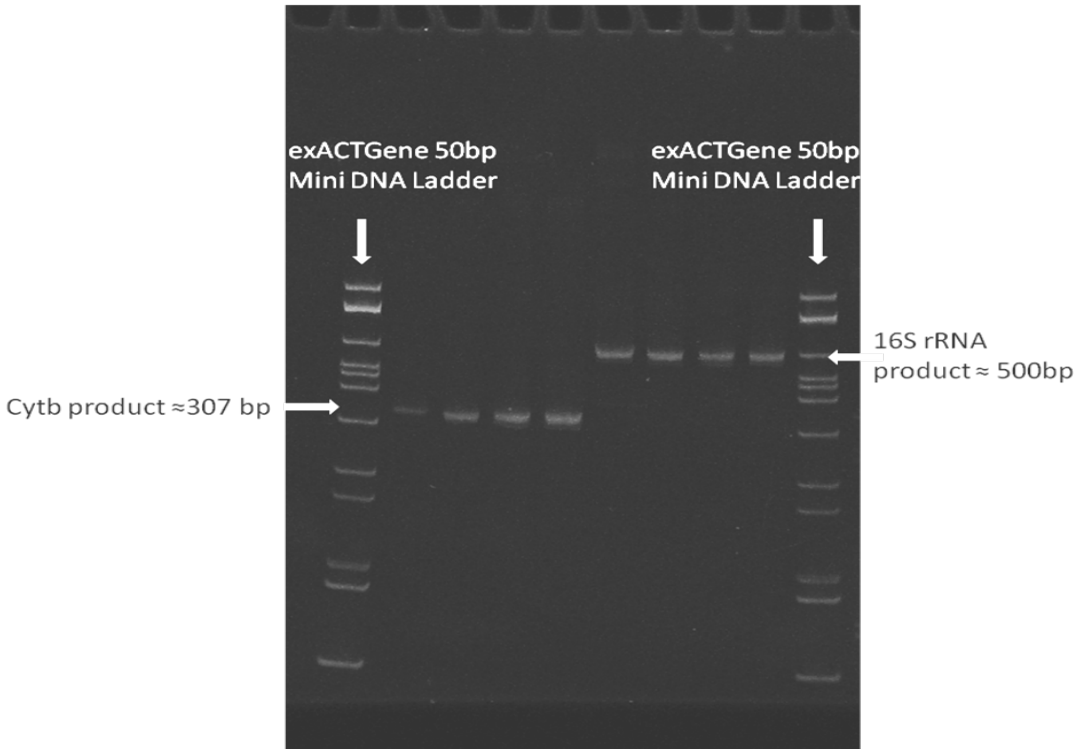

Figure A-2. Gel picture of PCR products of *C. brevis* amplified mtDNA for 16S rRNA and Cytb genes. Approximate sizes of the products are shown in number of base pairs using the exACTGene Mini Ladder as a measurement aid.

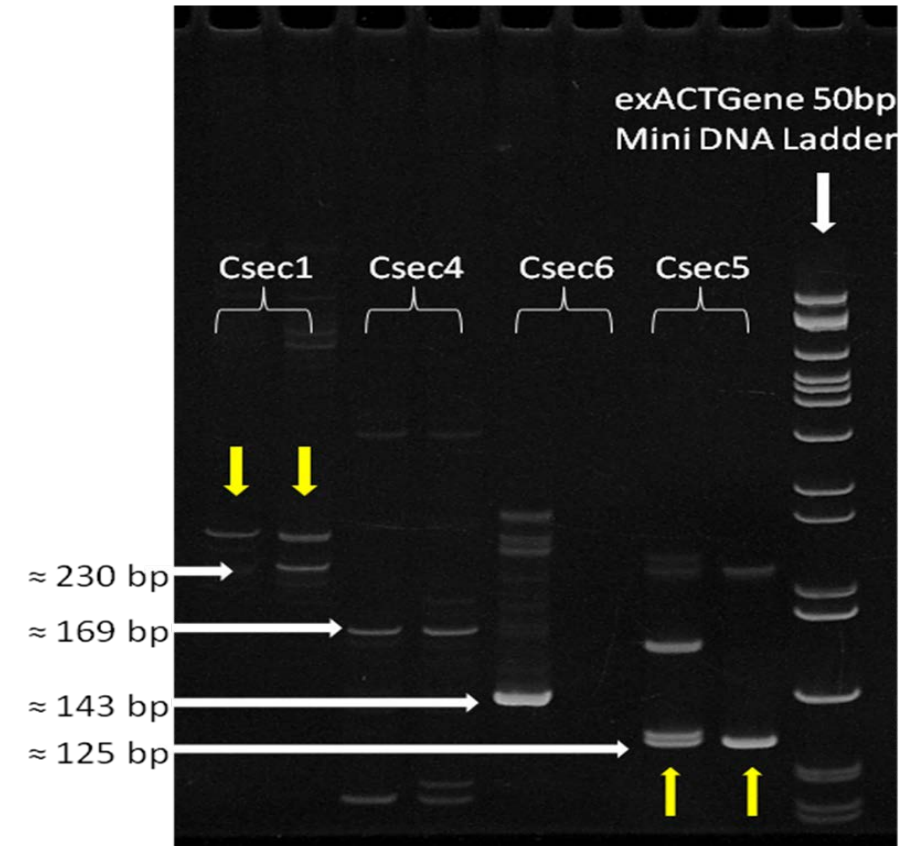

Figure A-3. Gel picture of amplified PCR products for four microsatellite loci (Csec1, 4, 5, and 6). White arrows indicate the approximate base pair size of the alleles, and yellow arrows indicate examples of a homozygote (single band) and a heterozygote (two bands) for the same loci.

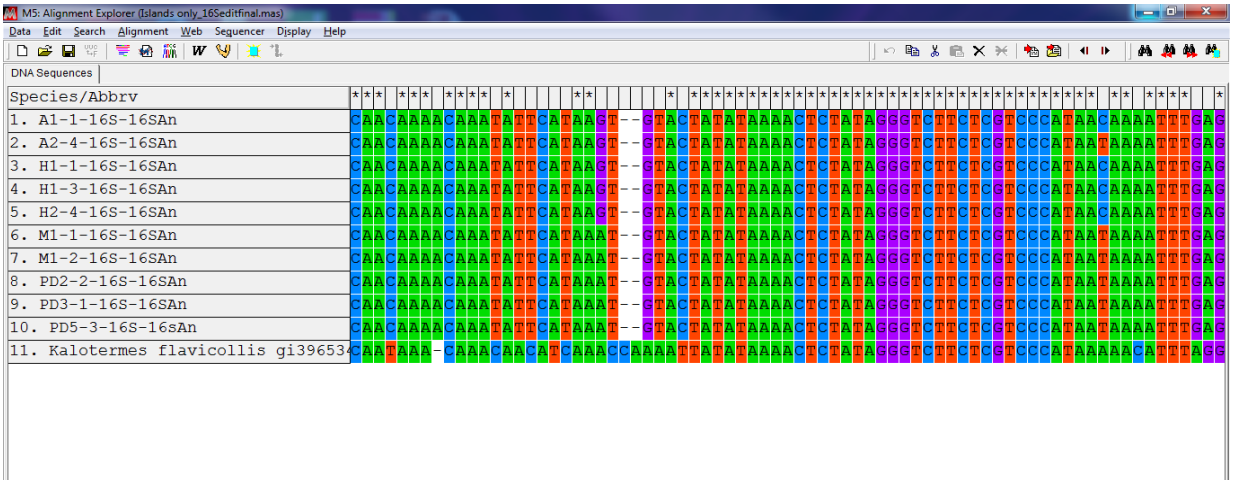

Figure A-4. Example of an alignment session using MEGA 5.0 where the sequences have been aligned using ClustalW method. The asterisks indicate matches in the sequences.

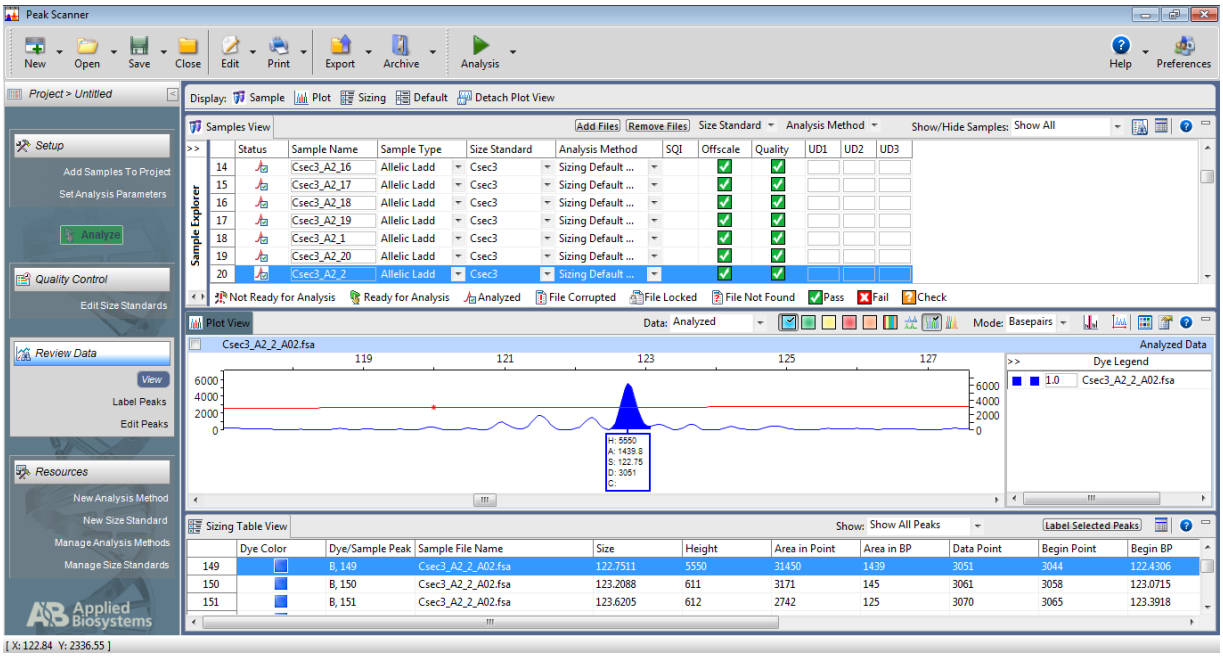

Figure A-5. Example of scoring the base pair size of an allele on the PeakScanner software. The peak is highlighted and it shows an homozygote with the information about the number of base pairs provided on the table at the bottom of the screen.

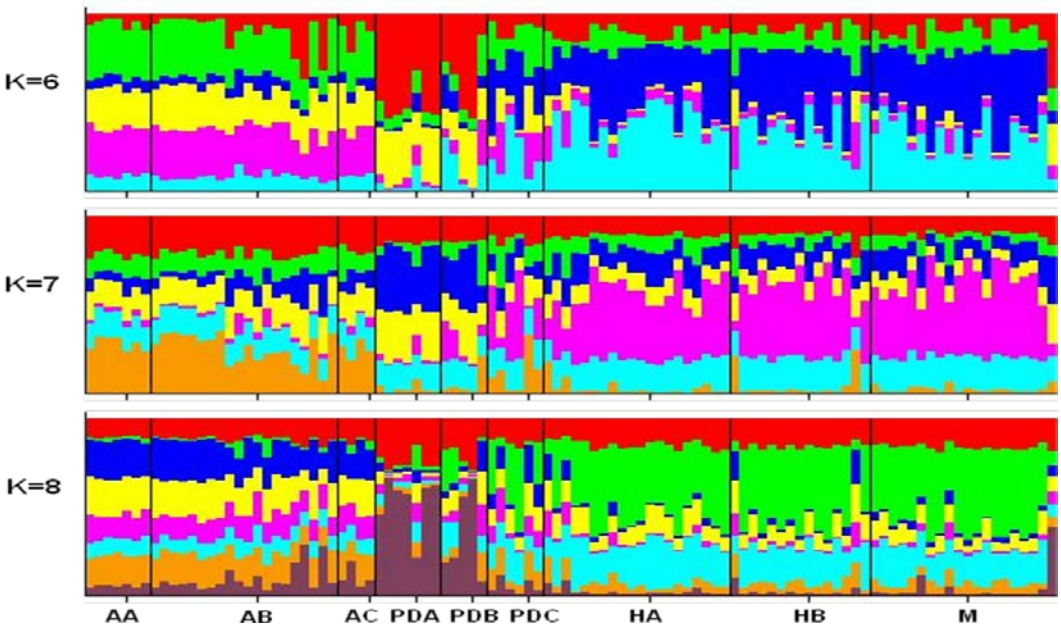

Figure A-6. Assignments of 104 *C. brevis* individuals sampled from 9 sites to genetic clusters inferred from STRUCTURE simulations (based on average membership coefficient, Q, derived from 5 microsatellite loci). Each individual is represented by a single vertical line, with the length of each color segment representing the individual's genomic membership to that cluster. Black lines separate sample sites. Clusters K=6 to 8 are shown. Labels identify the subpopulations from each Island from where samples were collected.

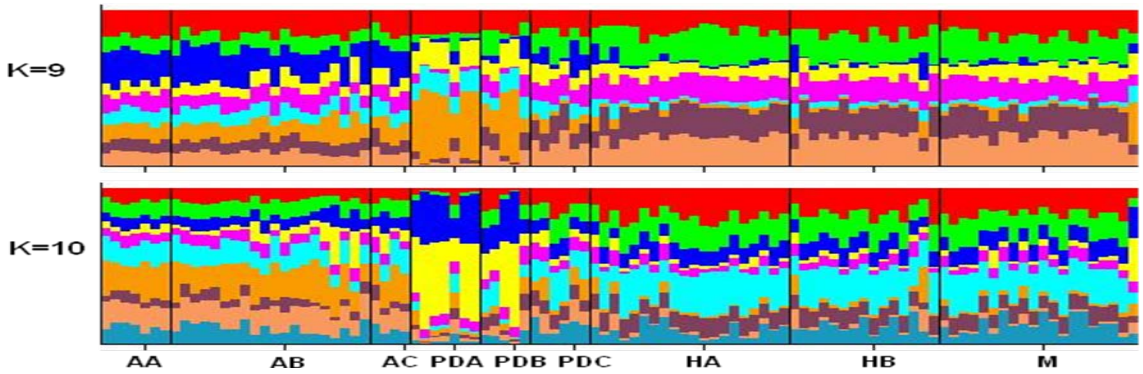

Figure A-7. Assignments of 104 *C. brevis* individuals sampled from 9 sites to genetic clusters inferred from STRUCTURE simulations (based on average membership coefficient, Q, derived from 5 microsatellite loci). Each individual is represented by a single vertical line, with the length of each color segment representing the individual's genomic membership to that cluster. Black lines separate sample sites. Clusters K=9 and K=10 are shown. Labels identify the subpopulations from each Island from where samples were collected.

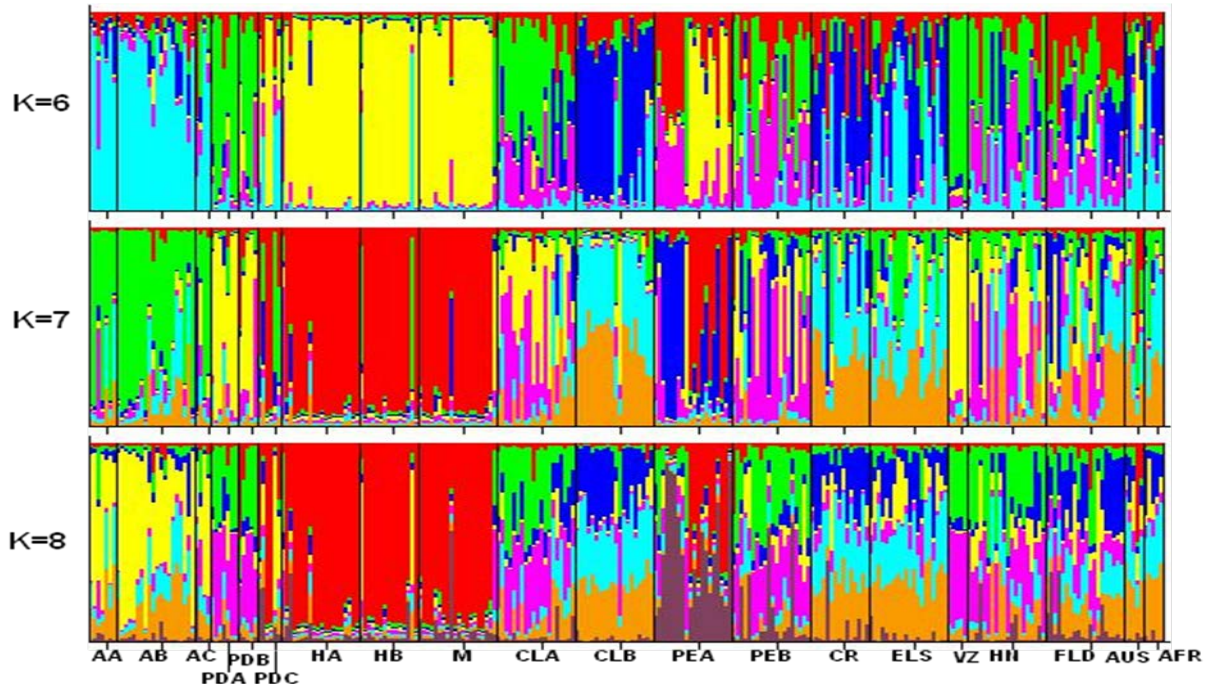

Figure A-8. Assignments of 274 *C. brevis* individuals sampled from 20 sites to genetic clusters inferred from STRUCTURE simulations (based on average membership coefficient, Q, derived from 5 microsatellite loci). Each individual is represented by a single vertical line, with the length of each color segment representing the individual's genomic membership to that cluster. Black lines separate sample sites. Clusters K=6 to 8 are shown. Labels identify the subpopulations from each subpopulations from where samples were collected.

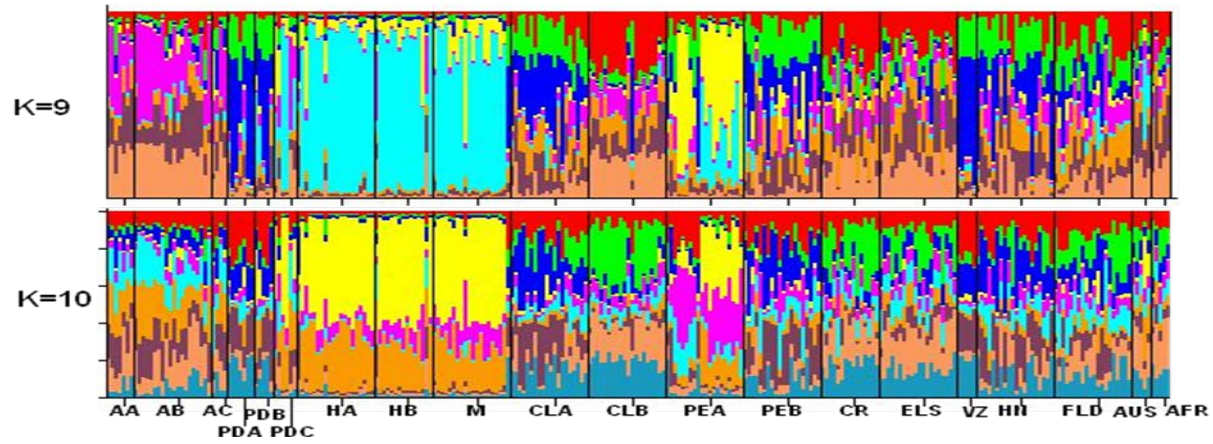

Figure A-9. Assignments of 274 *C. brevis* individuals sampled from 20 sites to genetic clusters inferred from STRUCTURE simulations (based on average membership coefficient, Q, derived from 5 microsatellite loci). Each individual is represented by a single vertical line, with the length of each color segment representing the individual's genomic membership to that cluster. Black lines separate sample sites. Clusters K=9 and K=10 are shown. Labels identify the subpopulations from each subpopulation from where samples were collected.

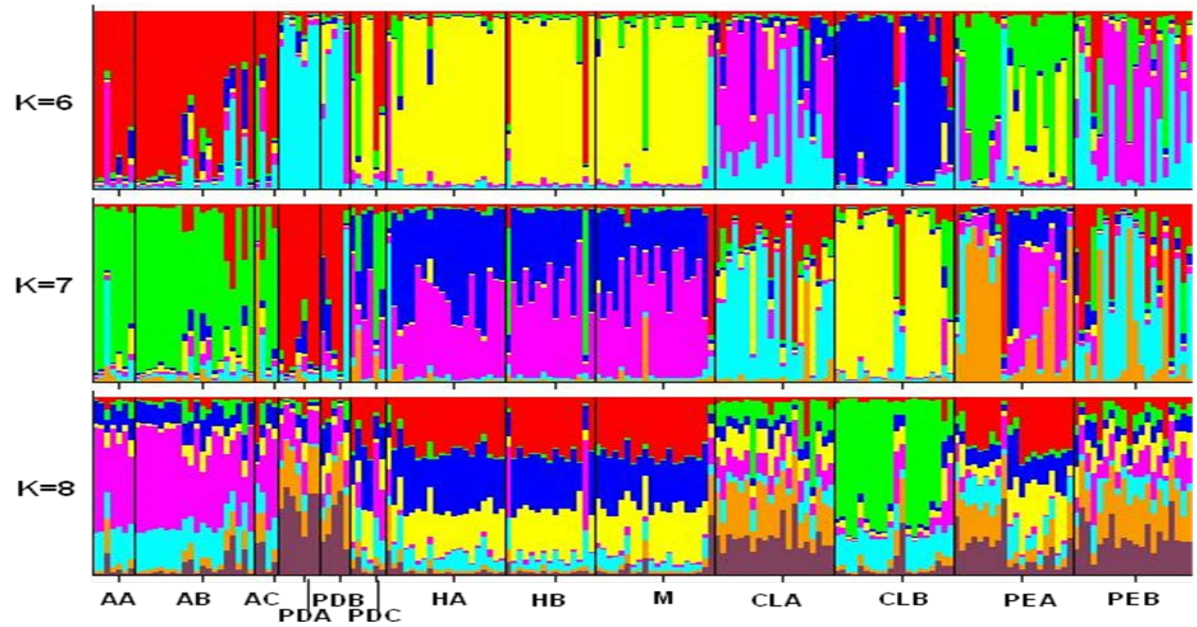

Figure A-10. Assignments of 184 *C. brevis* individuals sampled from 13 sites to genetic clusters inferred from STRUCTURE simulations (based on average membership coefficient, Q, derived from 5 microsatellite loci). Each individual is represented by a single vertical line, with the length of each color segment representing the individual's genomic membership to that cluster. Black lines separate sample sites. Clusters K=6 to 8 are shown. Labels identify the subpopulations from the Azores and the endemic region from where samples were collected.

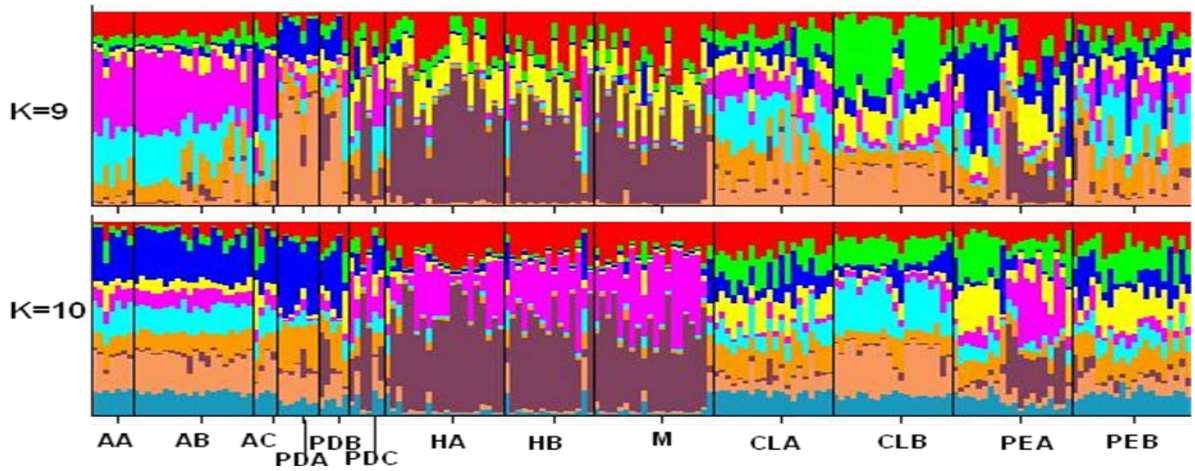

Figure A-11. Assignments of 184 *C. brevis* individuals sampled from 13 sites to genetic clusters inferred from STRUCTURE simulations (based on average membership coefficient, Q, derived from 5 microsatellite loci). Each individual is represented by a single vertical line, with the length of each color segment representing the individual's genomic membership to that cluster. Black lines separate sample sites. Clusters K=9 and 10 are shown. Labels identify the subpopulations from the Azores and the endemic region from where samples were collected.

APPENDIX B
EXTRA TABLES

Table B-1. Primer sequence for mitochondrial genes used for amplification. Adapted from Legendre et al. 2008.

Gene	primers	Sequence (5'→3')	Source
16S rRNA	16SAr	CGC CTG TTT ATC AAA AAC AT	Xiong and Kocher, 1991
	16SF	TTA CGC TGT TAT CCC TAA	Kambhampati, 1995
Cytb	cytb612	CCA TCC AAC ATC TCC GCA TGA TGA AA	Kocher et al., 1989
	cytb920	CCC TCA GAA TGA TAT TTG GCC TCA	Kocher et al., 1989

Table B-2. Primer sequence for microsatellite locus used for amplification. Adapted from Fuchs et al. 2003.

Locus	primers	Sequence (5'→3')
Csec1	Forward	AACGCTTGGATTATGGGTTC
	Reverse	TATCTGTCTGTCTGTCTGTCG
Csec3	Forward	TGTACTTCTGACATCTCAGG
	Reverse	GTCGGTACGGTCCACTTTGC
Csec4	Forward	TGTTAGAAGGCTACCAGCGC
	Reverse	TCTTTCCTCTGCGAACTGTC
Csec5	Forward	TGAAAGCCAGTGGGGCAGCTGC
	Reverse	TGCCTACAGTCAGAGCTCAAGC
Csec6	Forward	ACAGTTTGATCAGTGCCTTGG
	Reverse	ACTGGCATTAGGGTTAGGTAC

Table B-3. Variation sites for the 22 haplotypes of *C. brevis* sequences for the combined Cytb and 16SrRNA genes for the island samples. Dots are nucleotides equal to the first row. Numbers indicate the position of the variation sites on the combined sequences.

Haplotypes	7	10	12	13	14	15	16	17	18	19	21	23	31	35	44	47	51	56	74
AA-1	C	C	T	A	G	G	A	A	T	C	C	A	A	T	A	T	C	C	T
AA-3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AB-1	T	.	G	G	T	.	T	T	.	G	T	G	.	C	G	C	T	T	C
AB-4	.	A	.	.	.	.	T	.	.	.	.	.	.	.	G	.	.	.	.
AB-5	T	.	G	G	A	.	T	T	A	A	T	G	.	C	G	C	T	T	C
AC-2	T	.	G	G	A	.	T	T	A	G	T	G	.	C	G	C	T	T	C
PDA-2	T	.	.	G	.	T	.	C	.	T	T	G	.	C	G	C	T	T	C
PDA-3	T	.	G	G	A	T	T	G	.	T	T	G	C	C	G	C	T	T	C
PDA-5	T	.	.	G	.	T	.	C	.	T	T	G	C	C	G	C	T	T	C
PDB-1	T	.	.	G	.	T	.	C	.	T	T	G	.	C	G	C	T	T	C
PDB-2	T	.	.	G	.	T	.	C	.	T	T	G	.	C	G	C	T	T	C
PDC-1	T	.	A	G	.	T	.	C	.	T	T	G	.	C	G	C	T	T	C
HA-1	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.
HA-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HA-3	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.
HA-4	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.
HA-5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HB-2	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.
HB-4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
M-1	T	.	.	G	.	T	.	C	.	T	T	G	.	C	G	C	T	T	C
M-2	T	.	.	G	.	T	.	C	.	T	T	G	.	C	G	C	T	T	C
M-9	T	.	.	G	.	T	.	C	.	T	T	G	.	C	G	C	T	T	C

Table B-4. Variation sites for the 22 haplotypes of *C. brevis* sequences for the combined Cytb and 16SrRNA genes for the island samples. Dots are nucleotides equal to the first row. Numbers indicate the position of the variation sites on the combined sequences.

	92	107	110	122	128	143	146	155	170	191	203	206	257	258	272	275	278	280
AA-1	T	T	C	T	G	T	C	T	C	T	C	C	T	T	A	A	A	T
AA-3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AB-1	C	C	T	C	A	C	T	C	T	C	T	A	C	.	.	.	.	.
AB-4	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	G	.
AB-5	C	C	T	C	A	C	T	C	T	C	T	A	C	.	.	.	.	.
AC-2	C	C	T	C	A	C	T	C	T	C	T	A	C	.	.	.	.	.
PDA-2	C	C	T	C	A	C	T	C	T	C	T	A	C	.	.	.	.	.
PDA-3	C	C	T	C	A	C	T	C	T	C	T	A	C	.	.	.	.	.
PDA-5	C	C	T	C	A	C	T	C	T	C	T	A	C	.	.	.	.	.
PDB-1	C	C	T	C	A	C	T	C	T	C	T	A	C	.	.	.	.	.
PDB-2	C	C	T	C	A	C	T	C	T	C	T	A	C	.	.	.	.	.
PDC-1	C	C	T	C	A	C	T	C	T	C	T	A	C	.	.	.	.	.
HA-1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HA-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	G
HA-3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HA-4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HA-5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HB-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
HB-4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.
M-1	C	C	T	C	A	C	T	C	T	C	T	A	C	.	.	.	.	.
M-2	C	C	T	C	A	C	T	C	T	C	T	A	C	.	.	.	.	.
M-9	C	C	T	C	A	C	T	C	T	C	T	A	C	.	T	C	.	.

Table B-5. Variation sites for the 22 haplotypes of *C. brevis* sequences for the combined Cytb and 16SrRNA genes for the island samples. Dots are nucleotides equal to the first row. Numbers indicate the position of the variation sites on the combined sequences..

	327	328	345	349	350	372	396	397	401	405	434	475	585	592	646	647	652	654
AA-1	A	T	G	A	G	T	T	T	T	C	G	C	T	A	T	C	T	C
AA-3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
AB-1	.	C	A	G	A	C	C	C	C	T	A	T	.	.	.	.	.	.
AB-4	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
AB-5	.	C	A	G	A	C	C	C	C	T	A	T	.	.	.	.	.	.
AC-2	.	C	A	G	A	C	C	C	C	T	A	T	.	.	.	.	.	.
PDA-2	.	C	.	.	.	C	C	C	C	T	A	T	.	T	.	.	.	.
PDA-3	.	C	A	G	A	C	C	C	C	T	A	T	.	T	.	.	.	.
PDA-5	.	C	A	G	A	C	C	C	C	T	A	T	.	.	.	.	.	.
PDB-1	.	C	.	.	.	C	C	C	C	T	A	T	.	.	.	.	A	.
PDB-2	.	C	A	G	A	C	C	C	C	T	A	T	.	.	.	.	.	.
PDC-1	.	C	A	G	A	C	C	C	C	T	A	T	.	.	.	.	.	.
HA-1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
HA-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HA-3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	A	.	.
HA-4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HA-5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HB-2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HB-4	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
M-1	G	C	A	G	A	C	C	C	C	T	A	T	G	.	.	.	.	.
M-2	.	C	A	G	A	C	C	C	C	T	A	T	.	.	.	.	.	.
M-9	.	C	A	G	A	C	C	C	C	T	A	T	.	.	.	.	.	.

Table B-6. Distance matrix of estimates of evolutionary divergence between the combined sequences of Cytb and 16S rRNA of *C. brevis* for all subpopulations sampled. The number of base differences between sequences is shown. The analysis involved 67 nucleotide sequences.

	AFR2	AFR3	AFR4	AFR5	AUS1	AUS2	AUS5	CLA1	CLA2	CLA3	CLA4	CLA5	CLB1	CLB2
AFR2														
AFR3	29													
AFR4	33	4												
AFR5	17	39	40											
AUS1	26	4	6	38										
AUS2	27	3	5	37	3									
AUS5	29	2	4	39	2	1								
CLA1	11	30	34	24	27	28	30							
CLA2	10	27	31	22	24	25	27	2						
CLA3	8	25	29	23	22	23	25	8	7					
CLA4	13	34	36	23	31	32	34	14	13	12				
CLA5	15	15	19	29	13	14	15	16	13	11	20			
CLB1	29	7	7	39	7	8	7	30	27	25	32	14		
CLB2	29	4	4	39	4	5	4	30	27	25	32	15	3	
CLB3	30	6	6	40	6	7	6	31	28	26	33	15	1	4
CLB4	29	7	7	39	7	8	7	30	27	25	32	14	0	3
CLB5	29	7	7	39	7	8	7	30	27	25	32	14	0	3
CR1	26	7	7	36	3	6	5	27	24	22	29	16	8	5
CR2	11	22	24	23	20	21	22	12	9	7	14	9	21	20
CR3	29	4	4	39	4	5	4	30	27	25	32	15	3	2
CR4	18	15	17	30	13	16	15	22	20	16	21	20	16	15
ELS1	31	4	4	41	4	5	4	32	29	27	34	17	5	2
ELS3	30	5	5	40	3	6	5	31	28	26	33	16	6	3
ELS4	32	7	7	40	5	8	7	33	30	28	35	18	8	5
ELS5	27	8	8	37	5	6	8	28	25	23	30	15	9	6

Table B-7. Distance matrix of estimates of evolutionary divergence between the combined sequences of Cytb and 16S rRNA of *C. brevis* for all subpopulations sampled. The number of base differences between sequences is shown. The analysis involved 67 nucleotide sequences.

	AFR2	AFR3	AFR4	AFR5	AUS1	AUS2	AUS5	CLA1	CLA2	CLA3	CLA4	CLA5	CLB1	CLB2
FLD1	123	134	135	129	132	133	134	129	127	124	124	131	135	134
FLD3	13	37	39	24	34	35	37	19	20	17	19	24	35	35
HN1	29	4	4	39	4	5	4	30	27	25	32	15	3	2
HN3	29	4	4	39	4	5	4	30	27	25	32	15	3	2
HN4	45	20	20	54	20	21	20	46	43	41	48	31	19	18
HN5	30	5	5	40	5	6	5	31	28	26	33	16	4	3
PEA3	29	4	4	39	4	5	4	30	27	25	32	15	3	2
PEA4	14	21	21	28	19	20	21	18	16	14	17	10	18	17
PEB1	31	4	4	41	4	5	4	32	29	27	32	17	5	2
PEB2	31	4	4	41	4	5	4	32	29	27	34	17	5	2
PEB4	31	4	4	41	4	5	4	32	29	27	32	17	5	2
PEB5	31	4	4	41	4	5	4	32	29	27	32	17	5	2
VZ1	14	33	35	28	30	31	33	19	18	16	21	19	31	31
VZ5	12	27	29	25	24	25	27	16	15	10	18	17	25	25
AA1	31	4	4	41	4	5	4	32	29	27	34	17	5	2
AA3	30	3	3	40	3	4	3	31	28	26	33	16	4	1
AB1	8	34	36	19	31	32	34	14	15	13	16	20	32	32
AB2	8	34	36	19	31	32	34	14	15	13	16	20	32	32
AB4	31	9	9	41	9	10	9	32	29	27	34	16	4	5
AB5	10	35	37	23	32	33	35	17	18	15	19	22	33	33
AC2	8	34	36	19	31	32	34	14	15	13	16	20	32	32
PDA2	9	34	36	20	31	32	34	15	16	13	17	20	32	32
PDA3	9	35	37	20	32	33	35	15	16	14	17	21	33	33
PDA5	8	33	35	19	30	31	33	14	15	12	16	19	31	31
PDB1	9	34	36	20	31	32	34	15	16	13	17	20	32	32
PDB2	8	33	35	19	30	31	33	14	15	12	16	19	31	31
PDC1	8	33	35	19	30	31	33	14	15	12	16	19	31	31

Table B-8. Distance matrix of estimates of evolutionary divergence between the combined sequences of Cytb and 16S rRNA of *C. brevis* for all subpopulations sampled. The number of base differences between sequences is shown. The analysis involved 67 nucleotide sequences.

	AFR2	AFR3	AFR4	AFR5	AUS1	AUS2	AUS5	CLA1	CLA2	CLA3	CLA4	CLA5	CLB1	CLB2
HA1	32	5	5	42	5	6	5	33	30	28	35	18	6	3
HA2	32	5	5	42	5	6	5	33	30	28	35	18	6	3
HA3	33	6	6	43	6	7	6	34	31	27	36	19	7	4
HA4	31	4	4	41	4	5	4	32	29	27	34	17	5	2
HA5	30	3	3	40	3	4	3	31	28	26	33	16	4	1
HB1	31	4	4	41	4	5	4	32	29	27	34	17	5	2
HB2	32	5	5	42	5	6	5	33	30	28	35	18	6	3
HB3	32	5	5	42	5	6	5	33	30	28	35	18	6	3
HB4	32	5	5	42	5	6	5	33	30	28	35	18	6	3
HB5	30	3	3	40	3	4	3	31	28	26	33	16	4	1
M1	9	34	36	20	31	32	34	15	16	13	17	20	32	32
M2	8	33	35	19	30	31	33	14	15	12	16	19	31	31
M5	8	33	35	19	30	31	33	14	15	12	16	19	31	31
M7	8	33	35	19	30	31	33	14	15	12	16	19	31	31
M9	10	35	37	21	32	33	35	14	15	14	18	21	33	33

Table B-9. Distance matrix of estimates of evolutionary divergence between the combined sequences of Cytb and 16S rRNA of *C. brevis* for all subpopulations sampled. The number of base differences between sequences is shown. The analysis involved 67 nucleotide sequences.

	CLB3	CLB4	CLB5	CR1	CR2	CR3
CLB4	1					
CLB5	1	0				
CR1	7	8	8			
CR2	22	21	21	17		
CR3	2	3	3	5	20	
CR4	15	16	16	10	15	13

Table B-10. Distance matrix of estimates of evolutionary divergence between the combined sequences of Cytb and 16S rRNA of *C. brevis* for all subpopulations sampled. The number of base differences between sequences is shown. The analysis involved 67 nucleotide sequences.

	CLB3	CLB4	CLB5	CR1	CR2	CR3	CR4	ELS1	ELS3	ELS4	ELS5	FLD1	FLD3	HN1	HN3	HN4
ELS1	4	5	5	5	22	2	15									
ELS3	5	6	6	4	21	3	14	1								
ELS4	7	8	8	6	23	5	16	3	2							
ELS5	8	9	9	7	20	6	15	4	3	5						
FLD1	135	135	135	130	127	133	124	135	134	133	133					
FLD3	36	35	35	32	18	35	25	37	36	38	34	130				
HN1	2	3	3	5	20	0	13	2	3	5	6	133	35			
HN3	2	3	3	5	20	0	13	2	3	5	6	133	35	0		
HN4	18	19	19	21	36	16	29	18	19	21	22	147	51	16	16	
HN5	3	4	4	6	21	1	14	3	4	6	7	132	36	1	1	17
PEA3	2	3	3	5	20	0	13	2	3	5	6	133	35	0	0	16
PEA4	19	18	18	20	11	17	20	19	18	20	17	128	22	17	17	33
PEB1	4	5	5	5	22	2	15	2	3	5	6	135	37	2	2	18
PEB2	4	5	5	5	22	2	15	2	3	5	6	133	37	2	2	18
PEB4	4	5	5	5	22	2	15	2	3	5	6	135	37	2	2	18
PEB5	4	5	5	5	22	2	15	2	3	5	6	135	37	2	2	18
VZ1	32	31	31	30	17	31	26	33	32	34	29	132	20	31	31	47
VZ5	26	25	25	24	13	25	18	27	26	28	23	126	18	25	25	41
AA1	4	5	5	5	22	2	15	2	3	5	6	135	37	2	2	18
AA3	3	4	4	4	21	1	14	1	2	4	5	134	36	1	1	17
AB1	33	32	32	29	14	32	21	34	33	35	30	127	7	32	32	47
AB2	33	32	32	29	14	32	21	34	33	35	30	127	7	32	32	47
AB4	5	4	4	10	23	5	18	7	8	10	11	137	35	5	5	21
AB5	34	33	33	30	16	33	23	35	34	36	32	130	7	33	33	48
AC2	33	32	32	29	14	32	21	34	33	35	30	127	9	32	32	47
PDA2	33	32	32	29	14	32	21	34	33	35	30	126	9	32	32	47
PDA3	34	33	33	30	15	33	22	35	34	36	31	127	8	33	33	48

Table B-11. Distance matrix of estimates of evolutionary divergence between the combined sequences of Cytb and 16S rRNA of *C. brevis* for all subpopulations sampled. The number of base differences between sequences is shown. The analysis involved 67 nucleotide sequences.

	CLB3	CLB4	CLB5	CR1	CR2	CR3	CR4	ELS1	ELS3	ELS4	ELS5	FLD1	FLD3	HN1	HN3	HN4
PDA5	32	31	31	28	13	31	20	33	32	34	29	127	8	31	31	46
PDB1	33	32	32	29	14	32	21	34	33	35	30	127	9	32	32	47
PDB2	32	31	31	28	13	31	20	33	32	34	29	127	8	31	31	46
PDC1	32	31	31	28	13	31	20	33	32	34	29	127	8	31	31	46
HA1	5	6	6	6	23	3	16	3	4	6	7	136	36	3	3	19
HA2	5	6	6	6	23	3	16	3	4	6	7	136	38	3	3	19
HA3	6	7	7	7	24	4	17	4	5	7	8	137	37	4	4	20
HA4	4	5	5	5	22	2	15	2	3	5	6	135	35	2	2	18
HA5	3	4	4	4	21	1	14	1	2	4	5	134	36	1	1	17
HB1	4	5	5	5	22	2	15	2	3	5	6	135	35	2	2	18
HB2	5	6	6	6	23	3	16	3	4	6	7	136	36	3	3	19
HB3	5	6	6	6	23	3	16	3	4	6	7	136	36	3	3	19
HB4	5	6	6	6	23	3	16	3	4	6	7	135	38	3	3	19
HB5	3	4	4	4	21	1	14	1	2	4	5	134	36	1	1	17
M1	33	32	32	29	14	32	21	34	33	35	30	126	9	32	32	47
M2	32	31	31	28	13	31	20	33	32	34	29	127	8	31	31	46
M5	32	31	31	28	13	31	20	33	32	34	29	127	8	31	31	46
M7	32	31	31	28	13	31	20	33	32	34	29	127	8	31	31	46
M9	34	33	33	30	15	33	22	35	34	36	31	129	10	33	33	48

Table B-12. Distance matrix of estimates of evolutionary divergence between the combined sequences of Cytb and 16S rRNA of *C. brevis* for all subpopulations sampled. The number of base differences between sequences is shown. The analysis involved 67 nucleotide sequences.

	HN5	PEA3	PEA4	PEB1	PEB2	PEB4	PEB5	VZ1	VZ5	AA1	AA3	AB1	AB2	AB4	AB5	AC2
PEA3	1															
PEA4	18	17														
PEB1	3	2	19													
PEB2	3	2	19	2												
PEB4	3	2	19	0	2											
PEB5	3	2	19	0	2	0										
VZ1	32	31	18	33	33	33	33									
VZ5	26	25	18	27	27	27	27	12								
AA1	3	2	19	2	2	2	2	33	27							
AA3	2	1	18	1	1	1	1	32	26	1						
AB1	33	32	17	34	34	34	34	16	15	34	33					
AB2	33	32	17	34	34	34	34	16	15	34	33	0				
AB4	6	5	20	7	7	7	7	32	27	7	6	32	32			
AB5	34	33	20	35	35	35	35	18	16	35	34	4	4	33		
AC2	33	32	17	34	34	34	34	16	15	34	33	2	2	34	6	
PDA2	33	32	18	34	34	34	34	17	15	34	33	3	3	34	6	3
PDA3	34	33	18	35	35	35	35	17	16	35	34	1	1	33	5	3
PDA5	32	31	17	33	33	33	33	16	14	33	32	2	2	33	5	2
PDB1	33	32	18	34	34	34	34	17	15	34	33	3	3	34	6	3
PDB2	32	31	17	33	33	33	33	16	14	33	32	2	2	33	5	2
PDC1	32	31	17	33	33	33	33	16	14	33	32	2	2	33	5	2
HA1	4	3	20	3	3	3	3	34	28	3	2	33	33	6	34	35
HA2	4	3	20	3	3	3	3	34	28	3	2	35	35	8	36	35
HA3	5	4	21	4	4	4	4	35	29	4	3	34	34	7	35	36
HA4	3	2	19	2	2	2	2	33	27	2	1	32	32	5	33	34
HA5	2	1	18	1	1	1	1	32	26	1	0	33	33	6	34	33
HB1	3	2	19	2	2	2	2	33	27	2	1	32	32	5	33	34

Table B-13. Distance matrix of estimates of evolutionary divergence between the combined sequences of Cytb and 16S rRNA of *C. brevis* for all subpopulations sampled. The number of base differences between sequences is shown. The analysis involved 67 nucleotide sequences.

	HN5	PEA3	PEA4	PEB1	PEB2	PEB4	PEB5	VZ1	VZ5	AA1	AA3	AB1	AB2	AB4	AB5	AC2
HB2	4	3	20	3	3	3	3	34	28	3	2	33	33	6	34	35
HB3	4	3	20	3	3	3	3	34	28	3	2	33	33	6	34	35
HB4	4	3	20	3	3	3	3	34	28	3	2	35	35	8	36	35
HB5	2	1	18	1	1	1	1	32	26	1	0	33	33	6	34	33
M1	33	32	18	34	34	34	34	17	15	34	33	3	3	34	6	3
M2	32	31	17	33	33	33	33	16	14	33	32	2	2	33	5	2
M5	32	31	17	33	33	33	33	16	14	33	32	2	2	33	5	2
M7	32	31	17	33	33	33	33	16	14	33	32	2	2	33	5	2
M9	34	33	19	35	35	35	35	18	16	35	34	4	4	35	7	4

Table B-14. Distance matrix of estimates of evolutionary divergence between the combined sequences of Cytb and 16S rRNA of *C. brevis* for all subpopulations sampled. The number of base differences between sequences is shown. The analysis involved 67 nucleotide sequences.

	PDA2	PDA3	PDA5	PDB1	PDB2	PDC1	HA1	HA2	HA3	HA4	HA5
PDA3	2										
PDA5	1	3									
PDB1	2	4	1								
PDB2	1	3	0	1							
PDC1	1	3	0	1	0						
HA1	35	34	34	35	34	34					
HA2	35	36	34	35	34	34	4				
HA3	36	35	35	36	35	35	3	5			
HA4	34	33	33	34	33	33	1	3	2		
HA5	33	34	32	33	32	32	2	2	3	1	
HB1	34	33	33	34	33	33	1	3	2	0	1

Table B-15. Distance matrix of estimates of evolutionary divergence between the combined sequences of Cytb and 16S rRNA of *C. brevis* for all subpopulations sampled. The number of base differences between sequences is shown. The analysis involved 67 nucleotide sequences.

	PDA2	PDA3	PDA5	PDB1	PDB2	PDC1	HA1	HA2	HA3	HA4	HA5	HB1	HB2	HB3	HB4	HB5
HB2	35	34	34	35	34	34	2	3	3	1	2	1				
HB3	35	34	34	35	34	34	2	3	3	1	2	1	0			
HB4	33	34	34	35	34	34	4	2	5	3	2	3	4	4		
HB5	33	34	32	33	32	32	2	2	3	1	0	1	2	2	2	
M1	2	4	1	2	1	1	35	35	36	34	33	34	35	35	35	33
M2	1	3	0	1	0	0	34	34	35	33	32	33	34	34	34	32
M5	1	3	0	1	0	0	34	34	35	33	32	33	34	34	34	32
M7	1	3	0	1	0	0	34	34	35	33	32	33	34	34	34	32
M9	3	5	2	3	2	2	36	34	37	35	34	35	36	36	34	34

Table B-16. Distance matrix of estimates of evolutionary divergence between the combined sequences of Cytb and 16S rRNA of *C. brevis* for all subpopulations sampled. The number of base differences between sequences is shown. The analysis involved 67 nucleotide sequences.

	M1	M2	M5	M7
M2	1			
M5	1	0		
M7	1	0	0	
M9	3	2	2	2

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BIOGRAPHICAL SKETCH

Maria Teresa Monteiro da Rocha Bravo Ferreira was born in 1982, in Lisbon, Portugal, to a middle class family of a school teacher and a computer technician. Being a school teacher daughter she quickly learned the discipline and love for learning. Being a straight-A student, she never gave grievance to her parents and completed her early studies with honors.

Between the years of 1994 and 2000, she attended Liceu Camões, a very respected and traditional high school in Lisbon. There, she developed her love for science by taking several different laboratory courses in chemistry and biology. In 2000, she graduated from High School and went on to be a freshman in college pursuing a Biology major. At the Animal Biology Department at the Faculdade de Ciências da Universidade de Lisboa (Science College of the University of Lisbon), she joined the small entomology group in 2003 doing volunteer work in insect capturing and sorting. She did a year study in the Azorean Isle of São Miguel working with Diptera diversity in her final year in college. She graduated from college in July 2005, but continued her volunteer work with the entomology group until early 2006.

In 2006, she moved to the Azorean Isle of Terceira where she began work as a technician on a project to determine management tools for the *Cryptotermes brevis* infestation in the Azores. She worked in this project until the end of that year.

In 2007, she entered the graduate program at the University of Florida in the Department of Entomology and Nematology where she graduated with a master's degree in August 2008. She is currently seeking her PhD in entomology at the University of Florida.