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Systematics

Determining the Origin of the Coffee Berry Borer Invasion of Hawaii

ERIC G. CHAPMAN,[1,2] RUSSELL H. MESSING,[3] AND JAMES D. HARWOOD[1]

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ABSTRACT The coffee berry borer, *Hypothenemus hampei* (Coleoptera: Curculionidae: Scolytinae), was first discovered in coffee farms on the Big Island of Hawaii in 2010, after over 200 yr of borer-free coffee production. Because there are multiple pathways by which *H. hampei* could have entered Hawaii from >50 coffee-producing nations that harbor the pest, determining the invasion route requires genetic analyses. A previous study identified 27 *H. hampei* cytochrome *c* oxidase subunit I haplotypes from around the world using phylogenetic analyses to identify putative species. We sequenced cytochrome *c* oxidase subunit I from specimens collected in Hawaii and conducted phylogenetic and haplotype network analyses to trace the route of invasion. We conducted a network analysis to trace the most likely pathway that *H. hampei* could have taken to Hawaii and a phylogenetic analysis to assess clade support for broader groupings in the network analysis that are unlikely to have recently hybridized. The Hawaiian haplotype was identical to a haplotype from six Latin American countries, and our network analysis suggests the most likely route of invasion was from Kenya to Uganda to Latin America to Hawaii. Most coffee shipments from Latin America are fumigated, arrive on Oahu, and are processed before being shipped to other islands. Therefore, it is likely that *H. hampei* was accidentally transported to the Big Island by farm workers or other travelers from Latin America who carried borer-infested seeds in their clothing or luggage, or else by small quantities of illegally imported beans, although improper fumigation of shipments from Latin America remains a possibility.

KEY WORDS *Hypothenemus hampei*, Scolytinae, introduced species, network analysis, COI

The accidental introduction of exotic species into areas outside of their current range, largely facilitated by the ease of world travel in recent decades, has caused and continues to cause major economic and ecological problems throughout the world. While only a small percentage of introduced species find suitable habitats to survive and reproduce, those that do can reach high population densities due to a number of factors, including (but not limited to) a lack of natural enemies, and a lack of resistance in their newfound hosts or prey. Of course, some areas are much more prone to invasion than others due to the presence of a wide variety of habitats, mild climate, and large international ports of entry. For example, subtropical Florida, with multiple ports of entry, is now home to >1,000 exotic insect species that cause >US$1 billion in agricultural and structural damage annually (Simberloff et al. 1997).

In order to stop the spread of pest species, it is important to understand invasion pathways that are often not readily apparent, especially for widespread pests with multiple possible origins. The use of genetic markers to trace the origin of invasive arthropods has been well documented (e.g., hemlock wooly adelgid from Japan to eastern North America: Havill et al. 2006; spread of the pine shoot beetle in the Mediterranean Basin: Horn et al. 2006; avocado thrips from Mexico to California: Rugman-Jones et al. 2007; horse chestnut leaf-miner dispersal across Europe: Valde et al. 2009; multiple introductions of the red tomato spider mite into the Old World from South America: Boubou et al. 2011). These studies illustrate the usefulness of genetic data to trace the area of origin of invasive species, and thereby infer invasion pathways. Understanding the means by which exotic pest species spread gives port inspectors a better chance of preventing their introduction.

For two hundred years, the Hawaiian Islands have been home to a thriving coffee-growing industry, which expanded from its origins on the Kona Coast to include farms on all major islands, international exports, value-added products, and agri-tourism. The industry currently exceeds 6,000 acres of farmland, with a farm gate value of 32 million dollars. Coffee is the fifth largest crop (by cash receipts) in the state, and an economic mainstay of the Big Island of Hawaii (National Agricultural Statistics Service [NASS] 2011).

Horticultural and pest management practices for Hawaii's coffee industry have long been successful, given the absence of major insect and mite pests on most farms in most years. However, this changed in 2010 with the first reported records of the coffee berry

[1] Department of Entomology, University of Kentucky, S-225 Agricultural Science Center N., Lexington, KY 40546-0091.
[2] Corresponding author, e-mail: ericgchapman@gmail.com.
[3] University of Hawaii at Manoa, Kauai Agricultural Research Center, 7370 Kuamoo Rd., Kapaa, HI 97646.

borer, *Hypothenemus hampei* (Ferrari 1867) (Coleoptera: Curculionidae: Scolytinae), an invasive species that has come to threaten the existence of coffee as a viable agricultural commodity in the state (Messing 2012). The coffee berry borer is the most widespread and destructive insect pest of coffee worldwide (Damon 2000, Jaramillo et al. 2006). With origins in tropical Africa, this pest has spread to every major coffee-growing region in the world, reportedly causing >US$500 million in damage annually (Vega et al. 2003, Vega 2004), which now appears to be a conservative estimate, as it's been estimated to cause US$215–358 million annual damage in Brazil alone (Oliveira et al. 2013). *H. hampei* lives and feeds deep inside the coffee seed, making it an extremely difficult insect to control with conventional insecticides. Biological control solutions explored to date have had only marginal impact at best (e.g., Damon 2000, Baker et al. 2002, Jaramillo et al. 2005).

A quarantine of incoming coffee plants and plant parts (seeds, cuttings, rootstock, etc.) has been in effect in Hawaii since 1888 to protect the industry, but the law was amended in the 1970s to allow bulk unprocessed fumigated green coffee to be imported for the purpose of mixing with high-quality local beans (subsequently sold as "Kona Coffee," though it contains only 10% Kona beans; Kona Coffee Farmers Association [KCFA] 2013). Some growers have voiced concern that imported beans may have been the source of the beetle invasion (KCFA 2010), while others hypothesized that migrant farm workers may have inadvertently carried beetle-infested seeds on their clothing (Kona Coffee Blog 2010: http://konacoffeeblog.wordpress.com, last accessed 30 March 2015). Given this lack of understanding of beetle invasion, determining the geographic source of *H. hampei* in Hawaii could help address these competing scenarios. For example, if the beetles are determined to originate from Vietnam or Indonesia (both major coffee producers that export green coffee to Hawaii), they are unlikely to have been introduced by farm workers who hail mainly from Latin America. Thus, knowledge of the beetles' origin may help us to better understand invasion routes and processes.

While *H. hampei* occurs in >50 countries throughout the tropical world (Vega et al. 2003), it is not a single, uniform, or panmictic population. Rather, Gauthier (2010) demonstrated that *H. hampei* comprises a complex of discrete genetic populations, each of which may represent a separate sibling species. With distinctive genetic markers known for each of the five major geographic clusters of *H. hampei* identity, genetic testing of recently invasive specimens from Hawaii would enable us to match these markers and thus pinpoint the origin of the invasion. *H. hampei* was first detected as a coffee pest in Africa in 1901 (Le Pelley 1968) and had spread to Java, most Asian coffee-growing nations, and Brazil by 1925 (Gauthier 2010)—after which the world-wide coffee trade undoubtedly increased with increased ease of world travel. Thus, there is a high likelihood that multiple introductions of *H. hampei* may have occurred in some areas, with potential for hybridization (i.e., reticulation). Therefore, due to the potential for multifurcating and reticulating relationships among populations (Posada and Crandall 2001), a network analysis has the potential to uncover pathways that may be unclear via phylogenetic analyses for intraspecific genetic data.

In this study, we sequenced the barcode region of cytochrome *c* oxidase subunit I (COI; Hebert et al. 2003) of recently introduced Hawaiian specimens, added it to Gauthier's (2010) COI data set, and conducted a network analysis to gain insight into the probable source population of one of the Islands' newest and most economically important invasive pests. We also conducted phylogenetic analyses to assess clade support for broader groupings that were not likely to have recently hybridized. Our network analysis is the first such analysis of *H. hampei* haplotypes and yields insights into the pathways the pest has followed as it spread from Africa into most coffee-growing nations around the world.

Materials and Methods

Specimen Collection. Specimens of *H. hampei* were collected from coffee plantations at six widely separated areas on the Big Island of Hawaii: 1) Kona (GPS coordinates: 19.4955° N, 155.8917° W, 23 May 2011); 2) Captain Cook, Kona (19.4661° N, 155.8987° W, 14 February 2014); 3) Kainaliu, Kona (19.5345° N, 155.9247° W, 18 February 2014); 4) Hawi (20.2484° N, 155.8240° W, 24 February 2014); 5) Pahala (19.1961° N, 155.4979° W, 18 February 2014); 6) Hilo (19.7278° N, 155.1100° W, 18 February 2014). At each farm, beetle specimens were dissected from 10 berries, each berry from a separate plant (10 widely separated trees randomly selected in each plantation). Because the specimens in each berry are the presumed progeny from a single female, one specimen from each berry was set aside for sequencing, and the remainder of the specimens were stored as vouchers. When only one specimen was found in a berry, its legs were removed for extraction and the remainder of the specimen was vouchered. Vouchers are stored at the University of Kentucky and the University of Hawaii at Manoa. Specimens of two other invasive beetles in Hawaii, *Hypothenemus obscurus* (F.) (Coleoptera: Cucurlionidae) (Hawaii: Pahala: Macadamia nut farm, 19.1905° N, 155.4732° W) and *Xylosandrus compactus* (Eichhoff) (Coleoptera: Cucurlionidae) (Hawaii: Honaunau: Macadamia nut farm; 19.4526° N, 155.5589° W) were sequenced for phylogenetic rooting purposes.

It was noted that specimens of *H. hampei* from Hawaii showed observable morphological variation. Some specimens had slightly shorter and thicker setae on the elytra, making them appear morphologically intermediate between *H. hampei* and *H. obscurus* (although they were classified as *H. hampei* by Bernarr Kumashiro, Hawaii Dept. of Agriculture State Taxonomist). Special attention was paid to the genetic markers of these "intermediates." All specimens were preserved immediately in 95% ethanol and stored at −20°C until DNA extraction.

DNA Extraction, PCR, and Sequencing. Total DNA was extracted from crushed whole specimens

(when multiple specimens were available from the same berry) or beetle legs using QIAGEN DNeasy Tissue Kits (QIAGEN Inc., Chatsworth, CA) following the manufacturer's animal tissue protocol. COI was amplified from scolytines with the primers *LCO-1490* (Folmer et al. 1994) and *HCO-700ME* (Breton et al. 2006), which typically produce a 710 bp amplicon in arthropods (658 bp between primers). PCR reactions (50 µl) consisted of 1U Takara buffer (Takara Bio Inc., Shiga, Japan), 0.2 mM of each dNTP, 0.2 µM of each primer, 1.25U Takara Ex Taq, and template DNA (2 µl of total DNA, concentration unknown). PCR reactions were carried out in C1000 thermal cyclers (Bio-Rad Laboratories, Hercules, CA). The PCR cycling protocols were 94°C for 1 min followed by 50 cycles of 94°C for 50 s, 40°C for 45 s, 72°C for 45 s, and a final extension of 72°C for 5 min. Reaction success was determined by electrophoresis of 10 µl of PCR product in 1.5% SeaKem agarose (Lonza, Rockland, ME) stained with ethidium bromide (0.1 mg/µl). Reactions that yielded significant product were purified with a QIAGEN MinElute PCR purification kit according to the manufacturer's guidelines. Cycle sequencing reactions were carried out in both the forward and reverse directions using labeled dideoxynucleotides (ABI Big-Dye Terminator mix v. 3.0, Applied Biosystems, Foster City, CA, ABI sequencer) in an ABI 9700 thermal cycler. The separation of cycle sequencing reaction products was done by Applied Biosystems 3730XL or 3730 DNA Analyzers at the University of Kentucky Advanced Genetic Technologies Center (Lexington, Kentucky) and Beckman-Coulter Genomics (Danvers, Massachusetts). COI sequences of *H. hampei*, *H. obscurus*, and *X. compactus* were submitted to GenBank (Accession nos. KF724881–KF724883).

Sequence Alignment and Analysis. Bidirectional sequences were aligned using Geneious (v. 6.1.5; created by Biomatters: http://www.geneious.com/), and MAFFT (Katoh et al. 2005) was used to conduct multiple sequence alignments which contained no indels.

A Bayesian inference (BI) phylogenetic analysis was conducted with MrBayes (v. 3.1.2; Huelsenbeck and Ronquist, 2001, Ronquist and Huelsenbeck, 2003) on a 400-bp COI data set containing all 27 unique *H. hampei* COI haplotypes from Gauthier (2010) plus one exemplar from the 55 identical sequences that were generated from the Hawaiian specimens. One sequence each from *X. compactus* and *H. obscurus* were used for rooting purposes. Two independent simultaneous BI searches were run for 10 million generations (GTR+G+I model; Rodriguez et al. 1990), saving a tree every 500 generations, with four search chains each (temp = 0.01) to facilitate independent searches arriving at similar log-likelihood peaks/topologies. We used the most complex model available (GTR+G+I) as per recommendations of Huelsenbeck & Rannala (2004) for Bayesian analyses. The average standard deviation of the split frequencies was <0.004 at the end of the run. The 10,000 post-burn-in trees, determined by examination of the log probability of observing the data by generation plot with Tracer (v. 1.5 Rambaut and Drummond 2009), were used to calculate the majority rule consensus tree to assess nodal support.

A maximum likelihood (ML) tree search (eight search replicates) and a 200 replicate ML bootstrap analysis (Felsenstein 1985) were also conducted with (v. 2.0; Zwickl, 2006) using the same evolutionary model as the BI analysis and using the default settings. We used the software TCS 1.21 (Clement et al. 2000), a statistical parsimony method (Templeton et al. 1992) of network construction, to examine *H. hampei* COI haplotype relationships. We conducted two TCS analyses, one using the default settings, and a second with the confidence limit reduced to 90% (from a default of 95%).

Results

We were able to obtain COI sequences from 55 of the 60 specimens extracted, with an average sequence length (after trimming the primers) of 652 bp. Despite the observed morphological variation and wide separation among localities in the Hawaiian specimens, all 55 individuals of *H. hampei* (including the morphological "intermediates") represented a single COI haplotype, which was identical to Gauthier's (2010) AM-10N (GU133361) haplotype from Costa Rica. This haplotype was also found in Colombia, El Salvador, Mexico, Nicaragua, and the Dominican Republic (see GenBank accession GU133361); thus, we refer to this as the Latin American (Am-10N) haplotype. The Hawaii haplotype was one base-pair divergent from another haplotype from Costa Rica (CR1-COL1, GU133362), and those from Guatemala (GUA-3N, GU133360), Nicaragua (SM1, GU133363), and Uganda (NGO-2N, GU133343), and two base-pairs divergent from a Brazilian haplotype (Bra-2N, GU133358) and a Ugandan haplotype (KAW8, GU133344). Both the BI MAP tree (Bayesian maximum *a-posteriori* tree (= tree of highest posterior probability); Fig. 1) and ML tree (bootstrap values plotted on Fig. 1) show a sister relationship between the Hawaii and Latin American (AM-10N) haplotypes, although this relationship was not strongly supported by high bootstrap (BS) or posterior probability (PP) values. However, both BI and ML analyses supported a clade (BI PP = 0.94, MLBS = 74) composed of the Hawaii and all Central and South American haplotypes in the analysis, plus one haplotype from Uganda (NGO-2N; Fig. 1). This clade (minus the Hawaii haplotype) was also supported in the analyses in Gauthier (2010), albeit with a different arrangement of terminal taxa (i.e., countries).

Under the default setting of a 95% confidence limit (CL) which set the connection limit at eight steps, the network (TCS) analysis produced three unconnected networks composed of 1) all Ethiopian haplotypes, 2) a single haplotype from Java (KALIB1), and 3) all remaining haplotypes. Reducing the CL to 90% raised the CL to 12 steps and connected the lone Java haplotype to the non-Ethiopian network (Fig. 2). This network connected the Hawaiian and a single Latin American (Am-10N) haplotype to haplotypes from Costa Rica, Guatemala, Nicaragua, and Uganda by

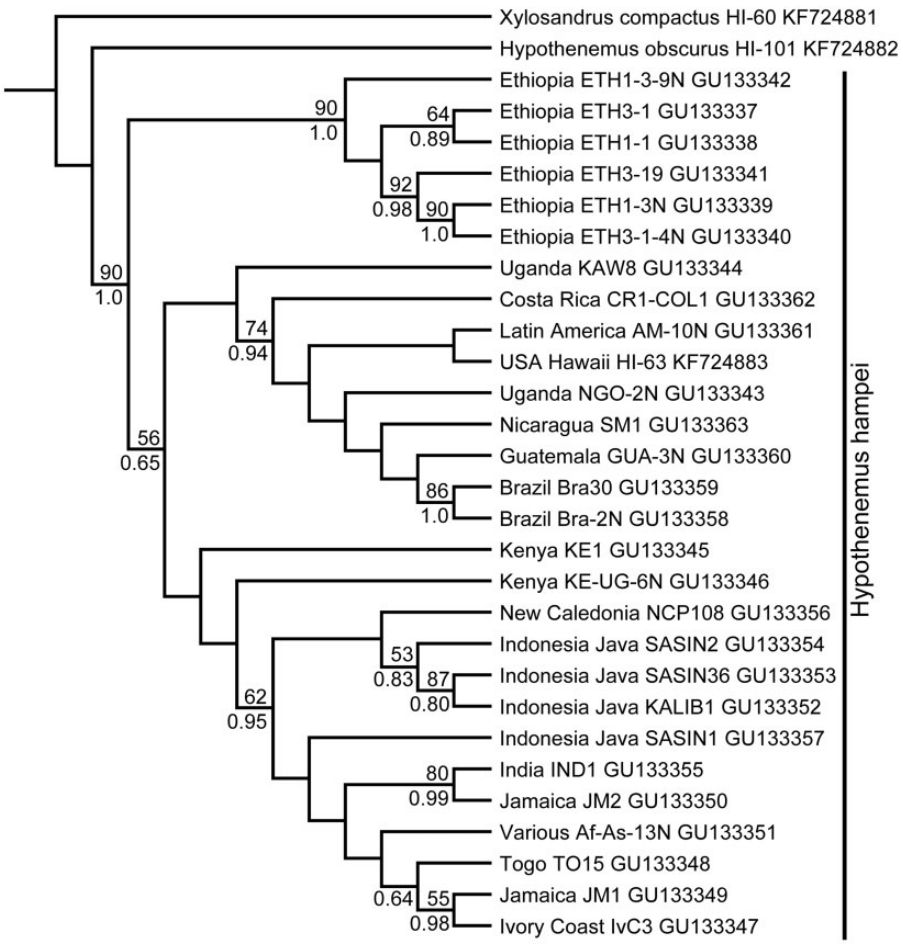

Fig. 1. Tree of highest posterior probability from a Bayesian phylogenetic analysis of 27 *H. hampei COI* haplotypes under the GTR+G+I substitution model. ML bootstrap values and posterior probabilities are plotted above and below the nodes, respectively. Specimen numbers from Gauthier (2010) and GenBank numbers are cited for each terminal. Note: the OTU labeled "Various AF-AS-13N" represents identical haplotypes from Ivory Coast, Togo, Cameroon, India, and New Caledonia.

single steps each. The shading on Figure 2 highlights the three well-supported major clades found by the phylogenetic analyses (Fig. 1), demonstrating the congruence between the major groups in the network and phylogenetic analyses. The data set used in this study is available from the senior author upon request and all sequences are available from GenBank.

Discussion

Hawaii is the most isolated landmass on Earth. Despite this geographic isolation, it is often referred to as the "invasive species capital of the world." With millions of tourists visiting annually, coupled with high levels of military transport and extensive importation of agricultural and ornamental produce, there is ample opportunity for small, invasive species to remain undetected. This is exacerbated by a lack of adequate inspection for goods and tourists entering the islands (whereas, ironically, there is a robust inspection program for people *leaving* the islands for the U.S. mainland). Approximately 20 exotic arthropod species become established in Hawaii every year; many of these become serious pests with profound ecological and economic impacts (Kumashiro et al. 2001, Messing and Wright 2006). The coffee berry borer is one such invasive pest, and is already causing severe economic disruption on a local scale.

We observed morphological variation in the *H. hampei* specimens sequenced from Hawai; some specimens had slightly shorter and thicker setae on the elytra, making them appear morphologically intermediate between *H. hampei* and *H. obscurus*. However, we only found a single haplotype among these 55 specimens, and their COI sequence clearly identified them as *H. hampei* (as did other morphological characters). The uncorrected p-distance between the Hawaii COI haplotype and *H. obscurus* is 12.5% (i.e., 70 differences among the 558 bp fragment), so it is virtually impossible that accidental mutations of an *H. obscurus* population arrived at the Hawaiian haplotype. One possibility is that the "intermediate" individuals were the

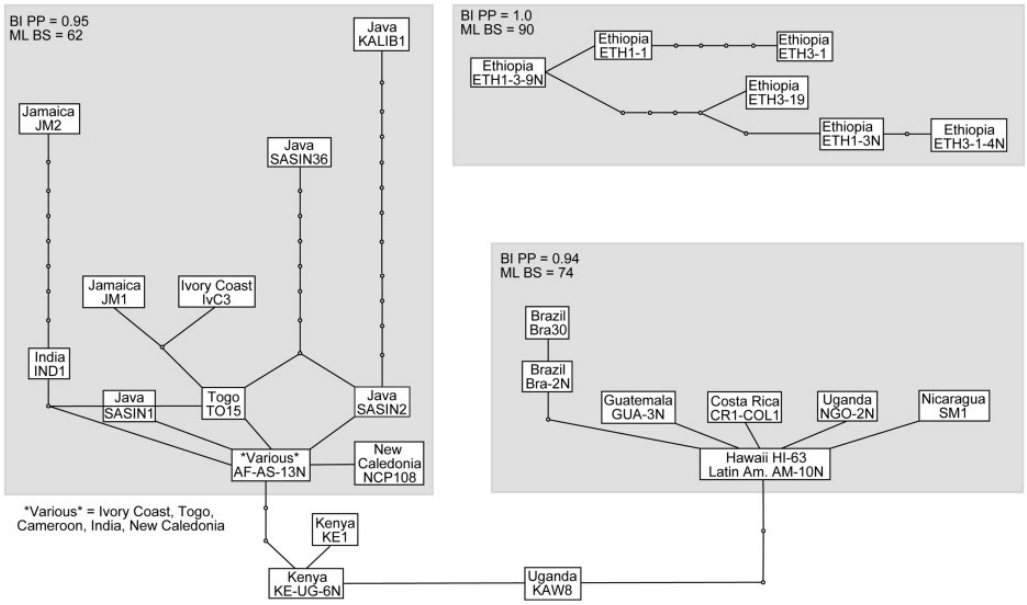

Fig. 2. TCS haplotype network showing relationships among populations of *H. hampei* sampled from around the world. The Hawaii and Latin America (AM-10N) haplotypes were identical; only one sequence was present in the analyzed dataset. Each line segment represents a single nucleotide change between connected populations (e.g., there are five differences between ETH1-1 and ETH3-1). Under a 95% confidence limit (CL), the Java (KALIB1) haplotype was separated from the other two networks, but connected to the larger network when the CL was relaxed to 90% (the analyses were otherwise identical). The shaded areas correspond to clades with high nodal support values in Figure 1, highlighting the general congruence between the two analysis methods. BI and ML support values are shown in the upper left corner of each shaded region (clade).

product of introgressive hybridization between a female *H. hampei* and a male *H. obscurus*, with subsequent generations back-crossing with *H. hampei*. However, such a hybridization event would have no effect on the conclusions of this study; it remains highly likely that the single observed haplotype had only a single source population, and that introgression, if it did occur, happened after *H. hampei* was introduced into Hawaii. Furthermore, the lack of variation in the Hawaii haplotype is likely due to a combination of 1) a single introduction in 2008 or preceding years, 2) not enough time for COI mutations to occur, and 3) the morphological variation is not controlled by COI (which functions in electron transport).

H. hampei is well established in every coffee-growing region of the world (with the possible exceptions of Nepal and Papua New Guinea: Burbano et al. 2011). Due to its small size, concealed life cycle, and ability to remain alive in coffee beans for several months (Baker and Barrera 1993), it was not unexpected that the pest would eventually arrive in Hawaii. However, quarantines are in place for just this reason, and it is important to identify the origin of these invasive pests to better understand how and where the detection system failed. With identification of genetic relationships of Hawaiian *H. hampei* to other populations around the world, it is possible to make inferences as to the most likely source of this introduction and likely routes of invasion. Our phylogenetic and network analyses convincingly suggest that Central and South America were colonized from a source population in Kenya through Uganda, and that Latin America was the source of the Hawaiian population of *H. hampei*. These results are generally congruent with those of Benavides et al. (2005) who conducted neighbor-joining cluster analysis on amplified fragment length polymorphisms (AFLP) to track the movements of *H. hampei* from Africa to the Americas, although their analysis suggested that Central American populations likely came from source populations in Brazil. In any case, the population discovered in Hawaii was most likely from Latin American stock. Furthermore, our network analysis supports Gauthier's (2010) conclusion that Ethiopian populations are distinct, as they formed a network not connected to the remaining populations. Our network analysis also supports a grouping of populations from India, Ivory Coast, Jamaica, Java, New Caledonia, and Togo; interestingly, one Ivory Coast haplotype (AF-AS-13N; Figs. 1 and 2) appears to be a key population in the spread of these populations. This haplotype is in a more derived position in our (Fig. 1) and Gauthier's (2010) phylogenetic analyses, illustrating the strength of network over phylogenetic analyses in situations where populations have undoubtedly interbred.

There are several routes by which *H. hampei* is considered likely to have reached Hawaii. First, the state imports >3,000 tons of green coffee beans each year. By law, this coffee must be fumigated with methyl bromide before arrival in the islands to kill any insect pests on or in the beans. Methyl bromide fumigation is

generally considered the most effective treatment and is the standard for international trade in green coffee (Hollingsworth et al. 2013). However, some green coffees for home roasters may escape this fumigation process if they are imported through regular mail services, private carriers, or by hand-carrying. Even though importation without fumigation is illegal, the rules may not be generally known in the mainland USA or Latin America. People who ship or receive small quantities may be unaware of the law, or may disregard the law.

Another possible route of *H. hampei* invasion is via farm workers, tourists, or Hawaii residents who travel to Hawaii after visiting, working, or residing in coffee-growing areas of Latin America. Many workers on Big Island coffee plantations, particularly during harvest and pruning (all Kona coffee on over 600 farms is harvested by hand), are from Latin America. As these workers would likely have spent time working in coffee farms in their home or neighboring countries (all of which are infested by *H. hampei*), it is plausible that a stray infested seed may have lodged in a boot, backpack, or other equipment of even the most careful worker, and inadvertently been transported to Hawaii. A single coffee seed can harbor up to 200 *H. hampei* in several life stages (Jaramillo 2008). Mating takes place among siblings within the seed (Bustillo et al. 1998) and adult beetles can survive in a semi-inactive state for months in old berries (Baker and Barrera, 1993), therefore easily surviving intercontinental or trans-oceanic transport.

Populations of *H. hampei* mainly cluster by geographic region (Gauthier 2010), so it is likely that genetic analyses can accurately pinpoint the source population of the infestation in Hawaii. Our phylogenetic and network analyses suggest that the source of the Hawaiian population was either from Central America or Uganda (Figs. 1 and 2). Considering that 1) Latin America is the source of the majority of migrant coffee workers; 2) Hawaii did not import traceable amounts of coffee from Uganda in the years immediately preceding detection (2008 and 2009); and 3) the Hawaii haplotype is identical to a haplotype from Latin America, we conclude that Latin America is the most likely source of the invasion.

The question remains whether *H. hampei* was introduced by way of imported beans or migrant workers or other travelers. Costa Rica was the second largest supplier of green coffee to Hawaii in 2008 and 2009, accounting for roughly 19% by weight of all imports. However, >90% of green coffee imported to Hawaii goes directly to Oahu, where *H. hampei* was only found quite recently, several years after the initial invasion; while only a very small percentage is imported to the Big Island where the invasion first occurred. If the source of *H. hampei* were from legal coffee bean shipments, *H. hampei* might be thought more likely to establish on Oahu before the Big Island, but this conclusion must be tempered by the fact that major commercial coffee roasters on Oahu occur in industrial urban areas, with very few if any coffee plants in close proximity. Furthermore, all legal shipments are fumigated. In combination, the evidence is more consistent with an inadvertent introduction via migrant workers or illegally imported coffee beans from Latin America, although we cannot rule out the possibility of improperly fumigated legal imports.

In summary, we conclude that the most likely source of the Hawaiian *H. hampei* population was a single introduction from Latin America and that the means of introduction was from coffee workers or visitors from that region. However, we cannot rule out the possibility that the source was from improperly fumigated coffee shipments from the region. Additionally, we were limited to Gauthier's COI data set, and therefore unable to test source populations that were not sampled in that study (e.g., Vietnam). Furthermore, because Gauthier's (2010) microsatellites did not show the variation necessary to distinguish among many populations, new microsatellite markers would need to be developed in order to bolster our results. The addition of another gene or AFLP would also help, but there was insufficient viable DNA remaining from Gauthier's (2010) work to add meaningful data to our study. While adding additional data may bolster the robustness of our conclusions, the available evidence points to a single *H. hampei* introduction from Latin America.

Invasive species are a worldwide scourge, causing incalculable damage to natural ecosystems and significant economic losses to agriculture and forestry. Understanding the routes by which these species travel, and applying the political will to reduce these pathways, may make it possible to reduce the rate of invasions and mitigate the extent of damage in Hawaii and elsewhere.

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