Phylogeographic patterns of mitochondrial haplotypes and nuclear genotypes of solanum fruit fly *Bactrocera latifrons* (Diptera: Tephritidae) from Ryukyu Islands indicate multiple origins and inter-strain breeding of the invasive species in Japan.

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Research Article

Keywords: Tephritid fruit flies, host plant, invasive pest, phylogeographic analysis, SNP genotyping

Posted Date: January 26th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-3896312/v1

Additional Declarations:

The authors declare no competing interests.

Tables 1-2 are available in the supplementary files section.

Abstract

Invasive fruit fly, *Bactrocera latifrons* primarily utilizes Solanaceae fruit crops in Ryukyu islands, southwest Japan. The fly species was reported to have invaded Japan twice and their distinct host preferences suggest that the two populations may be different strains derived from remote geographic origins. In this study, we surveyed various populations of *B. latifrons* in Okinawa by sequencing multiple mitochondrial and nuclear loci, thereby extrapolating their invasion events and phylogenetic origins. We used live, dried or ethanol-fixed specimens of *B. latifrons* captured from 15 islands between 2004 and 2020. Successfully sequenced COI, COII and ND4 regions of mitochondrial DNA revealed two distinct haplotypes and the phylogenetic analyses with those from other countries indicated that the haplotypes were clustered into two major clades. The most abundant haplotype found on Okinawa Island and many other islands was closely related to those from Malaysia, suggesting that it may have invaded from Southeast Asia. On the other hand, a minor haplotype consisting of older specimens exclusively from Yonaguni Island formed an independent clade along with those from Taiwan. In addition, sequencing *cry1* fragment of the nuclear gene uncovered distinct genotypes associated with the two haplotypes. The comparative analyses of both mitochondrial and nuclear genes also indicated crosses and introgression of the two strains on Yonaguni Island recently. Our study clearly demonstrates two distinct haplotypes/genotypes of *B. latifrons* with alternative host preferences have likely originated from independent invasion pathways, and therefore suggests that their genetic backgrounds should be carefully considered for customized pest control measures.

Introduction

Many species expand their ranges or migrate long-distance through water or air (Pinsky et al., 2020; Reynolds et al., 2017). Insects are the most diversified group of animals in terrestrial ecosystems and severely impact our agriculture and economy (Grimaldi 2023; Savary et al. 2019). Insect migrations are performed by their own flights or caused by natural phenomena such as air currents or tropical storms, or artificial immigration with logistics (Drake and Farrow 1988; Pimentel et al. 2001). Since migratory insects initially establish in non-native areas as a small population and remain unnoticed, genetic diversity analysis would be an effective approach for the first estimation of the actual scale and history of invasion. Comparing the DNA sequences, genetic diversity, and population structures of invaded insects with those of native ranges using molecular markers may pinpoint origins of the invasive populations and resolve their migratory pathways (Corin et al. 2007; Barr et al. 2014; Martinez-Sañudo et al. 2018).

Tephritid fruit flies (Diptera: tephritidae) constitute a group of flies utilizing a wide range of fruits and fleshy vegetables, some of which are known as serious pests attacking many crops (White and Elson-Harris 1992). Those pest flies have invaded and caused tremendous agricultural and economic losses in many parts of the world by accompanying international trading crops, being transported by tourists, or directly flying from their native geographic regions. Such invasive fruit flies have been frequently reported throughout the globe. For example, in the last 20 years, the oriental fruit fly *Bactrocera dorsalis* (Hendel,

1912) has invaded (with or without establishment) Africa (Ekesi et al. 2015), Europe (Nugnes et al. 2018) and North America (Vargas et al. 2014; McInnis et al. 2017). The melon fly *Zeugodacus cucurbitae* (Coquillett 1899) has invaded African countries: Uganda (2009), Burundi (2010), Ethiopia (2010), Malawi (2010) and Mozambique (2013) (De Meyer et al. 2015). Thus, molecular barcoding and phylogeographic surveys of fruit fly populations have been undertaken to monitor the invasion histories of those pests (Jacquard et al. 2013; San Jose et al. 2018).

Bactrocera latifrons (Hendel) is one of the most serious insect pests primarily attacking solanum fruit crops such as eggplants, tomatoes, and peppers (Vargas et al. 2015). *Bactrocera latifrons* is native to Southeast Asia, southern China, Taiwan, India, and Sri Lanka, and invaded many parts of the world. In Hawaii, *B. latifrons* that was first discovered on Oahu Island (Vargas and Nishida 1985), having expanded its distribution, now are present in almost all islands of Hawaii (Liquido et al. 1994). On the other hand, in Africa, *B. latifrons* was first detected in Tanzania in 2006 (Mwatawala et al. 2007). Its distribution was expanded to Kenya in 2007 (Mwatawala et al., 2010), Burundi in 2016 (Ndayizeye et al. 2019) and in the Democratic Republic of Congo in 2020 (Ndayizeye and Balangaliza 2021). In Europe, the first finding of *B. latifrons* was reported in Italy in 2019 (Gargiulo et al. 2021). Despite being such a globally distributed species, comprehensive phylogeographic relationships of all the solanum fruit fly populations have not been studied thus far.

In Japan, *B. latifrons* has officially invaded twice. The first invasion was reported in Yonaguni Island in 1984 (Kaneta et al. 1985), and the second was reported in Okinawa Island, approximately 500 km east from Yonaguni Island, in 2010 (Kohama 2014). The first established population was eradicated in 2011 by the sterile insect technique (SIT) (Fukugasako and Okamoto 2012). However, the second established population rapidly expanded its distribution not only to the western islands and Yonaguni Island, where the species had been successfully eradicated, but also to the northern islands of Okinawa (Taniguchi et al. 2018; Okinawa Prefectural Plant Protection Center 2022). The exact relationship of *B. latifrons* populations and their origins following these records have been elusive throughout the history of monitoring this species in Japan.

Considering its invasion history, eradication in Yonaguni in 2011, more recent invasion to Okinawa in 2010, and the fact that distance between Yonaguni Island and Okinawa Island is over 500 km (Kohama 2014), the two populations may have invaded from different geographic origins. In addition, these two populations exhibited distinct host utilization patterns under the host plant surveys in the field (Kohama 2014; Taniguchi et al., 2018) and showed different oviposition preferences also in the laboratory (Hisaoka et al. 2023). Therefore, detailed molecular investigation of these populations may not only allow us to elucidate their genetic relationships, but also to infer the genetic associations of Japanese populations among this fly species distributed around the world. In this study, we employ molecular phylogenetic and genotyping techniques to investigate the genetic diversity and phylogenetic divergence of mitochondrial and nuclear genes of the population that has been first recorded and eradicated in Yonaguni Island, and of that later found in Okinawa Island. We also estimate the phylogeographic patterns of invasive *B. latifrons* and its expansive distribution history in Okinawa prefecture using specimens of *B. latifrons*

collected throughout Okinawa Islands over the past 20 years, compared with those obtained outside Japan in search for their origins.

Materials & Methods

Specimen collection

The specimens of *B. latifrons* were collected from field surveys and harvest host fruits between 2019 and 2022. In addition, specimens air-dried or fixed in ethanol were provided from Okinawa Prefectural Plant Protection Center and Okinawa Prefectural Agricultural Research Center. The specimens outside Okinawa were also provided from Okinawa Prefectural Plant Protection Center (14 specimens from Hawaii) or Taiwan Agricultural Research Institute (8 specimens from Taiwan). The total number of specimens was 286 and they were collected from host plants listed below; *Diplocyclos palmatus* (L.) C. Jeffrey, *Capsicum annuum* L. 'Grossum' group, *Capsicum annuum* 'Parvo-acuminatum', *Capsicum chinense* Jacq., *Capsicum frutescens* L., *Solanum americanum* Mill., *Solanum lycopersicum* L., *Solanum melongena* L., *Solanum spirale* auct. non Roxb., *Solanum torvum* Sw. The specimens also included flies reared at Okinawa Prefectural Agricultural Research Center and Tropical Biosphere Research Center, Univ. Ryukyus.

DNA extraction, amplification, and Sanger sequencing

Genomic DNA was extracted from live specimens using either the gastrointestinal tract or whole abdomen. DNA from dried or ethanol-fixed specimens was extracted from the whole insect body. We used the Gentra Puregene Tissue Kit or QIAamp DNA mini (QIAGEN, Venlo, Netherland) following manufacturer's recommended protocols. Initially, four different gene regions were amplified by PCR: the mitochondrial genes Cytochrome c Oxidase II (COII, 576 base pairs [bp]) and NADH-ubiquinone oxidoreductase chain 4 (ND4, 669 bp), and the nuclear gene *cryptochrome 1* (*cry1*, 700 bp) and *wingless* (732 bp). We either used primers previously applied on tephritid files or designed specific primers targeting these genes (Table S1). We used BIOTAQ™ DNA Polymerase (Meridian Bioscience, Memphis, TN, USA) or TaKaRa Ex Taq I (TaKaRa Bio Inc., Shiga, Japan) for all PCR reactions in 10 μL per reaction. The PCR temperature profile was 95°C for 2 min followed by 35 cycles of denature at 95°C for 30 s, annealing at 56°C for the COI, COII, *cry1* genes and *wingless* or 53°C for the ND4 gene for 30 s, and extension at 72°C for 1 min for all genes. When PCR amplification failed, we increased the number of cycles to 40 for the failed DNA samples. PCR products were treated with Exonuclease I (E. coli) (New England BioLabs Inc., Ipswich, MA, USA) and Alkaline Phosphatase (Shrimp) (SAP) I (TaKaRa Bio Inc., Shiga, Japan) and directly sequenced with SuperDye™ v3.1 Cycle Sequencing Kit (AdvancedSeq, Pleasanton, CA, USA) by Sanger method using the ABI PRISM 3130xl Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). After the initial genotyping, representative specimens were selected based on the obtained sequences according to the collection sites, host plants, mitochondrial and nuclear genetic sequences (Table 1). These specimens were further used for sequencing cytochrome c oxidase subunit I (COI, 1203 bp) region using the universal and specific primers, LCO1490 and P2V2 (Table S1).

All Sanger chromatograms were inspected and corrected by CLC Main Workbench (QIAGEN, Venlo, Netherland) and determined nucleotide sequences were deposited to DDBJ/GenBank/EMBL (Table S2).

Molecular phylogenetic analysis

Multiple alignments of the sequences were generated by the program MUSCLE (Edgar, 2004) within a software package of MEGA11 (Molecular Evolutionary Genetics Analysis Version 11) (Tamura et al., 2021). Ambiguously aligned sites were corrected manually and gaps were removed with MEGA11 or CLC Main Workbench (QIAGEN, Venlo) along with its chromatographic information. Multi loci phylogenetic analyses of representative sequences (Table 1, Table S2) of *B. latifrons* were conducted with the sequences of other regions previously determined (China: MG020799; Italy: MZ621833 ~ MZ621835; Malaysia: FJ903490, JX129505, KT881556; Yonaguni Is., Japan: AB687549) and the mitogenomes of closely related species, *Bactrocera albistrigata* (Meijere 1911) (MH374118), *Bactrocera arecae* (Hardy and Adachi 1954) (KR23325), *Bactrocera frauenfeldi* (Schiner 1868) (MZ520731) and *Bactrocera limbifera* (Bezzi, 1919) (NC037722). We concatenated all the available sequences of COI, COII, and ND4 for each specimen and then performed phylogenetic analyses using Maximum Likelihood (ML) and Bayesian inference (BI). The optimal substitution model was determined by ModelTest-NG v0.1.7 (Darriba et al. 2020) and assessed using the Akaike information criterion (AICc). We chose TIM2 + G, TN93 + G and TPM2uf + I for COI, COII, and ND4, respectively. In the ML analysis, the best ML tree was searched and created by RAxML-NG (Kozlov et al. 2019) based on the concatenated sequences partitioned into three gene regions containing 2568 sites and 308 patterns in total, with 50 random starting trees. Bootstrap support values were calculated from 1000 replicates. In the BI analysis, with MrBayes v3.2.7a using BEAGLE library (Ronquist et al., 2012; Ayres et al., 2012), two independent runs with 16 simultaneous Markov chains were performed for 10,000,000 generations producing 7,500 trees in total (nperts = 6, sample freq = 1,000, burnin = 2,501, mcmc temp = 0.2 for each run). The trees were used to generate a majority consensus tree and calculate the posterior probabilities. Additionally, we performed phylogenetic analysis of COI region only using the above representative sequences and the sequences from Thailand (MN119737 ~ MN119924), Italy and Malaysia. The optimal evolutionary model TIM2 + G was chosen and ML/BI trees were inferred or generated as above. The resulting trees and statistical values were edited and illustrated by FigTree v1.4.4 (Rambaut 2018) and Adobe Illustrator 2023 (Adobe Inc., San Jose, USA).

Genotyping of deteriorated specimens

Initial PCR amplification targeting longer amplicons have failed in many specimens potentially caused by degraded DNA due to long-term storage. For such specimens, PCR reactions were performed again for short regions of COI, COII, and *cry1* (300–500 bp). For these analyses, we chose the barcoding primers for COI, and designed specific primers for COII and *cry1* (Table S1). When we observed amplicons, direct sequencing was performed to obtain those marker sequences. The specimens with clean chromatograms and determined sequences were analyzed as above and grouped based on their sequence identities in alignment with the longer representative sequences, and then the specific single nucleotide polymorphism (SNP) sites were identified and summarized (Table S3, S4 and S5).

Results

Sequencing of mitochondrial genes

After the extraction of all DNA samples from the live, dried or ethanol-preserved specimens of *B. latifrons*, partial sequences for the mitochondrial COII and ND4 regions were first determined. Those sequences were unambiguously divided into three main patterns, namely haplotypes among the two genes. The first pattern mainly consisted of the specimens from Yonaguni Island and Taiwan (hereafter, haplotype A), the second pattern of Ryukyu Islands excluding Yonaguni Island (hereafter, haplotype B), and the third pattern of Hawaii (hereafter, haplotype C). Specifically, the genetic pattern was detected at 5 sites in the COII region (Table S3) and 8 sites in the ND4 region (Table S4). Our screening efforts showed haplotype A was detected among a large fraction of specimens collected on Yonaguni Island, all specimens collected/reared in Taiwan and three specimens on Okinawa Island in 2012, 2013, and 2015 (sample numbers: H173 (2012), H175 (2013), A006 (2015)). On the other hand, haplotype B was detected among specimens collected throughout Okinawa Prefecture except for Yonaguni Island and the three specimens above, and 16 samples collected on Yonaguni Island (sample numbers: H007, H009, H039, H106, H107, H108, H109, H110, H111, H116, H117, H139, H143, H145, H155, 407) (Table S2). All specimens collected in Hawaii exhibited haplotype C, unique sequences compared with the two above (Table S2).

Phylogenetic relationships of distinct haplotypes in *Bactrocera latifrons*

For further estimation of genetic bases of invaded *B. latifrons* in Japan, we inferred molecular phylogenetic trees based on the nucleotide sequences of mitochondrial COI, COII and ND4 of 24 representative specimens covering genetic diversity of all the specimens collected from Ryukyu Islands along with four from Hawaii and Taiwan. These sequences were subjected to the analyses with those of *B. latifrons* from other parts of the world and of several other *Bactrocera* spp. published to date. All specimens of *B. latifrons* were grouped into two major clades distinct from other *Bactrocera* spp. (Fig. 1). The first clade includes the flies from Yonaguni Island and Taiwan (haplotype A) (88% bootstrap support; 0.91 posterior probability) (Fig. 1). The second clade (haplotype B) comprised almost all the specimens from Ryukyu Islands, and a small number of specimens from Yonaguni (Fig. 1). They formed a larger clade with the sequences of China, Hawaii, Italy and Malaysia (Fig. 1). First, this group diverged with the flies of Malaysia (KT881556) and China (MG020799), and the others, with a relatively high bootstrap support (82%) in the ML analysis but a low probability in the BI. Second, this group was further divided into a few groups, sequences including the haplotype B of Okinawa and Malaysia, Italy, and haplotype C from Hawaii with high bootstrap values (71–93%). In addition to the above, a maximum likelihood phylogenetic tree was constructed using nucleotide sequences of COI region only, from the same representative samples and Thailand (Kunprom and Pramual 2019). *Bactrocera latifrons* populations sampled in Thailand were placed near the Italian invasive population and haplotype B (Figure S1).

Geographic distribution of two haplotypes in Ryukyu Islands

By sequencing mitochondrial genes of samples from Ryukyu Islands, we mapped the mtDNA haplotypes and obtained a clear geographic pattern of their distributions (Fig. 2A). Almost all individuals of haplotype A (n = 75) were observed in Yonaguni Island. Exceptionally, only three individuals were distributed in Okinawa Island. However, these individuals were collected before 2015 and recent specimens exhibited only haplotype B (Table S2). The distribution of haplotype B has been detected all over Ryukyu Islands (n = 166) while only Yonaguni Island harbored a small fraction of haplotype B individuals at 18% (16/91) (Fig. 2A).

SNP genotyping of nuclear gene *Cryptochrome-1*

For nuclear gene genotyping, we first identified mutative 5 polymorphic sites in *cry1* gene from more than 10 specimens including both mitochondrial haplotypes (Table 2) while partial sequence of another nuclear gene *wingless* 732 bp in length had no polymorphism among 8 samples [accession number LC788695-LC788702]. Focusing on two specific sites of *cry1* allele, there were distinguishable differences between most haplotype A and haplotype B individuals initially identified by the mitochondrial genes. Other three sites in *cry1* have no distinct patterns among haplotypes nor populations. We categorized most haplotype A having two heterozygotic sites with single nucleotide polymorphisms (SNPs) as genotype A, while most haplotype B with homozygosity in the same two sites as genotype B. More precisely, for majority of genotype A on Yonaguni Island, the 261st and 438th base within 700 bp sequence showed C, G, or S (double peak of C and G) and A, G or R (double peak of A and G), respectively (Table 2). On the other hand, in genotype B, the 261st and 438th base of the same region are fixed as C and G, respectively (Table 2). At the populations level, one specimen from Ishigaki Island in 2020 (H153) and two from Okinawa Island in 2012 (H173, H174) detected genotype A as an exception while all the other individuals in the same islands were detected to be genotype B (Table 2, Table S2).

Population dynamics of *Bactrocera latifrons* in Yonaguni Island

According to our thorough phylogenetic and genotyping analyses throughout Ryukyu Islands, only Yonaguni Island maintains two distinct genetic groups classified into alternative haplotype/genotypes especially since 2018. Therefore, we specifically followed annual statistics of that population based both on haplotypes and genotypes (Fig. 2B). Of note, for mitochondrial gene, only haplotype A was detected in all specimens in 2004 before the eradication project initiated, and between 2018 and 2019 after the re-invasion. Subsequently, however, haplotype B suddenly emerged at the rate of 43% in 2020 (Fig. 2B) and continued to be present in 2021 at 42%. On the other hand, for nuclear gene *cry1*, genotype B had been already present at 31% of all the specimens in 2018 when their mitochondrial haplotype showed only A. Then, the specimens from both 2019 and 2020 exhibited genotype B at 43%, slightly increasing from 2018 (Fig. 2B). However, the rate of genotype B decreased to 25% in 2021, while mitochondrial haplotype B remained stable. Overall, the annual statistics of haplotype and genotype frequencies clearly indicated that early haplotype/genotype A invaders in the Yonaguni Island gradually decreased but co-existed with late haplotype/genotype B ones since 2018, and the mating between these two genetic populations seems to have started thereafter.

Discussion

Our detailed phylogenetic and genotyping analyses unequivocally revealed three major clades and haplotypes of *B. latifrons*. Many of the specimens collected in Yonaguni Island were assigned to the single clade (haplotype A), identical to the sequences of flies from Taiwan and a few exceptions from Okinawa Island. On the other hand, almost all specimens collected in Ryukyu Islands excluding Yonaguni Island were assigned to the major single clade (haplotype B). This haplotype is most closely related to or nearly identical with the flies from Italy and Malaysia. In addition, the old specimens collected in Hawaii were assigned to unique haplotype C which forms a larger clade with haplotype B, suggesting similar evolutionary origins for these two haplotypes. Our comparative analyses and annual transition of frequencies in mitochondrial haplotypes and nuclear genotypes in Yonaguni Island also indicated frequent crosses and introgression of the two genetically distinct strains.

Phylogenetic origins of invasive *B. latifrons*

Our study clearly showed that the initial population found on Yonaguni Island (specimens from 2004 before the eradication project) is haplotype A whose sequence is identical to those of several individuals in Taiwan (Fig. 1, Table 1, Table S2). In addition, the geographical proximity between Yonaguni Island and Taiwan suggests that haplotype A may have originated from Taiwan. On the other hand, all specimens collected in Ryukyu Islands except for Yonaguni Island after 2016 exhibited only haplotype B (Fig. 1, Table 1, Table S2). Haplotype B mitochondrial sequences are clearly distinct from haplotype A and closely related to the flies from Italy, Malaysia and haplotype C of Hawaii (Fig. 1). This suggests that haplotype B and C may have been introduced from Malaysia or nearby Southeast Asian countries via dispersion or more likely passive transportation to many parts of the world.

Furthermore, we found distinct heterozygosity/homozygosity patterns in the allele of nuclear gene *cry1* (Table 2). The heterozygosity between the two alleles was maintained in haplotype A individuals, which suggests that genotype A in Yonaguni Island may retain more genetic diversity in the smaller population rather than the expanding populations of genotype B. Genotype A complex on the single island may have resulted from repeated crosses among populations of multiple invasions and genetic backgrounds. In support of this, combinations of haplotypes and genotypes were heterogenous in Yonaguni Island, and influx of genes likely occur between the two strains (Table 2, Fig. 2B). It suggests that multiple populations may have often invaded, co-existed and crossed in recent Yonaguni Island whereas rather a uniform population of genotype B seems to have quickly expanded and dominated on the other islands of Ryukyus. Of note, the genetic diversity in SNP patterns was small in laboratory-maintained haplotype C flies in Hawaii, suggesting a single origin and a small founding population there.

Genetic invasion history of *B. latifrons* into Ryukyu Islands and host preferences

Based on our detailed genetic analyses on decades of *B. latifrons* specimens, we re-annotate the invasion history of *B. latifrons* into Japan by referring to their genetic identities as below (Fig. 3). In 1984, haplotype A of *B. latifrons* was first discovered on Yonaguni Island. This haplotype was known to prefer

laying eggs on eggplants in the laboratory and utilize *Solanum melongena* from the field host plant surveys (Kohama 2014). The haplotype A population was eradicated successfully by sterile insect technique in 2011. However, in 2010, just one year before the eradication, haplotype B flies suddenly appeared on Okinawa Island. It was characteristic of this population that female flies mainly utilized bush red pepper *C. frutescens*, which was only rarely used by haplotype A (Kohama 2014; Table S2). After the establishment, it rapidly expanded its distribution throughout the whole Ryukyu Islands before it would take the center stage pest control, and then again invaded Yonaguni Island in 2018. To our surprise, however, the genetic investigation suggested haplotype A was already present at 100% on Yonaguni Island at almost the same time of the detection of genotype B. It is not clear how and why two genotypes and haplotype A appeared at the same time in 2018. Even under the pressure of influx by haplo/genotype B in the island, both haplo/genotypes still seem to coexist and cross-mate at present. Moreover, not only haplotype B individuals but also recent haplotype A flies have often utilized bush red pepper in Yonaguni Island, suggesting that crossing of the two strains may have genetically altered their host utilization and preferences (Table S2).

While almost all samples in the Ryukyus besides Yonaguni exhibited haplo/genotype B, we noticed a few exceptional specimens of haplo/genotype A appearing on Okinawa Island in 2012 and 2015 and a single genotype A fly later on Ishigaki Island in 2020 (Table S2). These exceptions suggest that a very small number of haplotype A flies were also present in Okinawa Island several years ago or the other islands recently. For this finding, we first consider two hypotheses which can explain why haplotype A had existed on Okinawa Island. First, *B. latifrons* of haplotype A invaded Okinawa Island in the same manner and origin as Yonaguni Island, or directly migrated from Yonaguni Island. Although 113 specimens between 2016 to 2021 were sequenced, they were basically dominated by haplotype B. Therefore, even if there were minor invasions from Taiwan and/or Yonaguni Island and a small population of haplotype A flies may have been able to colonize and co-exist, they would be quickly obscured by its predecessors. Secondly, *B. latifrons* of haplotype A may have already invaded and established before the first discovery on Okinawa Island in 2010 and their presence only remained unnoticed. Haplotype A in Yonaguni commonly utilized wild host plants such as *S. americanum* or *D. palmatus* (Kohama 2014), so, in the circumstances of this host preference, the infested wild fruits would be more likely overlooked in regular host plant surveys. It should be noted, however, although many host plant surveys were conducted in Okinawa Island before the eradication of more polyphagous tephritid species, namely *B. dorsalis* and *Z. cucurbitae*, the solanum fruit fly had never been found until its discovery on Yonaguni Island in 1984 (Kanada et al. 1985). Therefore, these two fly species might have outperformed and undermined the growth of *B. latifrons* populations in Ryukyus Islands in the past, but, thanks to rich host plant resources available after the eradication of those competitors, the new pest fly could have made its way to the arena. On the other hand, one irregular specimen with genotype A recently collected on Ishigaki island may have accidently flown or been brought from Yonaguni Island, geographically close to each other (about 130 km).

A minor haplo/genotype A remains within Yonaguni Island

Our survey of annual transitions in Yonaguni Island indicated there are co-existing two different haplotypes and genotypes of *B. latifrons* only recently. All the specimens of 2004, 2018 and 2019 were assigned to mitochondrial haplotype A, but then nuclear genotype B and mitochondrial haplotype B appeared from 2018 and 2020, respectively. A fraction of genotype B in 2018 and its gradual increase later suggest that haplo/genotype B invaded and remained cryptic in Yonaguni Island initially, but repeated crosses and/or population growth of the strain could have occurred, given the ample availability of unfavorable host plants for major haplotype A. However, the exact invasion history and genetic structure of both strains are still unknown, as genotype A is again increasing in 2021. Also, our preliminary analysis only using the non-functional synonymous variations on a single allele *cry1* is obviously insufficient and awaits genome-wide association study based on more scalable sampling efforts, phenotyping as well as its whole genome sequences (Jiang et al. 2022). On our end, we found a very few cases of haplo/genotype A co-existing with B or never in many islands besides Yonaguni, suggesting that the haplo/genotype B individuals may have unidentified adaptive advantages over haplo/genotype A. Thus, the former flies outperform or limit the expansion of the latter in other islands outside of Yonaguni. Further molecular investigations of extant flies in the island are required for us to understand how two distinct genetic populations of the pest merge or exclude one another, and to design future pest control measures based on the precise identification of insect genotypes.

Global distribution of *B. latifrons* keeps expanding

Bactrocera latifrons is known to have originated in tropical Asia and has invaded many parts of the world (Fig. 4). In Japan, as described previously and shown by the current study, *B. latifrons* invaded into Ryukyu Islands at least twice or more. The fly populations having invaded into Hawaii, Italy, and Okinawa Island were phylogenetically associated haplotypes of Southeast Asian origin (haplotype B, C and closely related ones: haplotype B complex) more versatile and invasive, while only the populations of Taiwan and that found in Yonaguni Island were assigned to locally fixed haplotype A (Fig. 1, 3). While previous studies employing inconsistent molecular markers have not uncovered such global inter-relationships of this fly species (Nakahara et al. 2005; Kunprom and Pramual 2019; Gargiulo et al. 2021), our results successfully provide the basis for wider phylogeographic research on their invasion pathways.

In consideration of host plants, the fly population of haplotype A utilized mostly wild plants such as *S. americanum* (Shimizu et al., 2007), whereas haplotype B or cross-mated haplotype A flies frequently attack commercial crops such as bush red pepper and tomato *S. lycopersicum* (Okianwa Prefectural Plant Protection Center, 2022). *Bactrocera latifrons* in its native region mainly utilizes Solanaceae fruit crops, but also occasionally utilize *Citrus* and *Syzygium* fruit crops (Allwood et al. 1999). In addition, several cases of rare host exploitation of Cucurbitaceae plants have been reported in the invaded areas (Liquido et al. 1994; Shimizu et al. 2007; Mziray et al. 2010) which are not utilized by *B.latifrons* in its native habitat (Allwood et al. 1999) and the same is true for individuals assigned haplotype A and B (Shimizu et al. 2007; Okianwa Prefectural Plant Protection Center, 2022). In recent years, presumptive haplotype B flies have been reported to also utilize tropical fruits such as *Mangifera indica* L. and *Amygdalus persica* (L.) Batsch (Okianwa Prefectural Plant Protection Center 2022), and we have seen the

expansion of host plants by haplotype A flies after mating with haplo/genotype B (Table S2). In summary, *B. latifrons* tends to expand its host utilization in the invaded areas, therefore, we need to watch out for the risk of establishment even in areas with few Solanaceae host plants. In particular, haplotype B complex strains are presumed to be expanding their distribution globally, including unnoticed artificial immigration with their host plants of commercial crops. Accordingly, haplotype B complex flies may entail genetic characteristics that make them prone to becoming pests and drive the evolution of novel host utilization pattens by mating with other strains. The mechanisms and genetic bases leading to such host expansion in *B. latifron*s are yet to be identified; however, the host exploitation may be enabled not only by insects' own genomes but also through the acquisition of symbiotic gut microorganisms that can detoxify secondary metabolites of plants, recently reported in many insects (Itoh et al. 2018). Further research is needed to validate these hypothesizes involving both insect genetics and microbiota. Based on the current study, additional investigations shall be conducted to comprehensively identify haplo/genotypes of *B. latifrons* in their native and invasive habitats to elucidate more in-depth invasion history and host adaptation of the insect for future pest control programs.

Declarations

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Funding

This study was supported by the Japan Society for the Promotion of Science (JSPS), Grant-in-Aid for JSPS Fellows (No. 20J23739) to TH, Grant-in-Aid for Scientific Research (KAKENHI grant No. 21K05602) to AH and Grant-in-Aid for Challenging Research (Exploratory) (KAKENHI grant No. 18K19217) to YM.

Author Contributions

TH, AH and YM conceived the project. Material preparation and experiments were performed by TH, RS, TM, HI, Y-BH, AH and YM. Data collection, analyses and interpretations were performed by TH, RS, AH and YM. The first draft of the manuscript was written and revised by TH, AH and YM with critical inputs from K-IT and TN. All authors read and commented on the manuscripts, and approved the final version before submission.

Acknowledgments

We thank Okinawa Prefectural Plant Protection Center, Okinawa Prefectural Agricultural Research Center, and Taiwan Agricultural Research Institute for providing fly specimens. This study was supported by

Grant-in-Aid for JSPS Fellows (No. 20J23739), Grant-in-Aid for Challenging Research (Exploratory) (No. 18K19217), and Grant-in-Aid for Scientific Research (No. 21K05602) from the Japan Society for the Promotion of Science. This study was mainly conducted at, and also supported by Collaborative Research of Tropical Biosphere Research Center, University of the Ryukyus.

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Figures

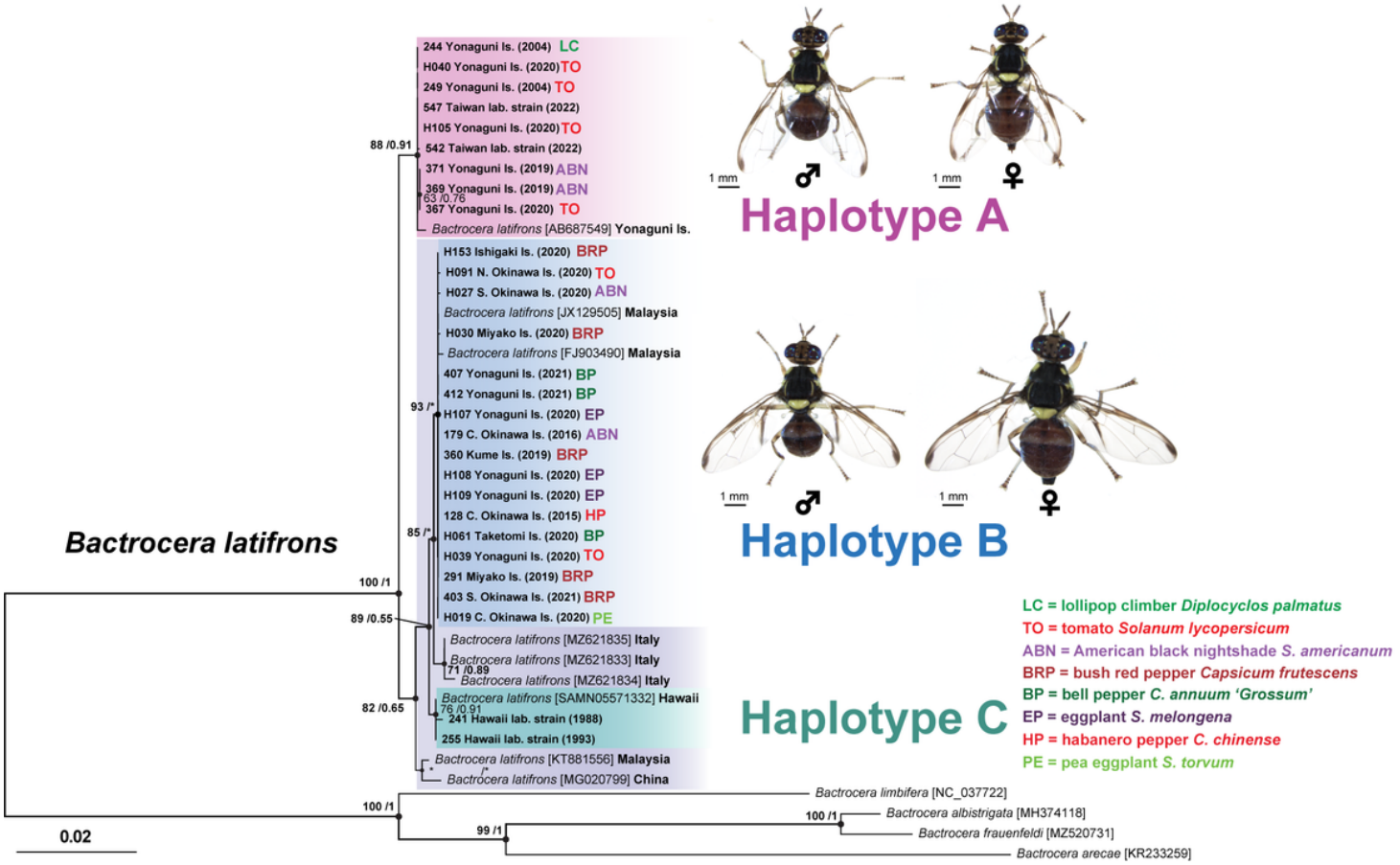

Figure 1

Phylogenetic relationships of representative specimens of *B. latifrons*invading the Ryukyu islands based on the concatenated COI, COII, and ND4 sequences (2568 sites). A maximum-likelihood phylogeny is shown while Bayesian inference produced essentially the same topology. The bootstrap support value of Maximum Likelihood and posterior probability of Bayesian inference are indicated on each node. Asterisks indicate support values less than 50% or 0.5. For each taxon, the number indicates specimen identification number, followed by the locality, (collection year) and host plant. Fully spelled common names and species for abbreviated host plants are indicated at the bottom right. Accession numbers of the publicly available sequences obtained from Genbank/EMBL/DDBJ are indicated in brackets. Photographs of laboratory-reared adult flies of haplotype A and B are also shown (left, male; right female).

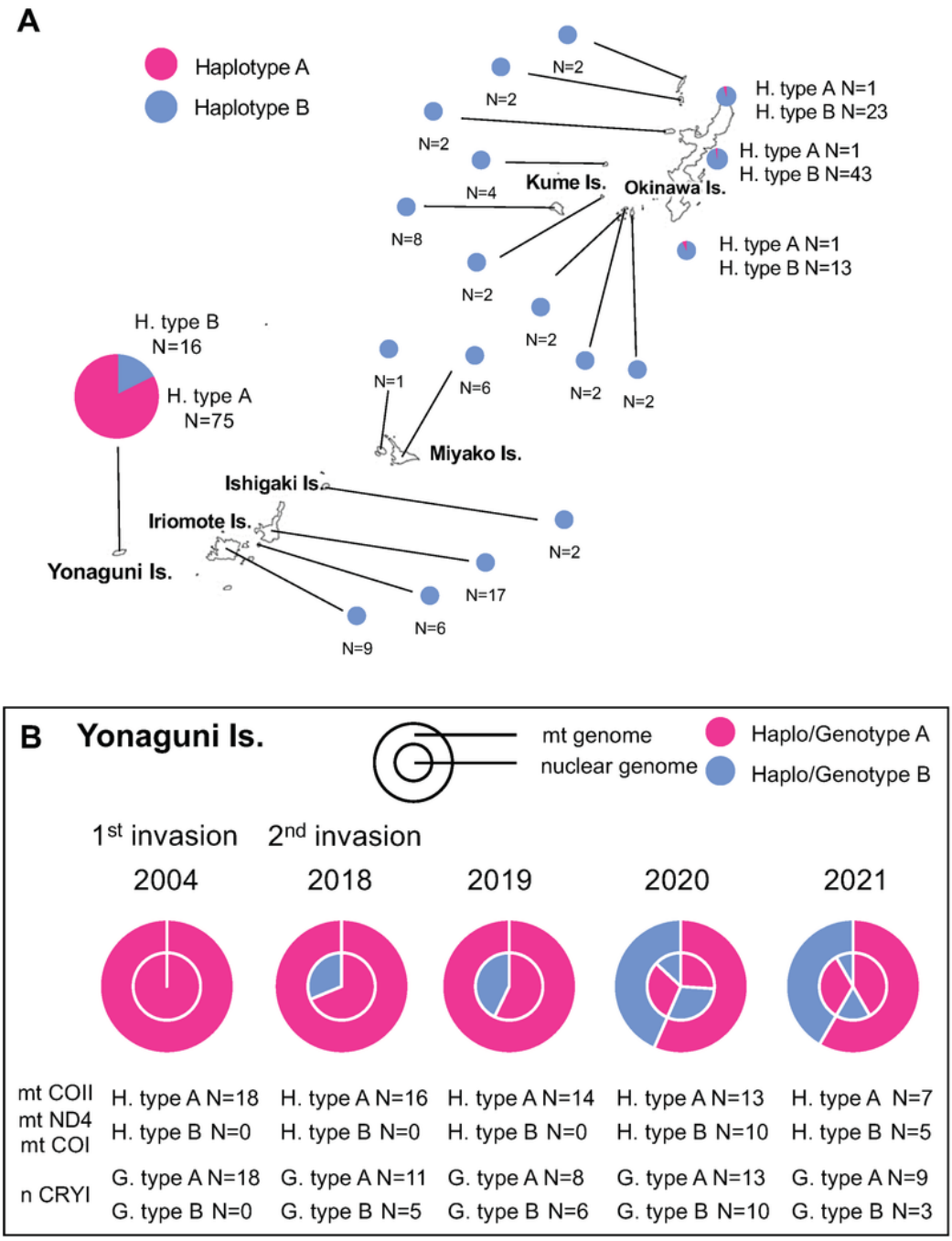

Figure 2

A: Geographic distribution of haplotypes based on sequences of mitochondrial genes COII and ND4 of *B. latifrons* invading the Ryukyu Islands. The magenta and blue areas indicate Haplotype A and Haplotype B, respectively. B: The graph below shows yearly transition of the percentage of haplo/genotypes determined by mitochondrial COII and ND4 and nuclear gene *cry1*in Yonaguni Island. The magenta and blue areas in the graphs indicate Haplo/Genotype A and Haplo/Genotype B, respectively.

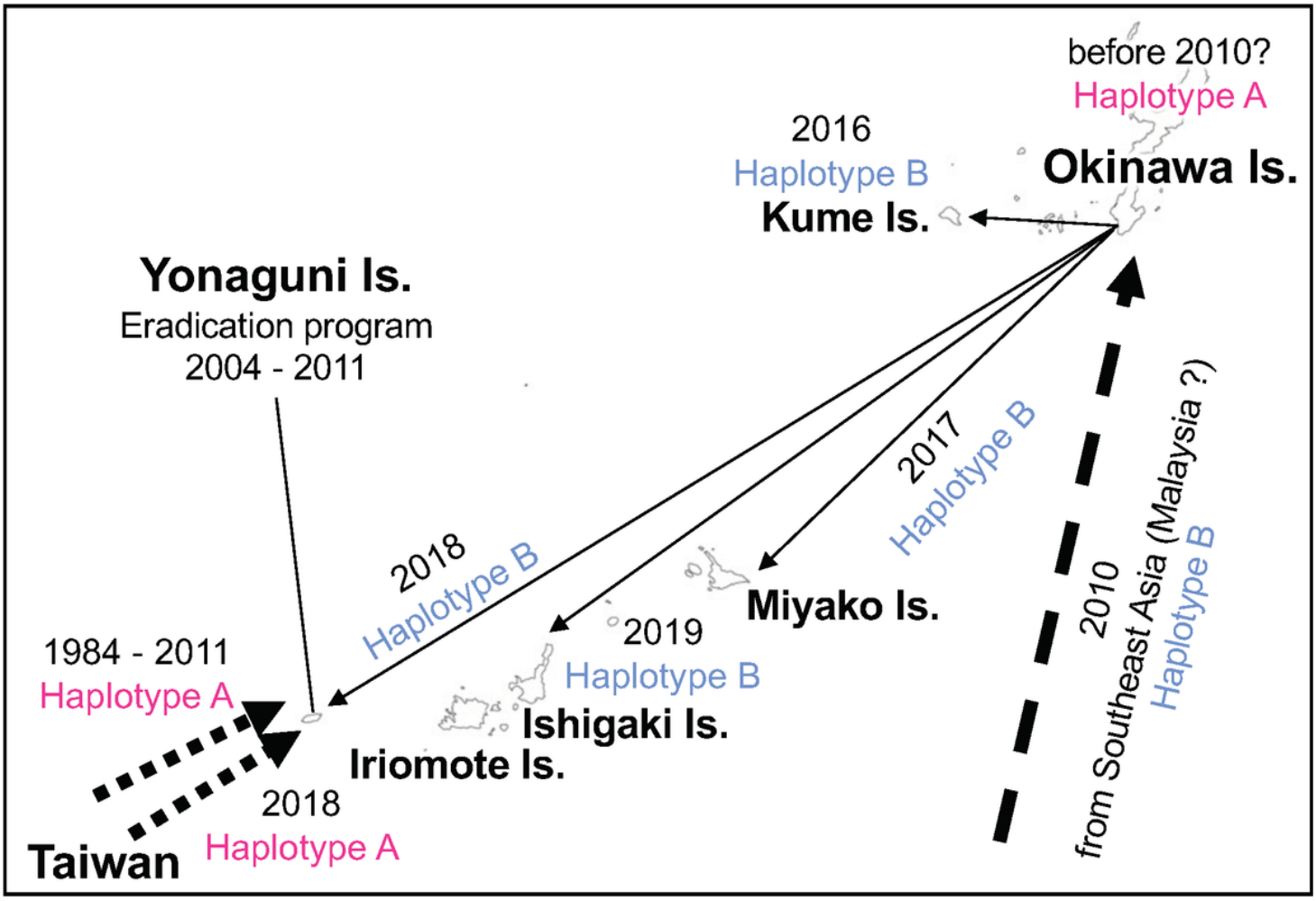

Figure 3

The invasion history of *Bactrocera latifrons* into the Ryukyu islands.

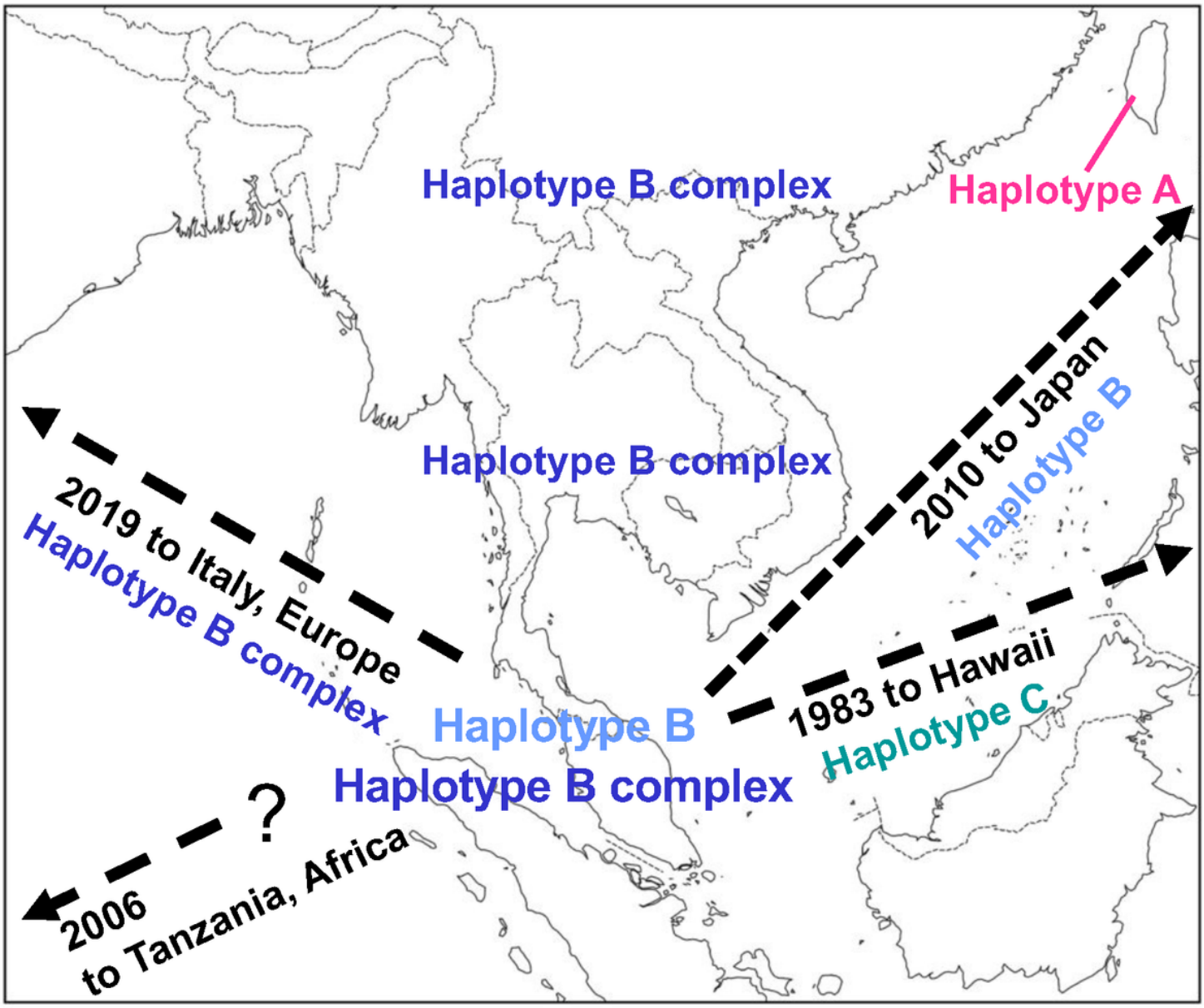

Figure 4

The global distribution and invasion history of *Bactrocera latifrons*. Haplotype A is only disturbed in Taiwan and Yonaguni Island. Haplotype B invading Okinawa is distributed in Malaysia, and other haplotypes closely related to haplotype B and C (haplotype B complex) which are presumably derived from Southeast Asia frequently invade other parts of the world.

Supplementary Files

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