Invasion genetics and development of rapid diagnostics of insect pests on traded plants

INAUGURALDISSERTATION

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

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aus Trubschachen (BE), Schweiz

Wädenswil, 2019

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von Prof. Dr. Jürg Utzinger, Dr. Jürg E. Frey und Prof. Dr. Alexander Mathis

Basel, den 21. Mai 2019

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Acknowledgements

- My warmest thanks go to my three supervisors Dr. Jürg E. Frey, Prof. Dr. Jürg Utzinger and PD. Dr. Pie Müller for their strong support and guidance over the past 3 years. You allowed me to conduct my PhD project in a very inspiring environment – and gave me always the possibility to bring in own ideas – thank you very much for all your efforts!
- A special thank goes to my external referee Prof. Dr. Alexander Mathis for his willingness to review my PhD thesis.
- An enormous thank to all people who contributed substantially to the different projects: Dr. Andreas Bühlmann, Dr. Andreas von Felten, Mrs. Beatrice Frey, Mrs. Cornelia Heusser, Mr. Daniel Frei, Mr. Hanspeter Diem, Dr. Jennifer Tomlinson, Dr. Michael Andreou, Mr. Morgan Gueuning and Prof. Dr. Neil Boonham.
- Sincerest thanks to Mrs. Elisabeth Razavi and Mr. Markus Oggenfuss for their support during my laboratory work.
- Special thanks to Mr. Markus Bünter for excellent discussions regarding plant health regulations.
- A final thank goes to my family – especially Friederike and Maurus – for their enormous patience and strong support throughout sometimes demanding times of my PhD!

Abbreviations

ABC = Approximate Bayesian Computation

AMOVA = Analysis of molecular variance

BOLD = Barcode of life data system

CO1= Cytochrome *c* oxidase subunit 1

ddRAD = Double-digest restriction-site associated DNA sequencing

DNA = Deoxyribonucleic acid

ELISA = Enzyme-linked immunosorbent assay

EU = European Union

FAO = Food and Agricultural Organization of the United Nations

FREQ = Frequency

GBS = Genotyping-by-sequencing

IBD = Isolation by distance

IPC = Internal positive control

IPPC = International Plant Protection Convention

IUCN = Union for the Conservation of Nature and Natural Resources

K = Number of population clusters

LAMP = Loop-mediated isothermal amplification

MNPD = Mean number of pairwise differences

N = Number

NAC = Negative amplification control

NGS = Next-generation sequencing

NPPO = National Plant Protection Organisation

NUMTS = Nuclear mitochondrial pseudogenes

PAC = Positive amplification control

PCA = Principal component analysis

PCR = Polymerase chain reaction

PCR-RFLP = PCR restriction fragment length polymorphism

PhD = Doctor of Philosophy

POE = Point of entry

RAD = Restriction-site associated DNA sequencing

RNA = Ribonucleic acid

SD = Standard deviation

SNP = Single nucleotide polymorphism

SPPS = Swiss Plant Protection Service

SSR = Simple sequence repeats

USA = United States of America

UTM = Universal Transverse Mercator

VAR = Variance

WGS = World geodetic system

WTO = World Trade Organization

bp = Base pair

fg = Femtogram

h = Hour

min = Minute

mm = Millimetre

µM = Micromole

µl = Microlitre

π = Nucleotide diversity

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Summary

Background: Global trade of plant products is a major driving force for the unintended spread of economically harmful insect pests. This PhD thesis aimed at (i) developing and implementing molecular tools for the on-site identification of invasive insect pests at points of entry (POEs) for plant import products as a prevention measure; and (ii) investigating the invasion history of the mosaic leafhopper *Orientus ishidae*, a potential vector of grapevine Flavescence dorée phytoplasma.

Methods: To achieve the first goal, loop-mediated isothermal amplification (LAMP)-based genetic assays for the rapid on-site identification of *Bemisia tabaci*, *Thrips palmi* and several invasive fruit flies of the genera *Bactrocera* and *Zeugodacus* were developed. Using publicly available DNA sequences, LAMP primers were designed to specifically target a fragment of the mitochondrial cytochrome *c* oxidase subunit 1 gene.

To address the second goal of this PhD thesis, the invasion genetics of *O. ishidae* was studied, an invasive insect species that spread from its native range from in East Asia to North America in the first half of the 20th century and only recently colonised Europe. Possible source populations and invasion pathways were investigated by assessing the genetic structure of 41 *O. ishidae* populations from Asia, Europe, and North America based on a mitochondrial marker and 641 single nucleotide polymorphisms (SNPs) generated by double digest restriction-site associated DNA (ddRAD) sequencing.

Results: Validation performed under laboratory and on-site conditions demonstrated the robustness and reliability of the developed LAMP identification assays. Analysing 319 insect specimens, the overall diagnostic test efficiency was 98% and the overall diagnostic test specificity was 100%. The small number of false-negative results (2%) originated either from previously unknown biotypes, not included in the initial primer design, or from handling errors during LAMP preparation.

The results from the molecular genetic analyses of *O. ishidae* revealed a clear genetic separation between a native population from Asia and the non-native populations from Europe

and North America. Among the non-native populations, only faint signals of spatial genetic structuring were found. However, when comparing non-native populations from Europe and North America, elevated levels of admixture of genetically distant mitochondrial haplotypes were observed for European populations.

Conclusion: Characterised by high analysis speed (<1 h) and simplicity in use (only 1 pipetting step), the validated LAMP assays were found to be suitable identification tools for on-site application by plant health inspectors. Since completion of the validation phase, the developed identification assays are routinely deployed in the phytosanitary import control process of Switzerland.

The considerable genetic separation between native and non-native populations of *O. ishidae* together with the strikingly high genetic similarity of European and North American populations suggest an invasion scenario in which North American populations served as source for the European invasion. A slightly reduced genetic structure combined with increased admixture of genetically distant mitochondrial haplotypes furthermore indicate that the European colonisation history was shaped by multiple introductions from North America, complemented by frequent intra-European gene flow. Taken together, it is hypothesised that the overall genetic complexity of non-native populations was strongly driven by frequent international trade of plants infested by *O. ishidae*.

Zusammenfassung

Hintergrund: Der weltweite Handel von pflanzlichen Produkten gilt als einer der Hauptgründe für die ungewollte Verbreitung von wirtschaftlich gefährlichen Insektenschädlingen. Das Ziel dieser Dissertation war (i) die Entwicklung und Implementierung eines molekularen Schnelltests zur Identifikation von invasiven Insektenschädlingen an Ersteintrittspunkten von pflanzlichen Importprodukten und (ii) die Untersuchung der Invasionswege der Mosaik-Zwergzikade *Orientus ishidae*, einem potentiellen Überträger der Phytoplasmen-Krankheit «Goldgelben Vergilbung» der Rebe.

Methoden: Für das Erreichen des ersten Ziels dieser Arbeit wurden «loop-mediated isothermal amplification (LAMP)»-basierte, genetische Tests zur Schnellidentifikation von *Bemisia tabaci*, *Thrips palmi* und mehreren Fruchtfliegen der Gattungen *Bactrocera* und *Zeugodacus* entwickelt. Die LAMP Primer wurden dabei so konstruiert, dass sie spezifisch ein Fragment des mitochondrialen «Cytochrome *c* oxidase subunit 1» Gens der jeweiligen Zielorganismen erkennen.

Im zweiten Teil dieser Arbeit wurde die Invasionsgenetik von *O. ishidae* untersucht. Diese invasive Insekten-Spezies stammt ursprünglich aus Ostasien, wurde in der ersten Hälfte des 20. Jahrhunderts nach Nordamerika eingeschleppt, und hat erst kürzlich Europa besiedelt. Um mögliche Ursprungspopulationen und Invasionswege der Zwergzikade zu identifizieren wurde die genetische Struktur von 41 *O. ishidae* Populationen aus Asien, Europa und Nordamerika miteinander verglichen. Die Analysen basierten dabei auf einem mitochondrialen Marker, sowie 641 Einzelnukleotid-Polymorphismen, welche mit «double-digest restriction-site associated DNA (ddRAD) sequencing» generiert wurden.

Resultate: Validierungen unter Labor- und «on-site»-Bedingungen zeigen, dass die entwickelten Schnelltests stabil und zuverlässig funktionieren. Bei der Identifikation von 319 Insekten-Proben mittels der neu entwickelten Schnelltests konnte eine diagnostische Test-Effizienz von 98%, sowie eine diagnostische Test-Spezifität von 100% festgestellt werden. Die wenigen falsch-negativen Resultate (2%) stammten einerseits aus Analysen von zuvor

unbekannten Schädlingsbiotypen, welche nicht in die ursprüngliche Entwicklung der Tests einbezogen wurden, und andererseits von Bedienungsfehlern in der Durchführung der Schnelltests.

Die molekulargenetischen Analysen von *O. ishidae* haben gezeigt, dass sich Populationen aus dem natürlichen Verbreitungsgebiet klar von den invasiven Populationen aus Europa und Nordamerika unterscheiden. Für die Populationen aus dem neu besiedelten Gebiet sind nur schwache Signale von geografisch-genetischer Strukturierung gefunden worden. Allerdings haben Vergleiche von Populationen aus Europa und Nordamerika gezeigt, dass europäische Populationen einen erhöhten Anteil an «Admixture» von genetisch weit distanzierten mitochondrialen Haplotypen aufweisen.

Schlussfolgerung: Insgesamt konnte gezeigt werden, dass die entwickelten Schnelltests dank ihrer Geschwindigkeit in der Durchführung (<1 h) und Einfachheit in der Handhabung (nur 1 Pipettier-Schritt) geeignete Werkzeuge zur Identifikation von regulierten Insekten-schädlingen an Ersteintrittspunkten von pflanzlichen Importprodukten darstellen. Seit Abschluss der Validierungsphase werden die entwickelten Schnelltests routinemässig in der phytosanitären Einfuhrkontrolle der Schweiz eingesetzt.

Die ausgeprägte genetische Trennung zwischen natürlichen und eingeschleppten Populationen von *O. ishidae* sowie die starke genetische Ähnlichkeit zwischen den europäischen und nordamerikanischen Populationen deuten auf ein Invasions-Szenario hin, in welchem nordamerikanische Populationen als Quelle für die europäische Invasion gedient haben. Zusätzlich lässt die leicht reduzierte genetische Struktur, sowie das lokale Zusammentreffen von genetisch weit entfernten mitochondrialen Haplotypen darauf schliessen, dass die europäische Besiedlung von *O. ishidae* durch mehrere Einschleppungen aus Nordamerika, sowie regelmässigen intra-europäischen Genfluss geprägt wurde. Insgesamt führten die ermittelten Resultate zur Hypothese, dass die komplexe genetische Struktur der invasiven Populationen stark geprägt wurde durch den internationalen Handel von mit *O. ishidae* befallenen Pflanzen.

1. Introduction

1.1. Biological invasions

1.1.1. Definition

The term biological invasion describes the process by which an organism is translocated from its native range to a new, often distant area, where it proliferates, spreads and successfully manages to persist (Mack et al., 2000). Traditionally, invasion processes are subdivided into three particular stages (Fig. 1.1): First, an arrival stage at which the organism is translocated to a new area distant from the native range. Second, an establishment stage at which the non-native organism successfully establishes a new population at a size that extinction is unlikely. Third, a spread stage at which the organism expands from the invaded area into new areas (Dobson and May, 1986; Liebhold et al., 1995; Liebhold and Tobin, 2008).

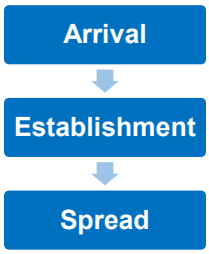

Figure 1.1. Three stages of the biological invasion process. Figure is adapted from Liebhold and Tobin (2008).

1.1.2. Translocation of non-native species

Biological invasions are not considered to be a novel phenomenon (Mack et al., 2000; Saccaggi et al., 2016). Already from the 16th century on, European explorers and settlers started to release numerous non-native plant and animal species across the world (Seebens et al., 2017). In contrast, many non-native plant species were introduced to Europe in the 19th century for domestic and ornamental purposes (Seebens et al., 2017). However, the geographic scope and the rate at which humans transport foreign species into new areas accelerated considerably during the past two centuries (Mack et al., 2000; Seebens et al., 2017). A recent study analysing first records of non-native organisms of the last 200 years

revealed that 37% of reported introduction events occurred in the period from 1970-2014 (Seebens et al., 2017). The observed increase is thought to be driven by the expanding international transport and commerce, consequences of ongoing globalisation (Mack et al., 2000; Bacon et al., 2012; Seebens et al., 2017). Because the transport volume of goods and people around the world is still expanding, it is expected that also the number of invasions will further increase (Bacon et al., 2012; Saccaggi et al., 2016).

1.1.3. Introduction pathways

The release of non-native organisms into a new area can occur intentionally such as for biocontrol agents or unintentionally as in the case of the introduction of pest species (Hulme et al., 2008; Saccaggi et al., 2016). Literature distinguishes between six major pathways for non-native species introduction with different levels of human influence. These are (i) unaided; (ii) corridor; (iii) stowaway; (iv) contaminant; (v) escape; and (vi) release (Fig. 1.2) (Hulme et al., 2008; Essl et al., 2015).

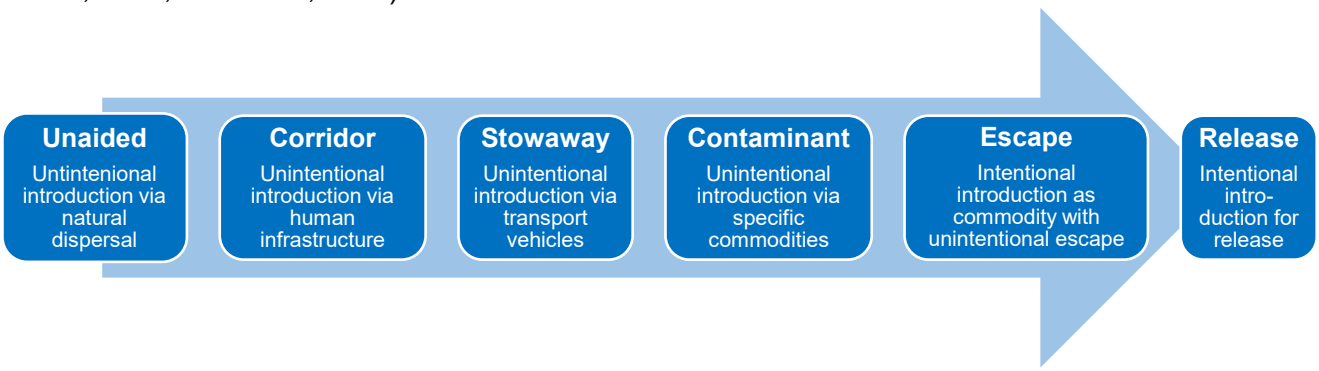

Figure 1.2. Six major introduction pathways for non-native species. Arrow indicates the increasing amount of human assistance in the invasion process. Figure is adapted from Hulme et al. (2008).

The importance of the individual introduction pathways varies strongly among different taxa. Aquatic plant invasions are frequently associated with the corridor pathway (e.g. via canal networks), whereas invasions of terrestrial plants are more often found to be associated with the intentional release for landscaping and pasture improvements (Hulme et al., 2008). In contrast, invasive arthropods are most often found to be introduced as contaminants of specific commodities (e.g. agricultural products, livestock and pets) and as stowaways of transport

vectors such as vehicles, containers, packaging material and passenger baggage (Kenis et al., 2007; Hulme et al., 2008; Saccaggi et al., 2016) .

1.1.4. Impacts

The unintentional translocation of species as by-product of globalisation has caused homogenisation in floras and faunas across the world and thereby reshaped biogeographic boundaries (Winter et al., 2009; Seebens et al., 2017). In the recent past, biological invasions were reported to have negatively affected native biodiversity, ecosystem functions, public health, as well as economy (Hulme, 2009; Pyšek and Richardson, 2010; Seebens et al., 2017). In many cases, more than one of these subject areas were affected and the induced processes are usually irreversible (Kenis et al., 2007). As an example, the yellow star thistle (*Centaurea solstitalis*) invaded 8,000,000 ha of California's grassland, resulting in a change of the local biodiversity and the ecosystem as a whole, and it led to a complete loss of the grassland productivity of this area (Pimentel et al., 2005). Biological invasions may also affect human health such as in the case of the pollen allergy-inducing invasive plant *Ambrosia artemisiifolia* (Pyšek and Richardson, 2010). The economic consequences of biological invasions can be subdivided into two categories: (i) direct economic losses due to reduction of potential yields in crop production or from fishery; and (ii) indirect economic losses resulting from costs invested for management measures such as quarantine, control and elimination (Mack et al., 2000). In a study from 2004, the annual US costs for total environmental damages and economic losses due to invasive species were estimated to be approximately US$ 120 billion (Pimentel et al., 2005; Marbuah et al., 2014).

1.2. Invasive insects

Insects are regarded as the most diverse and therefore largest group of living organisms on the Earth (Kenis et al., 2007; Feng et al., 2009). Hence, it is not surprising that they are also involved in a major part of the problems arising from biological invasion (Kenis et al., 2007). Early events of human-mediated movement of insect species already happened at the time, when Europeans conquered far distant corners of the world (Liebhold and Tobin, 2008). Upon

arrival at a new destination, settlers intentionally introduced domestic species such as the honey bee, but also insects unintentionally moved as stowaways on their vessels such as cockroaches (Liebhold and Tobin, 2008). Numerous studies reported a significant increase of insect introductions over the past 200 years (Hurley et al., 2016; Roques et al., 2016; Javal et al., 2017). The observed trend is thought to be directly linked with increasing global trade and transport, the homogenisation of host plant distribution, as well as with climate change (Hurley et al., 2016; Roques et al., 2016; Javal et al., 2017).

Exploring historical introduction records of non-native insects in Europe, Roques et al. (2016) recently reported that the annual establishing rate of non-native insects has almost doubled over the past 60 years, from an annual average of 10.9 species per year to an annual average of 19.6 species per year. Only 14% of the total introductions were intentional (mainly for biocontrol purposes), however, the majority of insect species were introduced accidentally (Roques et al., 2016). The pattern of mainly unintentional introductions of insect species observed in Europe is similar to those reported from other world regions (Kumschick et al., 2016; Roques et al., 2016). In general, the observed pattern differs from that seen in invasion processes of other invasive organisms such as plants and vertebrates, where species were more often introduced intentionally, e.g., for ornamental or domestic reasons (Kenis et al., 2007; Kumschick et al., 2016). Insect invasions further differ from those of plants and vertebrates, in that their introduction phase is mostly not detectable. In addition, whereas plant invasions are often characterised by a “lag-phase” after introduction, invasive insects can spread quickly across new areas (Kenis et al., 2007).

It is known that only a small fraction of the accidently translocated insect species can successfully establish and expand outside their native ranges (McCullough et al., 2006). Founder populations are often small and therefore at high risk of extinction (Liebhold and Tobin, 2008). Already in the 1930s, it was recognised that an invasive founder population must comprise a minimum number of individuals to survive in the invaded area (Allee, 1931; Liebhold and Tobin, 2008). This phenomenon, known as “Allee effect”, is thought to be driven by consequences of the lack of local mating partners (Berec et al., 2001), inbreeding

depression (Lande, 1998), as well as impaired cooperative feeding (Clark and Stanley, 1997; Liebhold and Tobin, 2008).

Nowadays, studies investigating establishment successes of invasive species often assess the propagule pressure, a composite measure incorporating the absolute number of arrived individuals (propagule size), together with the number of introduction events (propagule number) (Lockwood et al., 2005). In this concept, an increase in propagule size or propagule number is thought to be positively linked with an increase of the propagule pressure (Lockwood et al., 2005). A positive correlation of propagule pressure on non-native population establishment based on experimental and observational data was so far reported for species of several taxonomic groups, including insects released for biocontrol purposes (Lockwood et al., 2005; Simberloff, 2009).

It is widely accepted that the international trade of plants and plant products is one of the main drivers of the unintentional movement of invasive insects (Haack, 2001; Bacon et al., 2012; Liebhold et al., 2012). In the past, non-native insects were often found to be introduced on import commodities such as living plants, fruits, vegetables, cut flowers, seeds, wood packing materials or lumber (Haack, 2001; McCullough et al., 2006; Horton et al., 2013). An assessment of the non-native insect fauna of Austria and Switzerland estimated that approximately 43% of the analysed species were introduced via plant trade (Kenis et al., 2007; Liebhold et al., 2012). Examining different types of plant import commodities associated with non-native insect interceptions at European borders between 1995 and 2004, Kenis et al. (2007) reported that 29% of the foreign insects where found on cut flowers, 20% on vegetables, 15% on plants for planting and 11% on traded fruits. The remaining 25% of non-native insect interceptions were associated with import commodities containing aquarium plants, bonsai trees, seeds, stored food products, as well as wood and wood derivates (Kenis et al., 2007).

1.3. Invasive insect pests

1.3.1. Definition

Per definition, a pest species is defined as an organism that has the potential to disturb ecosystems resulting in significant ecological or economic harm (Kirk et al., 2013). In this PhD

thesis, the term “invasive insect pests” is used to refer to invasive insect species with the potential to cause substantial economic loss to agriculture.

1.3.2. Examples

Invasive insect pests can harm crops directly by feeding damage or indirectly by the transmission of plant pathogens such as bacteria, fungi and viruses (MacLeod et al., 2004; Chuche and Thiéry, 2014). An example of an invasive insect pest directly harming crops is given by the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae) (Fig. 1.3A) (Deole and Paul, 2018; Feldmann et al., 2019). Native to the Americas, *S. frugiperda* was accidentally introduced to Africa in 2016, where the species represents now a key pest of maize (Deole and Paul, 2018; Feldmann et al., 2019). In contrast, the melon thrips, *Thrips palmi* (Thysanoptera: Thripidae) (Fig. 1.3B) harms crops directly by feeding damage but also indirectly by the transmission of harmful viruses such as the watermelon silver mottle virus (MacLeod et al., 2004). Originating most probably from Southeast Asia, *T. palmi* invaded many tropical, subtropical and moderate regions of the world (MacLeod et al., 2004; Walsh et al., 2005).

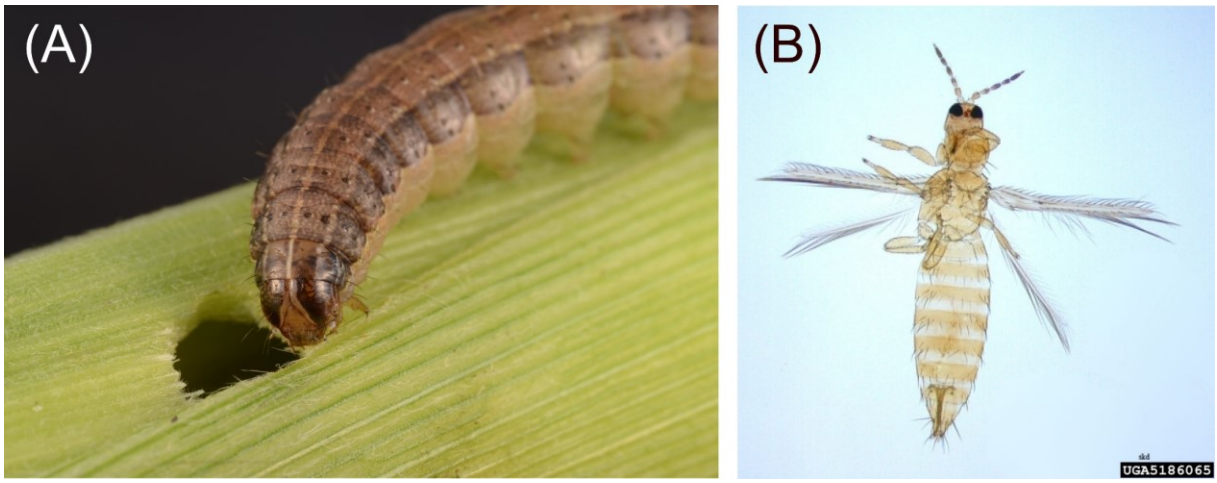

Figure 1.3. Examples of invasive insect pests. (A) Mature larva of the fall armyworm. Photograph by Lyle J. Buss, University of Florida, http://entnemdept.ufl.edu/creatures/field/fall_armyworm.htm. (B) Adult *Thrips palmi*. Photograph by Stan Diffie, University of Georgia, https://www.forestryimages.org/browse/detail.cfm?imgnum=5186065.

1.3.3. Economic impact

Globally, invasive insect pests are among the main vectors for crop damages (Ziska et al., 2011). The dimension can be illustrated using the example of the US state of Hawaii: in 1990, the number of identified native insect species was estimated to be 5,246 (Pimentel et al., 2005). An additional 2,582 insect species were estimated to originate from previous introduction events (Pimentel et al., 2005). Interestingly, those non-native insects accounted for 98% of the total pest insects (Pimentel et al., 2005). Focusing on the entire USA, the annual loss in crop production due to insect pests was found to be 13% when estimated in 2001 (Pimentel et al., 2005). Given the fact that approximately 40% of the US insect pests were non-native, an annual crop loss of US$ 13 billion was estimated to be caused by invasive insect pests (Pimentel et al., 2005). Moreover, additional costs of approximately US$ 500 million per year were invested for their control by pesticide applications (Pimentel et al., 2005). As a more specific example, Japan reported an average annual introduction of four non-native insects over the past 50 years, of which 74% became economic pests (Kiritani, 1998; Armstrong and Ball, 2005). The costs for the elimination of only two of those unintentionally introduced species, the oriental fruit fly *Bactrocera dorsalis* (Diptera: Tephritidae) and the melon fly *Zeugodacus cucurbitae* (Diptera: Tephritidae) was estimated to be equivalent to more than € 200 million (Kiritani, 1998; Armstrong and Ball, 2005).

1.3.4. Prevention

The management of introduction and spread of invasive insect pests can be divided into four parts, depending on the particular invasion step targeted: (i) prevention of introduction; (ii) early detection and fast response to recent introductions; (iii) elimination; and (iv) controlling of spread (Saccaggi et al., 2016). It is more and more accepted that the focus should be set on prevention measures, as they are more cost-effective than control and elimination measures conducted after successful insect pest establishment (Saccaggi et al., 2016). Prevention measures allow detecting foreign species at the initial stage of invasion events and provide therefore the possibility to implement rapid responses counteracting establishment and spread (Epanchin-Niell and Liebhold, 2015; Poland and Rassati, 2018).

Preventing the dispersal of invasive insect pests is a challenging task and requires cooperation of many different stakeholders involved in complex trade and transport processes (Garnas et al., 2016). International agreements such as the “International Plant Protection Convention (IPPC)” of the Food and Agricultural Organization of the United Nations (FAO) and the “Agreement on the Application of Sanitary and Phytosanitary Measures (SPS)” of the World Trade Organization (WTO) were formulated to mitigate the dispersal of harmful pests (Floyd et al., 2010; Saccaggi et al., 2016). In doing so, those agreements promote the adoption of phytosanitary regulations, while ensuring fairness of trade (Floyd et al., 2010; Saccaggi et al., 2016).

Phytosanitary regulations include prevention and quarantine measures such as post-harvest treatments (e.g. by exposure to heat), restrictions on type of goods to be imported and phytosanitary inspections of import commodities as a last line of defence (Bacon et al., 2012; Simberloff et al., 2013; Saccaggi et al., 2016). In general, phytosanitary inspections are conducted at the main points of entry (POEs) for import products, such as sea- and airports, and focus mainly on regulated invasive insects pests known to seriously harm agriculture (Bacon et al., 2012; Poland and Rassati, 2018). Import commodities suspected to harbour harmful insect pests are visually screened by plant health inspectors (Saccaggi et al., 2016; Blaser et al., 2018b; Poland and Rassati, 2018). In case of detection of a regulated pest, inspectors may prevent introduction directly by rejecting or destroying the infested imports (Blaser et al., 2018b; Poland and Rassati, 2018).

1.4. Identification of invasive insect pests

Once a suspicious insect species is intercepted, reliable and fast identification is needed to take a decision whether the infested consignment should be destroyed or not, as well as to inform decision makers (Saccaggi et al., 2016). In order to respect obligations associated with international treaties, destruction or refusal of infested cargo can only be implemented in case regulated pest species are intercepted (Floyd et al., 2010). However, the morphological differentiation between the vast range of regulated and non-regulated insect species is time-consuming and difficult, especially for plant health inspectors with limited taxonomic training

(Floyd et al., 2010; Saccaggi et al., 2016; Blaser et al., 2018b). It becomes even more difficult in case insects arrive at early development stages such as eggs or larvae (Floyd et al., 2010; Blaser et al., 2018b). Only few POEs are equipped with qualified taxonomists and species identification is therefore often outsourced to research institutions or private agencies, where specimens are analysed using morphological or molecular methods (Navia et al., 2010; Saccaggi et al., 2016; Blaser et al., 2018b).

1.4.1. Morphology-based identification

Morphological identifications are traditionally performed using a dichotomous key (Saccaggi et al., 2016). Such analyses depend on profound taxonomic knowledge and are mainly applicable for the identification of adult insect specimens (Armstrong and Ball, 2005; Saccaggi et al., 2016). This represents a major drawback of the method, as invasive insect pests are often intercepted at immature stages (Armstrong and Ball, 2005; Saccaggi and Pieterse, 2013; Saccaggi et al., 2016). Additional problems arise if insect specimens are damaged resulting in the lack of particular diagnostic characteristics needed for their accurate identification (Saccaggi et al., 2016). Recently, more user-friendly, interactive and multiple-choice-based taxonomic methods (e.g. online keys) were presented (Miller et al., 2014; Saccaggi et al., 2016). However, thus far, such keys have only be developed for few insect groups and their application still relies on a certain level of taxonomic experience (Saccaggi et al., 2016).

1.4.2. Molecular identification

Molecular methods represent powerful tools for the identification of invasive insect pests (Garnas et al., 2016). Compared to morphological identification, molecular assays do not depend on specific taxonomic knowledge and are generally not limited by the life stage of the intercepted insects (Saccaggi et al., 2016). In the recent past, a variety of different molecular identification methods were developed (Armstrong and Ball, 2005). These include antibody-based, protein-based and molecular genetic-based approaches (Armstrong and Ball, 2005). Antibody-based methods rely on the development of monoclonal antibodies specific for proteins of particular insect pest species, which can be applied for their identification in an

enzyme-linked immunosorbent assay (ELISA) (Symondson et al., 1999). In protein-based assays, electrophoretic profiles of salivary proteins are used to distinguish between morphologically similar insect species (Soares et al., 2000). Both antibody- and protein-based assays are not frequently used due to the difficulty to adapt them for the identification of different organisms, as well as their dependency on qualitatively high and fresh insect tissue (Armstrong and Ball, 2005).

1.4.3. Molecular genetic-based identification

Most of the molecular identification assays for insect pests are based on DNA amplification using polymerase chain reaction (PCR) (Armstrong and Ball, 2005). Compared to methods discussed above, this approach was proven to work also for low-quality samples such as incomplete or dried specimens. Nowadays, several different PCR-based methods are being used for insect pest identification, including species-specific PCR (Zhang et al., 2016), PCR restriction fragment length polymorphism (PCR-RFLP) (Armstrong et al., 1997), real-time PCR (Zhang et al., 2016) and DNA barcoding (Armstrong and Ball, 2005; Saccaggi et al., 2016; Zhang et al., 2016).

In species-specific PCRs, oligonucleotide primers hybridise to species-specific DNA regions and hence, initiate DNA amplification, an enzyme-driven process performed in consecutive thermal cycles (Yang and Rothman, 2004; Lauri and Mariani, 2009). The result of DNA amplification can be visualised by agarose gel electrophoresis using a DNA stain (e.g. ethidium bromide) (Lauri and Mariani, 2009). In case the expected DNA amplification product (amplicon) is present, the result of the test is considered to be positive (Lauri and Mariani, 2009). In PCR-RFLP analyses, the resulting amplicons are digested by restriction enzymes and differences in the length variation pattern of restriction fragments are used to differentiate between taxa (Armstrong et al., 1997; Arimoto et al., 2013). In contrast to PCR and PCR-RFLP, no agarose gel electrophoresis step is needed to validate the output of real-time PCR analyses (Lauri and Mariani, 2009). In this method, PCR reaction is performed using a reaction mix supplemented with fluorescent DNA stain and DNA amplification is performed in a thermal cycler able to detect and quantify fluorescence (Lauri and Mariani, 2009). During the PCR reaction, DNA

amplification can be quantified in real-time after each thermal cycle (Lauri and Mariani, 2009). In case dye quenched probes are used, this method even allows to identify several different taxa in a single assay (Lauri and Mariani, 2009; Zhang et al., 2016).

PCR-based identification tools such as those described above have the disadvantage that they are mostly developed specifically for particular taxonomic groups, and hence, there exists only limited potential to apply them to other species (Armstrong and Ball, 2005). In contrast, DNA barcoding represents an identification method that can easily be standardised between different laboratories and has the power to reliably identify the entire taxonomic range of insect pests using one single method (Armstrong and Ball, 2005; Floyd et al., 2010; Hodgetts et al., 2016). In this method, a "barcoding" sequence fragment of the mitochondrial cytochrome *c* oxidase subunit 1 gene (CO1) is amplified, using universal primers, and is subsequently sequenced (Armstrong and Ball, 2005; Floyd et al., 2010). The obtained "barcoding" sequence is then compared to a database containing reference sequence records of previously identified specimens, such as the Barcode of Life Data System (BOLD) or Q-bank (Sujeevan and Hebert, 2007; Bonants et al., 2013; Blaser et al., 2018b). The main weaknesses of this method are (i) the need for primer sequences that are specific for the target species – for thus far unknown species this information may be lacking and hence amplification may fail; and (ii) genetic differentiation on the barcoding fragment among some species may not allow to discriminate them reliably – such species need to be addressed as species groups (Armstrong and Ball, 2005; Boykin et al., 2012; Kirk et al., 2013).

1.4.4. Molecular genetic-based on-site identification

Due to the difficulty to morphologically identify the overwhelming range of different insect taxa associated with global trade, suspicious specimens intercepted during regular border import controls are mostly analysed in external laboratories (Saccaggi et al., 2016; Blaser et al., 2018b). The shipment of specimens to the laboratory as well as the subsequent molecular genetic analyses generally require at least 2-3 working days (Blaser et al., 2018b). Until results are available, the import products that were controlled are held at the POE (Mumford et al., 2016; Blaser et al., 2018b). Considering that invasive insects are mostly intercepted on

perishable import products such as cut flowers, fruits and vegetables, resulting import delays may cause substantial economic losses for exporters and importers alike (Kenis et al., 2007; Mumford et al., 2016; Blaser et al., 2018b). It has recently been reported that a delay of 24 h is already sufficient to adversely affect quality and thus value of such products (Mumford et al., 2016). Additionally, time delays resulting from pending identification results furthermore increase the risk of insect pest escape (Floyd et al., 2010).

Hence, a great need exists for on-site identification tools that can be performed directly at POEs (Blaser et al., 2018b). In order to be applicable, such on-site diagnostic methods need to be accurate, fast, and simple to perform. Furthermore, it should be possible to easily adjust them to different taxa in order to allow for standardisation (Blaser et al., 2018b).

1.4.5. Loop-mediated isothermal amplification-based identification

A method attracting more and more attention as suitable candidate for on-site identification of invasive insect pests is loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000; Huang et al., 2009; Poland and Rassati, 2018). Compared to PCR-driven analyses, this method is based on DNA amplification at a constant temperature using strand displacement polymerase, and hence, can be performed without unwieldy and costly thermo cyclers (Hodgetts et al., 2015; Poland and Rassati, 2018). Instead of using only one primer pair such as in PCR-based methods, LAMP includes a combination of three specific primer pairs rendering the method highly specific to its target organisms (Hodgetts et al., 2015). Furthermore, due to the robustness of the method against inhibitors, there is no need for any DNA purification step prior to DNA amplification (Kogovšek et al., 2015; Blaser et al., 2018b). LAMP is very fast; successful identification of plant pathogens has recently been reported to be possible within 20 min (Poland and Rassati, 2018). The method is especially promising for on-site identification, because amplification and subsequent read-out can be performed in laboratory-free environments using portable and battery-powered platforms such as Genie® II (Blaser et al., 2018b; Poland and Rassati, 2018). Such platforms allow to quantify DNA amplification in real-time in case SYBR Green-containing reaction mixes are used (Maeda et al., 2005; Hodgetts et al., 2015). Applied for the detection of plant pathogens such as bacteria

(Bühlmann et al., 2013; Hodgetts et al., 2015) and fungi (Tomlinson et al., 2010), as well as for the identification of insect pests (Huang et al., 2009), it has been shown that LAMP can be performed successfully with only basic laboratory training . Due to the mentioned properties including robustness, simplicity and speed, LAMP represents a promising method for on-site identification of invasive insect pests at POEs.

1.5. Invasion genetics of insect pests

1.5.1. Reconstructing routes of invasion

Understanding pathways followed by invasive insect pests is crucial for pest management and prevention of further spread (Estoup and Guillemaud, 2010; Correa et al., 2019). Outcomes of pest management strategies highly depend on reliable information of pest invasion histories (Estoup and Guillemaud, 2010). As an example, if a non-native species is found to be introduced repeatedly, it is more cost-effective to invest in prevention measures (e.g. import controls) than in management options such as elimination or containment (Estoup and Guillemaud, 2010). In case source populations and transport vectors of introduced species are known, it is furthermore possible to define quarantine measures precisely targeting the identified invasion pathway (Estoup and Guillemaud, 2010). Moreover, elucidating routes of invasion provides important information for the understanding of evolutionary and ecological processes underlying successful biological invasions (Estoup and Guillemaud, 2010; Javal et al., 2019).

Information on invasion pathways and source populations can be obtained using two different approaches: (i) direct methods, which are based on historical species observational records (presence/absence data); and (ii) indirect methods relying on population genetic data (Estoup and Guillemaud, 2010; Boissin et al., 2012). Observational data used for direct methods often originate from pest interception records of quarantine services (Boissin et al., 2012). However, an interception record does not per se imply that the captured insect has the potential to successfully establish in a certain area (Estoup and Guillemaud, 2010). Furthermore, observational data are often considered to be incomplete (Boissin et al., 2012). Due to these

limitations, it remains difficult to precisely elucidate invasion routes solely using direct methods (Estoup and Guillemaud, 2010; Boissin et al., 2012).

1.5.2. Invasion genetics

Indirect methods for retracing invasion histories and identification of source populations rely on analysing genetic patterns within and between populations based on molecular markers (Darling et al., 2008; Estoup and Guillemaud, 2010; Boissin et al., 2012). Population genetic patterns can vary strongly between different invasion scenarios (Garnas et al., 2016; Javal et al., 2019). It was shown that founder populations in invasive areas are often characterised by reduced genetic diversity resulting from the limited number of introduced genotypes (founder effect) and subsequent population bottlenecks (Dlugosch and Parker, 2008; Boissin et al., 2012; Javal et al., 2019). However, recent findings suggested that successful biological invasions often originate from multiple rather than single introduction events (Dlugosch and Parker, 2008; Javal et al., 2019). Recurrent and multiple introductions are thought to reduce impacts of founder and bottleneck effects by partially restoring genetic diversity (Dlugosch and Parker, 2008; Javal et al., 2019). Lately, several publications assessing invasion histories of non-native insect species reported “bridgehead” scenarios (Lombaert et al., 2010; Garnas et al., 2016; Javal et al., 2019; Lesieur et al., 2019). The bridgehead effect describes an invasion process in which a previously invasive population serves as a source for a secondary extra-range expansion (Lombaert et al., 2010; Garnas et al., 2016). Recurrent and multiple introductions, as well as impacts of bridgehead effects, may considerably complicate the population genetic structure of invaders (Garnas et al., 2016; Javal et al., 2019).

1.5.3. Invasion genetics of insect pests

Over the past several years, molecular methods were successfully applied to elucidate invasion histories of several insect pests (Estoup and Guillemaud, 2010). An early example was reported by Miller et al. (2005) deciphering the invasion route of the western corn rootworm *Diabrotica virgifera virgifera* (Coleoptera: Chrisomelidae) in Europe. First detected in former Yugoslavia, it was assumed for several years that subsequent introductions in France and Italy

resulted from intra-European invasion processes (Miller et al., 2005). However, based on molecular genetic analyses, Miller et al. (2005) demonstrated that the Western European populations originated from multiple North American introduction events rather than from Yugoslavian bridgehead populations (Estoup and Guillemaud, 2010; Kirk et al., 2013).

Later on, Pascual et al. (2007) assessed the genetic invasion pathway of the fruit fly pest *Drosophila subobscura* (Diptera: Drosophilidae) from Europe to the Americas (Estoup and Guillemaud, 2010). Results revealed an invasion history with a first introduction event in South America, followed by a bridgehead effect, in which South American populations served as source for the subsequent North American introduction (Pascual et al., 2007; Estoup and Guillemaud, 2010). The results stemming from the molecular analyses confirmed hypotheses formulated based on observational data (Pascual et al., 2007; Estoup and Guillemaud, 2010).

In a very recent example, Correa et al. (2019) investigated the worldwide genetic invasion history of the obscure mealybug *Pseudococcus viburni* (Hemiptera: Pseudococcidae). The study revealed that European populations most probably originated from South America (Correa et al., 2019). After their successful establishment, European populations served as bridgehead for the colonisation of North America, New Zealand and South Africa (Correa et al., 2019).

1.5.4. Molecular methods for reconstructing routes of invasion

Various analysis methods and software tools are available to assess invasion history based on molecular genetic markers (Estoup and Guillemaud, 2010; Kirk et al., 2013; Cristescu, 2015). These methods include traditional population genetics approaches such as phylogenetic trees, calculations of population genetic measurements (e.g. nucleotide diversity), analyses of molecular variance (AMOVAs), and parsimony networks (e.g. haplotype networks) (Meirmans, 2006; Estoup and Guillemaud, 2010; Leigh and Bryant, 2015). In addition, clustering approaches such as implemented in the software STRUCTURE (Pritchard et al., 2000) were demonstrated to be useful for assigning multi locus genotypes of invasive species into discrete genetic clusters (Cristescu, 2015; Roe et al., 2018; Javal et al., 2019). Moreover, STRUCTURE can describe levels of genetic admixture between different

populations and thus has the potential to inform about multiple introduction events (Pritchard et al., 2000; Javal et al., 2019). In principal component analyses (PCAs), genotypes are clustered using an alternative multivariate approach (Roe et al., 2018). Here, genetic variation is explained using a reduced selection of axes reflecting best the observed variation (Novembre and Stephens, 2008; Roe et al., 2018). In case such analyses reveal a clear clustering of an introduced population with a potential source population, conclusions about the possible invasion pathway can be drawn (Estoup and Guillemaud, 2010). Finally, a method gaining more and more attention for retracing invasion histories is Approximate Bayesian Computation (ABC) (Beaumont et al., 2002). ABC-based methods allow statistical testing of different invasion scenarios by estimating their likelihoods (Beaumont et al., 2002; Boissin et al., 2012). In order to increase accuracy, ABC models may also incorporate historical observational data (Boissin et al., 2012).

1.5.5. Molecular markers for reconstructing routes of invasion

The increased understanding of invasion histories over the past several years was strongly driven by advances in sequencing technologies resulting in the availability of more powerful markers for population genetic analyses (Davey et al., 2011; Garnas et al., 2016). Traditionally, studies reconstructing molecular invasion routes of insects were often based on mitochondrial CO1 data (Kirk et al., 2013; Garnas et al., 2016). Due to the haploid nature of mitochondrial DNA, CO1 sequence information can be obtained without extensive sequencing efforts (Hurst and Jiggins, 2005). An additional quality of the CO1 marker is its high evolutionary rate, which has the potential to resolve recent historical events (Hurst and Jiggins, 2005). Limitations arise from the strictly maternal inheritance of the mitochondrial genome – the observed population genetics patterns therefore correspond only to the population history of the female portion. Furthermore, in rare cases, the occurrence of nuclear mitochondrial pseudogenes (NUMTS) in the nuclear genome can confound the outcome of population genetic analyses (Hurst and Jiggins, 2005; Garnas et al., 2016).

In order to overcome these limitations, CO1 data were often combined with nuclear markers such as microsatellites (Kirk et al., 2013; Chown et al., 2015; Garnas et al., 2016). Also known

as simple sequence repeats (SSR), microsatellites are short (1-6 bp) tandem repeats frequently occurring in nuclear genomes of many organisms (Selkoe and Toonen, 2006). Despite high evolutionary rates in the repeat regions, such elements can be easily amplified by targeting conserved flanking regions (Selkoe and Toonen, 2006).

The recent advent of next-generation sequencing (NGS) has paved the way for studying invasion genetics based on genome-wide distributed single nucleotide polymorphisms (SNPs) (Davey et al., 2011; Garnas et al., 2016). High-throughput methods such as restriction-site-associated DNA sequencing (RAD) (Hohenlohe et al., 2010) and genotyping-by-sequencing (GBS) (Elshire et al., 2011) allow sequencing of large data sets of SNPs for hundreds of individuals at moderate costs (Davey et al., 2011; Chown et al., 2015). For both methods, specific restriction enzymes are used to sequence a representative subsample of the genome (Davey et al., 2011; Chown et al., 2015). Because RAD and GBS do not depend on a reference genome, both methods can easily be used to genotype non-model organisms (Hohenlohe et al., 2010; Elshire et al., 2011; Chown et al., 2015).

1.5.6. Limitations

In recent years, molecular genetics methods were widely applied to retrace invasion pathways of insect pest species (Miller et al., 2005; Kirk et al., 2013; Lesieur et al., 2019). In many cases, such analyses were shown to provide helpful insights into dispersal mechanisms important for pest management, as well as for the prevention of further introductions events (Miller et al., 2005; Kirk et al., 2013). However, while exploring invasion genetics with currently available methods, several drawbacks were identified (Kirk et al., 2013). These include insufficient power of some of the applied statistical models, limitations in describing population genetic processes using solely putatively neutral molecular markers and the appearance of complex and unexplainable genetic patterns (Kirk et al., 2013; Lesieur et al., 2019). In addition, due to the sometimes very condensed timescales of human-mediated species migration, molecular genetics approaches can fail to accurately resolve invasion dynamics (Fitzpatrick et al., 2012; Cristescu, 2015).

1.6. Goals and specific objectives of the PhD

The overarching goals of this PhD are (i) to develop rapid diagnostic tools for the on-site identification of invasive insect pests; (ii) to implement the tools in the regular phytosanitary control process of the Swiss Plant Protection Service (SPPS); (iii) to validate the tools under laboratory and on-site conditions; and (iv) to investigate the invasion genetics of a recently introduced insect pest.

There are four intertwined specific objectives of the PhD:

(i) To develop loop-mediated isothermal amplification (LAMP)-based assays for the rapid identification of the regulated insect pests *Bemisia tabaci* (Hemiptera: Aleyrodidae), *Thrips palmi* (Thysanoptera: Thripidae), and several fruit flies of the genera *Bactrocera* and *Zeugodacus* (Diptera: Tephritidae).

(ii) To implement the LAMP assays in the regular phytosanitary control process at the Swiss POE Zurich Airport. To achieve this, the assays were specifically adapted for on-site application by plant health inspectors with minimal laboratory training.

(iii) To validate the diagnostic accuracy of the LAMP assays under laboratory and on-site conditions.

(iv) To apply molecular genetics methods for retracing the invasion history of *Orientus ishidae* (Hemiptera: Cicadellidae), a potential leafhopper pest recently introduced to Europe.

2. From laboratory to point of entry: development and implementation of a loop mediated isothermal amplification (LAMP)-based genetic identification system to prevent introduction of quarantine insect species

Published in *Pest Management Science* (2018) doi:10.1002/ps.4866

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Research Article

Received: 6 October 2017 Revised: 12 January 2018 Accepted article published: 24 January 2018 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.4866

From laboratory to point of entry: development and implementation of a loop-mediated isothermal amplification (LAMP)-based genetic identification system to prevent introduction of quarantine insect species

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Abstract

BACKGROUND: Rapid genetic on-site identification methods at points of entry, such as seaports and airports, have the potential to become important tools to prevent the introduction and spread of economically harmful pest species that are unintentionally transported by the global trade of plant commodities. This paper reports the development and evaluation of a loop-mediated isothermal amplification (LAMP)-based identification system to prevent introduction of the three most frequently encountered regulated quarantine insect species groups at Swiss borders, *Bemisia tabaci*, *Thrips palmi* and several regulated fruit flies of the genera *Bactrocera* and *Zeugodacus*.

RESULTS: The LAMP primers were designed to target a fragment of the mitochondrial cytochrome *c* oxidase subunit I gene and were generated based on publicly available DNA sequences. Laboratory evaluations analysing 282 insect specimens suspected to be quarantine organisms revealed an overall test efficiency of 99%. Additional on-site evaluation at a point of entry using 37 specimens performed by plant health inspectors with minimal laboratory training resulted in an overall test efficiency of 95%. During both evaluation rounds, there were no false-positives and the observed false-negatives were attributable to human-induced manipulation errors. To overcome the possibility of accidental introduction of pests as a result of rare false-negative results, samples yielding negative results in the LAMP method were also subjected to DNA barcoding.

CONCLUSION: Our LAMP assays reliably differentiated between the tested regulated and non-regulated insect species within <1 h. Hence, LAMP assays represent suitable tools for rapid on-site identification of harmful pests, which might facilitate an accelerated import control process for plant commodities.

Supporting information may be found in the online version of this article.

Keywords: loop-mediated isothermal amplification; plant health inspections; point-of-entry diagnostics; quarantine organisms; evaluation

1 INTRODUCTION

The unintended spread of invasive insect species by global trade leads to considerable economic losses in agriculture.[1–3] Numerous insect species have been introduced into Europe, including harmful plant pests such as the western corn rootworm (*Diabrotica virgifera*) and the Colorado potato beetle (*Leptinotarsa decemlineata*).[1] As global trade is increasing, it is conceivable that the number of successful invasions of plant pests, as well as the scale of their impact, will also increase.[4,5] Invasive insects can be carried along with imported commodities such as agricultural goods, ornamental plants, nursery stocks, cut flowers, wooden products and packaging materials.[2,6,7] In addition, pests can unintentionally be vectored as stowaways in transport vehicles (e.g. ships, trains,

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and lorries), which assist the dispersal along trade networks, including anthropogenic corridors such as canals and railways.[2,8,9] Besides trade, international tourism, as well as changes in climate and land use also govern the movement of invasive species.[10]

International agreements such as the World Trade Organization (WTO) Agreement on the Application of Sanitary and Phytosanitary Measures (SPS) and the International Plant Protection Convention (IPPC) of the Food and Agricultural Organization of the United Nations (FAO) were concluded with the intention to prevent the spread and introduction of invasive species, as well as to promote the adoption of appropriate measures for their control.[1,11]

Within the European Union (EU), economically harmful plant pests, including insects, are regulated as quarantine organisms and are banned from import to the continent based on the European Council Directive 2000/29/EC.[1,12] This regulation also prevents the spread of such pests within the EU member states.[1,12] Switzerland as a non-EU member has ratified the same plant health regulations in the framework of the agreement between the EU and the Swiss Confederation on trade in agricultural products.[13] Inspections of plant consignments suspected to harbour quarantine organisms at points of entry (POEs), such as airports, seaports or other border controls, represent an important prevention measure against the introduction and movement of agricultural pests.[1]

In Switzerland, import inspections rely on visual examinations of plant products suspected to harbour quarantine organisms. Yet, morphological differentiation between harmful and non-harmful insects can be difficult. In particular, the early developmental stages (e.g. eggs and larvae) for which morphological keys are missing are challenging.[14] Suspicious insects are therefore sent to a reference laboratory (Agroscope, Wädenswil, Switzerland) where they are analysed using DNA barcoding, a method that accurately identifies insects without the need for extensive knowledge of morphological taxonomy. For identification by DNA barcoding, part of the mitochondrial gene cytochrome *c* oxidase subunit 1 (*COI*) is amplified and sequenced.[11,15,16] The resulting signature sequence is then queried against a database containing reference sequences for different species such as the publicly available Barcode of Life Data System (BOLD).[11,17] Because the method uses DNA instead of morphological characteristics, it can be equally well used for identification of taxa at all life stages.[11] Unlike traditional morphological identification, DNA barcoding also enables the identification of cryptic insect pest lineages.[18,19] However, although barcodes exist for well over 2 million different arthropod species, the method is limited by the fact that it can only identify specimens for which pre-existing reference barcode sequences are readily available.[11,17]

The shipment of samples to the Agroscope reference laboratory and the subsequent DNA barcoding analysis generally require 2–3 working days. This represents a major drawback of genetic diagnosis, as, in the meantime, the tested import consignments are blocked at the POE. Considering the fact that plant imports often are perishable commodities (e.g. fruits), the import delay due to the time between sampling and diagnosis can result in substantial economic losses for the importer. A promising approach to circumvent this delay is the use of rapid molecular on-site tests for species identification directly at the POE. The requirements for such an on-site identification system are, however, considerable. In addition to the feasibility of a test being performed rapidly by plant health inspectors with minimal laboratory training, high diagnostic specificity (true-negative rate) and sensitivity (true-positive rate) are pivotal to prevent the import of quarantine insect species and to meet obligations to the trade operators.

Loop-mediated isothermal amplification (LAMP) is a suitable technology for on-site analyses of organisms for which taxon-differentiating DNA or RNA sequences are known.[20] LAMP is highly specific as this method uses six primer pairs recognising eight distinct DNA regions.[21,22] Because of its isothermal nature and the robustness against inhibitors, LAMP tests can be performed in a simple and rapid manner in a laboratory-free environment.[22–24]

This paper reports on the development and evaluation of a LAMP-based identification system for quarantine insects and its successful implementation at the POE at Zurich Airport, Switzerland. The assay allows the molecular on-site identification of *Thrips palmi*, *Bemisia tabaci*, and several regulated fruit fly species from the genera *Bactrocera* and *Zeugodacus*. The fruit fly assay includes a group of members of the *Bactrocera dorsalis* species complex (*Bactrocera cacuminata*, *Bactrocera carambolae*, *Bactrocera dorsalis*, *Bactrocera papayae*, and *Bactrocera philippinensis*, hereafter the '*B. dorsalis* group'), as well as *Bactrocera latifrons* and *Zeugodacus cucurbitae*. These pest species were chosen as targets, because they account for >70% of the intercepted quarantine insect species over the past several years at the POE at Zurich Airport. The reported method has been designed for application by plant health inspectors with minimal laboratory training and can be performed within 1 h. As a result of its simplicity and the speed with which LAMP assays enable precise molecular diagnostics, this method represents a timely and promising new tool for National Plant Protection Organizations (NPPOs) and others in need of rapid identification of potential invasive pests on imported plant commodities.

2 METHODS

2.1 DNA extraction

For *T. palmi*, DNA was extracted from individual adults, for *B. tabaci* it was extracted from larvae and for the fruit flies it was extracted from approximately 1 mm^3 of larval tissue. For DNA extraction, tissue samples were added to 30 µl of an alkaline lysis solution [600 µM potassium hydroxide (Sigma-Aldrich Corp., St Louis, MO, USA) and 2 µM Cresol Red (Sigma-Aldrich Corp.)] and heated to 95 °C for 5 min on a heat block (Thermomixer Comfort; Eppendorf AG, Hamburg, Germany). The DNA extract was used directly for the LAMP reaction without any purification step.

2.2 LAMP primer design

LAMP assays for *T. palmi* and fruit flies of the genera *Bactrocera* and *Zeugodacus* were designed using publicly available sequences of an approximately 650-bp-long fragment at the 5′ end of the *COI* gene retrieved from the GenBank database.[25] For *B. tabaci*,

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Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

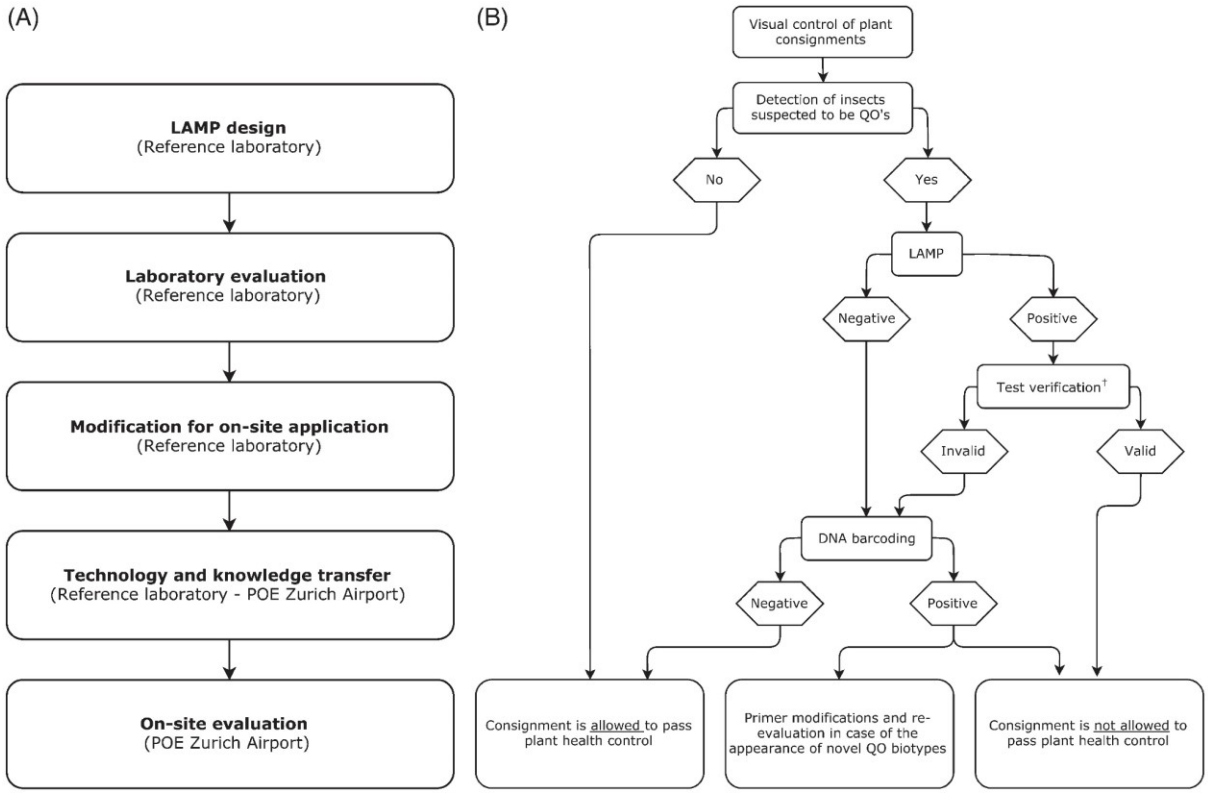

Figure 1. (A) Implementation procedure and (B) workflow of the LAMP-based identification system at the POE at Zurich Airport. †On-site test verification was performed by a control application inspecting results of the positive and negative controls, as well as melting temperatures of the LAMP amplification products. POE, point of entry; QO, quarantine organism.

as a result of the high level of sequence variation, a sequence fragment located at the 3′ end of *COI* was chosen as the target sequence for the LAMP assay. Primer design was performed using LAMPdesigner version 1.02 (Premier Biosoft International, Palo Alto, CA, USA) and Geneious versions R7 – 10.[26]

The fruit fly assay is designed as a combined LAMP test comprising one primer set targeting *B. latifrons* and *Z. cucurbitae*, and a second primer set targeting the *B. dorsalis* group (*B. carambolae*, *B. cacuminata, B. dorsalis, B. papayae*, and *B. philippinensis*). In order to simplify the protocol, the assay does not distinguish between the different fruit fly species targeted by the two primer sets. To ensure the specificity of this assay, sequences from the following closely related, non-target species were included in the primer design: *Anastrepha* spp. (11 species), *Bactrocera* spp. (five), *Ceratitis* spp. (12), *Dacus* spp. (32), and *Rhagoletis* spp. (five).

With the intention to cover the global sequence diversity observed for *B. tabaci* samples, a combined LAMP assay with three slightly different primer sets was designed. Closely related, non-target species included in the design of this assay were: *Aleurocanthus* spp. (two), *Aleurochiton aceris*, *Aleurodicus dugesii*, *Bemisia* spp. (three), *Neomaskellia andropogonis*, *Tetraleurodes acacia*, and *Trialeurodes* spp. (four).

The *T. palmi* LAMP test consists of only a single primer set and the following non-target species were included in the design: *Frankliniella* spp. (two), *Cephalothrips monilicornis*, *Scirtothrips* spp. (five), *and Thrips* spp. (two). Primers of all assays described in this study contain degenerated bases; the types and positions of the degeneracies are given in Supporting Information Table S1. They are available as commercial kits (OptiGene Ltd, Horsham, UK).

2.3 LAMP assays

LAMP reactions were performed in eight-well strips or 96-well plates. The reaction volume was 25 µl, containing 15 µl of Lyse n′ Lamp Isothermal Master Mix (OptiGene Ltd), 1.3 µM F3 and B3 primers, 13.3 µM FIP and BIP primers, 6.6 µM loopF and loopB primers and 2.5 µl of sample DNA extract. LAMP reactions were performed using Genie® II (OptiGene Ltd) or a 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) at 65 °C for 60 min. To determine the LAMP product melting temperature, samples were heated to 98 °C and cooled to 75 °C, while measuring fluorescence in real time.

As a negative amplification control, 2.5 µl of alkaline lysis solution (described above) was added to the reaction instead of DNA extract. Purified polymerase chain reaction (PCR) amplicons generated in the DNA barcoding approach (described below) were diluted to a concentration of 5 x 10^{-3} ng µl^{-1} in alkaline lysis solution (described above) and a volume of 2.5 µl was used as a positive amplification control. DNA concentrations of the positive amplification controls were measured using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.4 LAMP implementation and procedure at the POE

Individual steps in the development, implementation and evaluation of the LAMP assays at the POE at Zurich Airport are illustrated in Fig. 1A. After LAMP primer design, assays were evaluated for diagnostic accuracy under laboratory conditions by testing quarantine insect species intercepted between 2012 and 2015 at the POE at Zurich Airport and results were cross-validated by DNA

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Table 1. Results of LAMP assay evaluation performed under (A) laboratory and (B) on-site conditions at the POE at Zurich Airport

	LAMP assay	*N*	N_{TP}	N_{FP}	N_{TN}	N_{FN}	SEN (%)	SPE (%)	PPV (%)	NPV (%)	EFF (%)
A	Fruit fly[a]	117	57	0	60	0	100.0	100.0	100.0	100.0	100.0
	B. tabaci	67	62	0	2	3	95.4	100.0	100.0	40.0	95.5
	T. palmi	98	75	0	22	1	98.7	100.0	100.0	95.7	99.0
	Overall	282	194	0	84	4	98.0	100.0	100.0	95.5	98.6
B	Fruit fly[a]	14	9	0	4	1	90.0	100.0	100.0	80.0	92.9
	B. tabaci	13	13	0	0	0	100.0	n/c	100.0	n/c	100.0
	T. palmi	10	7	0	2	1	87.5	100.0	100.0	66.7	90.0
	Overall	37	29	0	6	2	93.6	100.0	100.0	75.0	94.6

N, number of analyses; N_{TP}, number of true-positive results; N_{FP}, number of false-positive results; N_{TN}, number of true-negative results; N_{FN}, number of false-negative results; SEN, sensitivity; SPE, specificity; PPV, positive predictive value; NPV, negative predictive value; EFF, test efficiency; n/c, not calculated.
[a] The fruit fly LAMP assay includes *B. latifrons/Z. cucurbitae*, as well as *the B. dorsalis* group (*B. carambolae*, *B. cacuminata*, *B. dorsalis*, *B. papayae*, and *B. philippinensis*).

barcoding. Thereafter, the LAMP protocol was further adapted to enable plant health inspectors with minimal laboratory training to successfully perform the method under on-site conditions. The resulting simplified protocol consists of only one single pipetting step, which has been achieved by the fabrication of pre-mixed LAMP kits, including all chemicals for the DNA amplification reactions. Furthermore, chemicals were stained with a dye (i.e. Cresol Red) to facilitate the handling of the small amount of liquid with the pipette (i.e. by enabling visual checking). LAMP kits were supplied by the Agroscope reference laboratory and stored at -20 °C.

After the technology transfer including the installation of a LAMP work station at Zurich Airport, plant health inspectors received basic laboratory training. Subsequent to the first LAMP round supervised by one of the investigators, plant health inspectors performed the LAMP tests independently. In order to evaluate the performance of the implemented identification system, LAMP results from the POE at Zurich Airport were cross-validated by DNA barcoding.

The workflow of the established identification system consists of visual inspections of incoming plant commodities followed by molecular identification using the LAMP assays in the case of the detection of insects suspected to be quarantine organisms (Fig. 1B). Each LAMP read-out is then checked for validity using a custom-written Microsoft® Excel® 2013 application available upon request from the corresponding author. The application checks the presence of amplification, the results of the controls and the expected melting temperature. The following lower and upper melting temperature threshold values were set: fruit fly assay, 80 and 85 °C; *B. tabaci* assay, 80 and 85.8 °C; and *T. palmi* assay, 78 and 84 °C. In the case of a valid positive result, the plant health inspector in charge can immediately destroy or reject the infested cargo.

In the case of a negative or invalid positive result, the DNA extract is sent to the Agroscope reference laboratory and is identified to species level through DNA barcoding. This control step ensures maximum test sensitivity, also preventing the introduction of unknown biotypes not included in the initial primer design. Such unknown biotypes can pose a risk for false-negative LAMP results, because the DNA amplification-based identification approach recognises only predefined targets. The addition of a sequencing step in the procedure also allows updating of the current LAMP assays by including new biotypes in the current LAMP primer set.

2.5 Analyses of diagnostic accuracy

In order to assess diagnostic accuracy, the following formulas were used to calculate sensitivity (true-positive rate), specificity (true-negative rate), positive predictive value (percentage of results that are true-positive), negative predictive value (percentage of results that are true-negative), and test efficiency (percentage of correct test results):

$$\text{Sensitivity (SEN)} = \frac{N_{TP}}{N_{TP} + N_{FN}} \times 100$$

$$\text{Specificity (SPE)} = \frac{N_{TN}}{N_{TN} + N_{FP}} \times 100$$

$$\text{Positive predictive value (PPV)} = \frac{N_{TP}}{N_{TP} + N_{FP}} \times 100$$

$$\text{Negative predictive value (NPV)} = \frac{N_{TN}}{N_{TN} + N_{FN}} \times 100$$

$$\text{Test efficiency (EFF)} = \frac{N_{TP} + N_{TN}}{N_{TP} + N_{TN} + N_{FP} + N_{FN}} \times 100$$

where *N* represents the number of analyses, N_{TP} the number of true-positive results, N_{TN} the number of true-negative results, N_{FN} the number of false-negative results, and N_{FP} the number of false-positive results.

2.6 DNA barcoding

All specimens included in the laboratory and on-site LAMP assay evaluation process were also subjected to DNA barcoding. PCR was carried out on a GeneAmp PCR System 9600 (PerkinElmer Inc., Waltham, MA, USA). The following primer pairs were used to amplify the 'Barcode of Life' fragment (i.e. the 5′ end of the *COI* gene) of *T. palmi* and the fruit fly specimens: Ron (GGAGCTCCTGACATAGCATTCCC) and C1-N-2353 (GCTCGTGTATCAACGTCTATTCC).[27,28] In order to amplify the barcode fragment of *B. tabaci* located at the 3′ end of the *COI* gene, the primers C1-J-2195 (5′-TTGATTTTTTGGTCATCCAGAAGT-3′) and TL2-N-3014 (5′-TCCAATGCACTAATCTGCCATATTA-3′) were used.[27,28] Reactions were run in reaction volumes of 20 µl with 1 × HotStarTaq Master Mix (Qiagen AG, Hilden, Germany), 0.4 µM

of each primer and 1 µl of DNA extract diluted 1:10 in molecular grade water. The PCR reaction was performed using the following cycling conditions: 15 min at 95 °C, followed by 45 cycles of 40 s at 95 °C, 15 s at 45 °C, ramping over 60 s to 60 °C and 2 min at 72 °C, and a final elongation step of 7 min at 72 °C. A clean-up step of the amplification product was performed using the NucleoFast® 96 PCR system (Marcherey-Nagel GmbH, Düren, Germany).

Linear amplification was carried out on a Labcycler (SensoQuest GmbH, Göttingen, Germany) in 10-µl reactions containing 1 × BigDye® Terminator v1.1 Ready Reaction Mix (Applied Biosystems), 0.2 µM of either forward or reverse primer (see above) and 1 µl of PCR product diluted 1:10 in molecular grade water. The linear amplification reaction was performed using the following cycling conditions: 15 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, 15 s at 45 °C and 2 min at 72 °C. The DyeEx 96 Kit (Qiagen AG) was used to remove unincorporated dye terminators. The amplicons were then sequenced on a 3130xl Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

Forward and reverse DNA sequences were assembled using Geneious versions R7–10.[26] The assembled sequences were then blasted for species identification against multiple publicly accessible databases, including GenBank, BOLD and Q-bank.[17,25,29] All sequences generated during the on-site evaluation step were uploaded to GenBank; accession numbers are shown in Supporting Information Table S2.

2.7 Sequence analyses

To assess the species-wide genetic diversity found in the on-site evaluation samples and to enable estimations of the risk of future false-negative results, the *COI* sequences of insect specimens analysed during on-site evaluation were compared to those retrieved from the GenBank database (accessed 15 June 2017). Sequences were aligned with MUSCLE using default parameters implemented in Geneious version 10.0.9.[25,26,30] To investigate whether the specimens analysed during on-site evaluation reflect the genetic diversity of larger data sets, genetic diversity indices such as the number of polymorphic sites (N_p), the number of haplotypes (h), haplotype diversity (H_d), nucleotide diversity (π) and the mean number of pairwise differences (MNPD) were estimated in DnaSP version 5.10.[31] *In silico* primer specificity analyses were performed using the primer testing function implemented in Geneious version 10.0.9.[26] Of note, the same software was used to generate pairwise genetic similarity matrices in order to assess the genetic similarity of the on-site evaluation specimens.[26]

3 RESULTS

3.1 Primer design and laboratory evaluation of the LAMP assays

The primer sets of the LAMP assays were designed based on the mitochondrial *COI* gene, where *in silico* analyses revealed taxa-specific regions for the target organisms.

In the first evaluation of the LAMP assays, a total of 282 insect specimens (fruit flies, $N = 117$; *B. tabaci*, $N = 67$; *T. palmi*, $N = 98$) suspected to be quarantine organisms were analysed by LAMP under laboratory conditions (Table 1A). Thereby, the fruit fly assay correctly identified *Z. cucurbitae* specimens from four different countries of origin, *B. latifrons* specimens from two different countries of origin and specimens from the *B. dorsalis* group from nine different countries of origin (Table 2A). Specimens from 13 non-target, closely related or morphologically similar species gave negative results in the same analysis (Table 2A). During the evaluation of the *B. tabaci* assay, specimens originating from eight different countries were correctly identified and two specimens from a closely related species gave negative results (Table 2B). Of note, the *T. palmi* assay was successfully tested for the identification of specimens originating from eight different countries (Table 2C). The same assay gave negative results when testing eight closely related, non-target species (Table 2C).

The test efficiency of the three individual assays ranged from 95.5% (*B. tabaci* assay) to 100% (fruit fly assay), and an overall test efficiency of 98.6% was calculated (Table 1A). Specificities were found to be 100% for all three tested LAMP assays (Table 1A). The overall test sensitivity was 98.0% and sensitivity was lowest in the *B. tabaci* test (95.4%) (Table 1A). During the first evaluation step, all tests showed a positive predictive value of 100%. A low negative predictive value was assigned to the *B. tabaci* test (40%) because of the low number of true-negative results (Table 1A). For the fruit fly and *T. palmi* assays, the negative predictive values were found to be 100 and 95.7%, respectively (Table 1A). Altogether, the overall negative predictive value was 95.5% (Table 1A). Mismatches in primer binding sites of false-negative *B. tabaci* and *T. palmi* biotypes were analysed and primer sets were modified (Table S3). When subsequently re-tested with the adapted primer sets, samples were correctly identified (data not shown).

3.2 On-site evaluation of the LAMP assays at the POE

A total of 37 insect specimens were analysed by LAMP under on-site conditions at the POE at Zurich Airport (Table 1B). The overall test efficiency was 94.6% and efficiency ranged from 90.0 to 100% in the individual assays (Table 1B). Specificity was calculated to be 100% for all assays (Table 1B). During on-site evaluation, sensitivity was lowest in the *T. palmi* assay (87.5%) and an overall sensitivity of 93.6% was calculated. Positive predictive values were found to be 100% for all assays. Negative predictive values for the fruit fly and *T. palmi* assays were 80.0 and 66.7%, respectively (Table 1B). The two false-negative samples were found to be positive when subsequently re-tested by the LAMP method in the Agroscope reference laboratory (data not shown). Analysing the pairwise genetic similarity matrix of the DNA barcoding fragment of tested fruit flies, false-negative *B. latifrons* sample no. 20496 was found to be genetically identical to sample no. 11524, which was correctly identified at the POE (Fig. S1A). The same was true for the false-negative *T. palmi* sample no. 11535, which was shown to be identical to the correctly identified sample no. 11529 (Fig. S1C).

Test performance of the on-site evaluation was assessed by analysing the duration until a positive result was available (time to positive) and melting temperatures of amplification products (Table 3). In order to separately investigate test performances of specimens from the *B. dorsalis* group and *B. latifrons*/*Z. cucurbitae*, results of the combined fruit fly assay were stratified (Table 3). Observed average times to positive (mean ± SD) ranged from 33.8 ± 11.6 min (*B. dorsalis* group) to 56.1 ± 5.6 min (*B. latifrons*/*Z. cucurbitae*) (Table 3). The melting temperatures were shown to extend from 80.1 ± 0.4 °C (*T. palmi*) to 82.2 ± 0.4 °C (*B. latifrons*/*Z. cucurbitae*) and were observed to be very similar for *T. palmi* and the stratified fruit fly samples (Table 3).

3.3 Sequence variation at primer binding sites

As a consequence of the lack of genetic information, it is virtually impossible to include the entire taxon-specific genetic diversity in the evaluation process of genetic tests, at least for non-model

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Table 2. Diversity and geographical origin of insect samples used for laboratory evaluation of the LAMP assays for (A) regulated fruit flies of the genera *Bactrocera* and *Zeugodacus*, (B) *B. tabaci* and (C) *T. palmi*. The *B. dorsalis* group includes *B. cacuminata*, *B. carambolae*, *B. dorsalis*, *B. papayae*, and *B. philippinensis*

	Species	Origin	LAMP		Species	Origin	LAMP
A	*Bactrocera dorsalis* group (5)	Cambodia	+	B	*Bemisia tabaci* (4)	Canary Islands	+
	Bactrocera dorsalis group (6)	Cameroon	+		*Bemisia tabaci* (1)	Dominican Republic	+
	Bactrocera dorsalis group (8)	India	+		*Bemisia tabaci* (20)	Israel	+
	Bactrocera dorsalis group (4)	Malaysia	+		*Bemisia tabaci* (13)	Malaysia	+
	Bactrocera dorsalis group (3)	Pakistan	+		*Bemisia tabaci* (14)	Morocco	+
	Bactrocera dorsalis group (3)	Sri Lanka	+		*Bemisia tabaci* (1)	Singapore	+
	Bactrocera dorsalis group (8)	Thailand	+		*Bemisia tabaci* (9)	Thailand	+
	Bactrocera dorsalis group (4)	Uganda	+		*Bemisia tabaci* (3)	Vietnam	+
	Bactrocera dorsalis group (1)	Vietnam	+		*Trialeurodes vaporariorum* (2)	Canary Islands	-
	Bactrocera latifrons (3)	Thailand	+				
	Bactrocera latifrons (2)	Vietnam	+				
	Zeugodacus cucurbitae (3)	Bangladesh	+	C	*Thrips palmi* (9)	Dominican Republic	+
	Zeugodacus cucurbitae (1)	Cambodia	+		*Thrips palmi* (16)	India	+
	Zeugodacus cucurbitae (3)	The Philippines	+		*Thrips palmi* (1)	Indonesia	+
	Zeugodacus cucurbitae (3)	Vietnam	+		*Thrips palmi* (11)	Malaysia	+
	Anastrepha fraterculus (3)	Argentina	-		*Thrips palmi* (19)	Pakistan	+
	Anastrepha oblica (3)	Dominican Republic	-		*Thrips palmi* (10)	Sri Lanka	+
	Anastrepha sp. (3)	Dominican Republic	-		*Thrips palmi* (6)	Thailand	+
	Anatrichus sp. (1)	Sri Lanka	-		*Thrips palmi* (4)	Vietnam	+
	Atherigona orientalis (9)	Sri Lanka	-		*Cephalothrips monilicornis* (1)	Sri Lanka	-
	Bactrocera kandiensis (2)	Sri Lanka	-		*Frankliniella intonsa* (1)	Vietnam	-
	Ceratitis capitata (5)	Egypt	-		*Frankliniella occidentalis* (3)	Canary Islands	-
	Ceratitis capitata (2)	Zimbabwe	-		*Haplothrips* sp. (4)	Thailand	-
	Ceratitis cosyra (7)	Cameroon	-		*Scirtothrips aurantii* (5)	Swasiland	-
	Ceratitis rosa (1)	Cameroon	-		*Scirtothrips dorsalis* (1)	Malaysia	-
	Dacus ciliatus (2)	Pakistan	-		*Thrips parvispinus* (2)	Uganda	-
	Drosophila ananassae (4)	Cameroon	-		*Thrips tabaci* (5)	Israel	-
	Rhagoletis cerasi (2)	Armenia	-				
	Zaprionus indianus (8)	India	-				
	Zaprionus indianus (8)	Dominican Republic	-				

Table 3. LAMP assay performances under on-site conditions at the POE at Zurich Airport. In order to investigate LAMP assay performances for individual fruit fly species groups, results of the combined fruit fly assay were stratified for the *B. dorsalis* group and *B. latifrons/Z. cucurbitae*

LAMP assay	N_{TP}	T_P (min) (mean ± SD)	T_M (°C) (mean ± SD)
B. dorsalis group[a]	6	33.8 ± 11.6	82.0 ± 0.3
B. latifrons/Z. cucurbitae	4	56.1 ± 5.6	82.2 ± 0.4
B. tabaci	13	38.4 ± 10.3	81.9 ± 0.4
T. palmi	8	38.0 ± 12.5	80.1 ± 0.4

N_{TP}, number of true-positive samples; T_P, time to positive; T_M, melting temperature; SD, standard deviation.
[a] Includes *B. cacuminata*, *B. carambolae*, *B. dorsalis*, *B. papayae*, and *B. philippinensis*.

organisms. However, comparative analyses of publicly available sequence information such as from GenBank may estimate how well the on-site evaluation results reflect the genetic diversity of larger data sets and the risk of producing false-negative results upon implementation of the methodology. For the following analyses, *B. latifrons* and *Z. cucurbitae* were treated as a single taxonomic unit, enabling estimates of the genetic diversity covered by the primer set of the combined LAMP assay.

The haplotype diversity (± SD) of on-site evaluation samples was found to be similar for all four species groups and ranged from 0.667 ± 0.204 for *B. latifrons/Z. cucurbitae* to 0.679 ± 0.122 for *T. palmi* (Table 4A). Compared with haplotype diversity values calculated for GenBank sequences (*B. dorsalis* group, $N = 995$; *B. latifrons/Z. cucurbitae*, $N = 1010$; *B. tabaci*, $N = 2476$; and *T. palmi*, $N = 243$), values of on-site evaluation samples ranged in the same order of magnitude (Tables 4A and B). The highest haplotype diversity (0.832 ± 0.004) was found for *B. tabaci* GenBank sequences (Table 4B). Nucleotide diversity (± SD) and MNPD (± SD) of the airport samples were found to range roughly in the same order of magnitude as nucleotide diversity values from GenBank sequences (Tables 4A and B). An exception was observed for the joint analysis of the two species *B. latifrons* and *Z. cucurbitae* (identified with the LAMP assay targeting both genetically well-separated species), where tenfold higher values ($\pi = 0.106 \pm 0.033$; MNPD = 10.0 ± 5.8) were detected compared with the GenBank sequences ($\pi = 0.019 \pm 0.002$; MNPD = 1.8 ± 1.0) (Tables 4A and B). The highest values of nucleotide diversity (0.139 ± 0.070) and MNPD (9.8 ± 4.5) for GenBank sequences were found for *B. tabaci* (Table 4B).

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Table 4. Variability and genetic diversity measures of concatenated LAMP primer binding sites from (A) samples tested during on-site evaluation and (B) sequences retrieved from the GenBank database

	LAMP assay	N	N_P	h	$H_d \pm$ SD	$\pi \pm$ SD	MNPD $\pm$ SD	L
A	*B. dorsalis* group[a]	6	2	3	0.733 ± 0.155	0.011 ± 0.002	1.1 ± 0.8	103
	B. latifrons/Z. cucurbitae[b]	4	15	2	0.667 ± 0.204	0.106 ± 0.033	10.0 ± 5.8	94
	B. tabaci	13	29	6	0.769 ± 0.103	0.086 ± 0.022	8.6 ± 4.3	101
	T. palmi	8	8	3	0.679 ± 0.122	0.026 ± 0.023	2.3 ± 1.4	100
B	*B. dorsalis* group[a]	995	32	45	0.647 ± 0.016	0.012 ± 0.001	1.2 ± 0.8	103
	B. latifrons/Z. cucurbitae[b]	1010	37	31	0.579 ± 0.012	0.019 ± 0.002	1.8 ± 1.0	94
	B. tabaci	2476	70	119	0.832 ± 0.004	0.139 ± 0.070	9.8 ± 4.5	101
	T. palmi	243	43	24	0.628 ± 0.030	0.049 ± 0.004	4.9 ± 2.4	100

N, number of individuals tested; N_P, number of polymorphic sites; h, number of haplotypes; H_d, haplotype diversity; π, nucleotide diversity; MNPD, mean number of pairwise differences; L, sequence length (bp) of analysed sequences SD, standard deviation.
[a] Includes *B. cacuminata*, *B. carambolae*, *B. dorsalis*, *B. papayae*, and *B. philippinensis*.
[b] *B. latifrons* and *Z. cucurbitae* were treated as one taxon, because both are identified by the same LAMP assay.

Despite high nucleotide diversity values in the primer binding sites, the designed LAMP primers containing degenerated bases were found to match 100% to all GenBank sequences of *B. tabaci* and the *B. dorsalis* group when tested *in silico*. For *T. palmi*, one mismatch (C/T) was found at position 17 (from the 3′ end) of the B3 primer and two mismatches (C/T) at positions 17 and 20 of the F3 primer (data not shown). Furthermore, primer mismatches at positions 9 (C/T) and 15 (C/G) of the B3 primer were found when analysing GenBank sequences of *B. latifrons/Z. cucurbitae* (data not shown). All described mismatches of *T. palmi* and *B. latifrons/Z. cucurbitae* found during *in silico* analyses have been observed in few individual samples during on-site evaluation at the POE at Zurich Airport without any impact on LAMP performance (data not shown).

4 DISCUSSION AND CONCLUSION

From a quarantine perspective, molecular diagnostics methods for the rapid identification of intercepted specimens are crucial to prevent the introduction and spread of morphologically indistinguishable pest species.[10,32] An ideal identification assay should be fast, reliable, easy to handle, affordable and suitable for on-site application.[32] This paper reports the successful development and on-site implementation of a LAMP-based system allowing the rapid identification (within 1 h) of three important and frequently intercepted quarantine insect species groups at a POE in Switzerland. The identification system was implemented to be performed by plant health inspectors with minimal laboratory training. The LAMP assays can be performed using simple and affordable equipment and the results are easy to interpret.

DNA amplification-based technologies such as the LAMP method can only identify specific target DNA sequences.[33] A comprehensive knowledge of the target sequence diversity is therefore crucial to ensure diagnostic reliability.[34] Unfortunately, available information is usually very limited for newly emerging quarantine organisms, even more so as import plant commodities originate from all over the world (Table 2). Rare false-negative LAMP results as a consequence of unknown single nucleotide polymorphisms (SNPs) at the primer binding sites are thus to be expected for all DNA amplification-based diagnostic tests and any identification system needs to take this into account.

In view of these points, the LAMP identification system for the POE at Zurich Airport was designed as a two-stage process (Fig. 1B). First, in the case of a positive LAMP result, the plant health inspectors can directly take action to prevent the introduction of the quarantine insect species. Second, in the case of a negative LAMP result, samples are sent to a reference laboratory where they are analysed by DNA barcoding. This procedure ensures maximum diagnostic sensitivity, which is needed to avoid the import of quarantine insect organisms and supports the further development of the LAMP assays in the case of the emergence of unknown insect biotypes.

In a first evaluation step, only four samples (1.4%) from a total of 282 analysed insect specimens gave false-negative results; all other results were correct. Sequence analyses of the false-negative samples revealed several new variant SNPs at the primer binding sites. Primer sets were therefore slightly adapted to accommodate these new variants and the modified LAMP assays were successfully revalidated using all available samples.

The evaluation of the LAMP-based identification system at the POE at Zurich Airport demonstrated that the LAMP assays are reliable for on-site diagnostics (Table 1B). Indeed, out of 37 analysed insect specimens, only two samples (5.4%) gave false-negative results and no false-positive results were identified (Table 1B). DNA sequences of both samples that gave false-negative results were found to be identical to DNA sequences from true-positively tested specimens (Figs S1A and C). Furthermore, both samples gave true-positive results when re-tested by the LAMP method in the Agroscope reference laboratory (data not shown). This observation suggests that the two identification failures may have been caused by a handling issue during the LAMP assay preparation. However, because negative LAMP results are routinely re-tested by DNA barcoding in the designed identification system, the import of quarantine insect species would be prevented in both cases.

Future adjustments to further enhance the diagnostic sensitivity could include testing specimens in duplicate and/or including an internal positive control (IPC). The latter measure would allow monitoring of each individual reaction separately and could consist of non-target control DNA spiked into the initial lysis solution.

During on-site evaluation, all specimens suspected to be *B. tabaci* were correctly confirmed (Table 1B). This demonstrates how well the plant health inspectors are trained in pre-identifying

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regulated insect quarantine organisms. A basic morphological knowledge is indeed crucial to select the appropriate LAMP assay for the identification of suspicious insects. In the case of the *B. tabaci* assay, because of the lack of any negative result during on-site evaluation, it was not possible to calculate diagnostic specificity and negative predictive value. Monitoring the test performance of this assay will therefore be an ongoing process.

In a comparative analysis, sequences generated during on-site evaluation were compared to all corresponding sequences currently available from the GenBank database in order to assess whether the observed genetic variability in the primer binding sites reflects the diversity of larger data sets. The nucleotide diversity values of the primer binding sites from the analysed *B. dorsalis* group, *B. tabaci*, and *T. palmi* specimens were found to range in the same order of magnitude as the values calculated for DNA sequences from the GenBank database (Table 4). In contrast, the nucleotide diversity value (0.106) and MNPD (10.0) calculated for the *B. latifrons/Z. cucurbitae* airport specimens were ten times higher than the values calculated for sequences from the GenBank database. The reason for the observed discrepancy is probably the low sample size, because only two specimens of each of these two genetically well-differentiated species were analysed (Table 4 and Fig. S1A). Yet, the results of the comparative analysis have to be interpreted with caution because of the relatively small sample size of the on-site evaluation samples as well as the fact that *B. latifrons* and *Z. cucurbitae* were treated as a single taxonomic unit. Furthermore, the composition of the GenBank entries for a particular species could also be biased because of overrepresentation of certain biotypes as a consequence of focal studies in specific areas.

The results of *in silico* primer specificity analyses revealed that the designed LAMP primers are suitable to detect all known haplotypes from numerous countries of origin of *B. tabaci*, *T. palmi* and several species of regulated fruit flies of the genera *Bactrocera* and *Zeugodacus*. The issue of the high within-taxon nucleotide diversity has been addressed by the application of degeneracy in primers, as well as the combination of multiple primer sets in the case of *B. tabaci*. Analysing the available sequences from the GenBank database, no primer mismatches were found either for the *B. dorsalis* group or for *B. tabaci*. Only a few mismatches distant from the 3′ end were found for some sequences of *T. palmi* and *B. latifrons/Z. cucurbitae*. As all observed mismatches were represented in the insect data set that was successfully analysed during the on-site evaluation at Zurich Airport, they seem to have no influence on the test performance of developed LAMP assays.

Further efforts towards improving the on-site identification system will focus on (i) expanding the range of diagnostic LAMP assays and (ii) developing on-site sequencing capabilities to eliminate the need for diagnostic core laboratories. Small next-generation sequencing-based systems such as the Oxford Nanopore technology are valuable candidates for on-site DNA/RNA sequencing.[35,36] Eventually, a sequencing-based technology may completely replace diagnostic assays which would eliminate the need for continuous development and evaluation of genetic tests. Furthermore, provided that sequencing is deep enough, information on characteristics such as pesticide resistance genes in arthropods or antibiotic resistance genes in bacteria may be acquired during the same process that identifies the species. Finally, accumulating sequence information of all intercepted specimens, together with the information on the geographical origin, will enable us to reconstruct invasion history in 'real time', thus deepening our understanding of how invasive species spread around the globe, enabling the development of new, more sustainable insect pest management strategies.

The successful molecular training of the plant health inspectors during the implementation of the LAMP-based identification system can be seen as a first step towards the future introduction of a sequencing-based on-site identification system. However, until novel sequencing technologies are ready to use for on-site application, the implemented LAMP assays represent fast and reliable identification tools for quarantine insect species.

ACKNOWLEDGEMENTS

The authors thank Annette Grendelmeier, Aurelia Drenovac, Cornelia Studer and Sven Moeller of the Swiss Federal Plant Protection Service at Zurich Airport for performing LAMP analyses at the POE. The authors also thank Beatrice Frey, Elisabeth Razavi, Seraina Vonzun, Daniel Frei and Markus Oggenfuss for participating in verification of the LAMP results and the prefabrication of the LAMP kits.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

3. A loop-mediated isothermal amplification (LAMP) assay for rapid identification of *Bemisia tabaci*

Published in *Journal of Visualized Experiments* (2018) doi:10.3791/58502

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Video Article

A Loop-mediated Isothermal Amplification (LAMP) Assay for Rapid Identification of *Bemisia tabaci*

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URL: https://www.jove.com/video/58502
DOI: doi:10.3791/58502

Keywords: Environmental Sciences, Issue 140, *Bemisia tabaci*, LAMP, loop-mediated isothermal amplification, point of entry diagnostics, plant health, rapid diagnostics, quarantine organisms

Date Published: 10/29/2018

Citation: Blaser, S., Diem, H., von Felten, A., Gueuning, M., Andreou, M., Boonham, N., Tomlinson, J., Müller, P., Utzinger, J., Frey, B., Frey, J.E., Bühlmann, A. A Loop-mediated Isothermal Amplification (LAMP) Assay for Rapid Identification of *Bemisia tabaci*. *J. Vis. Exp.* (140), e58502, doi:10.3791/58502 (2018).

Abstract

The whitefly *Bemisia tabaci* (Gennadius) is an invasive pest of considerable importance, affecting the production of vegetable and ornamental crops in many countries around the world. Severe yield losses are caused by direct feeding, and even more importantly, also by the transmission of more than 100 harmful plant pathogenic viruses. As for other invasive pests, increased international trade facilitates the dispersal of *B. tabaci* to areas beyond its native range. Inspections of plant import products at points of entry such as seaports and airports are, therefore, seen as an important prevention measure. However, this last line of defense against pest invasions is only effective if rapid identification methods for suspicious insect specimens are readily available. Because the morphological differentiation between the regulated *B. tabaci* and close relatives without quarantine status is difficult for non-taxonomists, a rapid molecular identification assay based on the loop-mediated isothermal amplification (LAMP) technology has been developed. This publication reports the detailed protocol of the novel assay describing rapid DNA extraction, set-up of the LAMP reaction, as well as interpretation of its read-out, which allows identifying *B. tabaci* specimens within one hour. Compared to existing protocols for the detection of specific *B. tabaci* biotypes, the developed method targets the whole *B. tabaci* species complex in one assay. Moreover the assay is designed to be applied on-site by plant health inspectors with minimal laboratory training directly at points of entry. Thorough validation performed under laboratory and on-site conditions demonstrates that the reported LAMP assay is a rapid and reliable identification tool, improving the management of *B. tabaci*.

Video Link

The video component of this article can be found at https://www.jove.com/video/58502/

Introduction

The whitefly *Bemisia tabaci* (Gennadius) is an invasive insect pest affecting the yield of many economically important crops including ornamental plants, vegetables, grain legumes, and cotton[1,2]. Beside damage caused through direct phloem-feeding, the homopteran species harms plants indirectly by the excretion of large amounts of honeydew onto the surfaces of leaves and fruits, as well as by the transmission of numerous plant pathogenic viruses[1,3,4]. Recent genetic studies comparing DNA sequences of the mitochondrial gene cytochrome *c* oxidase 1 (COI) revealed that *B. tabaci* is a species complex of at least 34 morphocryptic species[3,4]. Two highly invasive and damaging members within this complex, biotype B originating from the Middle East and the Asian Minor region, as well as biotype Q originating from the Mediterranean region, have been dispersed globally through international trading activities with plant products, particularly by the transportation of ornamentals[1,5,6]. Due to its worldwide pest status, the International Union for the Conservation of Nature and Natural Resources (IUCN) listed *B. tabaci* as one of the "world's 100 worst invasive alien species" and members of the species complex are regulated organisms by many countries[1,3,4].

In the European Union (EU), *B. tabaci* is listed in the Plant Health Directive 2000/29/EC Annex 1AI as a quarantine organism whose introduction from non-EU countries and its dissemination within the EU are banned[4]. An essential prevention measure against the spread of quarantine organisms is the inspection of plant shipments at points of entry (POEs) such as airports and seaports[7,8]. In the case a quarantine organism is found, the National Plant Protection Organization (NPPO) in charge takes action by either rejecting or treatment (including destruction)

of the infested shipment[9]. However, officers inspecting the imports often do not have the taxonomic expertise to accurately identify the vast range of pest species associated with global trade[9]. Especially the identification of immature life stages (*e.g.,* eggs and larvae) without distinct morphological keys is virtually impossible for non-taxonomists[8,9,10]. Consequently, to enable implementation of quarantine measures with minimal delay, there is a need for alternative, rapid on-site identification assays[9].

A candidate method is the loop-mediated isothermal DNA amplification (LAMP) technology that has recently been shown to be a suitable technology for the identification of plant pathogens[11,12,13]. LAMP is highly specific because the method uses at least two primer pairs recognizing six distinct DNA target sequences[14]. Due to the DNA strand displacement activity of the *Bst* DNA polymerase, LAMP reactions are performed under isothermal conditions[14]. Hence, in contrast to conventional polymerase chain reaction (PCR)-based assays there is no need for a thermal cycler[13,14]. Another advantage over PCR-based assays is its resilience against potential inhibitors in the DNA extract, circumventing the need for a DNA purification step[13]. Due to the protocol's speed and simplicity, LAMP may even be performed under on-site conditions using a portable, battery driven real-time detection device[8,15].

A LAMP assay was designed in response to the demand for a rapid on-site identification method for *B. tabaci*[8]. The overarching aim was to develop a protocol that can be performed by plant health inspectors with limited laboratory training. A strong focus was, therefore, set on optimizing speed and simplicity of the protocol. While existing diagnostic tests have generally been developed for the identification of one or several biotypes of *B. tabaci*, the novel LAMP assay covers the whole *B. tabaci* species complex[8,16,17,18]. The problem of the pronounced genetic within-taxon diversity of the complex was solved by using combinations of different primer sets and the application of degenerate primers[8]. The novel *B. tabaci* LAMP assay is designed in such a way that the primers target a fragment at the 3' end of the mitochondrial COI gene[8]. This gene presents a suitable target for animal diagnostic assays because it harbors regions conserved enough to ensure diagnostic sensitivity for a specific species, while discriminating enough between closely related organisms[19,20]. Furthermore, the COI gene is often used as a genetic marker in population genetic studies and as a signature sequence in DNA barcoding analyses, resulting in numerous DNA sequence entries in open source databases such as GenBank and BOLD[21,22]. Beside the publicly available COI sequences from *B. tabaci*, COI sequences from closely related species (*Aleurocanthus* spp. [N = 2], *Aleurochiton aceris*, *Aleurodicus dugesii*, *Bemisia* spp. [N = 3], *Neomaskellia andropogonis*, *Tetraleurodes acaciae*, and *Trialeurodes* spp. [N = 4]) were included in the primer design of this study and used to assess diagnostic sensitivity and specificity *in silico*[8].

Due to the accuracy of the method, its speed (<1 h) and the simplicity of the protocol, the assay has been shown to be suitable for on-site application when implemented as part of the import control procedure at a Swiss POE[8].

Protocol

1. Preparations

1. Preparing aliquots of alkaline DNA extraction solution.
    1. Produce a stock of alkaline DNA extraction solution using molecular grade water supplemented with 600 µM potassium hydroxide (KOH) and 2 µM Cresol Red.
    CAUTION: KOH is a strong base dissolved in water. Avoid spills, and skin and eye contact.
    2. Dispense 30 µL of alkaline DNA extraction solution (prepared in step 1.1.1) into 0.5 mL microcentrifuge tubes and store the aliquots at 4 °C.
    NOTE: Use the aliquoted DNA extraction solution within 1 year.

2. Preparing *B. tabaci* positive amplification control (PAC).
    1. Generate PCR amplicons of the LAMP target DNA fragment.
    NOTE: An introduction into general PCR principles and practices is given by Lorenz[23].
        1. Synthesize or obtain the primers C1-J-2195 (5'-TTGATTTTTTGGTCATCCAGAAGT-3') and TL2-N-3014 (5'-TCCAATGCACTAATCTGCCATATTA-3') amplifying a fragment of the mitochondrial COI gene[24,25].
        2. Set up the PCR reaction as described in **Table 1**. Use DNA extract (see step 2.1) of a reference *B. tabaci* specimen as DNA template.
        NOTE: Optionally, it is possible to extract the *B. tabaci* DNA for the PAC using a commercial kit according to the manufacturer's instructions.
        3. Program a thermal cycler using the following conditions: 15 min at 95 °C; 45 cycles of 40 s at 95 °C, 15 s at 45 °C, ramping over 60 s to 60 °C, 2 min at 72 °C; 7 min at 72 °C; hold at 4 °C.
        4. Clean the PCR amplification product using a commercial PCR clean-up kit according to the manufacturer's protocol and elute the final product in molecular grade water.
        5. Use a commercial kit with DNA-intercalating dye to measure the DNA concentration of the PCR amplification product according to the manufacturer's instructions and dilute with molecular grade water to a concentration of 1 ng/µL. Store the diluted PCR amplification product as PAC stock solution at -20 °C.
        NOTE: Use the PAC stock solution within 1 year.
        6. Supplement the PAC stock solution (prepared in step 1.2.1.5) with 0.6 µM KOH and dilute with molecular grade water to a concentration of 5 x 10^{-3} ng/µL. Store the product at 4 °C.
        NOTE: Use the PAC within 5 h for the preparation of the ready-to-use *B. tabaci* LAMP kits described in the next step.

3. **Preparing ready-to-use *B. tabaci* LAMP kit (protocol for 20 units)**
    1. Use scissors to cut 8-tube LAMP strips into two 4-tube LAMP strips.
    2. Label the tubes of the 4-tube LAMP strips according to the scheme shown in **Figure 1**.
    3. Prepare *B. tabaci* LAMP reaction mastermix (protocol for 80 reactions).

1. Add 1195.1 µL of ready-to-use GspSSD isothermal master mix (containing GspSSD polymerase, pyrophosphatase, magnesium sulfate, deoxynucleotides, double strand binding DNA binding dye) and 717.4 µL of *B. tabaci* LAMP primer mix to a 2 mL microcentrifuge tube. Briefly vortex and pulse centrifuge.
2. Dispense 22.5 µL of *B. tabaci* LAMP reaction mastermix (prepared in step 1.3.3.1) into each tube of the 4-tube LAMP strips (prepared in step 1.3.1) and pulse centrifuge.

4. Vortex the *B. tabaci* LAMP PAC (prepared in step 1.2) quickly and pulse centrifuge. Then, add 2.5 µL into the tube labelled with "PAC" of each 4-tube LAMP strip (**Figure 1**).
5. Close lids and store the ready-to-use *B. tabaci* LAMP kit units at -20 °C.
NOTE: Use them within 1 year.

2. On-site LAMP Analysis

1. DNA extraction
   1. Use sterile toothpicks to transfer the insect specimens into 0.5 mL microcentrifuge tubes containing 30 µL of DNA extraction solution (prepared in step 1.1.2).
   NOTE: Make sure that the insects are immersed in the extraction solution.
   2. Incubate the samples for 5 min at 95 °C in a thermomixer (300 rpm). Briefly vortex and pulse centrifuge.

2. *B. tabaci* LAMP assay
   1. Thaw a ready-to-use *B. tabaci* LAMP kit prepared in step 1.3. Vortex quickly and pulse centrifuge.
   NOTE: With each kit, it is either possible to test two different specimens or to analyze the DNA extract of one specimen in duplicate.
   2. Add 2.5 µL of sample DNA extract (prepared in step 2.1) into the tubes labeled "S1" and "S2" of the ready-to-use *B. tabaci* LAMP kit (**Figure 1**).
   3. Add 2.5 µL of pure alkaline DNA extraction solution (prepared in section 1.1) into the tube labeled "NAC" for the negative amplification control (**Figure 1**).
   4. Vortex the ready-to-use *B. tabaci* LAMP kit quickly and pulse centrifuge.
   5. Insert the ready-to-use *B. tabaci* LAMP kits into the LAMP analysis device (with real-time fluorescence measurement) or a real-time PCR platform and perform an isothermal DNA amplification analysis at 65 °C for 60 min.
   6. Measure the melting temperatures of DNA amplification products by heating up to 98 °C with a subsequent cooling step (ramp rate of 0.05 °C/s) to 75 °C, while measuring fluorescence in real-time.

3. LAMP assay read-out
   1. Validate the LAMP read-out manually as follows.
      1. If DNA amplifications were measured for the sample and the PAC, no DNA amplification was measured for the NAC, and the annealing temperature of the amplification products were between 80.0 and 85.5 °C, consider the LAMP results as POSITIVE (**Figure 2**).
      2. If there is no DNA amplification for the samples (*i.e.,* tubes labeled S1 and S2) but for PAC and NAC then consider the LAMP result as NEGATIVE (**Figure 2**).
      3. If DNA amplification was measured for the samples, but the annealing temperatures of corresponding amplification products were outside the range 80.0 – 85.5 °C, and/or PAC gave no DNA amplification, and/or NAC gave a DNA amplification, consider the LAMP result as INVALID (**Figure 2**).

   2. Optionally, validate the LAMP read-out using the LAMP validation application (**Supplemental file 1**).
      1. Define target species and define the number of tested samples. Click the *"Generate Report"* button.
      2. Transfer the read-out (DNA amplification yes/no, annealing temperature amplification product, results of PAC and NAC) from the on-site LAMP analysis device or real-time PCR platform to the corresponding input fields of the validation application. The result of the validation is immediately displayed after entering the data.

Representative Results

During the validation of the *B. tabaci* LAMP assay, insect specimens intercepted in the course of the regular Swiss import control process were analyzed[8]. The specimens originated from eight different countries (Canary Islands, Dominican Republic, Israel, Malaysia, Morocco, Singapore, Thailand, and Vietnam) and reflect the genetic diversity of *B. tabaci* found at European POEs[8]. All LAMP results were cross-validated by DNA barcoding[8].

From a total of 80 specimens analyzed by LAMP, 75 specimens (93.8%) were correctly identified as *B. tabaci* (true-positives), two specimens (2.5%) were correctly identified as not being *B. tabaci* (true-negatives), and three specimens (3.8%) were wrongly identified as not being *B. tabaci* (false-negatives) (**Table 2**)[8]. The correct-negative results originated from two *Trialeurodes vaporariorum* specimens, a non-regulated species at high risk to be confused with *B. tabaci* at POEs for plant products[8]. Based on these results, the following measurements of diagnostic accuracy were calculated: test specificity (true-negative rate), 100%; test sensitivity (true-positive rate), 96.2%; test efficiency (percentage of correct test results), 96.3% (**Table 2**)[8]. When assessing the analytical sensitivity (detection limit), the *B. tabaci* LAMP assay successfully amplified sample DNA diluted to 100 fg/µL across three technical replicates (**Table 3**).

A subset of the assays (N = 13) was performed under on-site conditions at the Swiss POE Zurich Airport by plant health inspectors using the ready-to-use *B. tabaci* LAMP kits[8]. When cross-validated in the reference laboratory, all results from on-site testing were found to be correct (test efficiency = 100%)[8]. Assessing the on-site LAMP assay performance, the average time to positive (time until a positive results was available) was 38.4 ± 10.3 min (mean ± standard deviation)[8]. A representative DNA amplification plot and the corresponding annealing derivative from a *B. tabaci* LAMP analysis performed under on-site conditions are shown in **Figure 3A and B**. In this example, sample one and two were correctly identified as *B. tabaci* indicated by DNA amplification after approximately 30 min (**Figure 3A**) together with the expected annealing temperatures at approximately 82 °C (**Figure 3B**).

Figure 1: Visualization of the experimental set-up of a ready-to-use *B. tabaci* LAMP kit described in the protocol. S1, sample 1; S2, sample 2; PAC, positive amplification control; NAC, negative amplification control. Please click here to view a larger version of this figure.

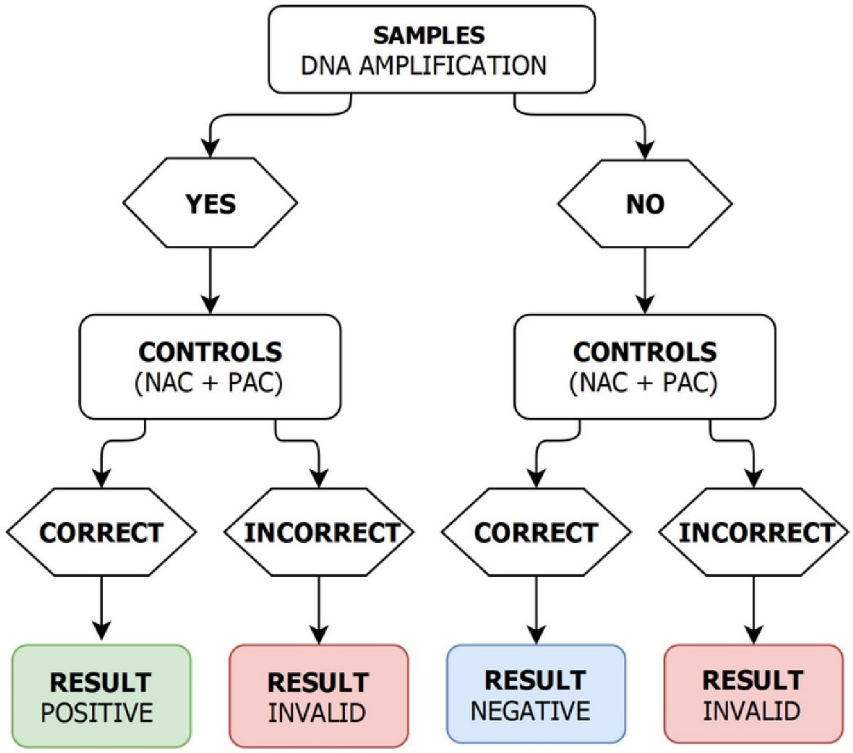

Figure 2: LAMP read-out validation schema. PAC: positive amplification control; NAC: negative amplification control. Please click here to view a larger version of this figure.

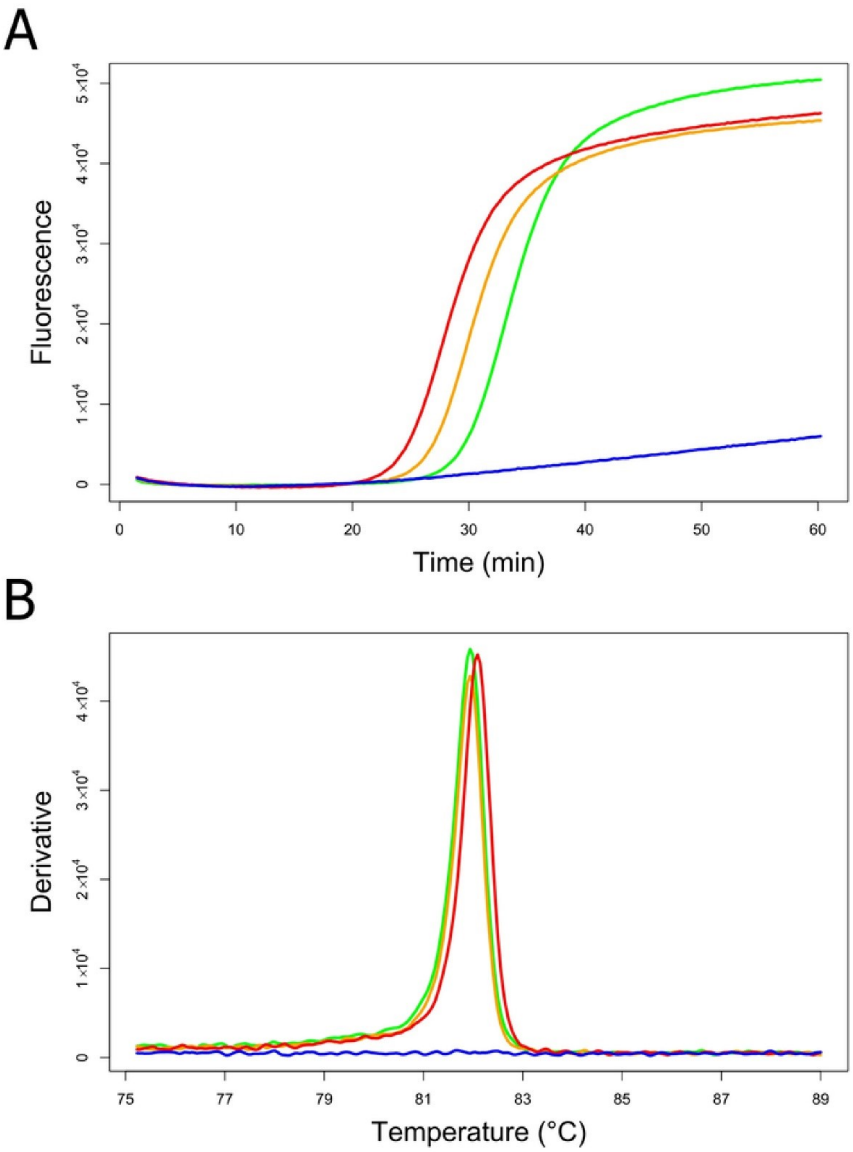

Figure 3: DNA amplification plot (A) and annealing derivative (B) of a *B. tabaci* LAMP analysis performed under on-site conditions. Fluorescence was measured in relative intensity units. Green line, sample 1; orange line, sample 2; blue line, negative amplification control (NAC); red line, positive amplification control (PAC). Please click here to view a larger version of this figure.

Component	Stock conc.	Final reaction conc.	Volume per reaction
Taq Polymerase Master Mix	2x	1x	10 µL
Primer C1-J-2195	20 µM	0.4 µM	0.4 µL
Primer TL2-N-3014	20 µM	0.4 µM	0.4 µL
Molecular Grade Water	-	-	8.2 µL
DNA Template	-	-	1 µL

Table 1: Preparation of PCR reaction mastermix for the *B. tabaci* positive amplification control. Components and concentrations needed to set up one PCR reaction. The final reaction volume is 20 µL. Primer sequences are shown in 1.2.1.1.

N	N_{TP}	N_{FP}	N_{TN}	N_{FN}	SEN (%)	SPE (%)	EFF (%)
80	75	0	2	3	96.2	100	96.3

Table 2: Results of the *B. tabaci* LAMP assay validation. N, number of analyses; N_{TP}, number of true-positive results; N_{FP}, number of false-positive results; N_{TN}, number of true-negative results; N_{FN}, number of false-negative results; SEN, diagnostic sensitivity; SPE, diagnostic specificity, EFF, test efficiency.

C_{DNA} (fg/µL)	N_{PR}	T_P (min) (mean ± SD)	T_A (°C) (mean ± SD)
1 x 10^5	3	33.5 ± 2.9	81.3 ± 0.1
1 x 10^4	3	30.7 ± 1.1	81 ± 0.0
1 x 10^3	3	40.4 ± 3.9	81.1 ± 0.1
1 x 10^2	3	50.7 ± 1.6	81.1 ±0.1
1 x 10^1	0	-	-
1 x 10^0	0	-	-

Table 3: Analytical sensitivity (detection limit) of the *B. tabaci* LAMP assay. Each dilution was tested in triplicates. C_{DNA}, DNA concentration per reaction; N_{PR}, number of positive replicates; T_P, time until a positive result was available; T_A, annealing temperature; SD, standard deviation.

Discussion

The ability to accurately identify potentially harmful organisms without time delay represents a critical aspect for the management of pest species[9,10,26]. Besides being rapid, for plant import products, an ideal pest identification method should be simple to perform on-site at POEs[8,26]. This paper reports the protocol of a novel LAMP assay for the rapid identification of *B. tabaci*, a quarantine insect organism frequently intercepted at European borders (https://ec.europa.eu/food/sites/food/files/plant/docs/ph_biosec_europhyt_annual-report _2016.pdf).

The rationale behind the development of the diagnostic test was to design an easy-to-follow protocol which can be performed during the plant import control procedure by plant health inspectors with minimal laboratory training. In order to make on-site testing as rapid and simple as possible, the protocol is divided into two parts, the preparation of a ready-to-use kit and the actual performance of the LAMP assay. The first part may be done in an external laboratory so that the plant health inspector can perform the DNA extraction and LAMP assay on-site with only one pipetting step.

Though only one step, pipetting small amounts of liquid may be challenging for users with little or no laboratory experience. To address this issue, a dye (cresol red) is added to the extraction solution so that the operator can visually confirm the small amount (*i.e.,* 2.5 µL) of DNA is correctly transferred to the respective tube. Another important simplification of the protocol is the validation application as it facilitates a reliable interpretation of the LAMP read-out (**Supplemental file 1**).

The novel *B. tabaci* LAMP assay has been validated under laboratory and on-site conditions by testing insect specimens intercepted during the regular import control process of Switzerland[8]. In total, 80 specimens from three continents, Africa, Eurasia, and North America, were analyzed by LAMP. Of the 80 specimens, only three (3.8%) were wrongly identified (false-negatives)[8]. When analyzing the primer target DNA sequences of the false-negative specimens, it was found that they were new *B. tabaci* haplotypes that have so far not been described[8]. Based on these results, the *B. tabaci* LAMP primer set has been modified and successfully re-validated[8].

One major limitation of any DNA amplification-based method including LAMP is that they only identify pre-defined target DNA sequences[8,27]. A comprehensive knowledge of the genetic variation found in the primer target sequence is therefore crucial to ensure diagnostic accuracy[8,27]. However, such information is often very limited, especially in the case of newly emerging pest species[8]. Though rare, false-negative results caused by mutations in the target sequence are expected[8]. In the case of the present *B. tabaci* LAMP assay, a solution for this problem is the combination with a DNA barcoding-based technology, a strategy realized in the course of the implementation of this diagnostic test at the POE Zurich Airport[8]. Here, all LAMP-negative results were re-analyzed by DNA barcoding in an external laboratory[8]. In case a novel pest haplotype not yet described is encountered, the LAMP primers can be modified using the DNA sequence generated in the barcoding process[8]. Thereby, the resulting loss of speed in case of a negative LAMP result is compensated for the maximum diagnostic accuracy ensured in this two-stage process[8].

The set-up costs for the current LAMP assay at a POE are approximately USD 25,000. With the increasing number of LAMP tests developed for plant pests (*e.g., Erwinia amylovora*, Flavescence dorée, and *Guignardia citricarpa*), such a one-time investment appears justified[13,15,28]. However, the protocol could potentially be modified to reduce these costs even further. For example, for the DNA extraction step at 95 °C the thermo mixer used here could be replaced by a less expensive water bath, or by performing this step directly in the real time LAMP device. Furthermore, the mixing steps on the vortex could probably be replaced by manually flicking the tubes, and in the DNA transfer step the pipettor might be replaced by sterile inoculation loops.

Future improvements for a rapid identification of *B. tabaci* and pest species in general could be an implementation of an on-site sequencing approach that would allow to perform DNA barcoding analyses at POEs. A promising candidate system for such an implementation is the nanopore sequencing technology. Indeed, the technology has recently been successfully implemented in an on-site DNA barcoding effort to assess the biodiversity of a rainforest[8,29,30]. An on-site DNA barcoding identification system can completely replace the need for the development

of targeted diagnostic tests and their validation. Also it allows collecting additional information about pest characteristics such as pesticide resistance genes[8]. Nevertheless, until novel sequencing technologies will be implemented routinely, the *B. tabaci* LAMP assay represents a rapid (<1 h) and accurate identification method.

Disclosures

The author Michael Andreou is a shareholder of OptiGene Limited that produces reagents and instruments used in this article. The other authors have nothing to disclose.

Acknowledgements

The authors are grateful to Annette Grendelmeier, Aurelia Drenovac, Cornelia Studer, Daniel Frei, Elisabeth Razavi, Markus Oggenfuss, Seraina Vonzun, and Sven Moeller for participating in the validation of the *B. tabaci* LAMP assay.

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4. Dispersal of harmful fruit fly pests by international trade and a loop-mediated isothermal amplification assay to prevent their introduction

Published in *Geospatial Health* (2018) doi:10.4081/gh.2018.726

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Geospatial Health 2018; volume 13:726

Dispersal of harmful fruit fly pests by international trade and a loop-mediated isothermal amplification assay to prevent their introduction

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Abstract

Global trade of plant products represents a major driving force for the spread of invasive insect pests. This visualisation illustrates the problem of unintended dispersal of economically harmful fruit fly pests (Diptera: Tephritidae) using geospatial tools and a time series of interception data from the Swiss import control system. Furthermore, it reports the development of a molecular diagnostic assay for rapid identification of these pests at points of entry (POEs) such as sea- and airports as a prevention measure. The assay reliably differentiates between target and non-target species within one hour and has been successfully evaluated for on-site use at a Swiss POE.

Video link

https://www.youtube.com/watch?v=d4Bv1E0pUoc

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Key words: Invasive fruit fly pests; Plant health inspections; Point of entry diagnostics; Loop-mediated isothermal amplification.

Acknowledgements: the authors would like to thank Annette Grendelmeier, Aurelia Drenovac, Cornelia Studer, Sven Moeller, Elisabeth Razavi, Seraina Vonzun, Daniel Frei and Markus Oggenfuss for participating in the evaluation of the fruit fly LAMP assay. Additionally, two anonymous referees are acknowledged for providing a series of useful comments and suggestions.

Contributions: SB, CH, AvF, MA, NB, JT, PM, JU, JEF and AB, conception of project; SB, HD, MG, BF and AB, analysis and interpretation of data; SB, manuscript writing; CH, HD, AvF, MG, MA, NB, JT, PM, JU, JEF, BF and AB, critical revision of manuscript; SB and CH, implementation of video; SB, CH, HD, AvF, MG, MA, NB, JT, PM, JU, JEF, BF and AB, final approval of manuscript and video prior to submission.

Conflict of interest: MA is a shareholder of OptiGene Limited that provided part of the materials used in this study.

Funding: none.

Received for publication: 23 July 2018.
Revision received: 12 October 2018.
Accepted for publication: 19 October 2018.

Licensee PAGEPress, Italy
Geospatial Health 2018; 13:726
doi:10.4081/gh.2018.726

Background

Introduction and dispersal of invasive insect pests into regions outside their native ranges can lead to substantial economic damage for local agriculture (Bacon *et al*., 2012; Horton *et al*., 2013). One of the primary means for the movement of non-indigenous insects is their inadvertent spread through global trade (Bacon *et al*., 2012; Horton *et al*., 2013). Invasive insects are vectored along major trading routes, as contaminants of a variety of import products, such as forest and agricultural goods, ornamental plants, nursery stocks, and also within packaging material (Horton *et al*., 2013; Blaser *et al*., 2018). Moreover, insect pests are carried along as stowaways of transport vehicles, such as aeroplanes, cargo trains, ships and trucks (Horton *et al*., 2013; Blaser *et al*., 2018). Additionally, the global spread of invasive pests is further facilitated through international tourism and environmental effects due to changes in climate and land use (Armstrong and Ball, 2005).

Fruit fly species of the family Tephritidae are among the economically most harmful invasive insect pests (Vargas *et al*., 2015). Due to their ability to feed on a wide range of fruits and fleshy vegetables and their high reproductive capacity, numerous species of this family have the potential to cause serious crop losses, and hence, constitute a worldwide threat for fruit and vegetable producers and traders (Vargas *et al*., 2015). *Bactrocera dorsalis*, the oriental fruit fly, is a prominent example of a highly invasive and destructive fruit fly pest (Theron *et al*., 2017). First recorded in Taiwan in 1912, the species dispersed throughout Southeast Asia, the Pacific region, and sub-Saharan Africa (Shi *et al*., 2010; Vargas *et al*., 2015; Theron *et al*., 2017). Several transient intro-

duction events were also reported from North America (Vargas *et al*., 2015). It was shown that *B. dorsalis* is not a single species, but rather forms a species complex, consisting of nearly 100 morphologically similar species (Kwasi, 2008; Schutze *et al*., 2015). Members of this complex have a host plant range including more than 250 species and varieties, among them commercially grown fruits (*e.g.* banana, guava and mango) traded on the global market (Shi *et al*., 2010; Vargas *et al*., 2015).

The economic impact of a fruit fly invasion on local horticulture can be exemplified using data from Ghana. After introduction and establishment of *Bactrocera invadens*, a member of the *B. dorsalis* complex, direct yield losses for fruit producers were estimated in excess of 40% (Kwasi, 2008; Badii *et al*., 2015). Additional indirect losses resulted from quarantine regulations imposed by importing countries such as import bans and costly monitoring and elimination programmes (Kwasi, 2008; Badii *et al*., 2015).

Biotic invasions are often initiated by a small number of individuals (Mack *et al*., 2000). While containment at that stage is comparatively simple, it is highly challenging and costly to contain successfully established communities (Mack *et al*., 2000). When analysing the entries (n = 211) of fruit fly elimination programmes recorded in the global eradication database (b3.net.nz/gerda) in 2014, the average costs per elimination were calculated to be about US$ 12 million (Suckling *et al*., 2014). However, the elimination of an invasive insect pest from a given area is challenging (Badii *et al*., 2015). Depending on the method used, elimination efforts can affect the environment and human health, especially when insecticides are being employed (Badii *et al*., 2015). Consequences of insecticide applications include chemical residues in crops, health problems of farmers and other community members due to insecticide exposure, contamination of water and soil, and decreases of frequency, relative abundance and diversity of native arthropod populations (De Barros *et al*., 2015; Sarwar, 2015).

Against this background, inspections of plant imports at points of entry (POEs), such as sea- and airports, are a crucially important and cost-effective control measure, as they prevent introduction of invasive, non-native pests (McCullough *et al*., 2006; Bacon *et al*., 2012; Poland and Rassati, 2018). Pest interception records from such inspections collected over time provide important information about the extent of human-mediated movement of plant pests by global trade and can inform about high risk invasion pathways of harmful pest species (McCullough *et al*., 2006; Holt *et al*., 2017). Beside information about pest abundance and origin, interception data have the power to inform about types of shipment associated with pest migration. Such information can be utilized by regulatory agencies to develop risk management measures mitigating the likelihood of pest introduction events (McCullough *et al*., 2006). Risk management measures can comprise refinements of inspection programmes as well as adoptions of international regulations and trade policies (McCullough *et al*., 2006). Evidence for pathway-associated pest movement can furthermore initiate in-depth pest risk analyses, including evaluations about the potential of a pest to establish outside its native range and estimations of accompanying economic and social impacts (Venette *et al*., 2010; Holt *et al*., 2017). Moreover, long-term interception data can reflect effects of novel trade policies, changes in market demand, efforts by exporters, and revisions of national regulations (McCullough *et al*., 2006).

Here, we use the format of a short video to communicate the issue of unintended spread of plant pests. We focus on the movement of harmful fruit flies, using a 7-year time series of interception data from Switzerland. As emphasised by Krieger and colleagues, a video-based approach has the potential to facilitate communication of complex geospatial correlations in an easy and understandable format that is readily accessible by different stakeholders (Krieger *et al*., 2012).

Inspecting Swiss plant imports between 2011 and 2017 revealed that there were 435 (0.6%) out of a total of 71,980 shipments that contained harmful insect pests. Among these, fruit flies of the family Tephritidae represented the most frequently intercepted taxonomic unit (n = 139, 32.0%) of all insect pest interceptions. The orders Hemiptera and Thysanoptera accounted for 106 (24.4%) and 105 (24.1%), respectively, while 67 (15.4%) of the intercepted insects were leaf-mining flies of the family Agromyzidae. The smallest contributions originated from interceptions of the orders Lepidoptera (n = 15, 3.5%) and Coleoptera (n = 3, 0.7%). Harmful fruit flies were intercepted on shipments originating from 19 different countries. The most common country of origin was Sri Lanka (23.7%), followed by Thailand (18.0%). India and Vietnam, each accounting for an additional 13.7% of the total fruit fly interceptions. The most common plant shipments associated with fruit fly interceptions were guava fruits (*Psidium guajava*, 27.5%), mango fruits (*Mangifera indica*, 26.1%), java apples (*Syzygium samarangense*, 16.0%) and peppers (*Capsicum* sp., 13.8%).

In the Swiss import control process, plant health inspections are based on visual examinations of incoming plant shipments suspected to harbour pest species (Blaser *et al*., 2018). Suspicious insects such as fruit flies are often encountered in the larval development stage, for which comprehensive morphological keys are missing, thus rendering morphological differentiation between harmful and non-harmful species challenging (Armstrong and Ball, 2005; Blaser *et al*., 2018). In order to ensure a reliable identification, the intercepted specimens are therefore sent to a reference laboratory where they are analysed by DNA-barcoding, an elaborate molecular identification method based on sequencing of a signature DNA-sequence, which is then queried against a reference database of sequences from previously identified specimens (Floyd *et al*., 2010; Blaser *et al*., 2018). The shipment of the specimens to the laboratory as well as their subsequent analysis requires two to three working days. In the meantime, the plant imports suspected to harbour pest species are hold back at the POE (Blaser *et al*., 2018). To circumvent such import delays, we developed a molecular on-site assay for the rapid identification of harmful fruit flies based on the loop-mediated isothermal amplification (LAMP) technology. The novel assay can be performed directly at POEs and results are available within only one hour. LAMP is a highly specific and robust identification method for species with previously known DNA or RNA sequences and suitable for on-site application because it can be performed in a laboratory-free environment after minimal training (Kogovšek *et al*., 2015).

Our assay is able to identify regulated fruit flies of the genera *Bactrocera* and *Zeugodacus*, namely *B. latifrons*, members of the *B. dorsalis* complex (*B. cacuminata*, *B. carambolae*, *B. dorsalis*, *B. papayae* and *B. philippinensis*), as well as *Z. cucurbitae*. These pests rank among the most destructive fruit fly species and are frequently intercepted at Swiss borders (Vargas *et al*., 2015).

The assay is designed in such a way that the primers target a sequence fragment of the mitochondrial gene cytochrome *c* oxidase 1. A detailed protocol of the method has been described elsewhere (Blaser *et al*., 2018). In brief, insect tissue is boiled for 5 min in an alkaline solution to extract the DNA. Subsequently, the

extraction product is transferred directly into the reaction tube containing all reagents needed for the LAMP reaction without the need of any purification step. The LAMP reaction is pursued at a constant temperature of 65 °C and its analysis can be performed in a battery-driven real-time LAMP device suitable for on-site application.

The fruit fly LAMP assay was initially evaluated for diagnostic accuracy under laboratory conditions with randomly selected fruit fly specimens intercepted during regular border controls and implemented in a second step as a part of the plant health control system at the Zurich Airport, one of the major POEs of Switzerland. For the assay evaluation, all results were rigorously cross-validated using DNA barcoding (Floyd *et al*., 2010; Blaser *et al*., 2018).

The results of the evaluation were partially described elsewhere (Blaser *et al*., 2018). A total of 143 fruit fly specimens originating from 16 different countries were analysed. Among these, 117 specimens were examined in a reference laboratory, whilst the remaining 26 specimens were analysed under on-site conditions at the Swiss POE Zurich Airport. During the evaluation, 78 fruit fly specimens (54.5%) were correctly identified as target species and 64 specimens (44.8%) correctly as non-target species. Only one specimen (0.7%) analysed at the POE was incorrectly identified as a target fruit fly specimen instead of a non-target species. Based on the results of the LAMP assay evaluation, we calculated a test sensitivity (true-positive-rate) of 98.7%, a test specificity (true-negative-rate) of 100% and a test efficiency (percentage of correct test results) of 99.3%.

Outlook

Geospatial maps visualising pest movement are effective tools to sensitise the community for the issue of the unintended spread of harmful invasive organisms along major trading networks. In this visualisation, we used pest interception data from the Swiss import control system to exemplify the problem of hitchhiking fruit flies associated with international trade of fruits and vegetables. We furthermore presented an on-site diagnostic test for rapid and accurate identification at POEs based on LAMP technology. After successful implementation of the LAMP assay for frequently intercepted fruit fly species, future efforts aim at expanding the target range of the LAMP assay to other harmful pest species associated with plant imports.

Overall aim

With this visualisation, we illustrate the problem of unintentional movement of harmful insect pests through global trade of plant products and present a new, rapid molecular on-site diagnostic test to prevent dispersal and introduction of harmful fruit fly pests. The visualisation is of particular interest to policy makers, plant health workers, producers of plant products and other stakeholders involved in the import and export of plant products, as well as to consumers of imported plant products.

Software

All geospatial elements of the visualisation were generated using the open-source software QGIS (version 2.14) based on *Natural Earth* vector maps published in the public domain.

If needed, illustrations were modified with the open-source vector graphics editor Inkscape (version 0.92). The final content visualisation was performed using Microsoft PowerPoint 2013 (Microsoft Corporation, Redmond, WA, USA).

Production of video was implemented using Camtasia Studio (version 9.0.5, TechSmith Corporation, Okemos, MI, USA).

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5. First evidence for a North American bridgehead effect in the European invasion of the mosaic leafhopper *Orientus ishidae*

Manuscript prepared for submission to *Biological Invasions*

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5.1. Abstract

Native to East Asia, the mosaic leafhopper *Orientus ishidae* (Matsumura, 1902) invaded North America in the 1920s and has recently spread across the European continent within less than 20 years. Recent studies indicated a potential vector competence of this species for the transmission of Flavescence dorée phytoplasma, the causing pathogen of the economically important grapevine yellows disease. Using a fragment of the mitochondrial cytochrome oxidase subunit 1 gene and 641 single nucleotide polymorphism markers generated by double-digest restriction-site associated DNA sequencing, we studied the genetic structure of native and non-native populations of *O. ishidae*, placing particular emphasis on Europe. Our findings revealed a strong genetic separation between native and non-native populations. The considerable genetic similarity between European and North American populations suggests a role of North America as bridgehead for the European invasion. Furthermore, slightly reduced structure and signs of admixture of genetically distant haplogroups in European populations indicate the occurrence of recurrent invasion events from North America, as well as gene flow between European populations. We hypothesise that the observed genetic structure of European populations was driven by frequent intra-continental trade of living plants such as ornamentals. Our study provides for the first time insight into the global invasion history of *O. ishidae* and hence, the findings may contribute to the prevention of future insect pest introductions.

Keywords: Bridgehead effect, Flavescence dorée phytoplasma, insect pest, invasion, *Orientus ishidae*, population genetics

5.2. Introduction

The rate of human-mediated spread of species beyond their native ranges has increased considerably in the recent past (Seebens et al., 2017; Javal et al., 2019; Lesieur et al., 2019). It is widely acknowledged that the increasing movement of goods and people across the world is the main driver for the growing number of introductions of non-native invasive species (Fisher et al., 2012; Garnas et al., 2012; Garnas et al., 2016). Once introduced and successfully established, invaders may become serious threats to the native biota, ecosystems, public health, as well as the economy (Kenis et al., 2009; Seebens et al., 2017; Lesieur et al., 2019). The invasion process can be subdivided into three phases; namely (i) an initial dispersal phase in which an organism moves from its native range into a new range outside of its home range; ii) a phase in which the invading organism establishes a self-sustaining population in the new range; and iii) a phase in which the organism spreads from the invaded range into surrounding areas (Kolar and Lodge, 2001; Puth and Post, 2005).

Insects represent a class of organisms for which a particularly large increase of successful introduction events has been observed (Roques, 2010; Roques et al., 2016). For instance, in Europe, the establishing rate of invasive, non-native insects was reported to have almost doubled when comparing the period from 1950 to 1974 (estimated annual average of 10.9 species) with that from 2000 to 2008 (estimated annual average of 19.6 species) (Roques, 2010; Roques et al., 2016).

Among the invasive insects are economically harmful pests such as the Western corn rootworm (*Diabrotica virgifera*) and the Colorado potato beetle (*Leptinotarsa decemlineata*), both causing considerable yield losses through direct feeding on crops (Roques et al., 2009; Bacon et al., 2012). Beside direct damages, non-native insect pests can also harm indirectly by vectorising plant diseases. An example is *Scaphoideus titanus*, a Nearctic leafhopper, which represents nowadays the main vector of Flavescence dorée phytoplasma, an intracellular bacterium highly pathogenic to several major grapevine cultivars in Europe (Papura et al., 2012).

The long-distance spread of invasive insect pests is often associated with the global trade of plant products (Haack, 2001; McCullough et al., 2006; Horton et al., 2013). Indeed, invasive pests are carried along trading networks as stowaways of import commodities such as agricultural goods, plants for planting, cut flowers, or wooden products and packaging material (Haack, 2001; McCullough et al., 2006). In addition, pests hitchhike along anthropogenic corridors (e.g. roads, railways and seaways) in transport vehicles such as lorries, trains and ships (Dobbs and Brodel, 2004; Hulme, 2009; Blaser et al., 2018).

Once arrived, the establishment of pest populations in new territories is facilitated by the homogenisation of landscapes due to urbanisation and intensive agriculture (Estoup and Guillemaud, 2010). For example maize crops provide relatively uniform habitats in many regions around the world (Estoup and Guillemaud, 2010). Such uniform and globally very similar landscapes greatly reduce the extent of evolutionary response needed for a successful adaptation (Estoup and Guillemaud, 2010; Papura et al., 2012; Seebens et al., 2017).

Profound knowledge of pest introduction pathways are key for the adoption of effective quarantine measures preventing pest introduction, as well as to define appropriate control strategies after their establishment. Pathways may be identified using direct methods based on historical observation data or indirectly by studying the population genetics of the invading insects (Estoup and Guillemaud, 2010; Garnas et al., 2016). While deciphering invasion routes and demography of invasive insect pests using molecular genetic approaches, previous studies revealed how complex and counter-intuitive such processes can be (Miller et al., 2005; Lombaert et al., 2010). However, the reconstruction of invasions pathways may be challenging due the stochasticity of demographic and genetic events (e.g. admixture and founder events) resulting in complex genetic signals (Guillemaud et al., 2010; Rius and Darling, 2014; Lesieur et al., 2019).

An example of a globally invasive insect species is represented by *Orientus ishidae* (Hemiptera: Auchenorrhyncha: Cicadellidae), an extremely polyphagous leafhopper feeding on many wild and cultivated shrubs and trees such as the common hazel (*Corylus avellana*) (Nickel 2010; Parise 2017), willows (*Salix* sp.) (Guglielmino 2005; Nickel 2010) and orchard

apple trees (*Malus domestica*) (Klejdysz et al. 2017). Probably overwintering in eggs laid on plant tissues, the mosaic leafhopper is thought to have one generation per year (univoltine) (Valley and Wheeler Jr 1985; Nickel 2010; Lessio et al. 2016).

Previous studies revealed a vector function of *O. ishidae* for the transmission of "*Candidatus Phytoplasma pruni*", the causing agent of the Peach X disease in North America (Rosenberger and Jones 1978). In addition, recent findings of *O. ishidae* specimens infected by "*Candidatus Phytoplasma vitis*"-related phytoplasma strains belonging to the 16SrV ribosomal group (Mehle et al. 2010; Gaffuri et al. 2011; Trivellone et al. 2015) and their experimental transmission in laboratory experiments (Lessio et al. 2016) indicated furthermore a potential role of this leafhopper species in vectorising grapevine Flavescence dorée in European vineyards. Interestingly, while studying ecology of Flavescence dorée phytoplasma in a vineyard agro-ecosystem of southern Switzerland, Casati et al. (2017) collected *O. ishidae* specimens on wild *C. avellana* and *Salix* sp. plants infected by "*Candidatus Phytoplasma vitis*"-related phytoplasma strains. Situated in close proximity to vineyards, infected wild host plants of *O. ishidae* therefore may serve as reservoirs for the grapevine Flavescence dorée disease (Casati et al. 2017).

O. ishidae is considered to originate from East Asia and was first described in Japan in 1902 (Matsumura, 1902). In 1919, the leafhopper was recorded for the first time outside its native range; in New Jersey, USA, where it was probably introduced in the egg stage on ornamental plants of the genus *Aralia* (Felt and Bromley, 1941). In 1955, *O. ishidae* was reported to be introduced also into southern Ontario, Canada (Hamilton, 1983). In 1967, the species occured in several additional U.S. states, namely District of Columbia, Long Island, Maryland, New Hampshire, New York, Ohio and Pennsylvania (Metcalf, 1967). In Europe, *O. ishidae* was first discovered in the northern part of Italy in 1998 (Guglielmino, 2005). Within 20 years, *O. ishidae* colonised many European countries ranging from southwestern France to southern Romania (see Table S8.4 for a detailed overview on occurrence reports from Europe) (Nickel, 2010; Chireceanu et al., 2017; Klejdysz et al., 2017). As for other invasive Auchenorrhyncha species, the rapid dispersal of *O. ishidae* in Europe is thought to be governed by the trade of fruit trees,

vine cuttings and ornamental plants, thereby translocating eggs associated with plant tissue (Mifsud et al., 2010). This hypothesis is supported by the fact that presence of *O. ishidae* was often reported from sub-urban and urban areas in proximity to trading networks (Günthart et al., 2004; Mifsud et al., 2010; Nickel, 2010; Klejdysz et al., 2017). However, the source of the European *O. ishidae* populations is unknown.

In the present study, we used the combination of a 573 bp long DNA marker fragment from the mitochondrial cytochrome oxidase subunit 1 (CO1) gene and a genome wide single nucleotide polymorphism (SNP) marker set generated by double-digest restriction-site associated DNA (ddRAD) sequencing (Peterson et al., 2012), to analyse population genetics across 41 *O. ishidae* populations from Asia, Europe, and North America. The objectives of this study were (i) to investigate the worldwide population genetic structure placing a particular emphasis on European populations; (ii) to assess the likely source of the European invasion; and (iii) to study whether the European invasion proceeded from one or multiple introduction events.

5.3. Materials and methods

5.3.1. Samples and DNA extraction

We analysed a total of 283 *O. ishidae* specimens originating from 41 sampling sites across three continents (Asia, Europe and North America) (Fig. 5.1, Table 5.1). Morphological species identification was confirmed by DNA barcoding (Hebert et al., 2003) using the mitochondrial CO1 marker described below. The final data set comprised mitochondrial CO1 data for 274 specimens and ddRAD data for 254 specimens (for the detailed list of specimens, see chapter 8.3.3.1). In order to reduce effects of unwanted bias during sample preparation (e.g. batch effects), specimens were initially randomised using the RAND function implemented in Microsoft® Excel® 2013. DNA extraction was performed nondestructively without any mechanical disruption step from whole specimens using the BioSprint 96 DNA Blood Kit (Qiagen AG, Hilden, Germany). The proteinase K digestion step was performed over night and the extracted DNA was eluted in a final volume of 100 µl Buffer AE. Visualisation of the sampling locations was performed using the R-package ggplot2 (R Core Team, 2016; Wickham, 2016).

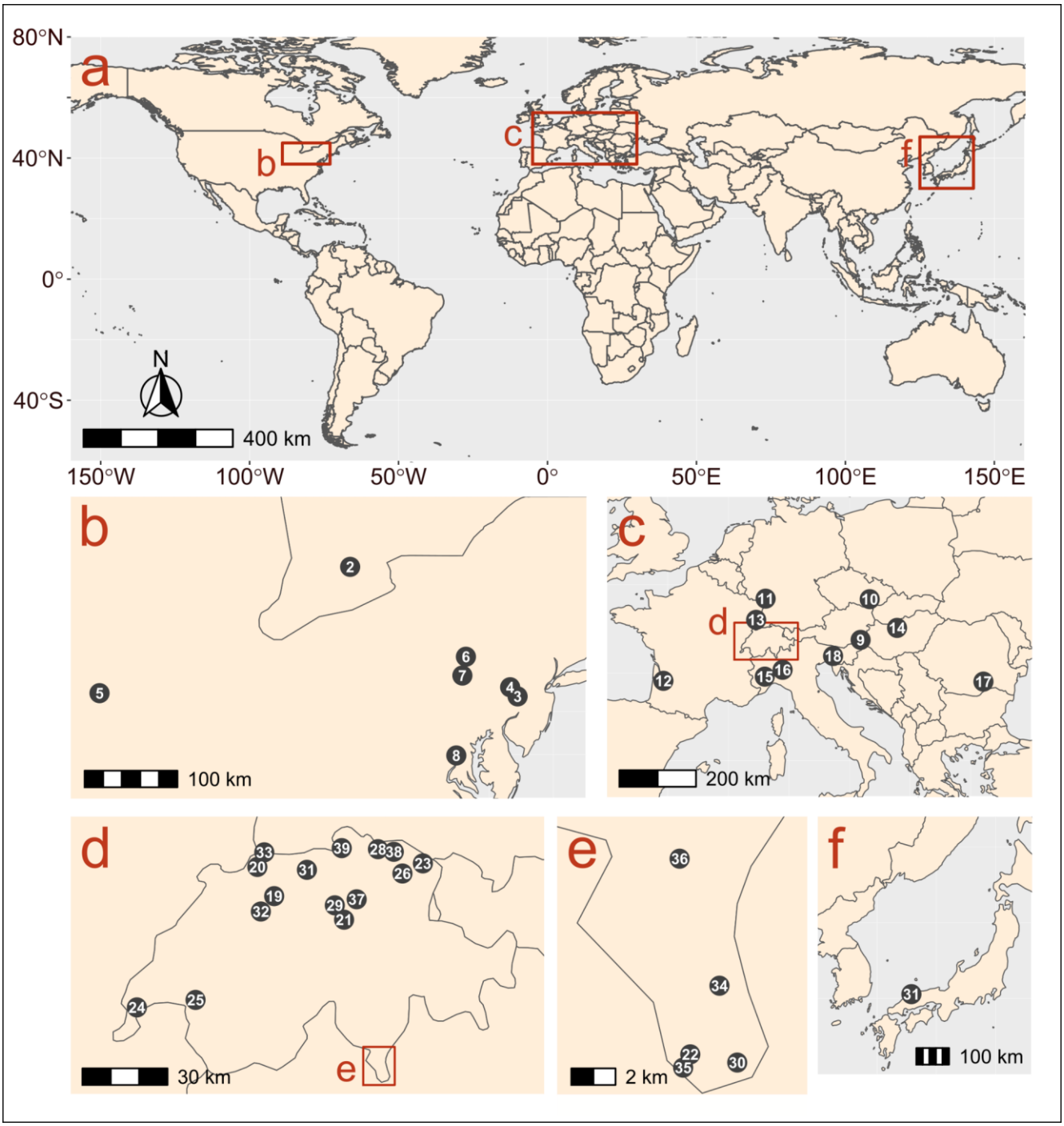

Figure 5.1. Distribution of sampling sites on **a** global scale, **b** North American scale, **c** European scale, **d** and **e** Swiss scale and **f** Japanese scale. Numbers correspond to sampling sites specified in Table 5.1.

Table 5.1. Specimens included in the CO1 and ddRAD analyses.

S_{SITE}	ID	Country	N_{RAD}	N_{COI}	Lat	Lon
1	JPN-UNN	Japan	4	5	35.283	132.900
2	CAN-CAM	Canada	7	7	43.369	-80.316
3	USA-DOY	USA	1	1	40.354	-75.109
4	USA-HE1	USA	1	1	40.568	-75.341
4	USA-HE2	USA	0	1	40.581	-75.330
5	USA-PAX	USA	2	2	40.430	-88.109
6	USA-PIC	USA	2	3	41.278	-76.716
7	USA-SHA	USA	2	2	40.840	-76.826
8	USA-TAK	USA	2	2	38.980	-77.015
9	AUT-BAI	Austria	6	7	46.872	15.929
10	CZE-BRN	Czech Republic	11	11	49.184	16.678
11	DEU-GOD	Germany	5	6	49.213	8.082
12	FRA-BOM	France	12	11	44.547	-0.356
13	FRA-HAT	France	2	1	48.011	7.300
14	HUN-BUD	Hungary	11	11	47.552	18.936
15	ITA-CAS	Italy	10	10	44.784	8.015
16	ITA-ROV	Italy	7	7	45.171	9.486
16	ITA-VAL	Italy	5	6	45.176	9.464
17	ROU-BUC	Romania	10	11	44.502	26.069
18	SVN-NOV	Slovenia	10	10	45.957	13.653
19	CHE-AAR	Switzerland	10	12	47.246	7.760
20	CHE-AES	Switzerland	11	11	47.468	7.573
21	CHE-ART	Switzerland	1	2	47.067	8.537
22	CHE-ARZ	Switzerland	9	11	45.868	8.937
23	CHE-BER	Switzerland	9	10	47.495	9.413
24	CHE-CHA	Switzerland	5	5	46.400	6.234
25	CHE-CHY	Switzerland	2	1	46.457	6.886
26	CHE-FLA	Switzerland	10	12	47.418	9.186
27	CHE-GIO	Switzerland	3	9	46.397	8.878
28	CHE-HER	Switzerland	1	1	47.603	8.914
29	CHE-HUE	Switzerland	11	11	47.176	8.434
30	CHE-MEN	Switzerland	2	3	45.860	9.003
31	CHE-OBE	Switzerland	13	13	47.446	8.124
32	CHE-OES	Switzerland	2	2	47.129	7.612
33	CHE-RIE	Switzerland	9	10	47.579	7.650
34	CHE-ROV	Switzerland	10	10	45.935	8.978
35	CHE-STA	Switzerland	12	12	45.855	8.927
36	CHE-TOR	Switzerland	0	1	46.059	8.922
37	CHE-WAE	Switzerland	12	12	47.222	8.677
38	CHE-WEI	Switzerland	1	1	47.583	9.090
39	CHE-WIL	Switzerland	11	10	47.611	8.511

S_{SITE}, position on map; *ID*, population identifier; N_{RAD}, number of specimens included in ddRAD data set; N_{COI}, number of specimens included in mitochondrial CO1 data set; Lat, latitude; Lon, longitude. Geographic coordinates are specified using the world geodetic system (WGS) 84. Sampling site of ITA-ROV and ITA-VAL, as well as of USA-HE1 and USA-HE2 were plotted together due to their close spatial proximities.

5.3.2. Mitochondrial DNA sequencing

As a marker for mitochondrial DNA diversity and population structure, a 701 bp fragment of the mitochondrial CO1 gene located at the 5' prime end of the gene was amplified using the

primers OI_Folmer_F (5'-CAAATCACAAAGATATCGG-3') and OI_Folmer_R (5'-TAAACTTCAGGGTGTCCGAAGAACC-3'). Those primers are a modified version of the primer combination LCO1490 and HCO2198 (Folmer et al., 1994).

Polymerase chain reaction (PCR) was carried out on a thermocycler (Senso-Quest GmbH, Göttingen, Germany) in a total reaction volume of 20 µl with 1x HotStarTaq Master Mix (Qiagen AG, Hilden, Germany), 0.4 µM of each primer and 1 µl of DNA extract. The cycling conditions were the following: 15 min at 95°C, followed by 35 cycles of 40 sec at 95°C, 15 sec at 45°C, 5 sec at 60°C and 2 min at 72°C. After a final elongation step for 7 min at 72°C, the reaction was hold at 10°C. The PCR product was cleaned using the NucleoFast® 96 PCR system (Marcherey-Nagel GmbH, Düren, Germany) according to the manufacturer's protocol. Linear amplification was performed on a thermocycler (Senso-Quest) in a total reaction volume of 10 µl containing 1x BigDye® Terminator version 1.1 Ready Reaction Mix (Applied Biosystems, Carlsbad, CA, USA), 0.2 µM of either forward or reverse primer (see above) and 0.3 µl of purified PCR product. For the linear amplification, the following cycling conditions were applied: 15 min at 95°C, followed by 35 cycles of 15 sec at 95°C, 15 sec at 45°C and 2 min at 72°C. The linear amplification product was separated from unincorporated dye terminators using the DyeEx 96 kit according to the manufacturer's protocol (Qiagen AG) and the clean product was sequenced on a SeqStudio Genetic Analyzer (Applied Biosystems). Forward and reverse sequences of each specimen were assembled using the de-novo assembly function implemented in Geneious® version 10.0.9 (Kearse et al., 2012) and aligned with the multiple alignment function of the same software.

5.3.3. ddRAD library preparation and sequencing

Library preparation was performed using a protocol modified from Peterson et al. (2012) and Lam et al. (2018) (for the detailed protocol, see chapter 8.3.1.1). In brief, extracted DNA was double-digested with the restriction enzymes EcoRI and NlaIII. Indexing of individual specimens was ensured (i) by ligating uniquely indexed adapter to the digested DNA fragments; and (ii) by amplifying the ligation products with uniquely indexed, adapter-matching Illumina PCR primers. After pooling samples into a single library, an automated size selection

step for fragments with a length of approximately 430 bp was performed using BluePippin (Sage Science, Beverly, MA, USA). The profile of the final library was then analysed using Fragment Analyzer (Agilent Technologies Inc., Santa Clara, CA, USA) and sequenced in six consecutive runs on an Illumina MiSeq platform using v3 kits (Illumina Inc., San Diego, CA, USA).

5.3.4. ddRAD sequence processing and SNP calling

After combining raw ddRAD sequencing data from the consecutive Illumina MiSeq runs, sequencing reads were demultiplexed and de-novo assembled using ipyrad version 0.7.28 (https://ipyrad.readthedocs.io/, accessed 10 November 2018). For this analysis, the minimal depth for statistical base calling was set to five, the minimal depth for majority-rule base calling to four, the maximum number of SNPs per locus to 80 (for forward and reverse reads each), the maximum number of insertions and deletions (indels) per locus to 32 (for forward and reverse reads each), and the minimum percentage of individuals per population sharing a locus was set to 10. Default settings were used for all other parameters. After the demultiplexing step, samples were additionally filtered by applying a cut off for weak read coverage (<100,000) resulting in the removal of 33 specimens from the ddRAD data set. Finally, the software vcftools version 0.1.15 (Danecek et al., 2011) was employed to remove SNPs with missing data higher than 50% and minor allele counts less than two.

The Bayesian simulation method of Beaumont and Balding implemented in BAYESCAN version 2.1 (Foll and Gaggiotti, 2008) was used to filter the data set for polymorphic loci under selection (Beaumont and Balding, 2004). The analysis was performed with 100,000 iterations, a burn-in of 50,000, a prior odds value of 10 and the number of pilot runs was set to 20. To avoid bias in the subsequent population genetic analyses, two significant outlier loci (q-value < 0.05) were removed from the data set (Fig. S8.2). The software PGDSpider version 2.1.1.5 (Lischer and Excoffier, 2011) was used to transform the final SNP data set in formats used for different population genetic analyses.

5.3.5. Population genetics

5.3.5.1. CO1 data set

Mitochondrial genetic diversity estimates, such as the number of polymorphic sites, the number of haplotypes, haplotype diversity, nucleotide diversity and the average number of nucleotide differences were calculated using DnaSP version 5.0 (Librado and Rozas, 2009). In order to investigate relationships between haplotypes, TCS networks (Clement et al., 2000) were drawn in PopART (Leigh and Bryant, 2015). Closely related haplotypes were summarised as haplogroups and geographic frequency distributions of haplogroups were plotted on a map using the R-packages “maps” (R Core Team, 2016) and “plotrix” (Lemon, 2006).

5.3.5.2. ddRAD data set

In order to assess the population structure, pairwise differentiation among populations was estimated for populations with a minimum sample size of four, using the F_{ST}–pairwise genetic distance method implemented in Arlequin version 3.5 (Excoffier and Lischer, 2010) with a significance assessment based on 1,023 permutations. The same software was applied to determine hierarchical levels of genetic structure among groups of populations from different geographic regions conducting hierarchical analyses of molecular variance (AMOVAs) (Excoffier et al., 1992). Significance testing of AMOVA was performed using 1,023 permutations. To investigate the genetic diversity among individuals, a principal component analysis (PCA) was performed using the R-package “adegenet” (Jombart, 2008; Jombart and Ahmed, 2011). Results of this multivariate and model-free method were plotted using the R-package “ggplot2” (Wickham, 2016).

A Bayesian clustering analysis was performed to identify genetically homogenous groups of individuals in the data set using STRUCTURE version 2.3.4 (Pritchard et al., 2000). The analysis was run with 10 independent replicates each comprising 100,000 iterations and 100,000 burn-in steps. The number of clusters (*K*) tested ranged from 1-10 and the most informative *K* was determined using the delta *K* estimation method of Evanno et al. (2005), implemented in Structure Harvester (Earl, 2012). Results from the independent replicates were

subsequently combined using the “FullSearch” algorithm of CLUMPP version 1.2.2 (Earl, 2012) and visualised using DISTRUCT version 1.1 (Rosenberg, 2004). The R-package “plotrix” (Lemon, 2006) was used to display cluster assignment probabilities on maps.

PartitionFinder version 2.0 (Lanfear et al., 2016) was applied to select the best substitution model for individual-based phylogenetic analyses. The “greedy” algorithm was employed together with Akaike information criterion corrected (AICC) to select among the following substitution models: (i) General time reversible (GTR); (ii) GTR + gamma distribution (GTR+G); and (iii) GTR + G + proportion of invariable sites (GTR+G+I). Based on the results of the PartitionFinder analysis, a maximum likelihood analysis using the GTR+G model was performed with RAxML version 8.2.12 (Stamatakis, 2014). Tree support was assessed by performing nonparametric bootstrapping with 1,000 iterations. The RAxML output was visualised using FigTree version 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree, accessed: 30 December 2019).

Population-based evolutionary relationships were investigated using the software TreeMix version 1.12 (Pickrell and Pritchard, 2012). The R-package “dartR” (Gruber et al., 2018) was used to transform the data set into the TreeMix format. The subsequent phylogenetic analysis was performed using a sliding windows size of 100 kb and tree support was calculated by performing 1,000 bootstraps using a python wrapper (https://github.com/mgharvey/misc_python/blob/master/bin/TreeMix/treemix_tree_with_bootstraps.py, accessed: 30 January 2019) together with the “summtrees.py” function of the python library DendroPy version 4.4.0 (Sukumaran and Holder, 2010).

In order to assess for associations between population structuring and geographic distances on the European continent, an isolation by distance (IBD) analysis was performed. First, coordinates of sampling sites were transformed from the world geodetic system (WGS 84) format to the Universal Transverse Mercator (UTM) system using the R-package “rgdal” (Bivand et al., 2014). Second, a mantel test (Mantel, 1967) with 100,000 permutations was performed with the R-package “adegenet” to test significance of association between Edward’s genetic distances and Euclidean geographic distances among the populations.

5.4. Results

5.4.1. Mitochondrial and genomic data

For the mitochondrial CO1 gene fragment, a trimmed alignment of 573 bp from 274 specimens was retained. The combined output of six MiSeq runs sequencing the same ddRAD library comprised 122 million reads. After ipyrad filtering, de-novo assembly, applying a minimal coverage cut-off and BAYESCAN filtering, a total of 153 ddRAD loci, including 641 SNPs for 254 specimens was recovered.

5.4.2. Genetic diversity and population structure

5.4.2.1. CO1 data set

Analyses of the mitochondrial CO1 sequence fragment from 41 sampling sites revealed 17 different haplotypes (Fig. S8.3a-c). Genetic diversity measures were only calculated for *O. ishidae* populations with a minimum sample size of three specimens. The number of polymorphic sites within the CO1 sequence fragments from each sampling site ranged from zero to 13 (Table S8.5). The number of haplotypes within each population was found to range from one to five, the haplotype diversity from zero to 0.9 and the nucleotide diversity from zero to 0.01 (Table S8.5). The average number of nucleotide differences ranged from zero to six (Table S8.5).

TCS networks represent the genetic relatedness, the relative abundance of the identified haplotypes, as well as their geographic occurrence at the global, European and Swiss scales (Fig. S8.3a-c). The haplotypes occurring with the highest frequencies were Hap_1 (47.5%, N=130), Hap_3 (20.4%, N=56), and Hap_6 (12.4%, N=34) (Table S8.6a). Eight haplotypes were found to be singletons (Fig. S8.3a, Table S8.6a, b).

In order to assess the geographic diversity, related haplotypes were grouped together resulting in six haplogroups (Fig. 5.2e, Table S8.6a). The haplogroups with the highest frequencies were haplogroup A (61.0%, N=167), haplogroup F (21.2%, N=58), and haplogroup B (6.9%, N=19) (Table S8.6b). Haplogroup A was clearly separated from the other haplogroups by five mutations to the nearest node connecting this haplogroup with haplogroups B and C

(Fig. 5.2e).The distribution of haplogroups indicated a clear separation between the native population from Japan and the non-native populations from North America and Europe, while only weak signals of spatial structuring were found within the invasive populations (Fig. 5.2a-d). Haplogroup C occurred only in the Japanese population (frequency=100%, N=5), whereas haplogroup E was only found in the European populations (frequency=4.4%, N=11) (Fig. 5.2a-d). Haplogroup A occurred in the US populations (frequency=33.3%, N=12) and represented the major haplogroup in Europe with a frequency of 65.2% (N=163) (Fig. 5.2a-d). Haplogroup B was only present in the US populations USA-HE1 and USA-HE2, as well as in some populations originating from Switzerland (Fig. 5.2a, b, d). In contrast, haplogroups D and F were more widely distributed and found to be present in the populations from North America and several European countries (Fig. 5.2a-d). In Switzerland, haplogroup B was only found in the populations north of the Alps, whereas haplogroups D and E occurred solely in the populations south of the Alps (Fig. 5.2d). Interestingly, the frequency of haplogroup A in Swiss populations was strikingly high (27.3-100%, N=1-13), except for the populations CHE-RIE (10.0%, N=10) and CHE-TOR (0%, N=1) (Fig. 5.2d).

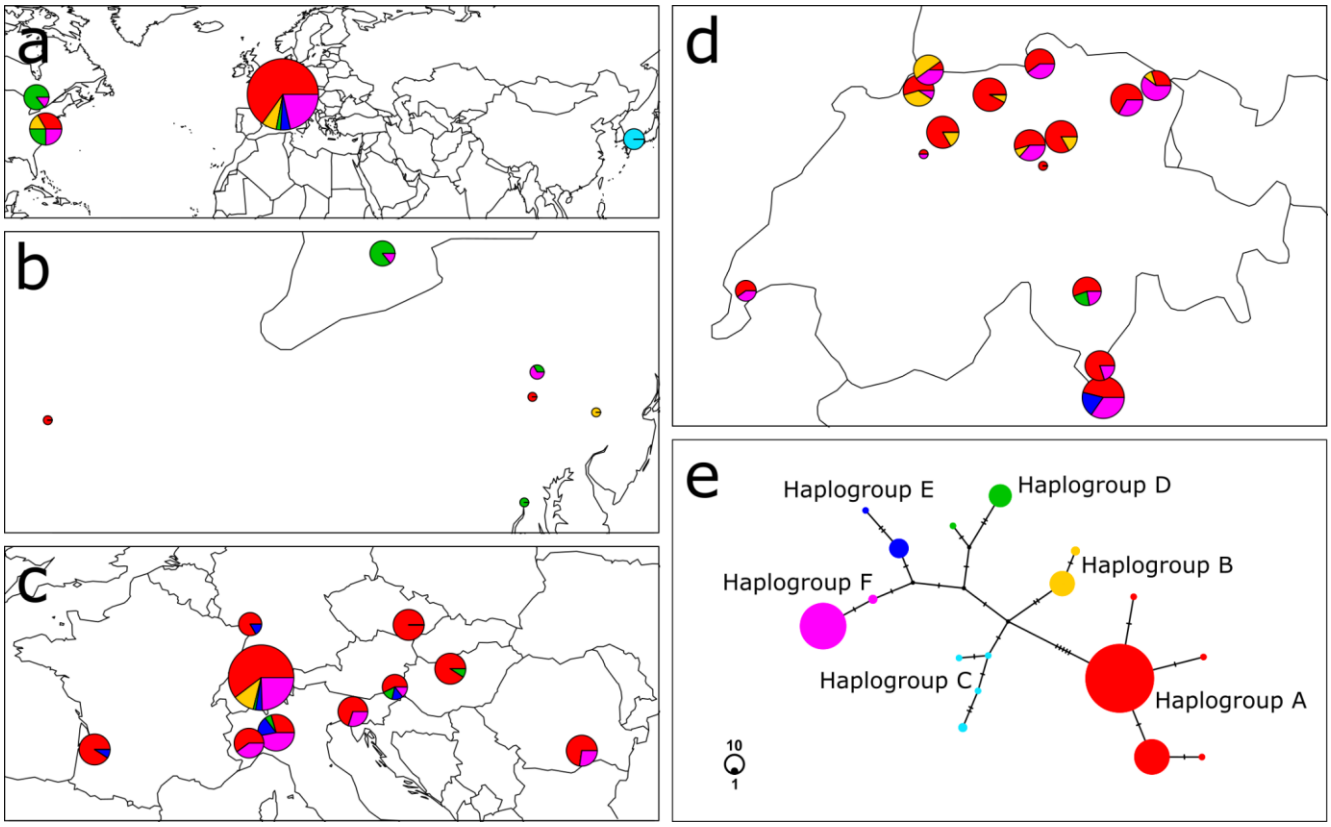

Figure 5.2. Spatial distribution of mitochondrial CO1 haplogroup frequencies at **a** global scale, **b** North American scale, **c** European scale and **d** Swiss scale. Colours correspond to haplogroups specified in the TCS haplogroup network. Frequencies were mapped for populations with a minimum sample size of two specimens. US populations USA-HE1 and USA-HE2, as well as Swiss populations CHE-ARZ, CHE-MEN and CHE-STA were combined due to their close geographic proximities. **e** TCS haplogroup network of a 573 bp mitochondrial CO1 gene fragment. Haplotypes are shown as pie charts representing proportional frequencies. Colours represent groups of related haplotypes.

5.4.2.2. ddRAD data set

Results of the AMOVA revealed limited partitioning of molecular variance among *O. ishidae* populations from different geographic regions. When comparing molecular variance between European, North American and Japanese populations, 19.6% ($p<0.001$) of the variation was found between groups, 3.6% ($p<0.001$) among populations within groups, and 76.7% ($p<0.001$) within populations (Table 5.2a). However, when comparing molecular variance of the Japanese population against the populations from Europe and North America, 53.5% ($p<0.05$) of variation was assigned to between-group variation, 2.2% ($p<0.001$) to among-populations-within-group variation, and 44.3% ($p<0.001$) to within-populations variation (Table

5.2b). In order to identify the potential origin of the European populations, partitioning of molecular variance was also tested between European and North American, as well as between European and Japanese populations. For European versus North American populations, the among-group variation was 1.4% (p=0.07), the among-populations-within-groups variation 4.5% (p<0.001) and the within-populations variation 94.2% (p<0.001) (Table 5.2c). However, testing molecular variance between European and Japanese populations revealed an among-groups variation of 54.1% (p<0.05), an among-populations-within-groups variation of 2.1% (p<0.001) and a within-population variation of 43.6% (p<0.001) (Table 5.2d).

Table 5.2. Analyses of molecular variance (AMOVAs) for the ddRAD data set. **a** molecular variance between European, North American and Japanese populations, **b** molecular variance of Japanese population versus populations from Europe and North America, **c** molecular variance between European and North American populations and **d** molecular variance between European and Japanese populations.

	Source of Variation	Df	Sum of squares	Variance components	Percentage of variation	Fixation index	P-value
a	Among groups	2	263.9	2.96	19.6%	0.233	<0.001
	Among populations within groups	36	676.7	0.55	3.6%	0.045	<0.001
	Within populations	469	5434.4	11.59	76.7%	0.196	<0.001
	Total	507	6375.1	15.10			
b	Among groups	1	236.9	14.01	53.5%	0.557	0.031
	Among populations within groups	37	703.7	0.57	2.2%	0.047	<0.001
	Within populations	469	5434.4	11.59	44.3%	0.535	<0.001
	Total	507	6375.1	26.17			
c	Among groups	1	27.0	0.17	1.4%	0.058	0.070
	Among populations within groups	36	676.7	0.55	4.5%	0.045	<0.001
	Within populations	462	5351.9	11.58	94.2%	0.014	<0.001
	Total	499	6055.6	12.30			
d	Among groups	1	235.5	13.97	54.1%	0.561	0.022
	Among populations within groups	30	581.8	0.54	2.1%	0.046	<0.001
	Within populations	442	5010.0	11.34	43.9%	0.521	<0.001
	Total	473	5827.3	25.85			

Df, degrees of freedom.

Calculations of pairwise fixation indices (F_{ST}) revealed an average value of 0.11 ± 0.01 (mean ± standard error) and 50% of all comparisons were statistically significant (Table S8.7). Comparatively high differentiation was found between the Japanese population JPN-UNN and populations from Europe and North America with F_{ST} values ranging from 0.62 to 0.75 (Table S8.7). Between European populations, the average F_{ST} value was 0.09 ± 0.004 (Table S8.7). The results of the PCA analysis agreed with the results from the AMOVA and F_{ST} calculations by clearly separating the Japanese from the of European and North American specimens with the first principal component (PC) explaining 9.6% of the total variance (Fig. S8.4a). PC2 and PC3 explained 3.7% and 2.7% of the total variance respectively, but identify neither additional genetic structure at the global nor at the European scale (Fig. S8.4a-c).

A cluster analysis using STRUCTURE indicated a *K*-value of three as the optimal model for the data (Fig. 5.3e). Congruent with the other analyses, there was a clear structure found between Japanese samples and samples from Europe and North America (Fig. 5.3a, f). While the samples from Japan were assigned solely to cluster I, European and North American specimens contained admixture of cluster I-III (Fig. 5.3a-d, f). A potential pattern of genetic structuring was identified for the populations CHE-AAR, CHE-AES, CHE-OBE, CHE-RIE and CHE-WAE originating from the north-eastern part of Switzerland, where probabilities of assignment to cluster III were strikingly higher (54.8-85.4%) compared to those of the other European populations (Fig. 5.3c-d, f). Furthermore, three of those populations, CHE-AAR, CHE-AES and CHE-OBE, harboured the highest probabilities (6.7-12.8%) of assignment to cluster I within Europe (Fig. 5.3c-d, f).

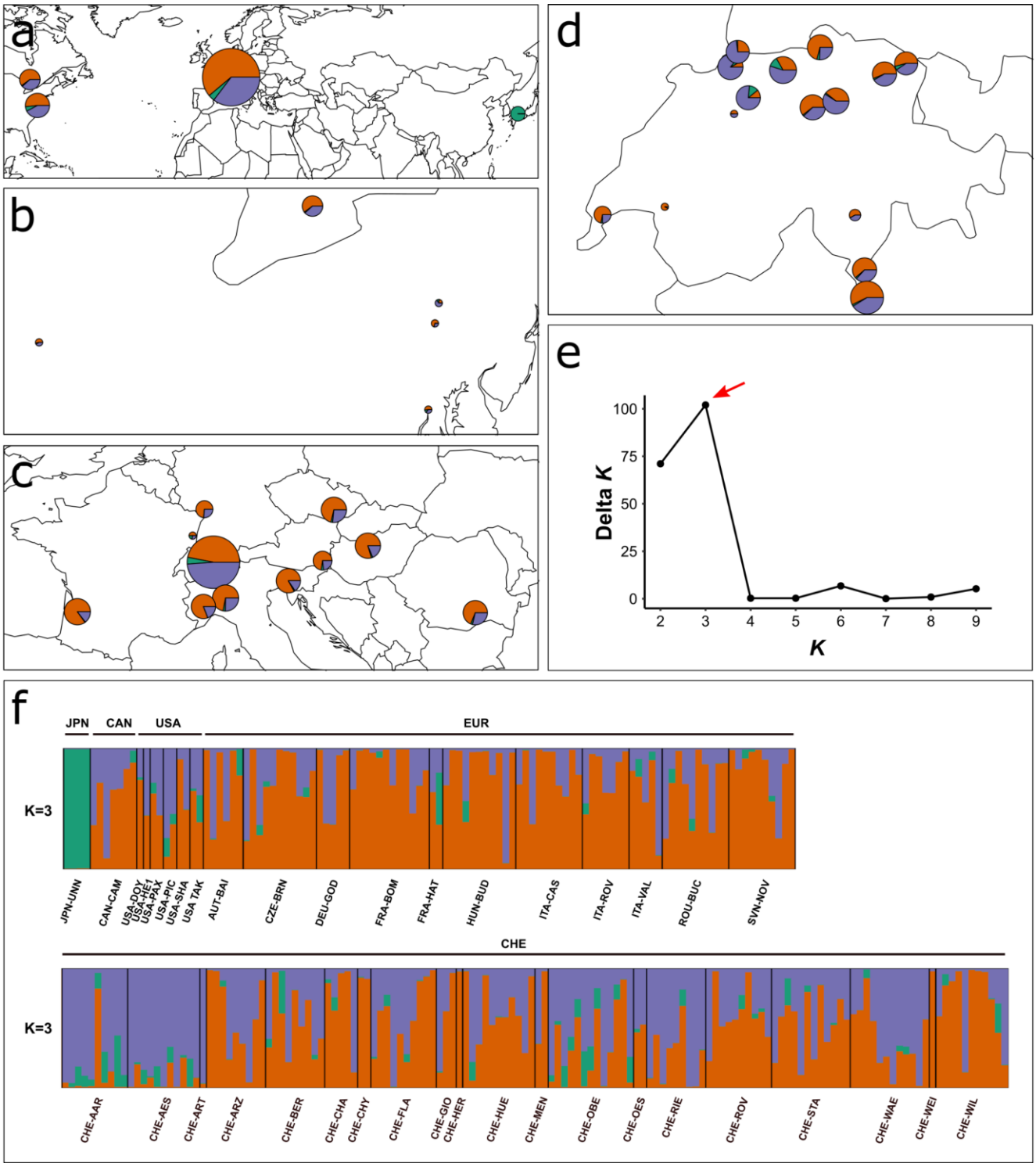

Figure 5.3. Distribution of clusters identified by STRUCTURE based on the ddRAD data set visualised at **a** global scale, **b** North American scale, **c** European scale and **d** Swiss scale. Probability plots are drawn as circles, which are proportional to the sample size. **e** STRUCTURE output for individual specimens. Each bar represents the composition of individual genotypes. Colours reflect the probability of the assignment to a certain cluster: cluster I, green; cluster II, orange; cluster III, purple. **f** STRUCTURE assessment of the optimal number of population clusters (*K*). The red arrow indicates the *K*-value explaining the largest

part of the present population structure. Delta *K* is calculated according to the method of Evanno *et al.* (2005).

The maximum likelihood based phylogenetic analysis performed by RAXML confirmed a clear separation (bootstrap support value=96) of the Japanese specimens from the North American and European samples (Fig. S8.5). Similar results were obtained when constructing a population-based maximum likelihood tree using TreeMix (Fig. 5.4). In this analysis, the Japanese population was separated from European and North American populations with a bootstrap support value of 100 (Fig. 5.4).

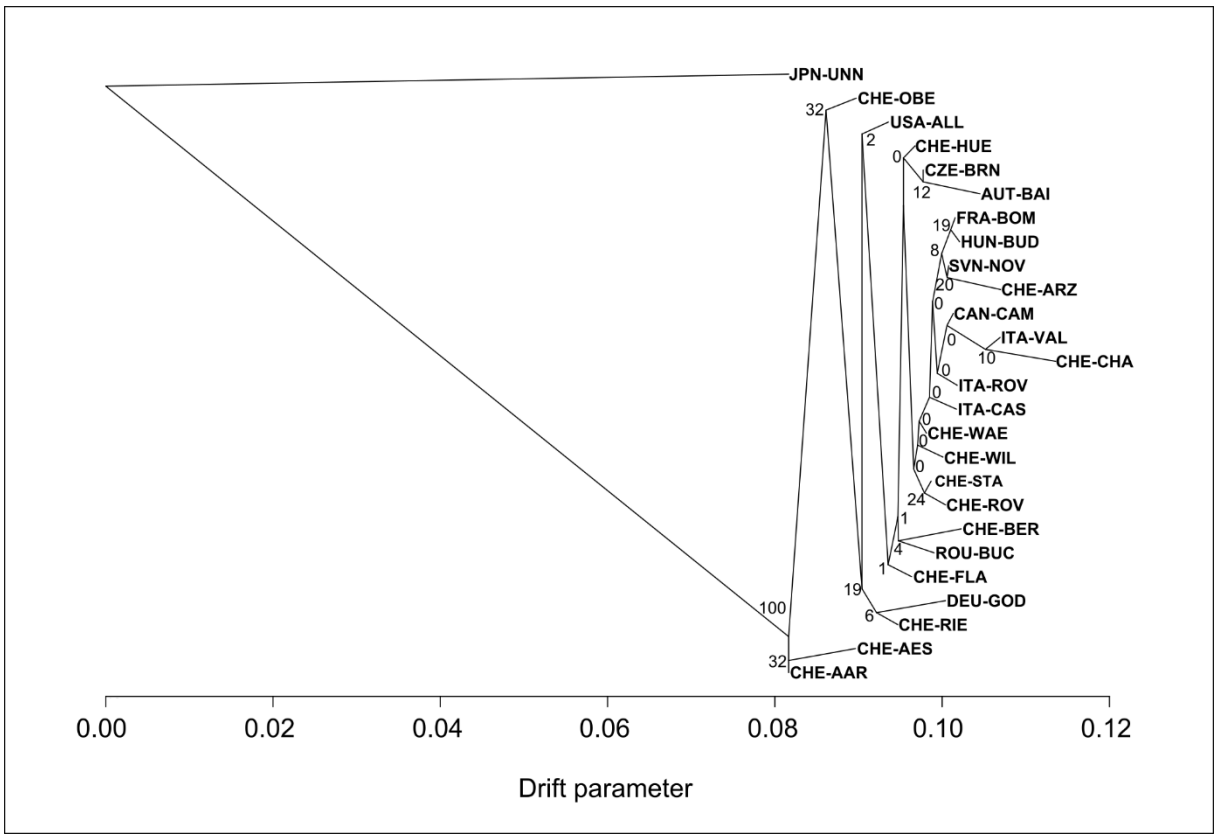

Figure 5.4. Results of a TreeMix phylogenetic analysis based on the ddRAD data set. Maximum likelihood tree of population relationships was generated for populations with sample size N>3. Due to samples sizes N< 3, US populations were summarised as USA-ALL. Branch lengths are proportional to the drift of each population. Population JPN-UNN was used as the outgroup to root the tree.

Indicated by the low bootstrap values, both approaches were not able to reliably resolve the phylogenetic relationships between individuals and populations originating from North America and Europe (Fig. 5.4, Fig. S8.5).

Finally, while investigating for signals of IBD on the European continent using the ddRAD data set, no significant correlation between genetic and geographic distances was detected (Mantel R=-0.62, p-value=0.96) (Fig. S8.6).

5.5. Discussion

Understanding population genetics of invasive pest species is crucial for the elaboration of appropriate quarantine, management, and biocontrol programmes (Garnas et al., 2016). Here, we assessed the population genetic structure of *O. ishidae* using both a mitochondrial CO1 marker and SNP data generated by ddRAD sequencing. Analysing populations from native (Asia) and invaded ranges (Europe and North America), our study is the first describing the worldwide genetic structure and relatedness of this potential pest species. We placed particular emphasis on European populations, where *O. ishidae* has been recently introduced.

Our ddRAD data demonstrated a clear genetic separation between invasive North American and European populations from the one from Japan situated in the native range of the species. These results were confirmed by the mitochondrial CO1 data. Drawing TCS haplogroup networks, Japanese specimens were assigned to only one haplogroup, which was not found in invasive populations from Europe and North America. The absence of closely shared ancestry between native and non-native populations shown by CO1 and ddRAD, however, might be an artefact of the limited sampling, including only one population from Asia. Hence, the results should be treated with caution when drawing region-wide conclusions.

Studying spatial population structuring between European and North American populations revealed a similar pattern. All mitochondrial haplogroups present in Europe were also found in North American, except of haplogroup B, which was unique to Switzerland (Fig. 5.2). The STRUCTURE analysis performed based on the ddRAD data identified for both regions a similar genetic pattern with admixture between clusters II and III together with only few signals from cluster I (Fig 5.3). However, whereas for invasive populations in European countries the

probability of assignment was always elevated for cluster II, five populations (CHE-AAR, CHE-AES, CHE-OBE, CHE-RIE, CHE-OBE and CHE-AES) located in the northern part of Switzerland showed an opposite pattern. Furthermore, two of those populations (CHE-AAR and CHE-OBE) showed a strikingly high admixture content of cluster I, compared to the other European populations. The observed deviation from the general European structure was also underlined by the distribution of the mitochondrial haplogroup B which, in Europe, was solely found in this particular area (Fig. 5.2). These findings suggest that populations from the northern part of Switzerland might have originated from another source than the remaining populations in Europe.

Considering the strong genetic similarity observed for European and Nord American populations and their substantial genetic separation from the native population from Japan, an invasion scenario in which North American populations served as source for the European invasion seems highly likely. The mechanism, by which a previously invasive population serves as source for new invasions has been termed a “bridgehead” effect and has recently been linked to large-scale invasions of other invasive insect pests, such as the Asian long-horned beetle (*Anoplophora glabripennis)* (Javal et al., 2019), the obscure mealybug (*Pseudococcus viburni*) (Correa et al., 2019) and the Western conifer seed bug (*Leptoglossus occidentalis*) (Lesieur et al., 2019). A bridgehead effect was first reported while studying the invasion of the harlequin ladybird beetle (*Harmonia axyridis*) (Lombaert et al., 2010) and the effect is increasingly considered as a driver for the accelerated spread of invasive species (Garnas et al., 2016). As suggested for other species, a bridgehead effect in *O. ishidae* could have been favoured by an evolutionary shift in the non-native North American populations increasing the invasive potential, by trading networks, or both (Garnas et al., 2016).

Our findings revealed that North American populations share mainly one or two closely related mitochondrial haplogroups per population. In contrast, the presence of genetically distant haplogroups (e.g. haplogroups A and F), together with the homogenous pattern found in the STRUCTURE and phylogenetic analyses, suggest that the structural pattern seen in Europe

originates from recurrent introduction events with gene flow between the introduced populations.

Similar findings with limited population structure have recently been reported from invasive populations of the spotted-wing fruit fly, *Drosophila suzukii* (Carvajal 2010; Adrion et al. 2014; Tait et al. 2017). As for *O. ishidae*, the extra-range dispersal of *D. suzukii* is thought to be anthropogenic as a result of the global trade (Cini et al. 2014; Tait et al. 2017) and strongly favoured by the fly's wide range of host plants (Walsh et al. 2011; Rota-Stabelli et al. 2013). Multiple introductions with gene flow between introduced populations such as supposed for *D. suzukii* and *O. ishidae* can maintain or even increase the genetic diversity in the invaded zone and have thereby the potential to counteract against genetic bottlenecks (Dlugosch and Parker 2008; Javal et al. 2019).

Furthermore, an increase in genetic diversity due to multiple introductions can strengthen the ability of an invasive species to adapt to selection pressures in the new environment (Dlugosch and Parker, 2008; Javal et al., 2019).

In the case of *O. ishidae*, dispersal is thought to be particularly driven by the trade of living plants, such as ornamentals and fruit trees (Mifsud et al., 2010). Italy, the country where *O. ishidae* was reported for the first time on the European continent, is a key import country for woody perennial plants within the EU (Eschen et al., 2015). First introduction events of *O. ishidae* from North America therefore might have occurred via direct plant imports. Recently, the complexity of the intra-European trade network of ornamentals was documented using the example of *Acer* spp. plants (Eschen et al., 2015). Only in 2009, Dutch tree nurseries exported plants with partially foreign origin to 26 other European countries. In this perspective, it seems likely that the homogenous genetic structure found among European populations of *O. ishidae* is governed by frequent intra-European trade of ornamentals. Genetic exchange between spatially separated populations by trade might be additionally favoured by the broad host plant range of this species.

Multiple introduction events and frequent exchange between populations as indicated by our analyses pose considerable challenges for potential pest management strategies of *O. ishidae*

within Europe. Same as for other highly invasive insect pests such as *D. suzukii*, elimination and containment measures would probably only have limited success due to the potential recurrence of the invasion events, as well as the wide spread of this species (Cini et al. 2012; Cini et al. 2014)

The results of our study highlight the problem of the non-intended movement of pest species by the transport of plant material, which is especially pronounced within the EU as there are no phytosanitary inspections of movements between the member countries (Eschen et al., 2015). The relatively short colonisation time of *O. ishidae* across Europe (<20 years) is in line with observations of other invasive insect species (e.g. *L. occidentalis*) that recently invaded the continent (Roques et al., 2016; Lesieur et al., 2019). The observed trend of increasing dispersal speed is thought to be strongly supported by the lack of EU internal controls. (Roques et al., 2016; Lesieur et al., 2019). Experience and lessons from previous invasions call for more stringent phytosanitary measures to avoid introductions and delay the spread of insect pests on the European continent.

Further efforts in studying the invasion history of *O. ishidae* should include a denser, worldwide sampling with larger sample sizes per population, particularly in the ancestral range (Asia) and regions where primary invasions occurred (North America), to unambiguously confirm the potential source(s) of non-native populations. In addition, repeated sampling in areas where invasion occurred recently is warranted to confirm the presence of gene flow between non-native populations and to test if recurrent invasions are in indeed at play. Our study revealed the need for a very high number of SNPs to elucidate the pathway of *O. ishidae* in recently invaded areas. Future studies should include large numbers of loci or even full genomes to better understand the invasion route(s) at fine scale. Finally, combining denser sampling and increased genetic resolution, different invasion scenarios might be tested using approximate Bayesian computation, a method that was recently used to successfully decipher pathways of other invasive insect pests (Correa et al., 2019; Javal et al., 2019; Lesieur et al., 2019).

5.6. Acknowledgements

The authors thank Masami Hayashi, Christian Linder, Patrik Kehrli, Thierry Castellazzi, Alberto Alma, Federico Lessio, Andrea Taddei, Christopher H. Dietrich, James Zahniser, Lawrence Barringer, Joel Kits, Constantina Chireceanu, Gabrijel Seljak, Gudrun Strauss, Helga Reisenzein, Igor Malenovsky, Michael Maixner, Sandor Koczor, Sylvie Malembic-Maher, the Equipe Mollicutes of the Institut National de la Recherche Agronomique (Bordeaux, France) and the Research Group Virology, Bacteriology and Phytoplasmology of Agroscope (Changins, Switzerland) for providing us samples of *O. ishidae*. The authors are also grateful to Valeria Trivellone for assistance with identification of sampling sites in Switzerland. A special thank is dedicated to Joël F. Pothier for his help in performing Fragment Analyzer analyses and BluePippin size selection while preparing the ddRAD library.

5.7. Author contributions

SB, MG, MJ, PM, JU and JEF, conception of project; SB, MG, DF and MJ, analysis and interpretation of data. SB, manuscript writing, MG, DF, MJ, PM, JU and JEF, critical revision of manuscript; SB, MG, DF, MJ, PM, JU and JEF, final approval of manuscript prior to submission.

5.8. Conflict of interest

The authors declare that they have no conflict of interest.

5.9. Data accessibility

Mitochondrial CO1 sequences used in this study were uploaded to the NCBI GenBank data base (Benson et al., 2008) (accession numbers MK778089-MK778362) and the ddRAD data set will be deposited in the Dryad data digital repository (https://datadryad.org/) once a manuscript number is available.

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6. Discussion and perspectives

6.1. On-site diagnostics of invasive insect pests

Phytosanitary import controls at POEs for plant products are key to prevent introduction and spread of invasive insect pests unintentionally translocated by global trade (Mumford et al., 2016; Saccaggi et al., 2016). In case suspicious insect specimens are intercepted, rapid identification is needed to ensure a timely import process, as well as to implement quarantine or elimination measures with minimal delay (Floyd et al., 2010; Mumford et al., 2016). However, visual differentiation between regulated and non-regulated species is exceedingly difficult and generally requires expert knowledge. Furthermore, if identification is outsourced to external specialist institutions, delays of up to several days are to be expected. Hence, there is a pressing need for novel on-site identification tools (Floyd et al., 2010; Saccaggi et al., 2016; Blaser et al., 2018b). The first part of this PhD was thus aimed at developing and validating rapid molecular identification assays for invasive insect pests that can be applied directly at POEs.

6.1.1. LAMP – a reliable and rapid tool for on-site identification

Already successfully deployed for detection of other plant pathogens (Hodgetts et al., 2015; Kogovšek et al., 2015), we were able to demonstrate that LAMP represents also a suitable candidate method for the on-site identification of insect pests. In doing so, we developed LAMP assays for the identification of the most commonly intercepted insect pests at Swiss POEs; namely, *B. tabaci*, *T. palmi* and several fruit fly species of the genera *Bactrocera* and *Zeugodacus*. After initial development, the assays were thoroughly validated under laboratory and on-site conditions at a Swiss POE. While analysing a total of 319 insect specimens, the overall diagnostic test efficiency was 98%. Rare false-negative results (2%) were shown to originate from previously undescribed pest biotypes not included in the initial primer design, as well as from mistakes during LAMP assay preparation. Similar to other LAMP diagnostics studies, the test specificity of our assays was strikingly high (overall diagnostic test specificity = 100%) (Mori and Notomi, 2009; Bühlmann et al., 2013; Hodgetts et al., 2015; Kogovšek et

al., 2015). Compared to conventional PCR-based identification methods using only one primer pair, the test specificity of LAMP seems to considerably benefit from the use of multiple primer pairs (2-3) reducing likelihood of cross-reactivity with non-target organisms (Mori and Notomi, 2009; Hodgetts et al., 2015).

Invasive insect pests such as *B. tabaci* or fruit flies of the genus *Bactrocera* successfully established in many regions across the globe (Stephens et al., 2007; Hadjistylli et al., 2016; Qin et al., 2018). Against this vastly variable genetic background, identification assays need to be sensitive for a wide range of invasive pest biotypes intercepted on plant imports from many different countries. In order to tackle the problems regarding high genetic diversity within individual species or species complexes, our identification assays were designed using degeneracy in primers. In addition, where needed to ensure diagnostic accuracy, several primer sets were combined in one assay. In case of *B. tabaci*, we were able to show that it is possible to combine up to three different primer sets within one LAMP assay without finding any negative impact on test performance. After first evaluating accuracy of primers by *in-silico* specificity analyses based on publicly available DNA sequences, laboratory validations revealed that the assays were able to successfully handle the genetic diversity of insect pests intercepted on internationally traded goods.

A major proportion of insects intercepted during phytosanitary import controls are immature, in overwintering forms, or damaged (Saccaggi et al., 2016). To be applicable, molecular on-site assays therefore need to have a low analytical sensitivity (detection limit) to ensure identification of small quantities of insect tissue. Using the example of *B. tabaci*, we have demonstrated that our LAMP assay is able to detect sample DNA diluted to 100 fg per µl. This corresponds to a 1:1,000 dilution of the total DNA extracted from *B. tabaci* larvae with a diameter less than 1 mm.

Rapid pest identification is crucial for prompt decision-making (Floyd et al., 2010; Saccaggi et al., 2016). We were able to show that all developed identification assays can be performed on-site within 1 h from tissue sampling to test result. During development and validation, LAMP reactions were conducted using GspSSD LF DNA polymerase. Very recently, a modified

version of this enzyme (GspSSD2.0 LF DNA) was released on the market (OptiGene, 2018). Announced as world's fastest LAMP polymerase (OptiGene, 2018), the enzyme was indeed found to excel with very short reaction times (Best et al., 2018). First trials using the novel polymerase for the identification of *T. palmi* were promising and suggest that the modified protocol has the potential to reduce reaction times of our insect pest identification assays by half (unpublished data). However, rigorous validation is required to assess test specificity and sensitivity, before the modified assays can be implemented in routine diagnostic processes.

6.1.2. Successful integration into plant health control system

In a recent review discussing new diagnostic technologies in plant biosecurity, Mumford et al. (2016) highlighted the importance of evaluating newly introduced methods in terms of how they are deployed rather than how they perform in stand-alone comparisons with existing methods. If directly compared to DNA barcoding regarding diagnostic accuracy, the developed LAMP assays for the on-site identification of insect pests would clearly perform less well than the sequencing-based method due to the rare occurrence of false-negative results. However, if the LAMP assays are correctly integrated into a multi-stage identification system, a diagnostic test sensitivity of 100 % can be ensured, while exploiting LAMP features such as speed, specificity, simplicity and cost-effectiveness.

To achieve this, we designed a two-stage identification system by which LAMP negatively tested specimens are cross-validated at an external institution by DNA barcoding, whilst LAMP positive results can directly be used for decision-making. The possibility of taking fast action when harmful insect pest are intercepted, provides important benefits. First, accelerated decision-making reduces the likelihood of pest escaping from POEs and allows the adoption of early management measures (Floyd et al., 2010). Second, timely diagnostic results accelerate the import process by reducing the waiting time of perishable import products (Hodgetts et al., 2016; Mumford et al., 2016). Third, our findings reveal the power of on-site identification in reducing costs for external analyses. Assessing the results of the on-site validation, we could demonstrate that plant health inspectors applied the assays mainly as a confirmatory test after careful visual species identification. Thus, 80% of tested specimens

were LAMP-positive. Hence, only every fifth specimen had to be sent to the external laboratory under the conditions of the two-stage identification process described above. Finally, beside direct prevention of introduction events due to false negatives, the sequencing step included in the cross-validation of negative LAMP results also allows to update the LAMP primer set in case novel insect pest biotypes are encountered that led to a false-negative test result.

6.1.3. Translational aspects – from laboratory to POE

Over the last decades, a vast range of scientific papers reporting development of novel diagnostic approaches for the identification of plant pathogens and pests were published (Mumford et al., 2016). Many of those approaches were successfully implemented for laboratory use, but only few of them are nowadays deployed for on-site diagnostics (Mumford et al., 2016). When assessing factors crucial for the successful uptake of novel on-site identification technologies into regular control systems, the two main drivers were found to be: (i) an existing need for a clearly defined application; and (ii) the early engagement of the end user in the development process (Mumford et al., 2016).

Considering those observations, our LAMP assays were specifically designed as a response to the existing need for rapid on-site identification of *B. tabaci*, *T. palmi* and several harmful fruit fly species which account for approximately 70% of the insect pest interceptions at Swiss POEs (Blaser et al., 2018b). The early involvement of plant health inspectors in designing the protocol was extremely helpful to tailoring the assays for optimal integration into the import control process. Frequent exchange with the end users furthermore allowed us to understand their fears associated with the handling of molecular assays and helped us figuring out how to best address them. The use of a stain for the visualisation of small amounts of liquid, as well as the development of a Microsoft Excel-based application to validate the LAMP output represent examples of modifications evolved from such interdisciplinary exchange.

After the technology transfer from the laboratory to the POE, plant health inspectors were individually trained to perform LAMP analyses. This knowledge translation step was recently further improved by publishing a step-by-step video protocol for the on-site identification of *B. tabaci* (Blaser et al., 2018a).

Finally, after successful technology and knowledge transfer, long-term support is key to ensure the persistence of diagnostic assays (Mumford et al., 2016). By transferring the protocols for the production of ready-to-use LAMP kits in the hands of a commercial entity (OptiGene Ltd), we were able to lay the foundation for the long-term availability of the developed assays.

6.1.4. Video publications – a new format for knowledge and information transfer

Peer-reviewed video journals are more and more considered as the next generation of scientific publishing (Pasquali, 2006, 2007; Stern, 2013). In the recent past, the use of scientific video publications was found to be particularly important in experimental studies to ensure reproducibility of protocols, but also for short communications to explain specific research findings to a wider audience (Krieger et al., 2012; Winkler et al., 2012; Xu et al., 2018).

In this PhD thesis, we exploited the format of peer-reviewed videos with two different intentions: (i) to publish a video protocol to support and improve reproducibility in the use of the developed LAMP assays; and (ii) to create a video sensitising stakeholders, policymakers and community about the dimension of global movement of fruit fly pests by international trade. The second video approach was furthermore used as a platform promoting the adoption of LAMP assays as prevention measure against insect pest introductions. In accordance with existing literature, we experienced that video articles allow including more information (e.g. colour, duration and motion) than conventional scientific articles, which is especially favourable for the publication of technical protocols (Pasquali, 2007). Although more time-consuming in the production phase, we additionally demonstrated that short communication videos have the potential to share scientific knowledge beyond the scientific community.

6.1.5. Perspectives of on-site identification

A drawback of LAMP is that this method can only identify a predetermined range of insect pest biotypes (Hodgetts et al., 2016). As for all DNA amplification-based methods, a profound knowledge of the genetic diversity within the target species is therefore crucial to prevent false-negative results (Blaser et al., 2018b). However, because of the limited availability of pest sequence information and considering that plant import products originate from a diverse and

constantly changing range of geographic regions, rare false-negative LAMP results due to primer mismatches have to be expected (Blaser et al., 2018b). In order to ensure diagnostic reliability during on-site testing, the developed LAMP assays were therefore integrated into the two-stage identification system described above.

The issue of false-negative results due to the emergence of previously unknown insect pest biotypes could be avoided by the development and implementation of on-site sequencing-based methods. Beside improved diagnostic reliability, such methods would remove the need for ongoing development and evaluation of molecular stand-alone tests targeting single or small groups of species (Hodgetts et al., 2016; Blaser et al., 2018b). Finally, next-generation based sequencing approaches may be designed without the need for specific primers, thus enabling its use on all pest species, including new, formerly not encountered ones.

A potential candidate platform for on-site sequencing applications is represented by the pocket-sized MinION sequencing device from Oxford Nanopore Technologies (Branton et al., 2008; Hoenen et al., 2016). Recently, this platform was successfully applied as on-site diagnostic tool during an Ebola virus outbreak in West Africa (Hoenen et al., 2016). Although constantly evolving, current Oxford Nanopore sequencing protocols still lack simplicity to be deployed by plant health inspectors with only limited laboratory training. First trials using the MinION sequencing platform for the identification of insect pests revealed the risk of carry-over contaminations in the current library preparation protocol (unpublished data). However, with VolTRAX, Oxford Nanopore Technologies recently released a fully automated library preparation system, which may bring the technology a further step forward towards simplified on-site sequencing (Oxford Nanopore Technologies, 2019). Nevertheless, in view of its simplicity and speed, LAMP will probably continue to be the rapid, reliable and robust on-site identification tool of choice in the foreseeable future.

6.2. Invasion genetics of *O. ishidae*

Understanding pathways followed by invasive species is crucial for their successful management and supports the prevention of new invasion events (Puth and Post, 2005; Wilson et al., 2009; Lombaert et al., 2010). In the second part of this PhD thesis, we applied molecular

methods to investigate the genetic invasion history of *O. ishidae*, a potential leafhopper pest that was unintentionally introduced from Southeast Asia to North America and only recently colonised Europe (Lessio et al., 2016).

6.2.1. Invasion history of *O. ishidae* in Europe

Using different population genetic approaches based on a mitochondrial CO1 marker and SNP data generated by ddRAD, we were able to demonstrate that invasive populations from Europe and North America are clearly separated from a native population of Japan. Overall, only little spatial structuring was found between and within European and North American populations. The strong genetic similarity found in populations from these geographically separated regions provides evidence that primary invasive populations from North America served as source for the European introduction. The observed pattern of secondary invasion is described as bridgehead effect and was recently reported for several other insect pest invasions (Correa et al., 2019; Javal et al., 2019; Lesieur et al., 2019). Secondary invasions may be favoured by evolutionary shifts in bridgehead populations increasing their invasiveness and are more and more considered to play a major role in the observed accelerated dispersal of invasive species. In-depth analyses of the mitochondrial haplotype distribution in Europe and North America furthermore revealed stronger spatial structuring within North American populations. Whereas primary invasive populations were found to be mainly composed of closely related haplotypes, the invasive populations from Europe were more diverse and characterised by co-occurrence of genetically distant haplotypes. These findings suggest that the European colonisation of *O. ishidae* originates from multiple introduction events and indicate furthermore the presence of strong gene flow among European populations. Multiple introductions could have enhanced the leafhoppers ability to adapt to new environments due to increased genetic diversity and may have counteracted against adverse genetic founder effects (e.g. genetic bottlenecks). Therefore, recurrent introduction events could have substantially contributed to the exceptionally fast colonisation observed for *O. ishidae* in Europe (< 20 years).

6.2.2. Consequences for management

It was hypothesised that the dispersal of *O. ishidae* is mainly vectorised by international trade of living plants (Felt and Bromley, 1941; Mifsud et al., 2010). Thereby, leafhopper eggs laid on tissues of translocated plants (e.g. ornamentals) are thought to serve as propagules for further spread (Felt and Bromley, 1941; Mifsud et al., 2010). It is known that intra-European spread of invasive insects via plant trade is strongly favoured by the absence of phytosanitary import controls between EU member countries (Roques et al., 2016). Our findings go along with those hypotheses and suggest that the complex genetic structure with gene flow between geographically distant *O. ishidae* populations indeed originates from frequent trade of living plants. From a pest management point of view, our results imply that elimination and control measures within Europe would most probably be useless due to the current wide distribution and the recurrence of introduction events. However, because the pest potential of *O. ishidae* is thought to mainly rely on its ability to transmit grapevine Flavescence dorée phytoplasma, quarantine measures such as phytosanitary trade controls could be adopted to reduce the risk of further spread of this economically harmful disease from infested areas.

6.2.3. Perspectives

To further deepen the understanding of the invasion history of *O. ishidae* on a global scale, as well as to reliably confirm source populations of European introductions, future studies should increase the sampling towards including more sites located in the ancestral range (Asia) and in regions where primary introductions occurred (North America). In addition, upcoming studies should use an enlarged SNP dataset in order to increase the resolution of the population genetic analyses. Due to the short evolutionary time since introduction and strong genetic exchange between populations, very high SNP numbers or even whole genomes will be needed to resolve the population structure of European populations at fine scale. Finally, forthcoming investigations could study associations between *O. ishidae* genotypes and different levels of vectorial phytoplasma transmission capacities – work that could directly impact future pest management strategies.

6.3. Thesis contribution to innovation, validation and application

Research and development activities at the Swiss TPH operate along the innovation chain, which is defined by three domains: (i) innovation; (ii) validation; and (iii) application (Swiss TPH, 2014). In this context, innovation includes basic research, development of novel tools, and the elaboration of novel concepts for epidemiology and public health. Once developed, suitability of novel tools and concepts is thoroughly validated under “field” conditions. Finally, successfully validated tools and concepts may be integrated into public health systems.

The present PhD thesis contributed to the first two domains of the chain, the specific contributions of the individual chapters are summarised in Table 6.1.

Table 6.1. Contribution of the PhD thesis to the Swiss TPH value chain of “innovation, validation and application”.

Chapter	Innovation	Validation	Application
Chapter 2	The LAMP-based identification assays for several regulated pest species were specifically developed for on-site application at POEs for plant import products.	Validation of the developed LAMP assays was performed in a first step under laboratory conditions at the Agroscope laboratory and in a second step under on-site conditions at the Swiss POE Zurich Airport.	
Chapter 3	To facilitate the knowledge transfer step to the end-user as well as to support their training, a scientific video publication visualising the developed LAMP protocol for *B. tabaci* was generated.		
Chapter 4	With the intention to sensitise policy makers, plant health workers, stakeholders involved in the international trade of fruits and consumers, the concept of a scientific video publication visualising the issue of the unintended spread of harmful fruit fly pests by global trade was developed. The publication was furthermore designed as a communication tool to promote the use of the developed LAMP identification assays as a prevention measure.		
Chapter 5	To improve the general understanding of insect pest invasions, to define pest management strategies, as well as to prevent future pest introduction events, we assessed the population genomic structure of *O. ishidae*, a leafhopper pest native to Asia that invaded North America and Europe.		

6.4. General conclusion

Rapid diagnostics at POEs for import products have the potential to reduce the ongoing global spread of invasive insect pests by international trade (Saccaggi et al., 2016). A promising candidate method for such on-site testing is represented by LAMP, a very robust and easy-to-perform molecular identification tool based on isothermal DNA amplification (Blaser et al., 2018b). In the first part of this PhD thesis, we evaluated the suitability of this method by designing LAMP assays for several regulated and harmful invasive insect pests. Validations under laboratory and on-site conditions at a Swiss POE for plant import products demonstrated the reliability of this method if correctly integrated into the control process. Manageable by plant health inspectors with little laboratory experiences within only 1 h, the developed assays were shown to represent powerful tools for the on-site identification of invasive insect pests. Since completing the validations, the novel LAMP assays are routinely used in the Swiss import control process. Successfully validated for several regulated insects pests, the method may be adapted for the on-site identification of other plant pests or human and veterinary pathogens.

The second part of this PhD thesis aimed at investigating the invasion history of *O. ishidae*, a leafhopper species suspected to vectorise the grapevine Flavescence dorée disease. Originating from East Asia, *O. ishidae* was introduced in the first half of the 20th century to North America and only recently invaded Europe. Using molecular genetic analyses, we showed that European populations of *O. ishidae* most probably originated from North American introduction events. The genetic pattern of European populations suggests the occurrence of multiple trans-Atlantic introductions and indicates the presence of frequent and recurrent intra-European gene flow. Overall, the observed population structure is thought to be strongly shaped by frequent movement of *O. ishidae* via international trade of living plants. To reduce the risk of future insect pest invasions via trade, more stringent phytosanitary inspections programmes of living plant imports at POEs are therefore needed.

6.5. Open research needs

In view of the experiences and findings from the present PhD thesis, the following research needs arise:

(i) To circumvent the problem of rare false-negative LAMP results due to undescribed pest biotypes, as well as to develop an identification system covering the whole range of pest species in one method, future diagnostics projects should focus on the development of on-site sequencing methods that can be deployed directly at POEs. Besides developing robust and rapid sequencing protocols for on-site application, efforts are also needed to design an automated bioinformatics pipeline analysing the generated sequencing data. Furthermore, to ensure precise pest identification, a reliable database of reference sequences needs to be established.

(ii) To confirm our result of the population genetic study of *O. ishidae*, as well as to resolve the pathways of its global invasion at a finer scale, future studies should be performed including (i) more sample sites (especially in the native range); and (ii) an increased SNP dataset.

(iii) To improve the understanding of the phytosanitary risk posed by *O. ishidae*, further transmission experiments should be performed to confirm the vector function of the leafhopper for specific Flavescence dorée-causing phytoplasma strains, as well as to assess the vector capacities of different *O. ishidae* genotypes.

(iv) Our findings suggest frequent intra-European exchange between geographically separated *O. ishdiae* populations via trade of living plants. Specific surveys of plant import products could be performed to confirm our findings and to identify the translocation pathways of *O. ishidae* at the species level of the traded plants. Such work has the potential to improve future phytosanitary inspection programmes.

7. References (chapters 1 and 6)

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8. Appendices

8.1. Supporting information chapter 2

8.1.1. Supplementary figures

	Anatrichus sp. (Sri Lanka, no. 11538)	*Zeugodacus cucurbitae* (Sri Lanka, no. 11514)	*Zeugodacus cucurbitae* (Vietnam, no. 11531)	*Bactrocera dorsalis* (Cameroon, no. 11512)	*Bactrocera dorsalis* (Cameroon, no. 11513)	*Bactrocera dorsalis* (Cameroon, no. 11519)	*Bactrocera dorsalis* (Malaysia, no. 20484)	*Bactrocera dorsalis* (Sri Lanka, no. 11549)	*Bactrocera dorsalis* (Thailand, no. 11504)	*Bactrocera latifrons* (Thailand, no. 20496)	*Bactrocera latifrons* (Vietnam, no. 11524)	*Ceratitis cosyra* (Cameroon, no. 11536)	*Ceratits capitata* (Zimbabwe, no. 11521)	*Rhagoletis cerasi* (Armenia, no. 11520)
Anatrichus sp. (Sri Lanka, no. 11538)														
Zeugodacus cucurbitae (Sri Lanka, no. 11514)	79.79													
Zeugodacus cucurbitae (Vietnam, no. 11531)	79.79	100.00												
Bactrocera dorsalis (Cameroon, no. 11512)	83.94	83.68	83.68											
Bactrocera dorsalis (Cameroon, no. 11513)	83.94	83.68	83.68	100.00										
Bactrocera dorsalis (Cameroon, no. 11519)	83.94	84.72	84.72	98.96	98.96									
Bactrocera dorsalis (Malaysia, no. 20484)	83.94	84.46	84.46	98.96	98.96	99.48								
Bactrocera dorsalis (Sri Lanka, no. 11549)	84.2	84.46	84.46	99.22	99.22	99.74	99.74							
Bactrocera dorsalis (Thailand, no. 11504)	83.68	84.46	84.46	99.22	99.22	99.74	99.22	99.48						
Bactrocera latifrons (Thailand, no. 20496)	81.09	80.57	80.57	86.01	86.01	86.53	86.01	86.27	86.27					
Bactrocera latifrons (Vietnam, no. 11524)	81.09	80.57	80.57	86.01	86.01	86.53	86.01	86.27	86.27	100.00				
Ceratitis cosyra (Cameroon, no. 11536)	83.68	83.42	83.42	82.9	82.9	83.16	83.16	83.42	82.9	81.61	81.61			
Ceratits capitata (Zimbabwe, no. 11521)	86.01	83.42	83.42	85.49	85.49	85.75	85.75	86.01	85.75	84.46	84.46	87.31		
Rhagoletis cerasi (Armenia, no. 11520)	85.49	84.72	84.72	85.75	85.75	86.27	85.75	86.01	86.01	83.42	83.42	84.2	88.6	

Figure S8.1. Pairwise genetic similarity matrices of insect specimens included in the on-site evaluation of (A) the fruit fly assay, (B) the *B. tabaci* assay, (C) the *T. palmi* assay based on a fragment of the mitochondrial CO1 gene. Numbers represent the percentage of bases that are identical. Length of the assessed CO1 fragments: fruit fly assay, 386 bp; *B. tabaci* assay, 521 bp; *T. palmi* assay, 364bp.

8.1.2. Supplementary tables

Table S8.1. Overview of types and positions of degeneracies used for LAMP primer design.

LAMP assay	**Primer**	**Degeneracy and position** (distant to 3' end)	**Primer length** (bp)
Bactrocera dorsalis group	B3	Y(P10)	26
Bactrocera dorsalis group	BIP	R(P11), W(P41)	50
Bactrocera dorsalis group	F3	no	22
Bactrocera dorsalis group	FIP	R(P1), Y(P31)	48
Bactrocera dorsalis group	LoopB	no	28
Bactrocera dorsalis group	LoopF	R(P10)	27
Bactrocera correcta / *Z. cucurbitae*	B3	W(P6), W(P9), M(P15)	25
Bactrocera correcta / *Z. cucurbitae*	BIP	Y(P4), Y(P7), R(P11), W(P13), W(P30), Y(P33), Y(P39), W(P42), M(P45), R(P48)	48
Bactrocera correcta / *Z. cucurbitae*	F3	W(P7), Y(P9), Y(P12), Y(P19), Y(P22)	25
Bactrocera correcta / *Z. cucurbitae*	FIP	Y(P7), R(P12), W(P13), W(P19), R(P27), R(P33), W(P36), K(P42), R(P48)	48
Bactrocera correcta / *Z. cucurbitae*	LoopB	Y(P4), W(P9), Y(P10)	23
Bactrocera correcta / *Z. cucurbitae*	LoopF	W(P4), K(P10), R(P16), R(P20)	21
B. tabaci	Set 1 B3	no	22
B. tabaci	Set 1 BIP	no	49
B. tabaci	Set 1 F3	R(P8)	20
B. tabaci	Set 1 FIP	Y(P26)	41
B. tabaci	Set 1 LoopB	no	27
B. tabaci	Set 1 LoopF	R(P4), R(P16)	30
B. tabaci	Set 2 B3	no	22
B. tabaci	Set 2 BIP	no	51
B. tabaci	Set 2 F3	no	22
B. tabaci	Set 2 FIP	no	45
B. tabaci	Set 2 LoopB	no	27
B. tabaci	Set 2 LoopF	no	30
B. tabaci	Set 3 B3	Y(P4), Y(P13)	22
B. tabaci	Set 3 BIP	Y(P8), R(P11), Y(P23), Y(P29), Y(P32),	49
B. tabaci	Set 3 F3	W(P2), Y(P11)	20
B. tabaci	Set 3 FIP	R(P15), R(P24), Y(P29), W(P32), Y(P35), R(P41)	41
B. tabaci	Set 3 LoopB	R(P25)	27
B. tabaci	Set 3 LoopF	R(P4), R(P28)	30
T. palmi	B3	R(P2), W(P4)	22
T. palmi	BIP	M(P26), R(P29), R(P35), Y(P41)	45
T. palmi	F3	R(P8)	21
T. palmi	FIP	R(P7), R(P22), W(P30)	42
T. palmi	LoopB	Y(P6)	31
T. palmi	LoopF	R(P12)	26

A, Adenine; *C*, cytosine; *G*, guanine; *T*, thymine; *M*, *A* or *C*, *R*, *A* or *G*; *W*, *A* or *T*; *Y*, *C* or *T*.

Table S8.2. GenBank accession numbers of partial CO1 sequences from insect specimens analysed during the on-site evaluation process.

Sample ID	GenBank accession number
no.11538	MG727962
no.11514	MG727963
no.11531	MG727964
no.11519	MG727965
no.11512	MG727966
no.11513	MG727967
no.20484	MG727968
no.11549	MG727969
no.11504	MG727970
no.20496	MG727971
no.11524	MG727972
no.11536	MG727973
no.11521	MG727974
no.11520	MG727975
no.20500	MG727976
no.20493	MG727977
no.20492	MG727978
no.11544	MG727979
no.11502	MG727980
no.20499	MG727981
no.20487	MG727982
no.20494	MG727983
no.20491	MG727984
no.20490	MG727985
no.11551	MG727986
no.20488	MG727987
no.11530	MG727988
no.11534	MG727989
no.11545	MG727990
no.11511	MG727991
no.11529	MG727992
no.11535	MG727993
no.20497	MG727994
no.11542_1	MG727995
no.11542_2	MG727996
no.11543	MG727997
no.11526	MG727998

Table S8.3. Primer mismatch analyses of false-negatively tested laboratory evaluation specimens.

LAMP assay	**Primer description**	**Type of mismatch and position** (distant to 3' end)	**Primer length** (bp)
B. tabaci	Set 1 B3	C/A (P4), C/A (P16), C/T (P22)	22
B. tabaci	Set 1 F3	C/T (P10), C/T (P19)	20
B. tabaci	Set 1 LoopB	G/A (P1), T/C (P19), A/G (P22), A/G (P27)	27
B. tabaci	Set 1 LoopF	A/T (P5), C/A (P7), C/A (P22), G/A (P28)	30
T. palmi	B3	A/T (P4)	22

A, Adenine; *C*, cytosine; *G*, guanine; *T*, thymine; bp, base pair.

8.2. Supporting information chapter 3

8.2.1. Materials list

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Materials List for:

A Loop-mediated Isothermal Amplification (LAMP) Assay for Rapid Identification of *Bemisia tabaci*

Simon Blaser[1,2,3], Hanspeter Diem[4], Andreas von Felten[4], Morgan Gueuning[1], Michael Andreou[5], Neil Boonham[6,7], Jennifer Tomlinson[6], Pie Müller[2,3], Jürg Utzinger[2,3], Beatrice Frey[1], Jürg E. Frey[1], Andreas Bühlmann[8]

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URL: https://www.jove.com/video/58502
DOI: doi:10.3791/58502

Materials

Name	Company	Catalog Number	Comments
B. tabaci LAMP primer mix	OptiGene Ltd.	on request	For preparation of read-to-use *B. tabaci* LAMP kit
Centrifuge MiniSpin	Eppendorf AG	5452000010	For several centrifugation steps
Cresol red (red dye)	Sigma-Aldrich Corp.	114472	Component of DNA extraction solution
Eppendorf ThermoMixer	Eppendorf AG	5382000015	For DNA extraction
Genie II (on-site LAMP analysis device)	OptiGene Ltd.	Genie® II	For LAMP analysis
Genie Strips (8-tube LAMP strips)	OptiGene Ltd.	OP-0008-50	For preparation of read-to-use *B. tabaci* LAMP kit
HotStarTaq Master Mix	Qiagen AG	203443	For generation of positive amplification control
Labcycler (Thermocycler)	SensoQuest GmbH, distributed by Witec AG	011-103	For DNA extraction
GspSSD Lyse n' Lamp Isothermal Mastermix	OptiGene Ltd.	ISO-001LNL	For preparation of read-to-use *B. tabaci* LAMP kit
Mini centrifuge Labnet Prism	Labnet International Inc.	C1801	For several centrifugation steps
NucleoFast 96 PCR	Marcherey-Nagel GmbH	743500.4	For clean-up of positive amplification control
Potassium hydroxide solution	Sigma-Aldrich Corp.	319376	Component of DNA extraction solution
Qbit Fluorometer 3	Thermo Fisher Scientific Corp.	Q33226	For measuring DNA conentration of positive control
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32854	For clean-up of positive amplification control
Safe-Lock Tubes 0.5 mL (microcentrifuge tube)	Eppendorf AG	0030 121.023	For DNA extraction
Safe-Lock Tubes 2.0 mL (microcentrifuge tube)	Eppendorf AG	0030 120.094	For preparation of read-to-use B. tabaci LAMP kit
Wood Toothpicks	VWR International LLC	470226-594	For DNA extraction
Vortex-Genie 2 (Vortex)	Scientific Industries Inc.	SI-0236	For several mixing steps

8.3. Supporting information chapter 5

8.3.1. Supplementary methods S1

8.3.1.1. ddRAD protocol

This is a modified version of protocols published by Peterson et al (2012) (*Double Digest RADseq: An Inexpensive Method for De Novo SNP Discovery and Genotyping in Model and Non-Model Species*) and Lam et al (2017) (*Phylogeography and population genomics of a lotic water beetle across a complex tropical landscape*). Sequences of ddRAD adapters and ddRAD PCR primers used for multiplexing are specified in chapter 8.3.2.

1.) Prepare ddRAD adapters

a.) Prepare hybridisation buffer (10x):

Hybridisation buffer (10x)	
UltraPure™ Tris-HCl (1M, Thermo Fisher Scientific, Waltham, MA, USA)	50 µl
NaCl (5M, Thermo Fisher Scientific)	50 µl
Ultrapure EDTA, ph8 (0.5M, Thermo Fisher Scientific)	10 µl
ddH2O	390 µl

b.) Produce 10 µM stock of hybridised P1 and P2 adapters

c.) Prepare hybridisation mix P1 and P2:

P1 hybridisation mix	
Adaptor_1* (100 µM)	10 µl
Adaptor_1b* (100 µM)	10 µl
hybridisation buffer (10x)	10 µl
ddH2O	70 µl

P2 hybridisation mix	
Adaptor_2.1* (100 µM)	10 µl
Adaptor_2.2* (100 µM)	10 µl
hybridisation buffer (10x)	10 µl
ddH2O	70 µl

d.) Vortex and centrifuge

e.) Use a thermoblock to perform the annealing process by heating the hybridisation mixes up to 97.5°C for 2.5 min, then cool them down to 21°C at a rate not higher than 3°C/min (=0.05°C/sec). Then, hold at 4°C.

2.) Double restriction enzyme digest

a.) Prepare Mastermix RE:

Mastermix RE-Digest	1x
CutSmart® Buffer (1x, New England BioLabs (NEB) Inc., Ipswich, MA, USA)	0.9 µl
NlaIII (10,000 units/ml, NEB)	0.1 µl
EcoRI-HF® (20,000 units/ml, NEB)	0.1 µl
H2O	1.9 µl

b.) Mix and quickspinn Mastermix RE-Digest

c.) Add 6 µl of sample DNA into each well of a 96-well plate

d.) Add 3 µl of Digest-Master mix plate by gently pipetting up and down

e.) Cover and seal plate, centrifuge, and incubate at 37°C for 3 h

f.) Heat kill enzyme by heating up to 65°C for 20 min

g.) Keep reaction at 4°C.

3.) Adaptor ligation

a.) Thaw P1 and P2 adapters

b.) Prepare Mastermix ADAPT-LIG:

Mastermix ADAPT-LIG	1x
Cut Smart Buffer (10x, NEB)	0.4 µl
ATP (10 mM, NEB)	1.3 µl
Adapter P2 (10 µM)	0.3 µl
T4 DNA Ligase (400’000 units/ml, NEB)	0.2 µl
ddH2O	1.4 µl

c.) Add 3.6 µl of Mastermix ADAPT-LIG to the RE-digestion product and mix by pipetting up and down

d.) Add 0.3 µl of Adapter P1 to each well

e.) Softly vortex and quickspinn

f.) Incubate at 16°C for 3 h in a thermocycler (Lid of cycler heated to 50°)

g.) Heat inactivate reaction at 65°C for 10 min

h.) Hold reaction at 4°C

4.) Ampure clean-up step

a.) Clean ligation product by performing an AMPure XP purification (Beckman Coulter Inc., Brea, CA, USA) with a beads ratio of 0.8x according to the AMPure XP manual

b.) Elute the cleaned ligation product in 40 µl of Elution Buffer (EB) from Qiagen

5.) RAD-PCR I (Perform in duplicate)

a.) Prepare Mastermix RAD-PCR I:

Mastermix RAD-PCR I	1x
Q5® Reaction Buffer (5x, NEB)	2 µl
dNTPs Solution Mix (10 mM, NEB)	0.2 µl
PCR RAD primer 1*	0.4 µl
GC enhancer	2 µl
Q5	0.1 µl
ddH2O	1.9 µl

b.) Dispense 6.6 µl of RAD-PCR I Mastermix into each well of a fresh 96-well plate

c.) Add 0.4 µl of indexed RAD-PCR primers 2 into each well of the 96-well plate

d.) Add 3 µl of cleaned ligation product into each well of the 96-well plate

e.) RAD-PCR I thermocycler protocol:

1	30 sec at 98°C
2	20 cycles of (20 sec at 98°C, 30 sec at 60°C, 40 sec at 72 °C)
3	10 min at 72°C
4	Hold at 4°C

6.) RAD-PCR II (Final cycle, perform in duplicates)

a.) Prepare Mastermix RAD-PCR II:

Mastermix RAD-PCR II	1x
Q5® Reaction Buffer (5x, NEB)	0.2 µl
dNTPs Solution Mix (10 mM, NEB)	0.2 µl
RAD PCR primer I	0.3 µl

b.) Add 0.7 µl of Mastermix RAD-PCR II to each RAD-PCR I product

c.) Add 0.3 µl of PCR primer II (indexed) to each RAD-PCR I product

d.) RAD-PCR II:

1	3 min at 98°C
2	2 min at 60°C
3	12 min at 72°C

7.) Pool amplicons from RAD-PCR II reaction performed in duplicate and verify amplification success

a.) Pool RAD-PCR II reaction products performed in duplicate

b.) Perform an agarose gel-electrophoresis to verify amplification success

8.) Pool samples and concentrate pools

a.) Pool always 48 samples in one tube (use 15 µl of each sample, final volume=720 µl)

b.) Speedvac each pool to a volume of ~200 µl

9.) Ampure cleaning-up step

a.) Clean ligation product by performing an AMPure XP purification (Beckman Coulter Inc.) with a beads ratio of 1.0x according to the AMPure XP manual

b.) Elute the cleaned ligation product in 40 µl of ddH2O

10.) Measure DNA concentration and pool combined ddRAD library

a.) Quantify DNA concentration of each pool with the Qubit 4 fluorometer (Thermo Fisher Scientific) according to the producers manual

b.) Pool sub-pools equimolarly into the combined ddRAD library

11.) Concentrate final ddRAD library

a.) Speedvac final ddRAD library to get a final concentration of ~140 ng/µl

12.) Analyse ddRAD library profile and perform size selection

a.) Analyse ddRAD library profile using the Fragment Analyzer (Agilent Technologies Inc., Santa Clara, CA, USA)

b.) Perform automated size selection using BluePippin with a 2% Agarose cassette (Sage Science, Beverly, MA, USA)

c.) Verify size selection success using Fragment Analyzer (Agilent Technologies Inc., Santa Clara, CA, USA)

13.) Sequence ddRAD library with Illumina Miseq

a.) Sequence ddRAD library in six consecutive Illumina MiSeq runs using the v3 kit (Illumina Inc., San Diego, CA, USA)

8.3.2. Supplementary methods S2

8.3.2.1. ddRAD PCR primers

Primer name	***Index***	***Oligo sequence***
RAD-PCR_1	No index	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG
RAD-PCR_2_Idx_1	ATCACG	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGC
RAD-PCR_2_Idx_2	CGATGT	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGC
RAD-PCR_2_Idx_3	TTAGGC	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGC
RAD-PCR_2_Idx_4	TGACCA	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGC
RAD-PCR_2_Idx_5	ACAGTG	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGC
RAD-PCR_2_Idx_6	GCCAAT	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGC
RAD-PCR_2_Idx_7	CAGATC	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGC
RAD-PCR_2_Idx_8	ACTTGA	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGTGC
RAD-PCR_2_Idx_9	GATCAG	CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAGACGTGTGC
RAD-PCR_2_Idx_10	TAGCTT	CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACGTGTGC
RAD-PCR_2_Idx_11	GGCTAC	CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCAGACGTGTGC
RAD-PCR_2_Idx_12	CTTGTA	CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGTGC

8.3.2.2. ddRAD adapters

Adapter name	***Barcode***	***Oligo sequence***
NlaIII_P2.1	no barcode	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCATG
NlaIII_P2.2	no barcode	/5Phos/AGATCGGAAGAGCGAGAACAA
EcoRI_P1.1_Idx_1	GCATG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCATG
EcoRI_P1.1_Idx_2	AACCA	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAACCA
EcoRI_P1.1_Idx_3	CGATC	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGATC
EcoRI_P1.1_Idx_4	TCGAT	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCGAT
EcoRI_P1.1_Idx_5	TGCAT	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGCAT
EcoRI_P1.1_Idx_6	CAACC	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAACC
EcoRI_P1.1_Idx_7	GGTTG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTTG
EcoRI_P1.1_Idx_8	AAGGA	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAAGGA
EcoRI_P1.1_Idx_9	AGCTA	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGCTA
EcoRI_P1.1_Idx_10	ACACA	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACACA
EcoRI_P1.1_Idx_11	AATTA	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAATTA
EcoRI_P1.1_Idx_12	ACGGT	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACGGT
EcoRI_P1.1_Idx_13	ACTGG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACTGG
EcoRI_P1.1_Idx_14	ACTTC	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACTTC
EcoRI_P1.1_Idx_15	ATACG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATACG
EcoRI_P1.1_Idx_16	ATGAG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATGAG
EcoRI_P1.1_Idx_17	ATTAC	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATTAC
EcoRI_P1.1_Idx_18	CATAT	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCATAT
EcoRI_P1.1_Idx_19	CGAAT	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGAAT
EcoRI_P1.1_Idx_20	CGGCT	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGGCT

EcoRI_P1.1_Idx_21	CGGTA	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGGTA
EcoRI_P1.1_Idx_22	CGTAC	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGTAC
EcoRI_P1.1_Idx_23	CGTCG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGTCG
EcoRI_P1.1_Idx_24	CTGAT	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTGAT
EcoRI_P1.1_Idx_25	CTGCG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTGCG
EcoRI_P1.1_Idx_26	CTGTC	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTGTC
EcoRI_P1.1_Idx_27	CTTGG	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTTGG
EcoRI_P1.1_Idx_28	GACAC	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGACAC
EcoRI_P1.1_Idx_29	GAGAT	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAGAT
EcoRI_P1.1_Idx_30	GAGTC	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAGTC
EcoRI_P1.1_Idx_31	GCCGT	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCCGT
EcoRI_P1.1_Idx_32	GCTGA	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCTGA
EcoRI_P1.2_Idx_1	GCATG	/5Phos/AATTCATGCAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_2	AACCA	/5Phos/AATTTGGTTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_3	CGATC	/5Phos/AATTGATCGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_4	TCGAT	/5Phos/AATTATCGAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_5	TGCAT	/5Phos/AATTATGCAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_6	CAACC	/5Phos/AATTGGTTGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_7	GGTTG	/5Phos/AATTCAACCAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_8	AAGGA	/5Phos/AATTTCCTTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_9	AGCTA	/5Phos/AATTTAGCTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_10	ACACA	/5Phos/AATTTGTGTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_11	AATTA	/5Phos/AATTTAATTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_12	ACGGT	/5Phos/AATTACCGTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_13	ACTGG	/5Phos/AATTCCAGTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_14	ACTTC	/5Phos/AATTGAAGTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_15	ATACG	/5Phos/AATTCGTATAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_16	ATGAG	/5Phos/AATTCTCATAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_17	ATTAC	/5Phos/AATTGTAATAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_18	CATAT	/5Phos/AATTATATGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_19	CGAAT	/5Phos/AATTATTCGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_20	CGGCT	/5Phos/AATTAGCCGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_21	CGGTA	/5Phos/AATTTACCGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_22	CGTAC	/5Phos/AATTGTACGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_23	CGTCG	/5Phos/AATTCGACGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_24	CTGAT	/5Phos/AATTATCAGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_25	CTGCG	/5Phos/AATTCGCAGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_26	CTGTC	/5Phos/AATTGACAGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_27	CTTGG	/5Phos/AATTCCAAGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_28	GACAC	/5Phos/AATTGTGTCAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_29	GAGAT	/5Phos/AATTATCTCAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_30	GAGTC	/5Phos/AATTGACTCAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_31	GCCGT	/5Phos/AATTACGGCAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
EcoRI_P1.2_Idx_32	GCTGA	/5Phos/AATTTCAGCAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

8.3.3. Supplementary methods S3

8.3.3.1. Individuals included into final CO1 and ddRAD data

Ind ID	Pop ID	CO1	NCBI No.	RAD	EcoRI adapter	RAD-PCR_2 primer
AUT-BAI-S0001	AUT-BAI	yes	MK778089	yes	EcoRI_Idx_29	RAD-PCR_2_Idx_5
AUT-BAI-S0002	AUT-BAI	yes	MK778090	yes	EcoRI_Idx_6	RAD-PCR_2_Idx_6
AUT-BAI-S0003	AUT-BAI	yes	MK778091	yes	EcoRI_Idx_10	RAD-PCR_2_Idx_11
AUT-BAI-S0004	AUT-BAI	yes	MK778092	no	n.a.	n.a.
AUT-BAI-S0005	AUT-BAI	yes	MK778093	yes	EcoRI_Idx_5	RAD-PCR_2_Idx_7
AUT-BAI-S0006	AUT-BAI	yes	MK778094	yes	EcoRI_Idx_16	RAD-PCR_2_Idx_10
AUT-BAI-S0007	AUT-BAI	yes	MK778095	yes	EcoRI_Idx_13	RAD-PCR_2_Idx_3
CAN-CAM-S0001	CAN-CAM	yes	MK778096	yes	EcoRI_Idx_30	RAD-PCR_2_Idx_2
CAN-CAM-S0002	CAN-CAM	yes	MK778097	yes	EcoRI_Idx_3	RAD-PCR_2_Idx_9
CAN-CAM-S0003	CAN-CAM	yes	MK778098	yes	EcoRI_Idx_15	RAD-PCR_2_Idx_8
CAN-CAM-S0004	CAN-CAM	yes	MK778099	yes	EcoRI_Idx_31	RAD-PCR_2_Idx_2
CAN-CAM-S0005	CAN-CAM	yes	MK778100	yes	EcoRI_Idx_19	RAD-PCR_2_Idx_1
CAN-CAM-S0006	CAN-CAM	yes	MK778101	yes	EcoRI_Idx_23	RAD-PCR_2_Idx_3
CAN-CAM-S0007	CAN-CAM	yes	MK778102	yes	EcoRI_Idx_4	RAD-PCR_2_Idx_3
CHE-AAR-R1000	CHE-AAR	yes	MK778103	yes	EcoRI_Idx_4	RAD-PCR_2_Idx_4
CHE-AAR-R1002	CHE-AAR	yes	MK778104	yes	EcoRI_Idx_12	RAD-PCR_2_Idx_4
CHE-AAR-R1004	CHE-AAR	yes	MK778105	yes	EcoRI_Idx_2	RAD-PCR_2_Idx_3
CHE-AAR-R1006	CHE-AAR	yes	MK778106	yes	EcoRI_Idx_12	RAD-PCR_2_Idx_1
CHE-AAR-R1007	CHE-AAR	yes	MK778107	yes	EcoRI_Idx_18	RAD-PCR_2_Idx_3
CHE-AAR-R1009	CHE-AAR	yes	MK778108	yes	EcoRI_Idx_8	RAD-PCR_2_Idx_3
CHE-AAR-R1012	CHE-AAR	yes	MK778109	no	n.a.	n.a.
CHE-AAR-R1018	CHE-AAR	yes	MK778110	yes	EcoRI_Idx_8	RAD-PCR_2_Idx_2
CHE-AAR-R1019	CHE-AAR	yes	MK778111	yes	EcoRI_Idx_3	RAD-PCR_2_Idx_10
CHE-AAR-R1020	CHE-AAR	yes	MK778112	yes	EcoRI_Idx_23	RAD-PCR_2_Idx_1
CHE-AAR-R1021	CHE-AAR	yes	MK778113	no	n.a.	n.a.
CHE-AAR-R1022	CHE-AAR	yes	MK778114	yes	EcoRI_Idx_20	RAD-PCR_2_Idx_2
CHE-AES-R0234	CHE-AES	yes	MK778115	yes	EcoRI_Idx_3	RAD-PCR_2_Idx_7
CHE-AES-R0235	CHE-AES	yes	MK778116	yes	EcoRI_Idx_15	RAD-PCR_2_Idx_5
CHE-AES-R0236	CHE-AES	yes	MK778117	yes	EcoRI_Idx_12	RAD-PCR_2_Idx_2
CHE-AES-R0247	CHE-AES	yes	MK778118	yes	EcoRI_Idx_6	RAD-PCR_2_Idx_10
CHE-AES-R0254	CHE-AES	yes	MK778119	yes	EcoRI_Idx_7	RAD-PCR_2_Idx_5
CHE-AES-R0255	CHE-AES	yes	MK778120	yes	EcoRI_Idx_4	RAD-PCR_2_Idx_1
CHE-AES-R0370	CHE-AES	yes	MK778121	no	n.a.	n.a.
CHE-AES-R0373	CHE-AES	no	n.a.	yes	EcoRI_Idx_24	RAD-PCR_2_Idx_9
CHE-AES-R0381	CHE-AES	yes	MK778122	yes	EcoRI_Idx_28	RAD-PCR_2_Idx_3
CHE-AES-R0390	CHE-AES	yes	MK778123	yes	EcoRI_Idx_14	RAD-PCR_2_Idx_9
CHE-AES-R0395	CHE-AES	yes	MK778124	yes	EcoRI_Idx_5	RAD-PCR_2_Idx_9
CHE-AES-R0404	CHE-AES	yes	MK778125	yes	EcoRI_Idx_19	RAD-PCR_2_Idx_11
CHE-ART-R1023	CHE-ART	yes	MK778126	yes	EcoRI_Idx_21	RAD-PCR_2_Idx_10
CHE-ART-R1024	CHE-ART	yes	MK778127	no	n.a.	n.a.
CHE-ARZ-R0688	CHE-ARZ	yes	MK778128	yes	EcoRI_Idx_6	RAD-PCR_2_Idx_8
CHE-ARZ-R0690	CHE-ARZ	yes	MK778129	yes	EcoRI_Idx_27	RAD-PCR_2_Idx_5
CHE-ARZ-R0692	CHE-ARZ	yes	MK778130	no	n.a.	n.a.
CHE-ARZ-R0693	CHE-ARZ	yes	MK778131	no	n.a.	n.a.
CHE-ARZ-R0694	CHE-ARZ	yes	MK778132	yes	EcoRI_Idx_11	RAD-PCR_2_Idx_4
CHE-ARZ-R0699	CHE-ARZ	yes	MK778133	yes	EcoRI_Idx_14	RAD-PCR_2_Idx_3
CHE-ARZ-R0700	CHE-ARZ	yes	MK778134	yes	EcoRI_Idx_5	RAD-PCR_2_Idx_1
CHE-ARZ-R0709	CHE-ARZ	yes	MK778135	yes	EcoRI_Idx_13	RAD-PCR_2_Idx_6
CHE-ARZ-R0711	CHE-ARZ	yes	MK778136	yes	EcoRI_Idx_2	RAD-PCR_2_Idx_11
CHE-ARZ-R0724	CHE-ARZ	yes	MK778137	yes	EcoRI_Idx_27	RAD-PCR_2_Idx_3
CHE-ARZ-R0729	CHE-ARZ	yes	MK778138	yes	EcoRI_Idx_17	RAD-PCR_2_Idx_1
CHE-BER-R0941	CHE-BER	no	n.a.	yes	EcoRI_Idx_19	RAD-PCR_2_Idx_7

CHE-BER-R0943	CHE-BER	yes	MK778139	no	n.a.	n.a.
CHE-BER-R0944	CHE-BER	yes	MK778140	no	n.a.	n.a.
CHE-BER-R0945	CHE-BER	yes	MK778141	yes	EcoRI_Idx_2	RAD-PCR_2_Idx_7
CHE-BER-R0947	CHE-BER	yes	MK778142	yes	EcoRI_Idx_25	RAD-PCR_2_Idx_2
CHE-BER-R0949	CHE-BER	yes	MK778143	yes	EcoRI_Idx_24	RAD-PCR_2_Idx_1
CHE-BER-R0951	CHE-BER	yes	MK778144	yes	EcoRI_Idx_14	RAD-PCR_2_Idx_2
CHE-BER-R0953	CHE-BER	yes	MK778145	yes	EcoRI_Idx_3	RAD-PCR_2_Idx_8
CHE-BER-R0954	CHE-BER	yes	MK778146	yes	EcoRI_Idx_24	RAD-PCR_2_Idx_10
CHE-BER-R0955	CHE-BER	yes	MK778147	yes	EcoRI_Idx_21	RAD-PCR_2_Idx_12
CHE-BER-R0957	CHE-BER	yes	MK778148	yes	EcoRI_Idx_17	RAD-PCR_2_Idx_12
CHE-CHA-R0873	CHE-CHA	yes	MK778149	yes	EcoRI_Idx_10	RAD-PCR_2_Idx_10
CHE-CHA-R0874	CHE-CHA	yes	MK778150	yes	EcoRI_Idx_1	RAD-PCR_2_Idx_10
CHE-CHA-R0875	CHE-CHA	yes	MK778151	yes	EcoRI_Idx_10	RAD-PCR_2_Idx_3
CHE-CHA-R0877	CHE-CHA	yes	MK778152	yes	EcoRI_Idx_8	RAD-PCR_2_Idx_1
CHE-CHA-R0878	CHE-CHA	yes	MK778153	yes	EcoRI_Idx_22	RAD-PCR_2_Idx_9
CHE-CHY-S0001	CHE-CHY	yes	MK778154	yes	EcoRI_Idx_21	RAD-PCR_2_Idx_2
CHE-CHY-S0002	CHE-CHY	no	n.a.	yes	EcoRI_Idx_15	RAD-PCR_2_Idx_9
CHE-FLA-R0918	CHE-FLA	yes	MK778155	yes	EcoRI_Idx_13	RAD-PCR_2_Idx_4
CHE-FLA-R0920	CHE-FLA	yes	MK778156	yes	EcoRI_Idx_15	RAD-PCR_2_Idx_10
CHE-FLA-R0922	CHE-FLA	yes	MK778157	no	n.a.	n.a.
CHE-FLA-R0924	CHE-FLA	yes	MK778158	yes	EcoRI_Idx_22	RAD-PCR_2_Idx_4
CHE-FLA-R0927	CHE-FLA	yes	MK778159	yes	EcoRI_Idx_12	RAD-PCR_2_Idx_6
CHE-FLA-R0928	CHE-FLA	yes	MK778160	yes	EcoRI_Idx_10	RAD-PCR_2_Idx_12
CHE-FLA-R0929	CHE-FLA	yes	MK778161	yes	EcoRI_Idx_5	RAD-PCR_2_Idx_6
CHE-FLA-R0930	CHE-FLA	yes	MK778162	yes	EcoRI_Idx_15	RAD-PCR_2_Idx_4
CHE-FLA-R0931	CHE-FLA	yes	MK778163	yes	EcoRI_Idx_23	RAD-PCR_2_Idx_5
CHE-FLA-R0932	CHE-FLA	yes	MK778164	no	n.a.	n.a.
CHE-FLA-R0933	CHE-FLA	yes	MK778165	yes	EcoRI_Idx_21	RAD-PCR_2_Idx_1
CHE-FLA-R0934	CHE-FLA	yes	MK778166	yes	EcoRI_Idx_3	RAD-PCR_2_Idx_6
CHE-GIO-R0125	CHE-GIO	yes	MK778167	no	n.a.	n.a.
CHE-GIO-R0128	CHE-GIO	no	n.a.	yes	EcoRI_Idx_32	RAD-PCR_2_Idx_1
CHE-GIO-R0129	CHE-GIO	yes	MK778168	no	n.a.	n.a.
CHE-GIO-R0130	CHE-GIO	yes	MK778169	no	n.a.	n.a.
CHE-GIO-R0131	CHE-GIO	yes	MK778170	no	n.a.	n.a.
CHE-GIO-R0132	CHE-GIO	yes	MK778171	no	n.a.	n.a.
CHE-GIO-R0133	CHE-GIO	yes	MK778172	no	n.a.	n.a.
CHE-GIO-R0840	CHE-GIO	yes	MK778173	yes	EcoRI_Idx_11	RAD-PCR_2_Idx_2
CHE-GIO-R0841	CHE-GIO	yes	MK778174	no	n.a.	n.a.
CHE-GIO-R0842	CHE-GIO	yes	MK778175	yes	EcoRI_Idx_10	RAD-PCR_2_Idx_5
CHE-HER-R1066	CHE-HER	yes	MK778176	yes	EcoRI_Idx_31	RAD-PCR_2_Idx_5
CHE-HUE-R1037	CHE-HUE	yes	MK778177	yes	EcoRI_Idx_15	RAD-PCR_2_Idx_2
CHE-HUE-R1038	CHE-HUE	yes	MK778178	yes	EcoRI_Idx_16	RAD-PCR_2_Idx_11
CHE-HUE-R1039	CHE-HUE	yes	MK778179	yes	EcoRI_Idx_22	RAD-PCR_2_Idx_10
CHE-HUE-R1040	CHE-HUE	yes	MK778180	yes	EcoRI_Idx_29	RAD-PCR_2_Idx_4
CHE-HUE-R1041	CHE-HUE	yes	MK778181	yes	EcoRI_Idx_26	RAD-PCR_2_Idx_4
CHE-HUE-R1042	CHE-HUE	yes	MK778182	yes	EcoRI_Idx_2	RAD-PCR_2_Idx_5
CHE-HUE-R1043	CHE-HUE	yes	MK778183	yes	EcoRI_Idx_9	RAD-PCR_2_Idx_7
CHE-HUE-R1044	CHE-HUE	yes	MK778184	yes	EcoRI_Idx_10	RAD-PCR_2_Idx_7
CHE-HUE-R1046	CHE-HUE	yes	MK778185	yes	EcoRI_Idx_32	RAD-PCR_2_Idx_5
CHE-HUE-R1047	CHE-HUE	yes	MK778186	yes	EcoRI_Idx_3	RAD-PCR_2_Idx_11
CHE-HUE-R1049	CHE-HUE	yes	MK778187	yes	EcoRI_Idx_18	RAD-PCR_2_Idx_12
CHE-MEN-R0883	CHE-MEN	yes	MK778188	yes	EcoRI_Idx_15	RAD-PCR_2_Idx_12
CHE-MEN-R0884	CHE-MEN	yes	MK778189	yes	EcoRI_Idx_25	RAD-PCR_2_Idx_5
CHE-MEN-R0885	CHE-MEN	yes	MK778190	no	n.a.	n.a.
CHE-OBE-R0890	CHE-OBE	yes	MK778191	yes	EcoRI_Idx_19	RAD-PCR_2_Idx_4
CHE-OBE-R0891	CHE-OBE	yes	MK778192	yes	EcoRI_Idx_7	RAD-PCR_2_Idx_4
CHE-OBE-R0892	CHE-OBE	yes	MK778193	yes	EcoRI_Idx_27	RAD-PCR_2_Idx_2
CHE-OBE-R0893	CHE-OBE	yes	MK778194	yes	EcoRI_Idx_13	RAD-PCR_2_Idx_2
CHE-OBE-R0899	CHE-OBE	yes	MK778195	yes	EcoRI_Idx_9	RAD-PCR_2_Idx_8

CHE-OBE-R0900	CHE-OBE	yes	MK778196	yes	EcoRI_Idx_14	RAD-PCR_2_Idx_6
CHE-OBE-R0902	CHE-OBE	yes	MK778197	yes	EcoRI_Idx_32	RAD-PCR_2_Idx_2
CHE-OBE-R0904	CHE-OBE	yes	MK778198	yes	EcoRI_Idx_30	RAD-PCR_2_Idx_4
CHE-OBE-R0905	CHE-OBE	yes	MK778199	yes	EcoRI_Idx_27	RAD-PCR_2_Idx_4
CHE-OBE-R0907	CHE-OBE	yes	MK778200	yes	EcoRI_Idx_24	RAD-PCR_2_Idx_4
CHE-OBE-R0909	CHE-OBE	yes	MK778201	yes	EcoRI_Idx_4	RAD-PCR_2_Idx_10
CHE-OBE-R0910	CHE-OBE	yes	MK778202	yes	EcoRI_Idx_5	RAD-PCR_2_Idx_10
CHE-OBE-R0911	CHE-OBE	yes	MK778203	yes	EcoRI_Idx_30	RAD-PCR_2_Idx_3
CHE-OES-R0888	CHE-OES	yes	MK778204	yes	EcoRI_Idx_9	RAD-PCR_2_Idx_11
CHE-OES-R0889	CHE-OES	yes	MK778205	yes	EcoRI_Idx_28	RAD-PCR_2_Idx_4
CHE-RIE-R0977	CHE-RIE	yes	MK778206	yes	EcoRI_Idx_2	RAD-PCR_2_Idx_4
CHE-RIE-R0984	CHE-RIE	yes	MK778207	yes	EcoRI_Idx_22	RAD-PCR_2_Idx_11
CHE-RIE-R0986	CHE-RIE	yes	MK778208	yes	EcoRI_Idx_24	RAD-PCR_2_Idx_5
CHE-RIE-R0988	CHE-RIE	yes	MK778209	no	n.a.	n.a.
CHE-RIE-R0992	CHE-RIE	yes	MK778210	yes	EcoRI_Idx_23	RAD-PCR_2_Idx_6
CHE-RIE-R0994	CHE-RIE	yes	MK778211	yes	EcoRI_Idx_5	RAD-PCR_2_Idx_12
CHE-RIE-R0995	CHE-RIE	yes	MK778212	yes	EcoRI_Idx_27	RAD-PCR_2_Idx_1
CHE-RIE-R0996	CHE-RIE	yes	MK778213	yes	EcoRI_Idx_20	RAD-PCR_2_Idx_9
CHE-RIE-R0997	CHE-RIE	yes	MK778214	yes	EcoRI_Idx_16	RAD-PCR_2_Idx_8
CHE-RIE-R0999	CHE-RIE	yes	MK778215	yes	EcoRI_Idx_22	RAD-PCR_2_Idx_6
CHE-ROV-R0649	CHE-ROV	yes	MK778216	yes	EcoRI_Idx_24	RAD-PCR_2_Idx_7
CHE-ROV-R0650	CHE-ROV	yes	MK778217	yes	EcoRI_Idx_32	RAD-PCR_2_Idx_3
CHE-ROV-R0651	CHE-ROV	yes	MK778218	yes	EcoRI_Idx_17	RAD-PCR_2_Idx_2
CHE-ROV-R0655	CHE-ROV	yes	MK778219	no	n.a.	n.a.
CHE-ROV-R0659	CHE-ROV	yes	MK778220	yes	EcoRI_Idx_28	RAD-PCR_2_Idx_2
CHE-ROV-R0665	CHE-ROV	yes	MK778221	yes	EcoRI_Idx_12	RAD-PCR_2_Idx_11
CHE-ROV-R0673	CHE-ROV	yes	MK778222	yes	EcoRI_Idx_20	RAD-PCR_2_Idx_12
CHE-ROV-R0678	CHE-ROV	no	n.a.	yes	EcoRI_Idx_9	RAD-PCR_2_Idx_3
CHE-ROV-R0681	CHE-ROV	yes	MK778223	yes	EcoRI_Idx_2	RAD-PCR_2_Idx_10
CHE-ROV-R0682	CHE-ROV	yes	MK778224	yes	EcoRI_Idx_18	RAD-PCR_2_Idx_4
CHE-ROV-R0683	CHE-ROV	yes	MK778225	yes	EcoRI_Idx_14	RAD-PCR_2_Idx_1
CHE-STA-R0732	CHE-STA	yes	MK778226	yes	EcoRI_Idx_25	RAD-PCR_2_Idx_6
CHE-STA-R0733	CHE-STA	yes	MK778227	yes	EcoRI_Idx_26	RAD-PCR_2_Idx_6
CHE-STA-R0735	CHE-STA	yes	MK778228	yes	EcoRI_Idx_6	RAD-PCR_2_Idx_3
CHE-STA-R0736	CHE-STA	yes	MK778229	yes	EcoRI_Idx_9	RAD-PCR_2_Idx_5
CHE-STA-R0737	CHE-STA	yes	MK778230	yes	EcoRI_Idx_11	RAD-PCR_2_Idx_5
CHE-STA-R0741	CHE-STA	yes	MK778231	yes	EcoRI_Idx_9	RAD-PCR_2_Idx_4
CHE-STA-R0742	CHE-STA	yes	MK778232	yes	EcoRI_Idx_16	RAD-PCR_2_Idx_3
CHE-STA-R0751	CHE-STA	yes	MK778233	yes	EcoRI_Idx_16	RAD-PCR_2_Idx_6
CHE-STA-R0752	CHE-STA	yes	MK778234	yes	EcoRI_Idx_23	RAD-PCR_2_Idx_4
CHE-STA-R0799	CHE-STA	yes	MK778235	yes	EcoRI_Idx_11	RAD-PCR_2_Idx_6
CHE-STA-R0805	CHE-STA	yes	MK778236	yes	EcoRI_Idx_18	RAD-PCR_2_Idx_5
CHE-STA-R0828	CHE-STA	yes	MK778237	yes	EcoRI_Idx_3	RAD-PCR_2_Idx_2
CHE-TOR-R0887	CHE-TOR	yes	MK778238	no	n.a.	n.a.
CHE-WAE-R1050	CHE-WAE	yes	MK778239	yes	EcoRI_Idx_7	RAD-PCR_2_Idx_12
CHE-WAE-R1051	CHE-WAE	yes	MK778240	yes	EcoRI_Idx_26	RAD-PCR_2_Idx_5
CHE-WAE-R1052	CHE-WAE	yes	MK778241	yes	EcoRI_Idx_22	RAD-PCR_2_Idx_2
CHE-WAE-R1053	CHE-WAE	yes	MK778242	yes	EcoRI_Idx_31	RAD-PCR_2_Idx_3
CHE-WAE-R1054	CHE-WAE	yes	MK778243	yes	EcoRI_Idx_20	RAD-PCR_2_Idx_4
CHE-WAE-R1055	CHE-WAE	yes	MK778244	yes	EcoRI_Idx_20	RAD-PCR_2_Idx_3
CHE-WAE-R1056	CHE-WAE	yes	MK778245	yes	EcoRI_Idx_4	RAD-PCR_2_Idx_9
CHE-WAE-R1058	CHE-WAE	yes	MK778246	yes	EcoRI_Idx_15	RAD-PCR_2_Idx_6
CHE-WAE-R1059	CHE-WAE	yes	MK778247	yes	EcoRI_Idx_12	RAD-PCR_2_Idx_5
CHE-WAE-R1060	CHE-WAE	yes	MK778248	yes	EcoRI_Idx_4	RAD-PCR_2_Idx_8
CHE-WAE-R1061	CHE-WAE	yes	MK778249	yes	EcoRI_Idx_17	RAD-PCR_2_Idx_10
CHE-WAE-R1064	CHE-WAE	yes	MK778250	yes	EcoRI_Idx_17	RAD-PCR_2_Idx_4
CHE-WEI-R0643	CHE-WEI	yes	MK778251	yes	EcoRI_Idx_28	RAD-PCR_2_Idx_5
CHE-WIL-R0959	CHE-WIL	yes	MK778252	yes	EcoRI_Idx_22	RAD-PCR_2_Idx_1
CHE-WIL-R0960	CHE-WIL	yes	MK778253	yes	EcoRI_Idx_31	RAD-PCR_2_Idx_1

CHE-WIL-R0961	CHE-WIL	yes	MK778254	yes	EcoRI_Idx_9	RAD-PCR_2_Idx_12
CHE-WIL-R0962	CHE-WIL	yes	MK778255	yes	EcoRI_Idx_3	RAD-PCR_2_Idx_1
CHE-WIL-R0964	CHE-WIL	yes	MK778256	yes	EcoRI_Idx_6	RAD-PCR_2_Idx_2
CHE-WIL-R0966	CHE-WIL	yes	MK778257	yes	EcoRI_Idx_8	RAD-PCR_2_Idx_11
CHE-WIL-R0968	CHE-WIL	no	MK778258	yes	EcoRI_Idx_7	RAD-PCR_2_Idx_11
CHE-WIL-R0970	CHE-WIL	yes	MK778259	yes	EcoRI_Idx_14	RAD-PCR_2_Idx_11
CHE-WIL-R0972	CHE-WIL	yes	MK778260	yes	EcoRI_Idx_11	RAD-PCR_2_Idx_7
CHE-WIL-R0975	CHE-WIL	yes	MK778261	yes	EcoRI_Idx_2	RAD-PCR_2_Idx_9
CHE-WIL-R0976	CHE-WIL	yes	MK778262	yes	EcoRI_Idx_11	RAD-PCR_2_Idx_11
CZE-BRN-S0001	CZE-BRN	yes	MK778263	yes	EcoRI_Idx_20	RAD-PCR_2_Idx_10
CZE-BRN-S0002	CZE-BRN	yes	MK778264	yes	EcoRI_Idx_10	RAD-PCR_2_Idx_1
CZE-BRN-S0003	CZE-BRN	yes	MK778265	yes	EcoRI_Idx_20	RAD-PCR_2_Idx_6
CZE-BRN-S0004	CZE-BRN	yes	MK778266	yes	EcoRI_Idx_3	RAD-PCR_2_Idx_12
CZE-BRN-S0005	CZE-BRN	yes	MK778267	yes	EcoRI_Idx_12	RAD-PCR_2_Idx_9
CZE-BRN-S0006	CZE-BRN	yes	MK778268	yes	EcoRI_Idx_32	RAD-PCR_2_Idx_4
CZE-BRN-S0007	CZE-BRN	yes	MK778269	yes	EcoRI_Idx_25	RAD-PCR_2_Idx_4
CZE-BRN-S0008	CZE-BRN	yes	MK778270	yes	EcoRI_Idx_18	RAD-PCR_2_Idx_8
CZE-BRN-S0009	CZE-BRN	yes	MK778271	yes	EcoRI_Idx_5	RAD-PCR_2_Idx_4
CZE-BRN-S0010	CZE-BRN	yes	MK778272	yes	EcoRI_Idx_3	RAD-PCR_2_Idx_5
CZE-BRN-S0011	CZE-BRN	yes	MK778273	yes	EcoRI_Idx_7	RAD-PCR_2_Idx_7
DEU-GOD-S2418	DEU-GOD	yes	MK778274	no	n.a.	n.a.
DEU-GOD-S2589	DEU-GOD	yes	MK778275	yes	EcoRI_Idx_10	RAD-PCR_2_Idx_9
DEU-GOD-S2590	DEU-GOD	yes	MK778276	yes	EcoRI_Idx_11	RAD-PCR_2_Idx_12
DEU-GOD-S2593	DEU-GOD	yes	MK778277	yes	EcoRI_Idx_23	RAD-PCR_2_Idx_8
DEU-GOD-S2617	DEU-GOD	yes	MK778278	no	n.a.	n.a.
DEU-GOD-S2619	DEU-GOD	yes	MK778253	yes	EcoRI_Idx_10	RAD-PCR_2_Idx_8
DEU-GOD-S2621	DEU-GOD	no	n.a.	yes	EcoRI_Idx_24	RAD-PCR_2_Idx_8
FRA-BOM-S0001	FRA-BOM	no	n.a.	yes	EcoRI_Idx_27	RAD-PCR_2_Idx_6
FRA-BOM-S0002	FRA-BOM	yes	MK778279	yes	EcoRI_Idx_20	RAD-PCR_2_Idx_5
FRA-BOM-S0003	FRA-BOM	yes	MK778280	yes	EcoRI_Idx_15	RAD-PCR_2_Idx_3
FRA-BOM-S0004	FRA-BOM	yes	MK778281	yes	EcoRI_Idx_17	RAD-PCR_2_Idx_11
FRA-BOM-S0005	FRA-BOM	yes	MK778282	yes	EcoRI_Idx_14	RAD-PCR_2_Idx_10
FRA-BOM-S0006	FRA-BOM	yes	MK778283	yes	EcoRI_Idx_24	RAD-PCR_2_Idx_11
FRA-BOM-S0007	FRA-BOM	yes	MK778284	yes	EcoRI_Idx_9	RAD-PCR_2_Idx_2
FRA-BOM-S0008	FRA-BOM	yes	MK778285	yes	EcoRI_Idx_6	RAD-PCR_2_Idx_4
FRA-BOM-S0009	FRA-BOM	yes	MK778286	yes	EcoRI_Idx_28	RAD-PCR_2_Idx_1
FRA-BOM-S0010	FRA-BOM	yes	MK778287	yes	EcoRI_Idx_8	RAD-PCR_2_Idx_9
FRA-BOM-S0011	FRA-BOM	yes	MK778288	yes	EcoRI_Idx_6	RAD-PCR_2_Idx_7
FRA-BOM-S0012	FRA-BOM	yes	MK778289	yes	EcoRI_Idx_21	RAD-PCR_2_Idx_6
FRA-HAT-S2578	FRA-HAT	yes	MK778290	yes	EcoRI_Idx_24	RAD-PCR_2_Idx_3
FRA-HAT-S2580	FRA-HAT	no	n.a.	yes	EcoRI_Idx_18	RAD-PCR_2_Idx_2
HUN-BUD-S0001	HUN-BUD	yes	MK778291	yes	EcoRI_Idx_26	RAD-PCR_2_Idx_3
HUN-BUD-S0002	HUN-BUD	yes	MK778292	yes	EcoRI_Idx_19	RAD-PCR_2_Idx_3
HUN-BUD-S0003	HUN-BUD	yes	MK778293	yes	EcoRI_Idx_17	RAD-PCR_2_Idx_6
HUN-BUD-S0004	HUN-BUD	yes	MK778294	yes	EcoRI_Idx_22	RAD-PCR_2_Idx_3
HUN-BUD-S0005	HUN-BUD	yes	MK778295	yes	EcoRI_Idx_19	RAD-PCR_2_Idx_6
HUN-BUD-S0006	HUN-BUD	yes	MK778296	yes	EcoRI_Idx_6	RAD-PCR_2_Idx_12
HUN-BUD-S0007	HUN-BUD	yes	MK778297	yes	EcoRI_Idx_29	RAD-PCR_2_Idx_1
HUN-BUD-S0009	HUN-BUD	yes	MK778298	yes	EcoRI_Idx_12	RAD-PCR_2_Idx_10
HUN-BUD-S0010	HUN-BUD	yes	MK778299	yes	EcoRI_Idx_11	RAD-PCR_2_Idx_3
HUN-BUD-S0011	HUN-BUD	yes	MK778300	yes	EcoRI_Idx_21	RAD-PCR_2_Idx_4
HUN-BUD-S0012	HUN-BUD	yes	MK778301	yes	EcoRI_Idx_30	RAD-PCR_2_Idx_5
ITA-CAS-S0001	ITA-CAS	yes	MK778302	yes	EcoRI_Idx_26	RAD-PCR_2_Idx_2
ITA-CAS-S0003	ITA-CAS	yes	MK778303	yes	EcoRI_Idx_22	RAD-PCR_2_Idx_8
ITA-CAS-S0004	ITA-CAS	yes	MK778304	yes	EcoRI_Idx_29	RAD-PCR_2_Idx_6
ITA-CAS-S0005	ITA-CAS	yes	MK778305	yes	EcoRI_Idx_22	RAD-PCR_2_Idx_5
ITA-CAS-S0006	ITA-CAS	yes	MK778306	yes	EcoRI_Idx_14	RAD-PCR_2_Idx_7
ITA-CAS-S0007	ITA-CAS	yes	MK778307	yes	EcoRI_Idx_5	RAD-PCR_2_Idx_8
ITA-CAS-S0008	ITA-CAS	yes	MK778308	yes	EcoRI_Idx_23	RAD-PCR_2_Idx_2

ITA-CAS-S0009	ITA-CAS	yes	MK778309	yes	EcoRI_Idx_5	RAD-PCR_2_Idx_5
ITA-CAS-S0010	ITA-CAS	yes	MK778310	yes	EcoRI_Idx_17	RAD-PCR_2_Idx_3
ITA-CAS-S0011	ITA-CAS	yes	MK778311	yes	EcoRI_Idx_11	RAD-PCR_2_Idx_1
ITA-ROV-S0001	ITA-ROV	yes	MK778312	yes	EcoRI_Idx_30	RAD-PCR_2_Idx_1
ITA-ROV-S0002	ITA-ROV	yes	MK778313	yes	EcoRI_Idx_16	RAD-PCR_2_Idx_5
ITA-ROV-S0003	ITA-ROV	yes	MK778314	yes	EcoRI_Idx_7	RAD-PCR_2_Idx_10
ITA-ROV-S0004	ITA-ROV	yes	MK778315	yes	EcoRI_Idx_7	RAD-PCR_2_Idx_9
ITA-ROV-S0005	ITA-ROV	yes	MK778316	yes	EcoRI_Idx_30	RAD-PCR_2_Idx_6
ITA-ROV-S0006	ITA-ROV	yes	MK778317	yes	EcoRI_Idx_4	RAD-PCR_2_Idx_2
ITA-ROV-S0007	ITA-ROV	yes	MK778318	yes	EcoRI_Idx_8	RAD-PCR_2_Idx_10
ITA-VAL-S0002	ITA-VAL	yes	MK778319	yes	EcoRI_Idx_23	RAD-PCR_2_Idx_10
ITA-VAL-S0003	ITA-VAL	yes	MK778320	no	n.a.	n.a.
ITA-VAL-S0004	ITA-VAL	yes	MK778321	yes	EcoRI_Idx_20	RAD-PCR_2_Idx_11
ITA-VAL-S0005	ITA-VAL	yes	MK778322	yes	EcoRI_Idx_12	RAD-PCR_2_Idx_12
ITA-VAL-S0006	ITA-VAL	yes	MK778323	yes	EcoRI_Idx_26	RAD-PCR_2_Idx_1
ITA-VAL-S0007	ITA-VAL	yes	MK778324	yes	EcoRI_Idx_16	RAD-PCR_2_Idx_1
JPN-UNN-S0001	JPN-UNN	yes	MK778325	yes	EcoRI_Idx_9	RAD-PCR_2_Idx_9
JPN-UNN-S0002	JPN-UNN	yes	MK778326	no	n.a.	n.a.
JPN-UNN-S0003	JPN-UNN	yes	MK778327	yes	EcoRI_Idx_18	RAD-PCR_2_Idx_9
JPN-UNN-S0004	JPN-UNN	yes	MK778328	yes	EcoRI_Idx_17	RAD-PCR_2_Idx_9
JPN-UNN-S0005	JPN-UNN	yes	MK778329	yes	EcoRI_Idx_24	RAD-PCR_2_Idx_6
ROU-BUC-S0001	ROU-BUC	yes	MK778330	yes	EcoRI_Idx_8	RAD-PCR_2_Idx_7
ROU-BUC-S0002	ROU-BUC	yes	MK778331	yes	EcoRI_Idx_24	RAD-PCR_2_Idx_12
ROU-BUC-S0003	ROU-BUC	yes	MK778332	no	n.a.	n.a.
ROU-BUC-S0004	ROU-BUC	yes	MK778333	yes	EcoRI_Idx_29	RAD-PCR_2_Idx_2
ROU-BUC-S0005	ROU-BUC	yes	MK778334	yes	EcoRI_Idx_19	RAD-PCR_2_Idx_12
ROU-BUC-S0006	ROU-BUC	yes	MK778335	yes	EcoRI_Idx_4	RAD-PCR_2_Idx_12
ROU-BUC-S0007	ROU-BUC	yes	MK778336	yes	EcoRI_Idx_18	RAD-PCR_2_Idx_1
ROU-BUC-S0008	ROU-BUC	yes	MK778337	yes	EcoRI_Idx_7	RAD-PCR_2_Idx_3
ROU-BUC-S0009	ROU-BUC	yes	MK778338	yes	EcoRI_Idx_16	RAD-PCR_2_Idx_7
ROU-BUC-S0010	ROU-BUC	yes	MK778339	yes	EcoRI_Idx_7	RAD-PCR_2_Idx_6
ROU-BUC-S0011	ROU-BUC	yes	MK778340	yes	EcoRI_Idx_13	RAD-PCR_2_Idx_7
SVN-NOV-S0001	SVN-NOV	yes	MK778341	yes	EcoRI_Idx_4	RAD-PCR_2_Idx_5
SVN-NOV-S0002	SVN-NOV	yes	MK778342	yes	EcoRI_Idx_2	RAD-PCR_2_Idx_8
SVN-NOV-S0004	SVN-NOV	yes	MK778343	yes	EcoRI_Idx_1	RAD-PCR_2_Idx_1
SVN-NOV-S0005	SVN-NOV	yes	MK778344	yes	EcoRI_Idx_10	RAD-PCR_2_Idx_4
SVN-NOV-S0006	SVN-NOV	yes	MK778345	yes	EcoRI_Idx_1	RAD-PCR_2_Idx_6
SVN-NOV-S0007	SVN-NOV	yes	MK778346	yes	EcoRI_Idx_19	RAD-PCR_2_Idx_10
SVN-NOV-S0008	SVN-NOV	yes	MK778347	yes	EcoRI_Idx_2	RAD-PCR_2_Idx_6
SVN-NOV-S0009	SVN-NOV	yes	MK778348	yes	EcoRI_Idx_13	RAD-PCR_2_Idx_12
SVN-NOV-S0011	SVN-NOV	yes	MK778349	yes	EcoRI_Idx_2	RAD-PCR_2_Idx_2
SVN-NOV-S0012	SVN-NOV	yes	MK778350	yes	EcoRI_Idx_23	RAD-PCR_2_Idx_11
USA-DOY-R1078	USA-DOY	yes	MK778351	yes	EcoRI_Idx_31	RAD-PCR_2_Idx_4
USA-HE1-R1076	USA-HE1	yes	MK778352	yes	EcoRI_Idx_13	RAD-PCR_2_Idx_1
USA-HE2-R1079	USA-HE2	yes	MK778353	no	n.a.	n.a.
USA-PAX-S0001	USA-PAX	yes	MK778354	yes	EcoRI_Idx_22	RAD-PCR_2_Idx_12
USA-PAX-S0002	USA-PAX	yes	MK778355	yes	EcoRI_Idx_15	RAD-PCR_2_Idx_7
USA-PIC-R1073	USA-PIC	yes	MK778356	no	n.a.	n.a.
USA-PIC-R1074	USA-PIC	yes	MK778357	yes	EcoRI_Idx_2	RAD-PCR_2_Idx_12
USA-PIC-R1075	USA-PIC	yes	MK778358	yes	EcoRI_Idx_8	RAD-PCR_2_Idx_6
USA-SHA-R1071	USA-SHA	yes	MK778359	yes	EcoRI_Idx_6	RAD-PCR_2_Idx_1
USA-SHA-R1072	USA-SHA	yes	MK778360	yes	EcoRI_Idx_18	RAD-PCR_2_Idx_6
USA-TAK-S0001	USA-TAK	yes	MK778361	yes	EcoRI_Idx_9	RAD-PCR_2_Idx_6
USA-TAK-S0002	USA-TAK	yes	MK778362	yes	EcoRI_Idx_13	RAD-PCR_2_Idx_10

8.3.4. Supplementary figures

Figure S8.2

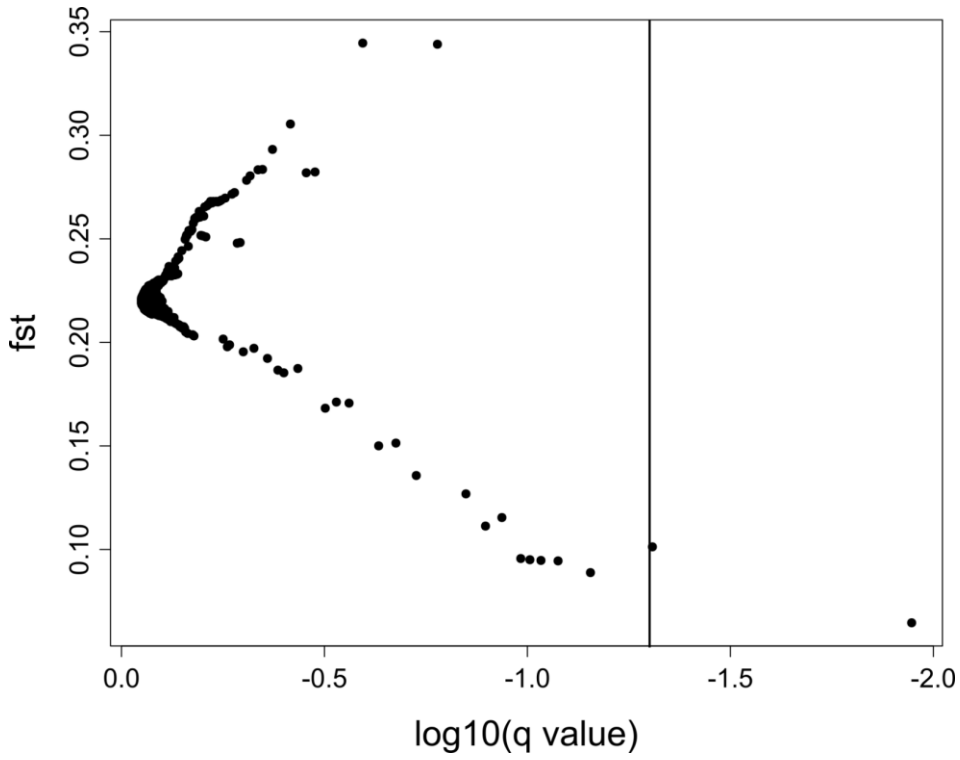
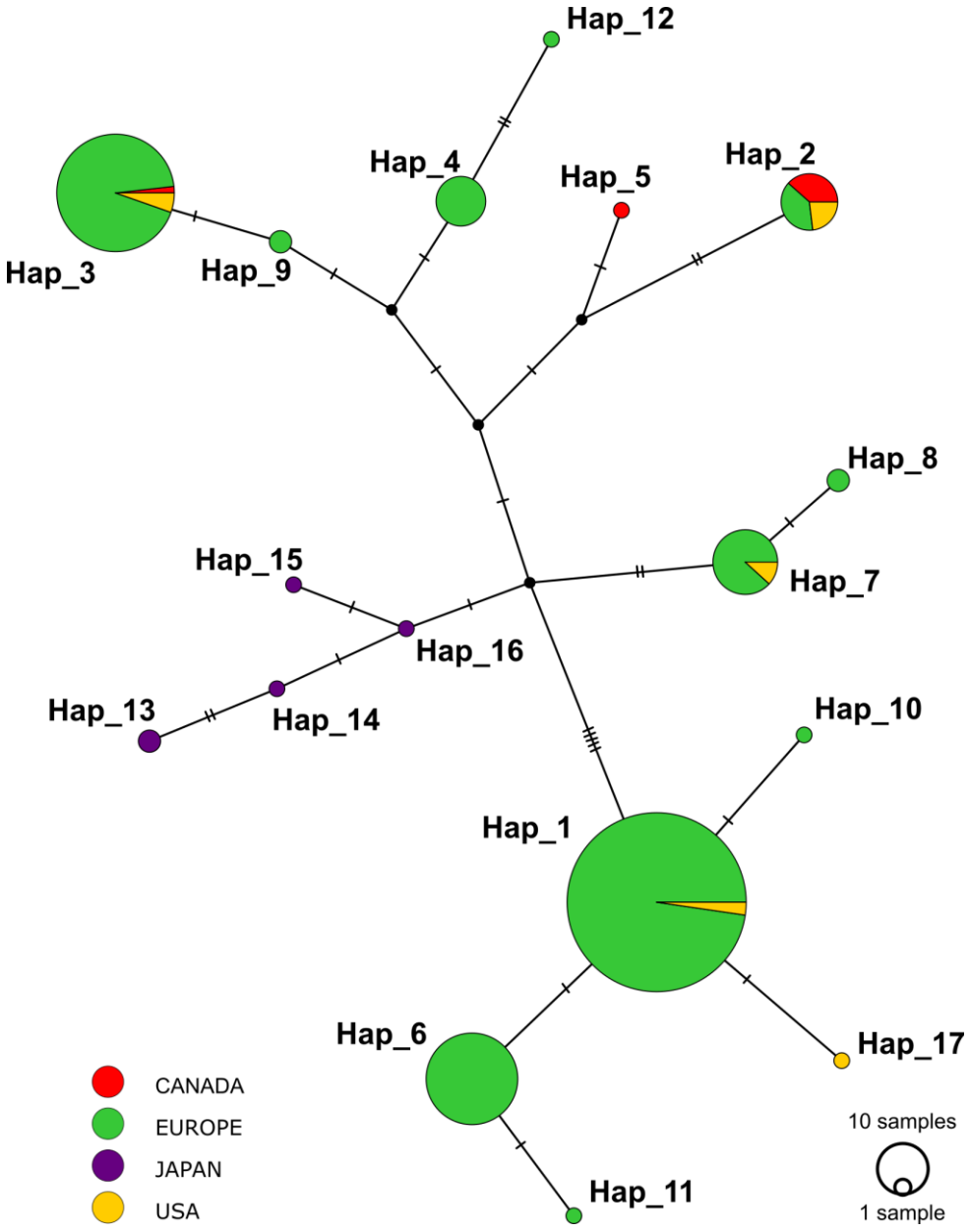


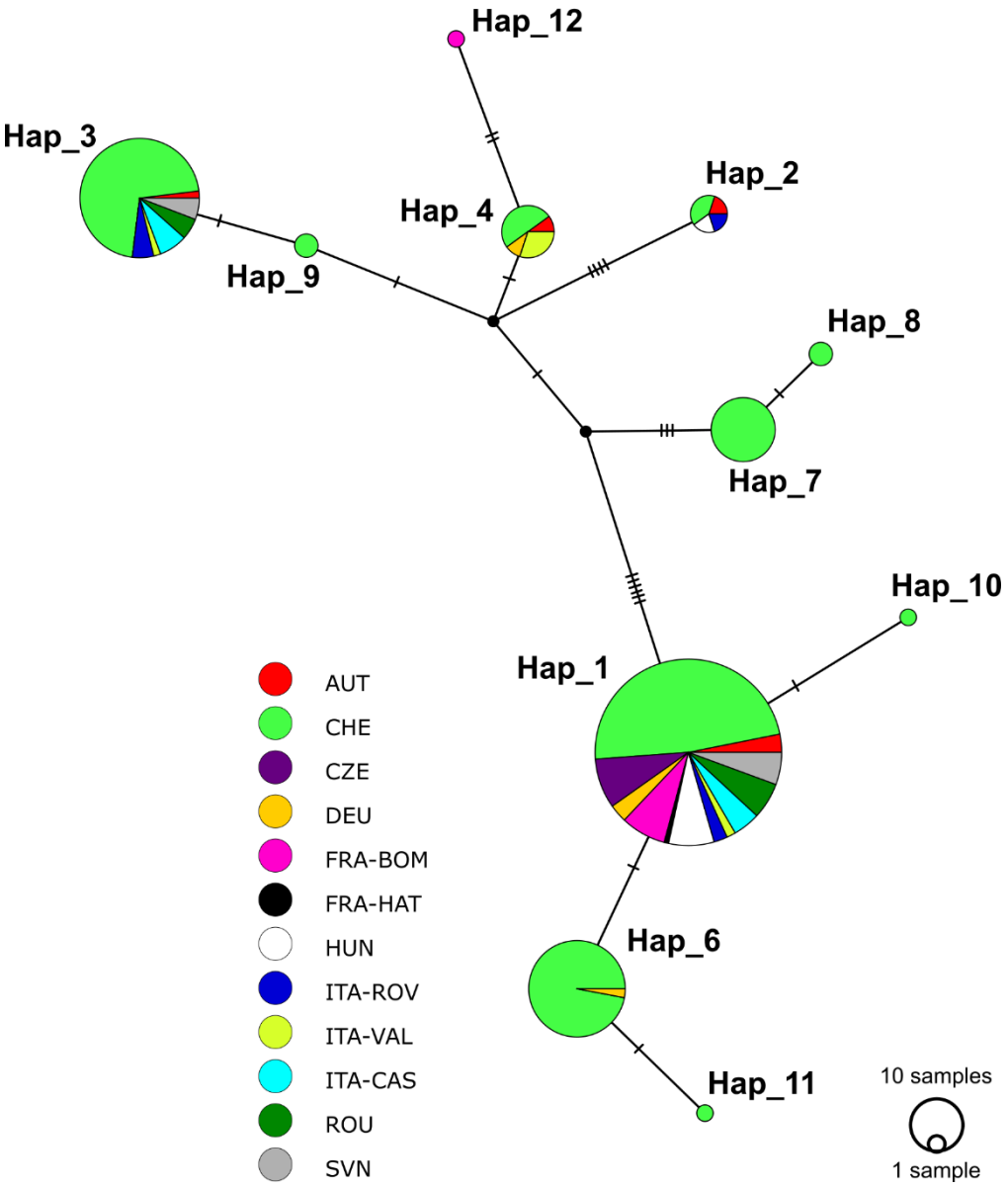
Figure S8.2. Plot of a Bayescan 2.1 analysis scanning all polymorphic loci of the ddRAD set. Plotting F_{ST} against log10 of the posterior odds (q-value) revealed two outlier markers under selection that were removed from the dataset.

Figure S8.3

a


Hap_12
Hap_4
Hap_5
Hap_2
Hap_3
Hap_9
Hap_8
Hap_15
Hap_7
Hap_16
Hap_13
Hap_14
Hap_10
Hap_1
Hap_6
Hap_17
Hap_11
CANADA
EUROPE
JAPAN
USA
10 samples
1 sample

b


Hap_12
Hap_3
Hap_2
Hap_4
Hap_9
Hap_8
Hap_7
Hap_10
Hap_1
Hap_6
Hap_11
AUT
CHE
CZE
DEU
FRA-BOM
FRA-HAT
HUN
ITA-ROV
ITA-VAL
ITA-CAS
ROU
SVN
10 samples
1 sample

c

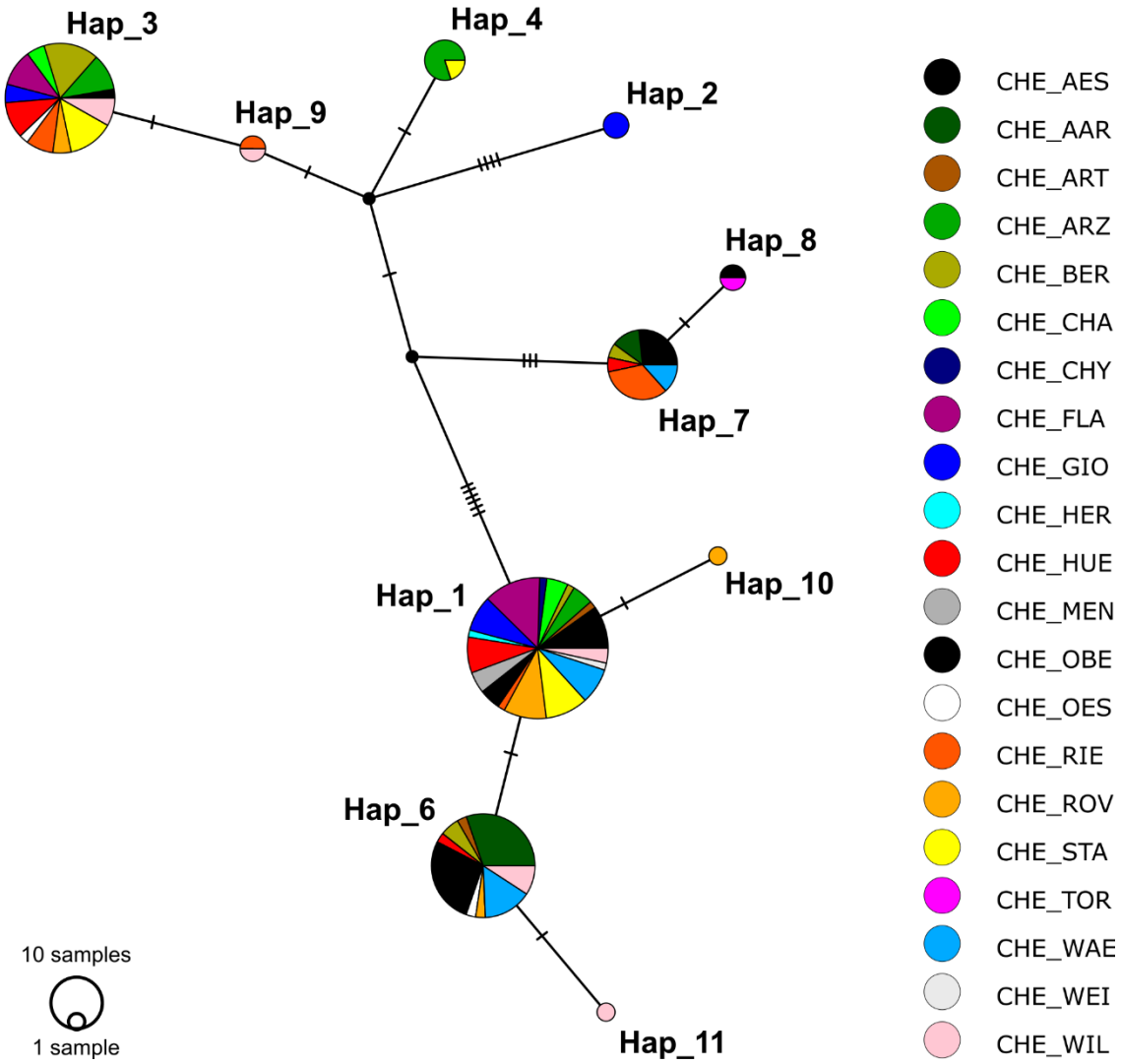
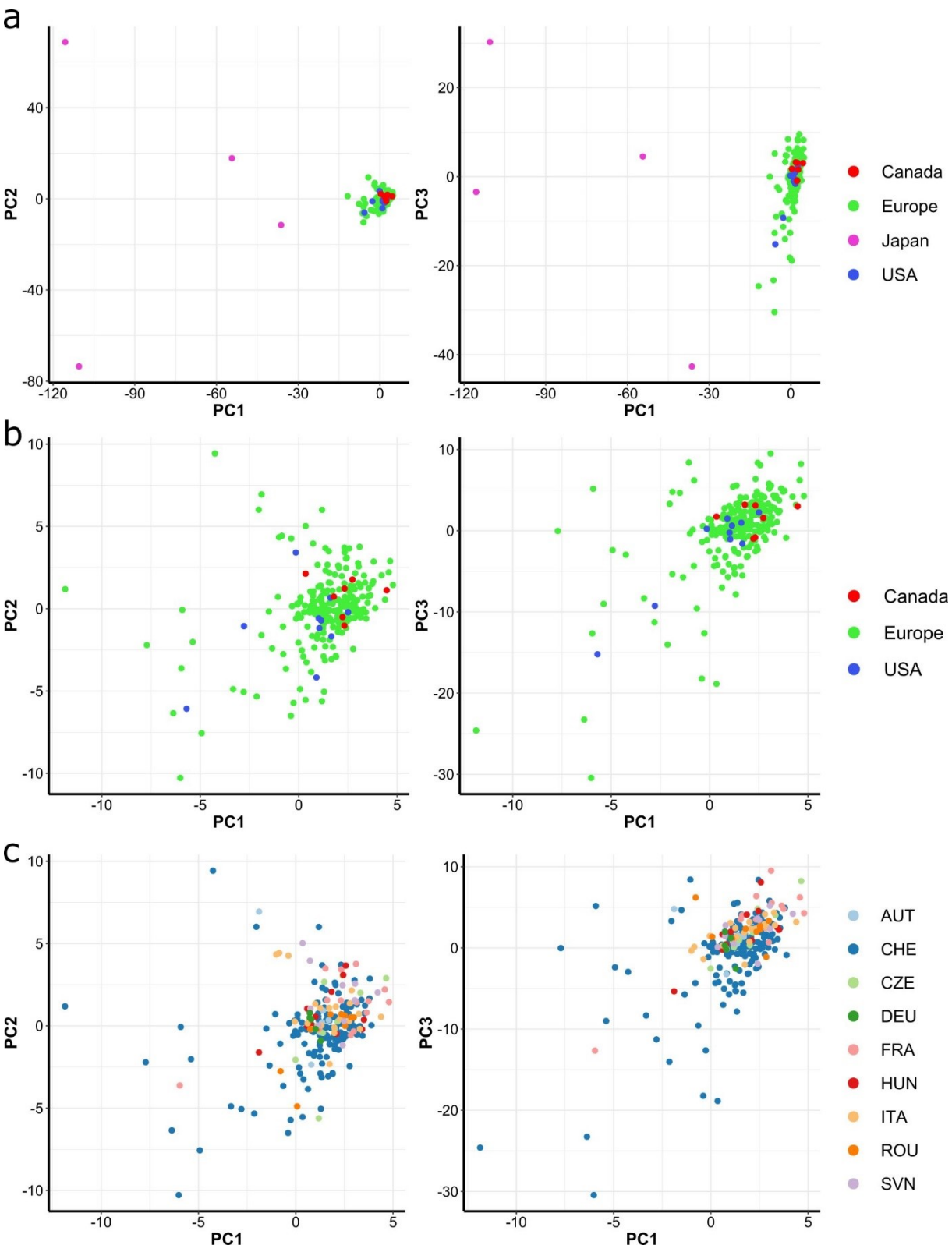


Figure S8.3. TCS haplogroup network of a 573 bp mitochondrial CO1 gene fragment on **a** Global scale, **b** European scale and **c** Swiss scale. Haplotypes are shown as circles, which are proportional to their frequencies.

Figure S8.4.


a
PC2
PC1
PC3
PC1
Canada
Europe
Japan
USA
b
PC2
PC1
PC3
PC1
Canada
Europe
USA
c
PC2
PC1
PC3
PC1
AUT
CHE
CZE
DEU
FRA
HUN
ITA
ROU
SVN

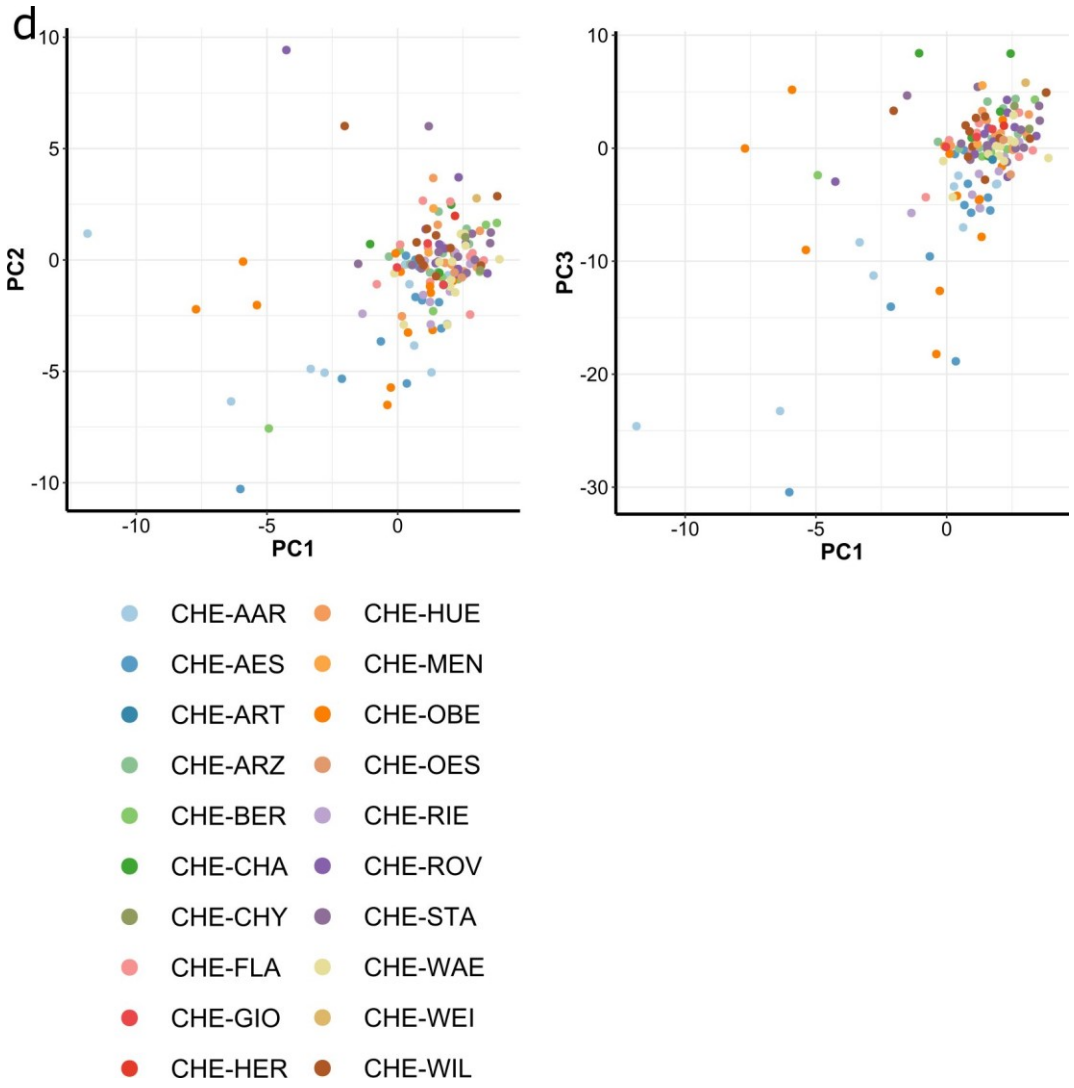


Figure S8.4. Principal component analysis (PCA) analysis plots based on individual ddRAD genotypes visualised on **a** global scale, **b** global scale without Japanese specimens, **c** European scale and **d** Swiss scale. PC1 explains 9.64%, PC2 3.67%, and PC1 2.72% of total variation.

Figure S8.5

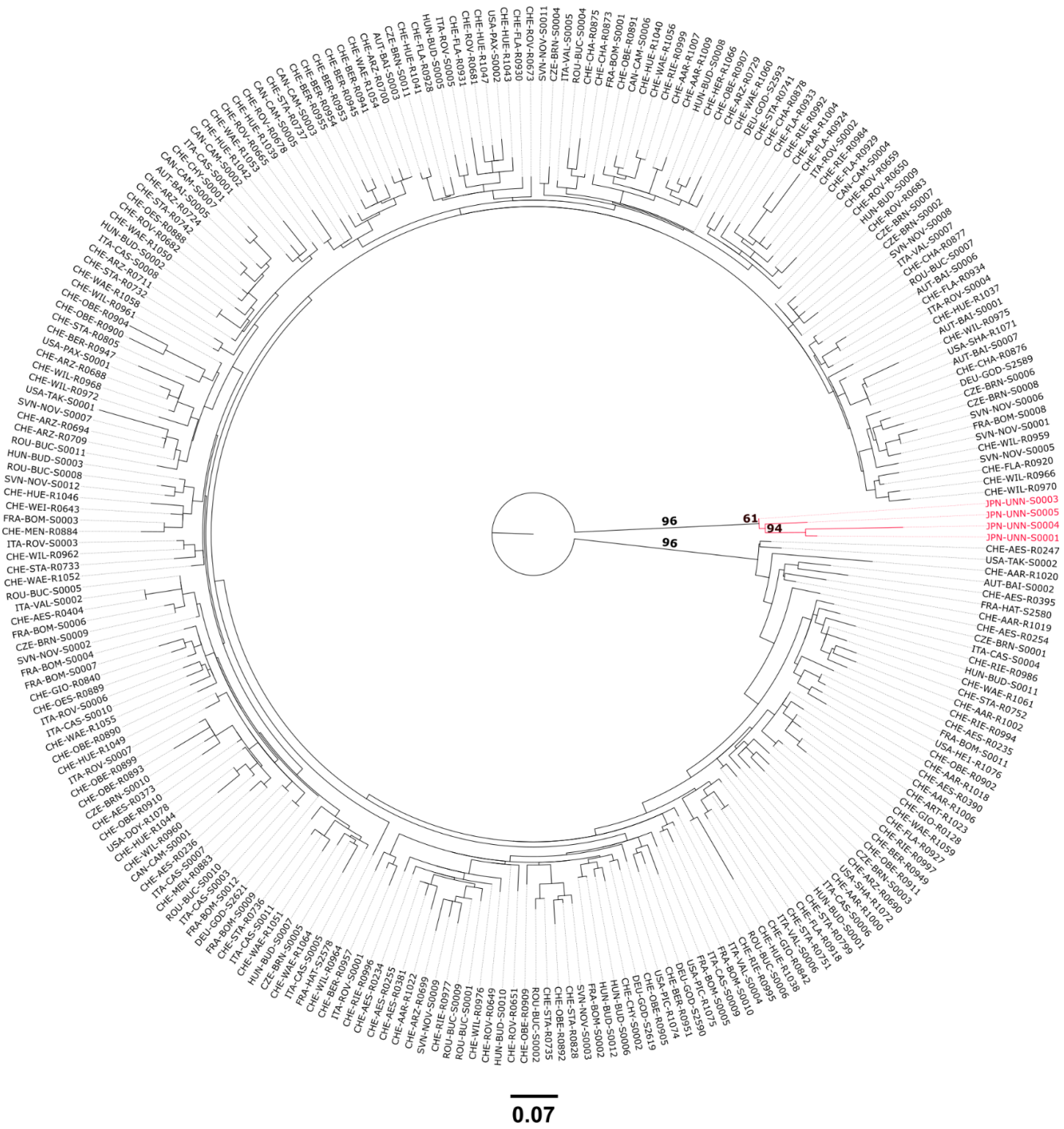


Figure S8.5. Maximum likelihood tree based on the ddRAD dataset generated by RAXML. Best topology was assessed by validating 1000 bootstrap replicates. Numbers represent bootstrap support values higher than 50. Scale bar indicates number of amino acid changes per site. Colours represent geographic origins of specimens: Black, Europe and North America; red, Japan.

Figure S8.6

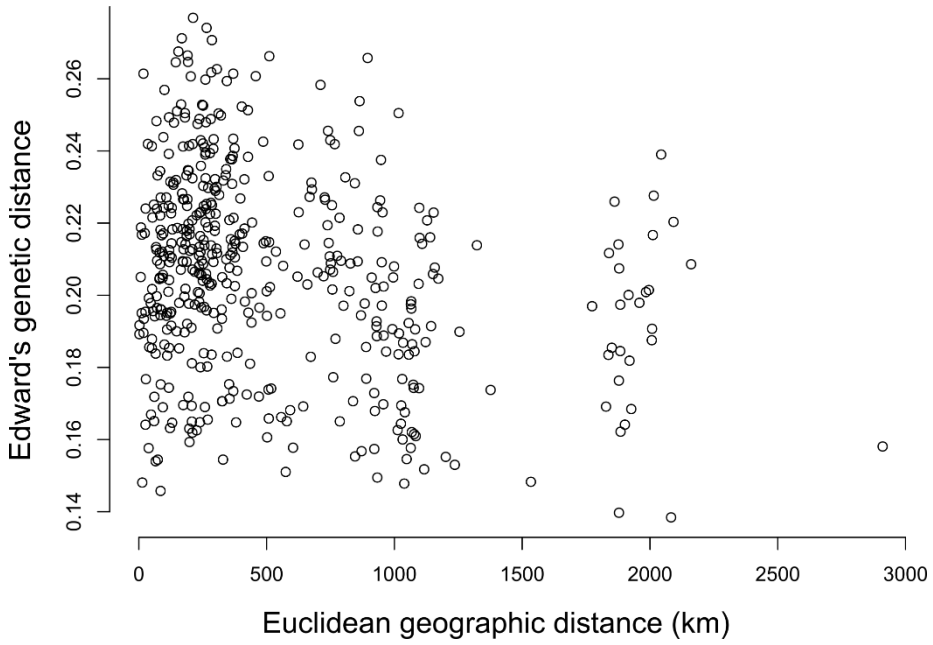


Figure S8.6. Results of an isolation by distance (IBD) analysis of European specimens based on the ddRAD dataset. Correlation between Edward's genetic distances and Euclidean geographic distances between populations were assessed using Mantel test implemented in the R-package Adegenet, Mantel R=-0.261, p-value=0.960.

8.3.5. Supplementary tables

Table S8.4. Occurrence reports of *O. ishidae* in Europe.

Country	Year	Reference
Italy	1998	(Guglielmino, 2005)
Switzerland	2000	(Günthart et al., 2004)
Germany	2002	(Nickel, 2010)
Slovenia	2002	(Seljak, 2004)
Czech Republic	2004	(Malenovsky and Lauterer, 2010)
Austria	2007	(Nickel, 2010)
Belgium	2008	(Anonymous, 2015)
France	2009	(Mifsud et al., 2010)
The Netherland	2009	(den Bieman and Klink, 2015)
Hungary	2010	(Koczor et al., 2013)
Spain	2012	(Anonymous, 2015)
Slovakia	2012	(Anonymous, 2015)
United Kingdom	2011	(Anonymous, 2015)
Poland	2014	(Klejdysz et al., 2017)
Romania	2016	(Chireceanu et al., 2017)

References

Anonymous (2015) *Orientus ishidae*: a potential phytoplasma vector spreading in the EPPO region. https://gd.eppo.int/reporting/article-4763. Accessed: 14 February 2019.

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den Bieman C, Klink Rv (2015) A considerable increase of the Dutch cicadellid fauna with fifteen species (Hemiptera: Auchenorrhyncha: Cicadellidae). Entomol Berichten 75:211-226.

Guglielmino A (2005) Observations on the genus *Orientus* (Rhynchota Cicadomorpha Cicadellidae) and description of a new species: *O. amurensis* n. sp. from Russia (Amur Region and Maritime Territory) and China (Liaoning Province). Marburger Entomol Publik 3:99-110.

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Nickel H (2010) First addendum to the Leafhoppers and Planthoppers of Germany. Cicadina 11:107-122.

Seljak G (2004) Contribution to the knowledge of planthoppers and leafhoppers of Slovenia (Hemiptera: Auchenorrhyncha). Acta Entomol Sloven 12:189-216.

Table S8.5. Genetic diversity measures of mitochondrial CO1 sequences.

ID	N_{COI}	N_{PS}	N_{HAP}	H_{DIV}	H_{DIV} (SD)	π	π (SD)	K	K (VAR)
AUT-BAI	7	13	4	0.714	0.181	0.010	0.002	5.619	2.617
CAN-CAM	7	7	3	0.524	0.209	0.004	0.002	2.381	0.581
CHE-AAR	12	10	2	0.300	0.147	0.005	0.003	3.030	0.470
CHE-AES	11	12	3	0.618	0.104	0.009	0.001	5.345	1.405
CHE-ART	2	1	2	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
CHE-ARZ	11	10	3	0.727	0.068	0.008	0.002	4.582	1.066
CHE-BER	10	13	4	0.644	0.152	0.009	0.002	5.356	1.573
CHE-CHA	5	9	2	0.600	0.175	0.009	0.003	5.400	3.697
CHE-CHY	1	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
CHE-FLA	12	9	2	0.485	0.106	0.008	0.002	4.364	0.886
CHE-GIO	9	12	3	0.667	0.132	0.010	0.002	5.667	1.970
CHE-HER	1	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
CHE-HUE	11	13	4	0.709	0.099	0.010	0.001	5.527	1.493
CHE-MEN	3	0	1	0.000	0.000	0.000	0.000	0.000	0.000
CHE-OBE	13	11	3	0.500	0.136	0.003	0.002	2.000	0.216
CHE-OES	2	10	2	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
CHE-RIE	10	12	4	0.711	0.117	0.008	0.002	4.400	1.106
CHE-ROV	10	11	4	0.644	0.152	0.006	0.002	3.600	0.777
CHE-STA	12	10	3	0.621	0.087	0.009	0.009	5.045	1.147
CHE-TOR	1	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
CHE-WAE	12	10	3	0.682	0.079	0.006	0.002	3.258	0.532
CHE-WEI	1	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
CHE-WIL	10	11	5	0.844	0.080	0.010	0.001	5.467	1.633
CZE-BRN	11	0	1	0.000	0.000	0.000	0.000	0.000	0.000
DEU-GOD	6	9	3	0.600	0.215	0.005	0.003	3.000	1.033
FRA-BOM	11	10	2	0.182	0.144	0.003	0.003	1.818	0.224
FRA-HAT	1	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
HUN-BUD	11	9	2	0.182	0.144	0.003	0.002	1.636	0.189
ITA-CAS	10	9	2	0.533	0.095	0.008	0.001	4.800	1.292
ITA-ROV	7	12	3	0.714	0.127	0.010	0.002	6.000	2.952
ITA-VAL	6	10	3	0.733	0.155	0.009	0.002	5.000	5.000
JPN-UNN	5	4	4	0.900	0.161	0.004	0.001	2.200	0.762
ROU-BUC	11	9	2	0.436	0.133	0.007	0.002	3.927	0.812
SVN-NOV	10	9	2	0.467	0.132	0.007	0.000	4.200	4.200
USA-DOY	1	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
USA-HE1	1	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
USA-HE2	1	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
USA-PAX	2	0	1	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
USA-PIC	3	6	2	0.667	0.314	0.007	0.003	4.000	4.190
USA-SHA	2	1	2	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
USA-TAK	2	1	0	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*

ID, Population identifier; N_{COI}, Number of CO1 sequences; N_{PS}, number of polymorphic sites, N_{HAP}, number of haplotype; H_{DIV}, haplotype diversity; H_{DIV} (SD), standard deviation of H_{DIV}; π, nucleotide diversity Pi; π (SD), standard deviation of π; K, number of nucleotide differences; *K (VAR)*, sampling variance of *K*.

Table S8.6. Frequency and haplogroup assignment of mitochondrial CO1 haplotypes. **a** Frequencies of haplotypes and haplogroup assignment. **b** Haplogroup frequencies.

a

H	N	FREQ	H_{GROUP}
Hap_1	130	47.45	A
Hap_2	13	4.74	D
Hap_3	56	20.44	F
Hap_4	10	3.65	E
Hap_5	1	0.36	D
Hap_6	34	12.41	A
Hap_7	17	6.12	D
Hap_8	2	0.72	D
Hap_9	2	0.72	F
Hap_10	1	0.36	A
Hap_11	1	0.36	A
Hap_12	1	0.36	E
Hap_13	2	0.72	C
Hap_14	1	0.36	C
Hap_15	1	0.36	C
Hap_16	1	0.36	C
Hap_17	1	0.36	A

b

H_{GROUP}	N	FREQ
A	167	60.95
B	19	6.93
C	5	1.82
D	14	5.11
E	11	4.01
F	58	21.17

H, haplotype; *N*, number of specimens; *FREQ*, frequency; H_{GROUP}, haplogroup.

Table S8.7. Pairwise F_{ST} estimates for populations with sample size N>3 based on the ddRAD dataset. Significant values are shaded in grey.

	AUT-BAI	CAN-CAM	CHE-AAR	CHE-AES	CHE-ARZ	CHE-BER	CHE-CHA	CHE-FLA	CHE-HUE	CHE-OBE	CHE-RIE	CHE-ROV	CHE-STA	CHE-WAE	CHE-WIL	CZE-BRN	DEU-GOD	FRA-BOM	HUN-BUD	ITA-CAS	ITA-ROV	ITA-VAL	JPN-UNN	ROU-BUC	SVN-NOV
AUT-BAI	-																								
CAN-CAM	0.109	-																							
CHE-AAR	0.077	0.14	-																						
CHE-AES	0.137	-0.209	-0.004	-																					
CHE-ARZ	0.164	0.008	0.013	0.116	-																				
CHE-BER	0.133	0.084	0.171	0.188	0.010	-																			
CHE-CHA	0.158	0.076	0.198	0.244	0.089	0.164	-																		
CHE-FLA	-0.050	0.049	0.108	0.116	-0.056	0.114	0.140	-																	
CHE-HUE	-0.039	0.036	0.136	0.170	-0.057	0.122	0.128	0.021	-																
CHE-OBE	0.036	0.128	0.031	0.090	-0.058	0.209	0.154	0.082	0.080	-															
CHE-RIE	0.021	0.066	0.028	0.064	0.052	0.098	0.176	0.050	0.056	0.070	-														
CHE-ROV	-0.060	0.030	0.107	0.139	-0.121	0.095	0.092	0.026	0.014	0.114	0.029	-													
CHE-STA	0.049	0.074	0.090	0.098	0.021	0.088	0.084	0.034	0.045	0.078	0.042	-0.052	-												
CHE-WAE	-0.005	0.011	0.053	0.077	-0.108	0.104	0.119	0.060	0.049	0.042	0.046	0.041	0.004	-											
CHE-WIL	-0.002	0.028	0.102	0.189	0.002	0.146	0.108	-0.013	-0.010	0.048	0.058	0.014	0.025	-0.029	-										
CZE-BRN	-0.107	0.071	0.120	0.115	-0.070	0.097	0.130	0.021	0.007	0.085	-0.013	0.024	0.031	0.038	0.002	-									
DEU-GOD	0.073	-0.069	-0.037	0.083	0.198	0.039	0.132	-0.082	-0.074	0.059	0.004	-0.190	0.007	-0.026	0.032	-0.137	-								
FRA-BOM	-0.131	0.042	0.190	0.195	-0.154	0.144	0.072	0.117	0.078	0.141	0.066	0.063	0.002	0.036	0.011	0.018	-0.166	-							
HUN-BUD	0.063	0.089	0.118	0.174	0.013	0.105	0.096	0.073	0.051	0.113	0.070	-0.036	0.014	-0.003	0.045	-0.057	-0.023	-0.092	-						
ITA-CAS	0.113	0.030	0.086	0.200	0.130	0.118	0.168	-0.002	0.004	0.092	0.124	-0.008	0.048	0.006	0.061	-0.005	0.163	-0.062	-0.031	-					
ITA-ROV	-0.095	-0.018	0.103	0.138	-0.127	0.06	0.061	0.038	0.000	0.059	0.029	-0.021	-0.050	-0.030	0.015	-0.039	-0.131	0.025	-0.035	-0.100	-				
ITA-VAL	0.040	0.022	0.010	0.095	0.066	0.086	0.032	-0.010	-0.006	0.080	0.021	-0.024	0.051	-0.013	0.007	-0.049	-0.027	-0.072	0.020	0.057	-0.045	-			
JPN-UNN	0.571	0.618	0.522	0.559	0.614	0.589	0.594	0.544	0.570	0.508	0.585	0.543	0.579	0.555	0.568	0.543	0.525	0.561	0.562	0.567	0.554	0.522	-		
ROU-BUC	-0.073	0.090	0.138	0.158	-0.126	0.099	0.059	0.080	0.057	0.112	0.050	0.013	0.010	0.039	0.042	0.026	-0.118	0.056	0.004	0.031	0.047	-0.048	0.565	-	
SVN-NOV	-0.066	0.003	0.155	0.131	-0.134	0.138	0.103	0.088	0.031	0.074	0.064	0.001	-0.006	0.021	-0.008	0.019	-0.088	0.010	-0.067	0.010	-0.009	-0.042	0.590	0.016	-