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Article

First DNA barcoding of a new alien species *Glycaspis brimblecombei* Moore, 1964 (Hemiptera: Aphalaridae) in Croatia with a distribution note

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Abstract
A new alien psyllid *Glycaspis brimblecombei*, native in Australia, was first discovered in Croatia in 2020. Its presence was also confirmed in the Botanical Garden of the Institute for Marine and Coastal Research (University of Dubrovnik) on the island of Lokrum, using DNA barcoding. Distribution and spreading of this alien species were noted as across the other continents so in Europe. The pest is considered as a threat to forestry, paper industry and could affect ornamental values of the eucalyptus species. It is important to continue with following research on this species because of the potential spreading and ascendant estimation.

Key words: *Eucalyptus* spp., red gum lerp psyllid, *COI* gene fragment, Lokrum, botanical garden.

Introduction

Insects are one of the most numerous invaders worldwide (Kenis & Branco 2010; Brockerhoff & Liebhold 2017) and make 87% of the non-native arthropods introduced in Europe (Roques 2010). Alien insect species appear to have a large increase rate in Europe in recent decades (Roques 2010, Kenis & Branco 2010; Smith *et al*. 2018). Non-native species, except for causing significant economic losses, may have impacts on natural processes in ecosystem functions (Simberloff 2001, 2011; Wardle & Peltzer 2017; Liebhold *et al*. 2017).

The red gum lerp psyllid *Glycaspis brimblecombei* (Moore, 1964) is a sap-sucking insect that specifically feeds on *Eucalyptus*. Native in Australia, this invasive species has spread to several countries and become a major *Eucalyptus* pest worldwide (Cuello *et al*. 2018). Outside its natural habitat, it was first recorded in the U.S.A. in 1998 (Brennan *et al.* 1999); thereafter, it was detected in Mexico (Cibrián 2002),

Chile (Sandoval & Rothmann 2002), Brazil (Wilcken *et al.* 2003), Argentina (Bouvet *et al.* 2005), Ecuador (Onore & Gara 2007), Venezuela (Rosales *et al.* 2008) and Peru (Burckhardt *et al*. 2008). It invaded Mediterranean Basin and its first record was in the Iberian Peninsula (Portugal and Spain) in 2007 (Hurtado & Reina 2008; Valente & Hoodkinson 2009; Prieto-Lillo *et al.* 2009). Following spread occurred in Italy (Laudonia & Garonna 2010), Morocco (Maatouf & Lumaret 2012), France (Cocquempot *et al.* 2012), Greece (Bella & Rapisarda 2013), Montenegro (Malumphy *et al*. 2013), Algeria (Reguia & Peris-Felipo 2013), Tunisia (Ben Attia & Rapisarda 2014), Israel (Spodek *et al*. 2015), Syria (Kaf and Mouhamed 2015), Turkey (Karaca *et al*. 2015), Gibraltar (Malumphy *et al*. 2020) and Croatia (Pintar *et al*. 2020). *Glycaspis brimblecombei* was also confirmed across Africa (Holis 2004; Sookar *et al*. 2013; Bush *et al*. 2016; Ndlela *et al*. 2018; Yirgu & Anjulo 2019; Wondafrash *et al*. 2020). It has been projected that there is a great potential to colonize in latitude between 20° and 40° in both hemispheres (de Queiroz *et al.* 2013).

Glycaspis brimblecombei infestations are recognizable by the presence of conical white shields known as lerps, each inhabited by a single nymph and attached to the foliage covering the surface of the leaves. These lerps are built from faecal excretions of the nymphs that harden upon exposure to the air (White 1972). The damage is caused by nymphs and adults sucking sap from the leaves which, in the case of heavy infestations, can lead to reduced growth and tree vigour (Collett 2000).

DNA barcoding represents a tool which can prevent incorrect identifications, most caused by phenotypic plasticity and genetic variability, to anticipate the presence of morphologically cryptic taxa and to enable identification of different life stages (Hebert *et al*. 2003). The standardized ~ 658 bp long fragment of the mitochondrial cytochrome oxidase gene subunit I (COI) was selected as a base because of its high interspecific and low intraspecific variability (Ratnasingham & Hebert 2013). In the present study, DNA barcoding was applied for the first time on alien species *G. brimblecombei* collected in Croatia.

Material and methods

Sampling and identification

During July and August 2020, samples were collected in the Botanical Garden of the Institute for Marine and Coastal Research (University of Dubrovnik) on the island of Lokrum, Dubrovnik-Neretva County (Figure 1B and 1C). The species was found in several life stages on various *Eucalyptus* species (Figure 2) and it was stored in absolute ethanol for further analyzes. *Eucalyptus* species, on which G. *brimblecombei* has been observed, were: *E. andrewsii, E. blakelyi, E. bridgesiana, E. camaldulensis, E. cephalocarpa, E. globulus* subsp. *bicostata, E. leucoxylon, E. macarthurii, E. mannifera, E. melliodora, E. nicholii, E. rudis, E. scoparia, E. tereticornis* and *E. viminalis*. Before DNA extraction, collected specimens were examined under binocular and photographed using Dino-Lite Digital Microscope Camera in the Laboratory of Tree Pathology at Faculty of Forestry and Wood Technology (Figure 3).

DNA extraction, gene amplification, sequencing and sequence analyzes

Total genomic DNA was extracted from whole specimens (4 larvae and 1 adult), using QIAamp DNA Micro Kit (Qiagen, Germany) according to the manufacturer's specifications, eluted in 50 µl of elution buffer and stored in a freezer until PCR amplification. The universal primer pair LCO-1490/HCO-2198 (Folmer *et al.* 1994) was used for amplification and sequencing of the mtCOI-5P gene fragment. As there are no publicly available sequences of mtCOI-5P gene fragment of species *G. brimblecombei*, for comparison with obtained DNA sequences, another set of primers was used: C1-J-2441 (alias Dick: 5'-CCTACAGGAATTAAAATTTTTAGATGATTAGC-3') and TL2-N-3014 (alias Pat: 5'-TCCATTGCACTAATCTGCCATATTA-3') (Simon *et al*. 1994) for amplification of the downstream gene fragment (mtCOI-3P) of approximately 520 bp in length. PCR amplifications were performed in a 20 µl reaction mixtures (for both primers sets), consisting 1 x DreamTaq™ reaction buffer with 2 mM $MgCl_2$ (Thermo Scientific), 0.2 mM dNTPs (Qiagen), 0.4 µM/µL of each primer, 0.75 U/µL of DreamTaq polymerase (Thermo Scientific) and 1 µl of eluted DNA. Thermal profiling for PCR consisted of: initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. PCR products were purified using Exonuclease I (0.05 U/µL), FastAP Thermosensitive Alkaline Phosphatase (0.025 U/µL) enzymatic system (Thermo Fischer Scientific). Sequencing of PCR products was performed by Macrogen Inc. (Amsterdam, Netherlands). Sequences were checked, edited and assembled from both directions in

program BioEdit v.7.2.5. (Hall 1999). Obtained sequences were submitted to BOLD (CROHP001-20 - CROHP005-20) and GenBank databases (MZ402612 - MZ402616 for mtCOI-5P gene fragment and MZ402609 - MZ402610 for mtCOI-3P gene fragment). BOLD IDs and GenBank accession number used in the phylogenetic analysis are given in Table 1.

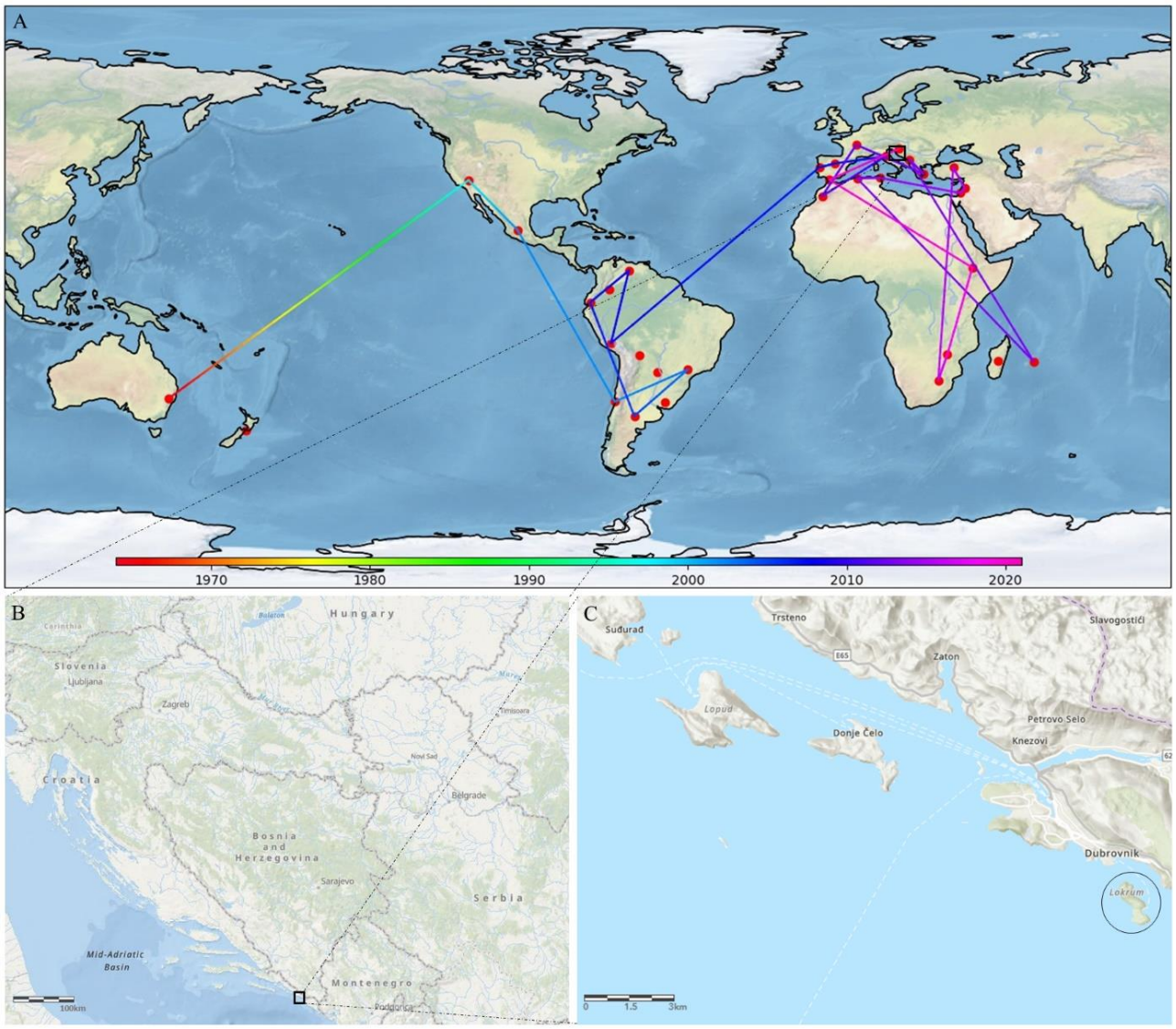

Figure 1. Distribution of species *Glycaspis brimblecombei*. **A.** Spread of the species *G. brimblecombei*. The coloration of the lines shows the spread of the species in the temporal scale (excluding localities for which the year of occurrence of the species is not known), **B.** and **C.** Location of sampling – Botanical Garden of the Institute for Marine and Coastal Research on the island of Lokrum.

DNA barcode region obtained from *G. brimblecombei*, Lokrum was compared with all DNA barcode sequences available in BOLD using BOLD's Identification Engine. As the sequences of DNA barcode region of *G. brimblecombei* are not publicly available, the phylogenetic analysis was conducted on the sequences of mtCOI-3P gene fragments retrieved from BOLD and GenBank. Sequences were collapsed to haplotypes in FaBox online toolbox (Villesen 2007) and uncorrected p-distances between haplotypes were calculated using MEGA-X (Kumar *et al*. 2018). Neighbor-joining (NJ) tree based on the K2P distance model was calculated in the same program and the robustness of the clades was assessed through 2000 bootstrap replicates.

A distribution map (Figure 1A) was drawn in PYTHON, package Cartopy (version 0.11.2., Met Office 2010-2015) with use of data from Natural Earth. Figure 1B and 1C were downloaded and edited from portal GeoAdriatic (Hydrographic Institute of the Republic of Croatia).

Figure 2. Symptomatic *Eucalyptus* spp. at sampling location: **A.** visible defoliation of top branches **B.** and **C.** leaves covered with lerps.

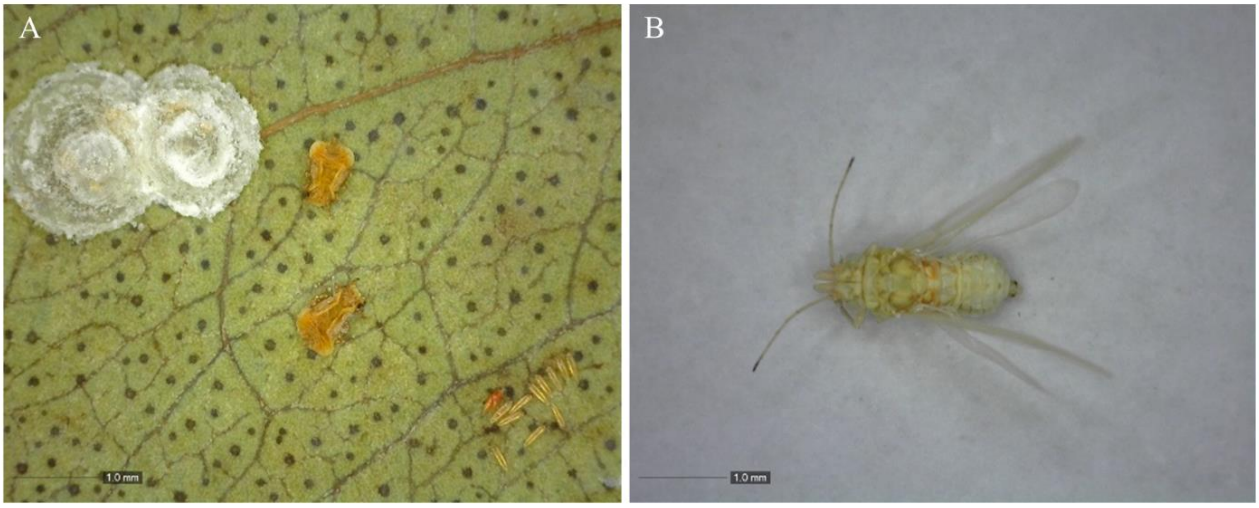

Figure 3. Developmental stages of *Glycaspis brimblecombei*: **A.** lerps, nymphs and egg cluster, **B.** adult (scale: 1 mm).

Table 1. Specimens and sequences used in the phylogenetic analysis for mtCOI-3P gene fragment. Sequenced samples from Croatia are marked in bold.

Species name	Country	BOLD sequence ID	GenBank accession number
Glycaspis brimblecombei	**Croatia**	**CROHP001-20**	**MZ402609**
		CROHP002-20	**MZ402610**
	Australia	GBMHH27415-19	KY923938
	Australia	GBMHH27416-19	KY923939
	Portugal	GBMHH27465-19	KY923988
Glycaspis sp.	Australia	GBMHH27422-19	KY923945
Glycaspis sp.2.	Australia	GBMHH23983-19	KU568252
Glycaspis sp.2.	Australia	GBMHH27475-19	KY923988
	Australia	GBMHH27477-19	KY924000

Results and discussion

Sequence of *G. brimblecombei* from Lokrum, Croatia was aligned with all sequences of *G. brimblecombei* species from BOLD (Figure 4) with the addition of one outgroup sequence *Calophya* sp. n. 1 (GBMHH20055-19, KM234305). All sequences from Croatia were collapsed into a single haplotype (homology of sequences = 100%) and the alignment of mtCOI-3P sequences was 520 bp long. Based on mtCOI-3P haplotype, BOLD Identification System identified specimens from Croatia as *G. brimblecombei* having an identical DNA barcode (100% sequence similarity) to the Portuguese specimen of the same species.

The compared intraspecific uncorrected pairwise genetic distances (p-distances) calculated for sequences among *G. brimblecombei* specimens based on mtCOI-3P gene fragment (0,2 – 0,4%) indicate genetic uniformity of species *G. brimblecombei* from different parts of the world. For the specimen *G. brimblecombei* from Australia (GBMHH27422-19) p-distance is 11,5% potentially suggesting erroneous determination. Interspecific uncorrected pairwise genetic distances (p-distances) among *G. brimblecombei*, Lokrum and *Glycaspis* species which are not determined to the species level is 16,2 – 18,4%.

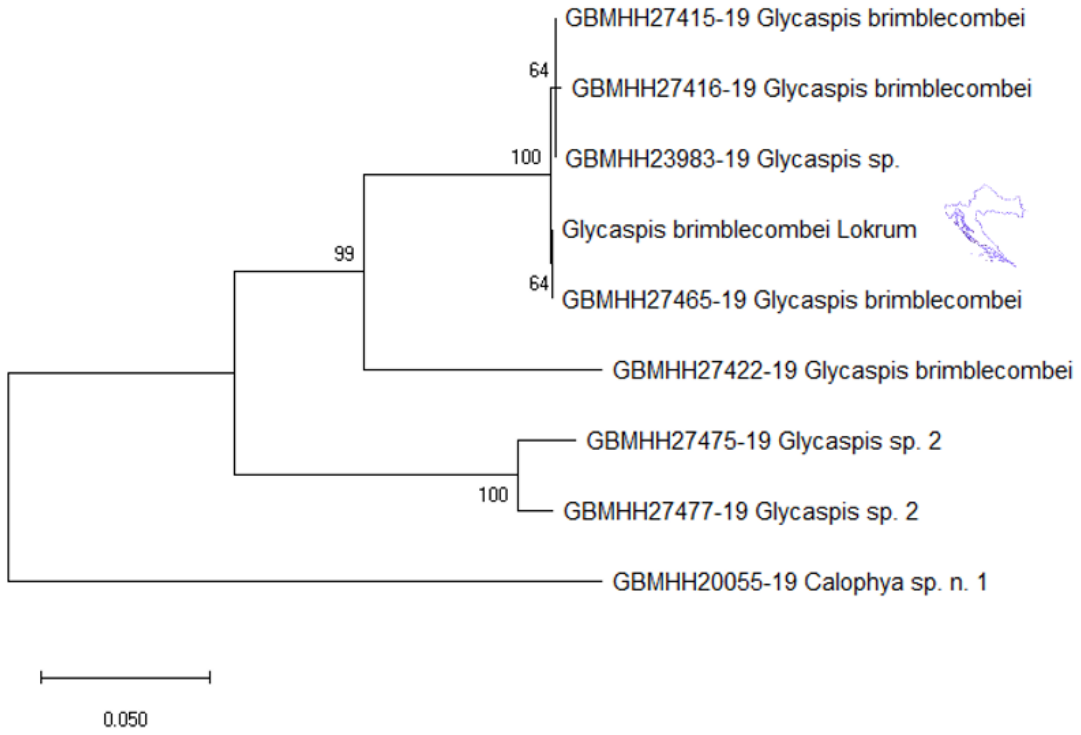

Figure 4. Neighbor-joining phylogenetic tree constructed by sequences of the mtCOI-3P of *Glycaspis brimblecombei* Lokrum, Croatia of *Glycaspis* species from BOLD and GenBank databases, based on Kimura-2-parameter distance model. Numbers at nodes are NJ bootstrap support values calculated from 2000 bootstrap replicates.

In conclusion, the finding of *G. brimblecombei* Moore, 1964 in Croatia was confirmed by molecular approach. Since the introduction of the concept of DNA barcoding, this method is involved in numerous projects which are already resulted in formation of comprehensive DNA barcode reference library and provide a base of knowledge primarily for biodiversity, taxonomy, phylogeography and phylogenetics (Elías-Gutiérrez *et al*. 2008; Pauls *et al*. 2010; Cárdenas *et al*. 2013; Kučinić *et al*. 2013, 2019, 2020; Dela Cruz *et al*. 2016; Franjević *et al*. 2016; Guo *et al*. 2016; Santos *et al*. 2016; De Barros Machado *et al*. 2017; Tyagi *et al*. 2017; Brehm *et al*. 2019; Huemer *et al*. 2020).

In studies on psyllid species (Hemiptera, Psylloidea) molecular methods were used mostly for study of vertical transmission of communities of bacteria and psyllids (Morrow *et al*. 2017). Study on species *G. brimblecombei* in Brazil revealed large genetic diversity among specimens from Brazil and specimens from Australia, region of origin (Santos *et al*. 2020). To date (accessed on November 25, 2020), data of 14 specimens of species *G. brimblecombei* from Australia, Portugal and Brazil are included in the Barcode of Life Data System (Ratnasingham & Hebert 2007).

The Mediterranean Basin is considered one of the main hotspots for biodiversity, climatic changes and, consequently, for biological invasions of alien organisms (Ponti *et al*. 2013). As *Eucalyptus* spp. are planted as ornamental solitary tree or in alleys in Mediterranean parts of Croatia, the alien species *G. brimblecombei* could present a threat to ornamental values as in urban areas so in special forest vegetation reserves such as Lokrum Island and its botanical garden. Future researches on *G. brimblecombei* should be focused on estimation of its damage and spreading across Croatia and establishing the presence of its natural enemy (parasitoid) since it is confirmed in Italy where it's probably accidentally introduced together with the host (Laudonia *et al*. 2014), as had already happened in Brazil (Berti-Filho *et al*. 2003), Spain (Perez-Otero *et al*. 2011) and Morocco (Bami 2011).

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