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TAKING EUROPE BY STORM: A FIRST INSIGHT IN THE INTRODUCTION AND EXPANSION OF *Dryocosmus kuriphilus* IN CENTRAL EUROPE BY mtDNA

EUROPA OSVOJENA U TRENU: PRVI UVID U PRODOR I ŠIRENJE KESTENOVE OSE ŠIŠKARICE (*Dryocosmus kuriphilus*) U SREDIŠNJOJ EUROPI PUTEM MITOHONDRIJSKE DNK

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Summary

The chestnut gall wasp, *Dryocosmus kuriphilus*, poses one of the latest additions to the long list of exotic pests that invaded Europe. After its introduction in Italy, chestnut gall wasp expanded rapidly in Europe in a very short period of time. Analysis of a polymorphic mtDNA locus from nine European populations verified the Chinese origin of this invasion. Moreover, the results revealed traces of a severe bottleneck during the phase of introduction that reduced considerably the genetic diversity. It was also shown that the rapid and successful post-introductory expansion was accomplished by a single mtDNA haplotype that has spread in three European countries. The paradox of successful establishment despite the absence of genetic diversity could be attributed to the synergistic effect of several agents. Uniparental propagation and general-purpose genotypes, lack of natural enemies and human-mediated transport seem to have facilitated the invasion and subsequent expansion of *D. kuriphilus* in Europe.

KEY WORDS: chestnut gall wasp, invasive pest, post-introductory expansion, population bottleneck

Introduction
Uvod

Invasive species are considered a major threat to biodiversity (Williamson 1996; Walker & Steffen 1997; Kamata & Gottschalk 2007; Scalera 2010) as well as an increasing economic concern (Vitousek et al. 1996; Wilcove et al. 1998; Pimentel et al. 2000). It is now widely recognized that biological invasions are favored by dispersal events linked to human activities (Lockwood et al. 2005), which provide vectors for dispersion, and could promote the introduction in new areas and accelerate their expansion rate (Shigesada et al. 1995). Invasive organisms include organisms of both flora (Chandler et al. 2008; Zhang et al. 2010) and fauna (Roman & Darling 2007; Zachos et al. 2007). Insects particularly, with their (usually) minute size, that helps them remain undetected, in concert with their flying ability can very easily be introduced to new areas, becoming invasive

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pests (Grapputo et al. 2005; Rubinoff et al. 2010; Bray et al. 2011). Europe has suffered several invasions of exotic insect species, some of which have become serious pests that cause considerable damage (Matson et al. 2007; DAISIE 2009; Kenis et al. 2009, Blank et al. 2010).

One of the latest insects that invaded Europe is *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera; Cynipidae), which is a global pest of chestnut (*Castanea)*. The chestnut gall wasp is a univoltine species, that reproduces parthenogenetically laying eggs in the buds of *Castanea* spp. during summer. A direct consequence of this *modus vivendi* is that even a single female is capable of establishing a population (Nohara 1956; Askew 1984). In addition to that, eggs and first instars that grow in chestnut buds, render this species practically undetected (Panzavolta et al. 2011). By attacking vegetative buds *D. kuriphilus* disrupts twig growth and reduces fruiting that can ultimately lead to losses of up to 70 % (Dixon et al. 1986; EPPO 2005). Being under this stress, chestnut trees exhibit a gradual decline in biomass coupled with a decline in the aesthetic and the amenity value (EFSA 2010).

Even though it is native to China, *D. kuriphilus* was first recorded in Japan in 1941 (Murakami 1980). This was the first invasion of the chestnut gall wasp, that occurred rapidly (in 25 years) (Oho & Umeya 1975) and had serious impact on chestnut production. In the years that followed, *D. kuriphilus* expanded into Korea and South Korea in 37 years (Aebi et al. 2006). Not late afterwards, it was introduced in America (Payne et al. 1975), putting at risk the American chestnut production (Cooper & Rieske 2007). In Europe, chestnut gall wasp was first reported from Piedmont in noth-west Italy in 2002 (Brussino et al. 2002), introduced with chestnut cultivars from China (Quacchia et al. 2008). Since then, *D. kuriphilus* expanded rapidly taking actually Europe by storm. By 2005 it has spread in Sicily, south eastern France and Corsica (Aebi et al. 2006) as well as in Slovenia (Knapič et al. 2010). A year later it was found in Hungary (Csóka et al., 2009) and Switzerland (Forster et al. 2009), arriving in Netherlands and Croatia (Matošević et al. 2010) in 2010.

As genetic polymorphisms accumulate over time, they enclose valuable information regarding the invasion history of a species, a reason that explains the wide application of molecular techniques in studies of invasive species (Fitzpatrick et al. 2012). Among different markers available, mitochondrial DNA markers are broadly selected in such investigations (Cognato et al. 2005; Bray et al. 2011), as they are expected to evolve rapidly, something that adds to their sensitivity (Avise 1994). Here, we employ a mtDNA marker, to study the genetic variability of *D. kuriphilus* for the first time after its introduction in Europe. Comparing our data, with previous results from China reveals not only the origin of the source population, but also the expanding potential of this pest species. Though preliminary, our research offers a first insight into the on-going invasion of Europe by D. *kuriphilus*, providing information that can be used to plan and improve management strategies.

Materials and Methods
Materijali i metode

Following the expanding distribution of *D: kuriphilus* in Europe, 96 specimens belonging to 9 different locations were sampled in two successive years (namely 2011 and

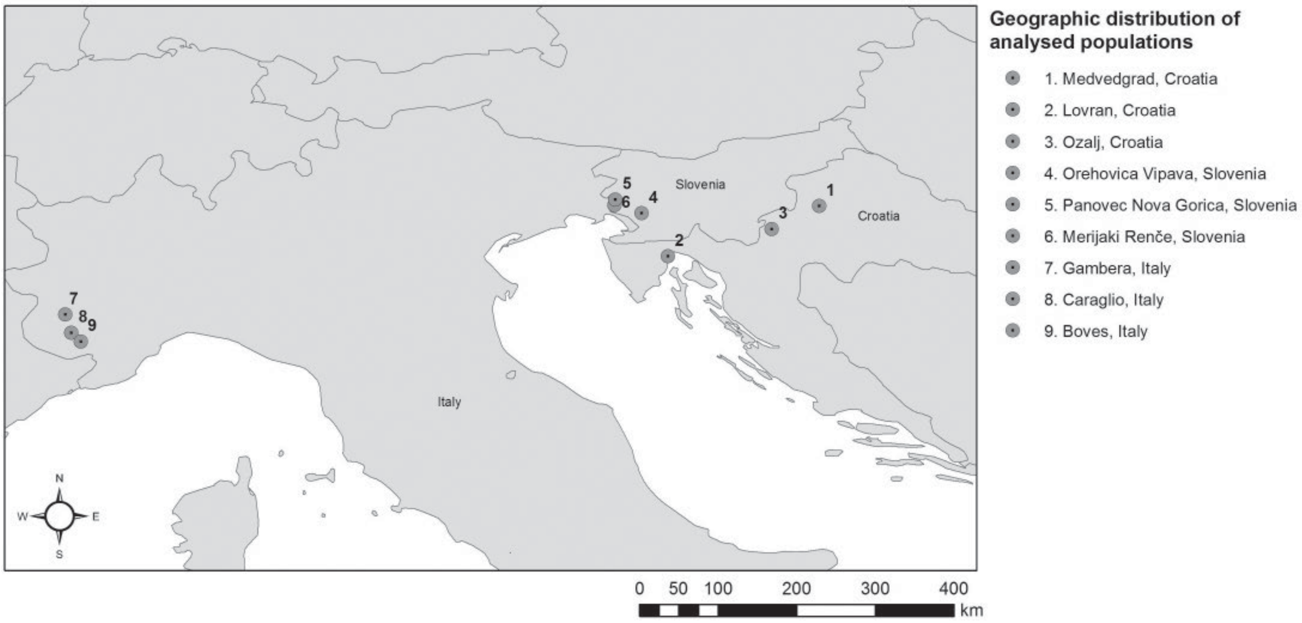

Figure 1. Geographic distribution of the populations analysed
Slika 1 Geografska rasprostranjenost analiziranih populacija

	Central Eur. HT	Italian HT (DQ28681)	FS (JF411595)	LBX (JF411598)	LZ (JF411594)	CC (JF411597)	SY (JF411596)
Central Eur. HT		0	0.16%	0.16%	0	2.08%	2.24%
Italian HT (DQ28681)	0		0.16%	0.16%	0	2.08%	2.24%
FS (JF411595)	1	1		0.32%	0.16%	2.24%	2.40%
LBX (JF411598)	1	1	2		0.16%	2.24%	2.40%
LZ (JF411594)	0	0	1	1		2.08%	2.24%
CC (JF411597)	13	13	14	14	13		0.16%
SY (JF411596)	14	14	15	15	14	1	

Table 1 Number of individuals per population analysed and haplotype assignment.
Tablica 1: Broj jedinki po analiziranoj populaciji i raspodjela haplotipova

2012) across 3 countries (Croatia, Slovenia and Italy; Figure 1 and Table 1). Specimens were immediately put into eppendorfs with pure ethanol (100 % EtOH) so that DNA would remain intact. DNA extraction was carried out with GenElute Kit (Sigma), following the protocol of the manufacturer. Amplification of an approximately 700bp locus from the mitochondrial Cytochrome Oxidase I (*Cox1*) gene was carried out with primers LCO2198 (5'-ggtc aac aaa tca taa aga tat tgg-3') and HCOI1490 (5'-taa act tca ggg tga cca aaa aat-3'), designed for the analysis of *D. kuriphilus* by Ács et al. (2007). Reactions were 25μl in volume, containing 0.2μl of MyTaq™ (BioLine, GmBH, Germany), 5μl of the provided 5x MyTaq™ Red Reaction Buffer (BioLine, GmBH, Germany), 0.75 μl of each primer (20μM), 8 μl of DNA extract and ddH$_2$O to the volume of 25μl. PCR conditions included an initial denaturation step at 94 °C for 3 minutes, followed by 40 cycles at 94 °C for 30 s, 48 °C for 30 s and 72 °C for 90s with a final extension step that lasted 7 minutes at 72 °C. PCR products were then purified with PureLink™ PCR Purification Kit (Invitrogen) and sent for sequencing with both primers at a commercially available service (Chemia SA, Larissa – Greece). Sequences were initially visualized by ChromasLite® (Technelysium Pty Ltd.) and aligned manually by eye. The haplotype retrieved was deposited in NCBI Genebank under the Ascession Number (KF308606). ClustalX (Thomson et al., 1997) was used to create an alignment of the haplotype with reference sequences acquired from NCBI GeneBank with Accession Numbers (JF411594, JF411595, JF411596, JF411597, JF411598 and DQ286810). Nucleotide diversity and pairwise nucleotide differences were calculated with MEGA version 5 (Tamura et al. 2011).

Results
Rezultati

A polymorphic locus of about 700bp in length from mtDNA's Cytochrome Oxidase One (*Cox1*) gene was amplified from 96 individuals. Alignment of a 623bp long locus revealed that every individual exhibits the same mitochondrial haplotype. Careful examination of the sequences together with comparison of the two loci obtained by both primers, verified the occurrence of only one haplotype in all populations from Italy, Slovenia and Croatia. When this unique European haplotype was further aligned with the only reference sequences of *D. kuriphilus* deposited in NCBI Genebank, it matched perfectly with the Italian haplotype under the Accession Number DQ286810 (Ács et al. 2007).

	FS (JF411595)	LBX (JF411598)	European HT	Italian HT (DQ28681)	LZ (JF411594)	CC (JF411597)	SY (JF411596)
FS (JF411595)		0.32%	0.16%	0.16%	0.16%	2.24%	2.4%
LBX (JF411598)	2		0.16%	0.16%	0.16%	2.24%	2.4%
European HT	1	1		0	0	2.08%	2.24%
Italian HT (DQ28681)	1	1	0		0	2.08%	2.24%
LZ (JF411594)	1	1	0	0		2.08%	2.24%
CC (JF411597)	14	14	13	13	13		0.16%
SY (JF411596)	15	15	14	14	14	1	

Table 2 Number of nucleotide differences (below diagonal) and % percentage nucleotide diversity among Chinese mtDNA haplotypes (Lu et al., 2012), Italian mtDNA haplotype (Ács et al., 2007) and the haplotype of the current analysis (central European HT). mtDNA haplotypes are described with names given by the authors and the NCBI GenBank Accession Number is provided in parenthesis.
Tablica 2: Broj razlika u nukleotidima (ispod dijagonale) i postotak nukleotidne raznolikosti između kineskih (Lu et al., 2012), talijanskih (Ács et al., 2007) i haplotipova mitohondrijske DNK analiziranih ovim istraživanjem (srednjeeuropski haplotipovi). Haplotipovi mitohondrijske DNK opisani su imenima koja su im dali autori uz pristupni broj iz NCBI GenBank (NCBI GenBank Accession Number) koji se nalazi u zagradama.

However, it was identical also with haplotype LZ (Accession Number JF411594) that was detected in China (Lu et al. 2011). Very close genetically were the Chinese haplotypes LBX and FS (Accession Numbers JF411598 and JF411595, respectively), whereas haplotypes CC and SY (Accession Numbers JF411597 and JF411596, respectively) are more distantly related (Lu et al. 2011) (Table 2).

Discussion
Rasprava

Molecular techniques are being now increasingly used in studies of invasive species, with applications that range from assessing genetic variation (Roderick 1996; Sakai et al., 001), identifying population origin (Cognato et al. 2005; Grapputo et al. 2005) or even developing control strategies (Conord et al. 2006; Fu et al. 2010). The chestnut gall wasp, *D. kuriphilus* was introduced in north-west Italy with chestnut cultivars from China in 2002 (Quachia et al. 2008). This Chinese origin of *D. kuriphilus* was verified by the molecular data. All the Italian individuals exhibited a single haplotype that was identical with a Chinese one that was already deposited in NCBI GenBank under the Accession Number JF411594 (Lu 2011), confirming that the founder population had a Chinese origin. Interestingly this Chinese haplotype was the most widespread one as it was found in several provinces of China (Henan, Jiangxi, Hunan and Fujian) (Lu et al., 2011). As this haplotype was retrieved once again in Italy (Ács et al. 2007), it can be easily deduced that *D. kuriphilus* expanded in the three investigated countries after a single introduction event, with a founding population of limited size or at least low genetic diversity. Should chestnut gall wasp have invaded Italy with multiple introductions, then genetic diversity would have been restored at least to the levels of native populations if not higher (Kolbe et al. 2004; Zalewski et al. 2011), giving rise to an admixture of haplotypes. However, not only was a single haplotype retrieved among the Italian individuals, but this haplotype was the same with the one retrieved five years before (Ács et al. 2007). Conclusively, this population bottleneck during the initial colonization of *D. kuriphilus* could only be indicative of a single colonization event that was additionally performed by few individuals (small founder population) (Russell et al. 2009). In any other case, multiple introductions and/or large founder numbers should soon have increased genetic variation (Stepien et al. 2005).

However, populations founded under such unfavorable conditions (single introduction of a small number of individuals) suffer so evidently from low genetic variation that might even lose their adaptive ability (Frankham & Ralls 1998; Saccheri et al. 1998; Duglosch & Parker 2008), putting ultimately their very existence in risk (Nei et al. 1975; Courchamp et al. 1999). One of the most efficient ways to overcome the defects of low population levels coupled with low genetic diversity is parthenogenesis. Uniparental propagation is highly adaptive as it permits small populations to quickly expand and efficiently exploit ephemeral resources (Niemela & Mattson 1996; Davis 2009). The advantage of parthenogenetic reproduction has been demonstrated in other organisms (e.g. *Potamopyrgus antipodarum*) that have been introduced succesfully, overcoming low genetic diversity at the phase of invasion through uniparental propagation (Dybdahl & Drown 2011). It thus seems logical to assume that the parthenogenetically reproducing chestnut gall wasp took advantage of the same biological feature (parthenogenesis) to compensate for the severe bottleneck it suffered during invasion and establishing the initial population in Italy.

In general, the post-introductory expansion of an invasive species is determined either by evolutionary adaptation or plasticity across a range of novel environments. Evolutionary adaptation facilitates the range expansion in invasive species particularly when there is sufficient genetic variation within the invading population for natural selection to act on (Garcia-Ramos & Rodriguez 2002; Maron et al. 2004; Bossdorf et al. 2005). This remark however, leads to a logical paradox: how can bottlenecked invasive species be successful when sometimes bottlenecked native species are indeed susceptible to extinction (Frankham 2005; Roman & Darling 2007)? Loss of genetic diversity during colonization has been observed in several invading insects (Kourti 2002; Bonizzoni et al. 2004; Grapputo et al. 2005; Tung et al. 2009; Rubinoff et al. 2010; Bray et al. 2011); nevertheless, in none of these cases was the invasion really interrupted. Based on our findings, *D. kuriphilus* can well be included among the above mentioned examples of succesful expansion despite the initial low genetic diversity during introduction. In less than ten years, chestnut gallwasp has dispersed from the point of introduction in Italy to several European countries (France, Slovenia, Switzerland, Croatia, Netherlands, Austria, Hungary), covering a distance of about 1200km to the south (Sicily) and 750 km to the east (Croatia). To our surprise, this expansion was accomplished by a unique mtDNA haplotype, identical to the one that invaded Europe in the first place. It thus seems possible that the paradoxically succesful post-introductory expansion of *D. kuriphilus* could possibly be attributed to phenotype plasticity that maintained high fitness over a broad range of environments (Richards et al. 2006). The concept that general-purpose genotypes are positively associated with invasiveness has been expressed before (Baker 1965). Conclusively, the fact that the single haplotype that expanded in the countries investigated is the very same with the most abundant one in China (Lu et al. 2011) argues for the hypothesis of a general-purpose genotype adapted to various environmental conditions.

Even though destructive insect invasions can generally progress rapidly (Johnson et al. 2006; Muirhead et al. 2006), *D. kuriphilus* became established and expanded in a noteworthy short time. The rate at which this pest dispersed indicates that species' movement is not accomplished only by its own flight (active or even wind-assisted flight), but is mostly human-mediated. This is observed as long distance dispersal when *D. kuriphilus* is trasported over long distances with infested plant material. Consequently, the assessment of the mean rate of spread of the invasion front at only 8 km/year (EFSA 2010), underestimates the actual expanding ability of the pest. As eggs and larvae grow in dormant buds (winter shoot and twigs for grafting and overwintering plants) inspection of plant material against infestation of *D. kuriphilus* is sometimes very difficult to be done properly. This fact has to be taken into careful consideration when planning an efficient strategy to limit the further expansion of *D. kuriphilus* into other European countries, as until now this pest seems to be very difficult to confine.

A severe population bottleneck during invasion does not seem to have compromised the ability of *D. kuriphilus* to establish and expand rapidly in Europe. Uniparental propagation, phenotype plasticity together with human mediated long-distance dispersal, lack of natural enemies and availibility of host plant have determined the invasion of the chestnut gall wasp in the European continent. Application of molecular methods to elucidate the genetic variation of this invasive pest has provided information not only on the introduction but most importantly its dispersal. Beside that, the general remarks on the on-going invasion of the chestnut gallwasp can be used to understand the mechanisms by which parthenogenetic organisms can become serious pests even without sufficient genetic variation.

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Sažetak

Invazivne vrste smatraju se jednom od najvećih prijetnji bioraznolikosti te su postale i ozbiljan ekonomski problem. Biološkim invazijama pogoduju ljudske aktivnosti koje su značajan vektor pri unosu i širenju novih vrsta. Jedna od nedavno unesenih vrsta kukaca u Europu je kestenova osa šiškarica (*Dryocosmus kuriphilus*) (Hymenoptera; Cynipidae) koji je globalni štetnik pitomog kestena (*Castanea*). Napada pupove, čime sprječava rast izbojaka i listova te smanjuje plodonošenje, što konačno može dovesti do smanjenja uroda od čak 70 %.

Kako se genetski polimorfizmi akumuliraju tijekom vremena, sadrže vrijedne informacije o povijesti invazije neke vrste, što objašnjava uporabu molekularnih tehnika u istraživanju invazivnih vrsta. Među različitim dostupnim markerima, mitohondrijska DNK (mtDNK) se često koristi u takvim istraživanjima. Usporedbom rezultata ovoga istraživanja s dosadašnjim rezultatima iz Kine, željelo se dokazati ne samo porijeklo izvorne populacije, nego i potencijal širenja (ekspanzije) ovoga štetnika u novom staništu. Ovo istraživanje koristi mtDNK markere za proučavanje genetske varijabilnosti kestenove ose šiškarice, prvi puta nakon njezinog unašanja u Europu.

Za potrebe ovog istraživanja uzorkovano je 96 jedinki s 9 različitih lokaliteta u 3 europske zemlje (Slika 1, Tablica 1) u dvije godine (2011. i 2012.). Analiza mitohondrijske DNK napravljena je prema važećim protokolima. Pregledom sekvenci i usporedbom dva lokusa, potvrđena je pojava samo jednog haplotipa u svim populacijama u Italiji, Sloveniji i Hrvatskoj. Taj je haplotip točno odgovarao i jedinom haplotipu pohranjenom

u NCBI Genebank, pod pristupnim brojem (Accession Number JF411594) iz Italije, te je bio i istovjetan s haplotipom LZ (Accession Number JF411594) koji je nađen u Kini.Vrlo su blizi i kineski haplotipovi LBX i FS, dok su haplotipovi CC i SY nešto udaljeniji (Tablica 2).

Kestenova osa šiškarica unesena je u sjeverozapadnu Italiju iz Kine 2002. godine sa sadnicama pitomog kestena. Kinesko porijeklo ovoga štetnika potvrđeno je i genetskim analizama. Sve talijanske jedinke pokazuju jedan haplotip, koji je istovjetan onom kineskom pohranjenom u NCBI GenBank, pod pristupnim brojem JF411594. To potvrđuje da je osnovna populacija unesena u Europu kineskog podrijetla. Zanimljivo je i da je to i najrasprostranjeniji haplotip u Kini, nađen u nekoliko provincija (Henan, Jiangxi, Hunan and Fujian).

Kako je taj haplotip još jednom nađen u Italiji, može se zaključiti da se kestenova osa šiškarica raširila u području istraživanja nakon samo jednog jedinog unosa, iz osnovne populacije ograničene veličine ili niske genetske raznolikosti. Ovo populacijsko usko grlo tijekom prve kolonizacije kestenove ose šiškarice upućuje na samo jedan slučaj unosa s nekoliko jedinki (mala osnivačka populacija). U slučaju višestrukog unosa i/ili velike osnivačke populacije, genetska varijabilnost brzo bi se povećala.

Jedan od najučinkovitijih načina savladavanja mana niskih gustoća populacije spojenih s niskom genetskom raznolikosti je partenogeneza. Aseksualni način razmnožavanja je vrlo prilagodljiv, jer dozvoljava malim populacijama da se brzo šire i učinkovito iskorištavaju ograničene resurse. Zbog toga je logično pretpostaviti da je kestenova osa šiškarica, koja se razmnožava partenogenetski, iskoristila tu biološku osobinu kako bi kompenzirala populacijsko usko grlo tijekom unosa i širenja osnovnih populacija u Italiji. Širenje, nakon unosa, neke invazivne vrste određeno je njezinom evolucijskom prilagodljivosti ili plastičnosti u novom području. Evolucijska prilagodljivost olakšava širenje vrste, posebice kada postoji dovoljno genetske varijabilnosti unutar populacije invazivne vrste, kako bi došlo do procesa prirodne selekcije. No to je i paradoks: kako može invazivna vrsta s populacijskim uskim grlom biti uspješna kada su takve vrste vrlo osjetljive na izumiranje? Gubitak genetske raznolikosti tijekom kolonizacije već je primijećena kod invazivnih vrsta, no niti u jednom slučaju nije došlo do prekida takve invazije (daljnjeg širenja). Na temelju rezultata ovoga istraživanja, kestenova osa šiškarica može se uključiti u takve primjere uspješnih invazija bez obzira na nisku genetsku raznolikost prilikom unosa. Za manje od deset godina ovaj se štetnik proširio od mjesta unosa u Italiji na više europskih zemalja (Francuska,Slovenija, Švicarska, Hrvatska, Nizozemska, Austrija, Mađarska) prevalivši udaljenost od 1200 km prema jugu (Sicilija) i 750 km prema istoku (Hrvatska). Na naše iznenađenje, ova ekspanzija uspješno je obavljena samo s jednim haplotipom mtDNK, istovjetnom onome koji je i unesen u Europu. Činjenica da je haplotip koji se proširio na području istraživanja isti kao i onaj najčešći u Kini, nameće hipotezu o univerzalnom genotipu prilagođenom različitim uvjetima okoliša. Populacijsko usko grlo nije utjecalo na sposobnost kestenove ose šiškarice da se udomaći i munjevito širi Europom. Aseksualno razmnožavanje, fenotipska plastičnost zajedno s brzim širenjem uz pomoć čovjeka, nedostatak prirodnih neprijatelja i široka dostupnost biljke domaćina, odredili su smjer i opseg invazije kestenove ose šiškarice u Europi. Primjena molekularnih metoda za objašnjavanje genetske varijabilnosti ovog invazivnog štetnika dala je informacije ne samo o njegovom unosu, nego i širenju. Rezultati ovoga istraživanja mogu se koristiti kako bi se bolje razumjeli mehanizmi kako partenogentski organizmi mogu postati ozbiljni štetnici i bez dovoljne genetske varijabilnosti.

KLJUČNE RIJEČI: kestenova osa šiškarica, invazivni štetnik, širenje nakon unašanja, populacijsko usko grlo