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Kadoić Balaško, Martina

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Martina Kadoić Balaško

GENOMIC CHANGES ASSOCIATED WITH INSECTICIDE RESISTANCE IN ECONOMICALLY IMPORTANT INSECT PESTS IN CROATIA

INTERNATIONAL DUAL DOCTORATE

Zagreb, 2022

University of Zagreb

Faculty of Agriculture

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Supervisors:
Prof. Renata Bažok, PhD
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Dr Katarina M. Mikac, PhD
Dr Gerrit van den Bergh, PhD

Zagreb, 2022

University of Zagreb
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Martina Kadoić Balaško

PROMJENE GENOMA POVEZANE S RAZVOJEM REZISTENTNOSTI NA INSEKTICIDE U EKONOMSKI VAŽNIH ŠTETNIKA U HRVATSKOJ

MEĐUNARODNI DVOJNI DOKTORAT ZNANOSTI

Mentori:
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I, **Martina Kadoić Balaško**, declare that I have composed solely by myself the thesis titled:

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With my signature I confirm that:

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Zagreb, 16.12.2022.

PhD Candidate signature

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Ja, **Martina Kadoić Balaško**, izjavljujem da sam samostalno izradila doktorski rad pod naslovom:

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Prof. Renata Bažok, Ph.D is full professor at University of Zagreb Faculty of Agriculture. She is a full professor and has been working in higher education for 29 years. She was the coordinator of the undergraduate Plant Protection program and the graduate study program in Plant Medicine. She has been the recipient of three fellowships, including a USDA/ARS, a Cochran, and a Fulbright.

She was involved in two TEMPUS projects and led/coordinated one TEMPUS project. She worked on two USDA/CRO projects and several national scientific projects. She was also the principal investigator in four national scientific projects and one FAO project. She coordinated a structural project jointly funded by the EU and Croatia (IPA 2007/ HR /16IPO/001-040511) and a project on the development of human potential in plant medicine (ESF project). She coordinates an Erasmus capacity building project that is currently developing a PhD study programme in Plant Health. She participated and is currently participating in two ERASMUS + Strategic Partnership projects focused on entrepreneurial skills development and innovative teaching methods for organic agriculture education. She was the principal investigator of two projects funded by the Croatian Science Foundation. Her research focuses on applied entomology, integrated pest management, crop protection and phytopharmacy. Under her supervision, six students have completed their dissertations and she is currently supervising five PhD students.

She is Editor in-chief of the Journal of Plant Protection and a member of the editorial boards of Agriculture by MDPI, Fragmenta Phytomedica by Croatian Plant Protection Society, and Nature. She received a medal from the Faculty of Agriculture at the University of Zagreb in 2014 for her scientific and professional achievements and the National Science Award of the Croatian Parliament - Annual Award for the Transfer of Scientific Results into practise in 2019. Since 1993, she has conducted research in the field of on integrated control of Colorado potato beetle, wireworms, sugar beet pests, oilseed rape pests, western corn rootworm and other maize pests. Her publications include more than 150 peer-reviewed journal articles and more than 180 miscellaneous (Web of Science Index Expanded - SCI -Expanded, 67 articles, total citations: 564, h-index: 14; Scopus, 74 articles, total citations: 637, h-index: 14). List of her publications is available at: https://www.researchgate.net/profile/Renata_Baok/contributions. Her current research interests include integrated pest management (IPM) in field crops (maize, sugar beet, potato) and insect resistance development. The general research focus is on the development of safe, effective, and economical methods IPM and the biological/ecological interactions between insect species and their environment. She is a vice-chair of the initiative COST TOP-AGRI -Network "Towards zero pesticide agriculture: European network for sustainability".

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Darija Lemić is associate professor at the Faculty of Agriculture, Division of Phytomedicine, Department of Agricultural Zoology (318090). She is actively involved in teaching 23 subjects in BS, MS, Specialist and PhD study. Her R&D competences are entomology, integrated pest management and phytomedicine. She is engaged in research of pest population genetics and geometric morphometric methods, integrated pest management, invasive species, phytopharmacy and the application of information technology in teaching and science. She is author or co-author book chapters (3), handbooks (3), 61 scientific papers cited in a1, 32 scientific papers cited in a2 and a3 databases and more than 137 conference papers. She actively participated in the implementation of scientific research projects (12), professional projects (6), teaching projects (4), Citizen Science project (1). She is a member of the Croatian Entomological Society, Croatian Genetic Society, Croatian Plant Protection Society, fellow of Royal Entomological Society, European Society for Evolutionary Biology, International organization for biological control, International Working Group for Ostrinia and other Maize Pests. She is one of the founders and leaders of the Entomology Group, an extracurricular activity at the Faculty of Agriculture. She was additionally trained in entrepreneurial skills programs (3). She is the recipient of numerous awards and recognitions (Silver Medal for Innovation, AgroArca ('22); DIGIAward plaque ('21); Exemplary work and special contribution to the work and promotion in and wellbeing of the Faculty (‘21), Certificate of Fellowship of Royal Entomological Society (’18), State Award for Science (’16), Award for exemplary work and special contribution to the work and promotion and wellbeing of the Faculty (’15).

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Academic qualifications

- Doctor of Philosophy in Population genetics & entomology, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Entomology 2002 - 2006
- B.A.S. in Marine Ecology (Honours 1), University of Canberra, School of Heritage and Natural Resource Management 2000 - 2001
- Bachelor of Applied Science in Ecology and Environmental Science, University of Canberra, School of Heritage and Natural Resource Management 1997 – 1999

Research position

- Senior Lecturer; School of Earth, Atmospheric and Life Sciences Faculty of Science, Medicine and Health

Selection of academic publications

- Gracanin, A., & **Mikac, K. M.** (2022). Camera traps reveal overlap and seasonal variation in the diel activity of arboreal and semi-arboreal mammals. *Mammalian Biology*, 1-15.
- Knipler, M. L., Dowton, M., Clulow, J., Meyer, N., & **Mikac, K. M.** (2022). Genome-wide SNPs detect fine-scale genetic structure in threatened populations of squirrel glider Petaurus norfolcensis. *Conservation Genetics*, 1-18.
- Knipler, M., Dowton, M., & **Mikac, K**. (2022). Limited genetic structure detected in sugar gliders (Petaurus breviceps) using genome-wide SNPs. *Australian Mammalogy*.
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Research overview

She works in the field of applied ecology, with specific focus on threatened species of the NSW South Coast/Illawarra Region. Her research group's focus is spotted tailed quolls and gliding possums (greater glider, sugar glider and squirrel glider). She also works on pest insects that threaten global food production. The insects that I focus on are mainly beetle and moth pests of corn and sugar beet.

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Academic qualifications

- PhD (December 8, 1997) University of Utrecht, The Netherlands.
- MSc (June 27, 1988) University of Utrecht (Major: Sedimentology; Minors: Vertebrate paleontology and Marine Geology
- BSc (May 24, 1982) University of Utrecht (Geology)

Research positions (last 15 years)

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- Australian Research Council Research Fellow (April 1, 2011-December 31, 2016); CAS; SEALS;UOW
- Australian Research Council Research Associate (2008-2011); School of Earth and EnvironmentalSciences, UOW

Selection of academic publications (last 10 years)

- Puspaningrum, M.R., **van den Bergh, G.D**., Chivas, A.R., Setiabudi, E., Kurniawan, I., 2020. Isotopic reconstruction of Proboscidean habitats and diets on Java since the Early Pleistocene: Implications for adaptation and extinction, *Quaternary Science Reviews*, **228**:106007
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Academic reviewer

PlosOne Biology; Integrative Zoology Journal; Journal of Human Evolution; Palaeogeography, Palaeoclimatology, Palaeoecology; Quaternary International; Quaternary Science Reviews; Zootaxa, Nature, Science, Current Anthropology, Folia Primatologica, Journal of Archaeological Science, Journal of Mammalogy; Australian Research Council (ARC), European Research Council (ERC)

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"Life is like a book that never ends.
Chapters close, but not the book itself."

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Abstract

The emergence of resistance (pest resistance to control measures) is a serious and growing problem in agricultural production that significantly reduces yields. Without effective control, 70% of food for human and livestock consumption is wasted. The western corn rootworm (*Diabrotica virgifera virgifera* LeConte) (WCR), Codling moth (*Cydia pomonella* L.) (CM), and Colorado potato beetle (*Leptinotarsa decemlineata* Say) (CPB) are the most important pests in Croatian agriculture, and these insects have developed resistance to various insecticides and established control strategies. There is a need to find effective methods for determining resistance that will allow early detection and the development and timely implementation of resistance control strategies.

In this study, two methods were used. The first was single nucleotide polymorphism (SNP) markers which were used to perform detailed population genetic analysis of the whole genome of the insects investigated. The second was geometric morphometric (GM) methods to analyze morphological variations related to resistance development. The aim of this dissertation was to analyze population genetic structure, differentiation, gene flow, distribution and adaptability of the three target insect pests by genotyping SNPs. In addition, morphometric analyzes were performed to examine phenotypic variation across populations investigated in Croatia.

For genetic analyzes, genomic DNA of WCR, CM and CPB was isolated and genotyped and the forewings (CM) or hindwing (WCR and CPB) size and shape difference were investigated for morphometric analyses. The data generated were analyzed using the statistical program R. The approaches used to analyze the genetic structure of WCR, CM and CPB populations inclluded: Bayesian-based models of population structure (STRUCTURE), principal component analysis (PCA), discriminant analysis of principal components (DAPC), neighborhood cluster analysis (NJ), and VanRaden Kinship matrix analyzes. To confirm the genetic results, forewing and hindwing morphology was examined using geometric morphometric techniques based on the venation patterns of 14 landmarks for WCR, 18 landmarks for CM, and 16 landmarks for CPB.

The results for WCR indicated that the combination of genetic and geometric morphometrics could be a reliable technique to detect differences between WCR populations. The results also showed that geometric morphometrics can be used as a biomarker for resistance detection as part of a larger integrated resistance management strategy for WCR. For CM, SNP markers did not show sufficient power to detect changes between populations based on the type of apple control method from which they were sampled. However, geometric morphometrics showed higher sensitivity in detecting population changes associated with different types of apple production/control and proved to be a reliable, accurate, and cost-effective biomarker. For CPB populations, low genetic variability was found using SNPs and the presence of a single panmictic population in the study area was noted. The results of GM for the CPB populations demonstrated morphological changes across geographic space in Croatia thus demonstrating the phenotypic plasticity of CPB.

The combined use of SNPs and GM to detect resistant variants is a novel approach where morphological traits can provide additional information about underlying population genetics and morphology can contain useful information about genetic structure. Findings from this thesis also provided new insights into an important and timely area of pest management, namely in testing methods of early detection of resistance and novel use of monitoring methods.

Keywords: Single nucleotide polymorphism, geometric morphometrics, resistance, resistance mechanism, genetic structure, genetic diversity, population structure, monitoring, control strategies, anti-resistance programs.

Prošireni sažetak (Extended summary in Croatian):

Naslov doktorske disertacije na hrvatskome jeziku (title of the doctoral thesis in Croatian)**:**

Promjene genoma povezane s razvojem rezistentnosti na insekticide u ekonomski važnih štetnika u Hrvatskoj

Pojava rezistentnosti na insekticide u kukaca ozbiljan je i rastući problem u poljoprivrednoj proizvodnji, koji može ugroziti učinkovito suzbijanje štetnika i zaštitu uzgajanih kultura. Uvođenje sintetskih insekticida za suzbijanje štetnih kukaca prije pedesetak godina izazvalo je veliki entuzijazam te se smatralo da su upravo oni održivo rješenje za sve probleme vezane uz proizvodnju i nestašicu hrane. Ipak, vrlo brzo pojavili su se problemi vezani uz negativne izravne i neizravne učinke na ljude i okoliš. Rezistentnost kukaca na diklor-difenil-trikloretan (DDT), nekada masovno primjenjivan sintetički insekticid, zabilježena je 1947. Od tada do danas utvrđena je rezistentnost brojnih štetnika na gotovo sve grupe insekticida na tržištu. Pojava rezistentnih populacija štetnika sve je veća, a time su povećani i gubitci u poljoprivrednoj proizvodnji. Iako se u svijetu koristi sve više insekticida, više od 500 vrsta kukaca, grinja i pauka razvilo je određenu razinu rezistentnosti. Nekoliko je načina razvoja rezistentnosti kukaca na insekticide: fiziološka, rezistentnost na mjestu djelovanja, morfološka i psihofizička rezistentnost. Bez obzira na tip rezistentnosti koju pojedini kukac razvija, ona proizlazi iz selekcije genetske modifikacije u jednome ili više gena koji se pojavljuju migracijom i/ili mutacijom.

Proizvodnja najvažnijih ratarskih (kukuruz i krumpir) i voćarskih (jabuka) kultura u Hrvatskoj ugrožena je brojnim štetnicima, od kojih su najznačajniji kukuruzna zlatica (*Diabrotica virgifera virgifera* LeConte – WCR), jabukin savijač (*Cydia pomonella* L. – CM) i krumpirova zlatica (*Leptinotarsa decemlineata* Say – CPB). Sve navedene vrste razvile su rezistentnost na insekticide i/ili strategije suzbijanja. Kukuruzna zlatica razvila je rezistentnost na 13 aktivnih tvari, ali ono što je još važnije, razvila je i otpornost na strategije suzbijanja (npr. plodored). Za jabukina savijača prijavljena su 196 slučaja rezistentnosti na 22 različite aktivne tvari. Krumpirova zlatica razvila je rezistentnost na čak 56 različitih aktivnih tvari te je službeno zabilježeno 306 slučajeva rezistentnosti diljem svijeta.

Pojavu i razvoj rezistentnosti moguće je spriječiti ili odgoditi pravovremenim djelovanjem, koje podrazumijeva monitoring pojave i ranog utvrđivanja rezistentnosti štetnika. Za monitoring i dokazivanje rezistentnosti uobičajeno se koriste metoda biotesta, biokemijski ili molekularni testovi, metode koje imaju određene prednosti, ali i nedostatke. Biotestovi često nisu dovoljno osjetljivi ili zahtijevaju velik broj živih kukaca (određivanje LD_{50}) te ljudskoga rada dok biokemijske i molekularne metode nisu dostupne za sve tipove rezistentnosti ili zahtijevaju specijaliziranu i skupu opremu. Antirezistentni programi obuhvaćaju tri osnovne komponente: monitoring kompleksa štetnika u polju i promjena u gustoći populacije, ekonomski prag štetnosti i višestruke integrirane strategije suzbijanja štetnika. Otkrivanje i monitoring rezistentnih populacija prvi je korak prema implementaciji antirezistentnih strategija i održivoj uporabi insekticida. Antirezistentne strategije mogu osigurati dugoročnu učinkovitost pojedinih djelatnih tvari u suzbijanju štetnika.

Polimorfizam pojedinačnoga nukleotida (engl. Single Nucleotide Polymorphism – SNP) novija je metoda analize cijeloga genoma. Uporaba SNP-a mogla bi pomoći u boljem razumijevanju populacijske genetike kukuruzne i krumpirove zlatice te jabukina savijača. Takvi podaci koji podrazumijevaju utvrđivanje promjene genoma povezane s razvojem rezistentnosti ključni su za provedbu antirezistentnih programa kao sastavnoga dijela integrirane zaštite bilja od štetnika.

S obzirom na navedeno postavljene su hipoteze istraživanja: (1) Otpornost štetnika na insekticide rezultat je genetskih mutacija kukaca; (2) Mutacije se mogu učinkovito detektirati na

populacijskoj razini i dokazati u promjenama SNPs-a unutar i između populacija pojedine vrste štetnika.

Ciljevi istraživanja u sklopu doktorske disertacije bili su: (1) Uspostavljanje SNP genotipa za svaku jedinku u populaciji i SNP biblioteke za kukuruznu i krumpirovu zlaticu te jabukinog savijača; (2) Analizom ukupne genske varijabilnosti pomoću SNP-a odrediti razlike između i unutar populacija kukuruzne i krumpirove zlatice te jabukina savijača; (3) Utvrditi vezu između genetskih mutacija i rezistentnosti kukaca na insekticide.

Poznavanje evolucijskih promjena i ukupne genetske raznolikosti populacija nekoga štetnika može pružiti korisne informacije za razumijevanje genetskih uzoraka povezanih sa svakim stupnjem razvoja otpornosti štetnika, tako da se praćenje i suzbijanje mogu prilagoditi rezistentnosti pojedine vrste štetnika. Utjecaj okoline na genotip organizma kompleksan je proces za koji je potrebno puno više vremena da se utvrdi nego utjecaj okoline na fenotip organizma. Iz toga razloga istraživanja utjecaja okoliša, kao što su klima, biljni domaćin, strategije suzbijanja i dr., na populaciju i pojedine jedinke štetnika trebaju se temeljiti i na fenotipskim, a ne samo na genotipskim karakteristikama. Često štetnik postaje otporan na insekticid razvijajući fiziološke promjene, stoga smo u istraživanje uključili i tehnike geometrijske morfometrije kojima smo analizirali morfološke karakteristike oblika kukca koje su pod direktnim utjecajem promjene genotipa (npr. krila). Metoda geometrijske morfometrije ima veliku „statističku osjetljivost“, pa se njezinom primjenom mogu otkriti male promjene u obliku morfoloških cjelina (krila) zaduženih za širenje populacija.

Pokazalo se da su morfološke osobine, kao što su veličine i oblika krila kukaca, prvi fizički pokazatelji promjena jer su pod utjecajem okolišnih i genetskih čimbenika, što ih čini idealnim za otkrivanje i praćenje rezistentnih populacija štetnika. Osim uporabe geometrijske morfometrije kao alata za praćenje, ovom je metodom također moguće dobiti važne informacije o osnovnoj ekologiji kukaca. Točnije, oblik i veličina krila ili tijela mogu se koristiti kao markeri (biljezi) populacije i pomoću njih mogu se detektirati razlike između nerezistentnih i rezistentnih populacija.

Tijekom istraživanja prikupljene su: populacije kukuruzne zlatice iz Amerike, koje su razvile otpornost na plodored te na određene *Bt* toksine, populacije jabukina savijača prikupljene iz ekoloških i integriranih voćnjaka i populacije krumpirove zlatice iz najvažnijih uzgojnih područja krumpira u Hrvatskoj, kao i laboratorijske nerezistentne populacije kukuruzne zlatice i jabukina savijača. Ukupno je obrađeno više od 500 jedinki navedenih vrsta (100 jedinki kukuruzne zlatice te 200 jedinki krumpirove zlatice i jabukina savijača). Iz svake jedinke izolirana je cjelovita genomska DNK. Na uzorcima svake vrste provedena je genotipizacija korištenjem tehnologije nizova raznolikosti (DArT). Dobiveni podaci analizirani su u statističkome programu R. Za analizu genetske strukture populacija kukuruzne i krumpirove zlatice te jabukina savijača korišteni su različiti pristupi: Bayesov model strukturiranja (STRUCTURE), analiza glavnih komponenti (PCA), diskriminantna analiza glavnih komponenti (DAPC), analiza genetske udaljenosti (NJ) pomoću filogenetskoga stabla i VanRaden Kinship analiza. Kako bi se potvrdili genetski rezultati, metodama geometrijske morfometrije (GM) određene su morfološke varijacije unutar i između populacija. Za analize korišteni su biološki definirani markeri, koji se postavljaju na fotografije odabranih dijelova tijela kukca (markeri se postavljaju na gornja ili donja krila). Na svakome krilu odabire se određen broj homolognih markera (specifičnih točaka) tipa 1, definiranih na čvorištima ili završetcima vena. Na kukuruznoj zlatici određeno je 14 specifičnih točaka, na jabukinom savijaču 18, a na krumpirovoj zlatici 16. Dobiveni rezultati analizirani su standardnim programima i procedurama geometrijske morfometrije. Ukupno je analizirano 775 krila kukuruzne zlatice, 363 krila jabukina savijača i 258 lijevih krila krumpirove zlatice.

Genetskim analizama populacija kukuruzne zlatice utvrđena su tri genetička klastera (STRUCTURE), što je također potkrijepljeno VanRaden analizom i analizom genetske udaljenosti (NJ). Rezultati istraživanja pokazali su da su se populacije rezistentne na Bt toksin

Cry34/35Ab1 i kombinaciju toksina Cry3Bb1_Cry34/35Ab1 odvojile od ostalih populacija. Rezultati GM kukuruzne zlatice potvrdili su rezultate genetskih analiza. Rezultati istraživanja pokazali su da jedinke rezistentne na Cry3Bb1_Cry34/35Ab1 toksin imaju šira i veća krila, a varijacije su primijećene na markerima 9 (središnji dio) i 14 (gornji rub krila). Jedinke rezistentne na Cry3Bb1 toksin imaju uža krila dok jedinke rezistentne na Cry34/35Ab1 toksin imaju manja krila s varijacijama na točkama 3 i 4 (vrh krila). Izduženiji oblik krila imaju jedinke rezistentne na plodored te su uočene varijacije na točkama 1 i 2 (vrh krila), kao i proširenje udesno na točki 9. Ovaj rezultat posebno je važan jer pokazuje da različiti Bt toksini različito djeluju na promjene u obliku krila.

Populacije jabukina savijača STRUCTURE grupirao je u dva klastera, a rezultati PCA analize bili su u skladu s tim. DAPC je odvojio jedinke u tri različite skupine. Međutim, rezultati su pokazali da genetska varijabilnost između populacija iz organskih i integriranih voćnjaka nije značajna. Za populacije jabukina savijača rezultati istraživanja pokazali su da se populacije štetnika iz prirode (iz ekološkoga i integriranoga uzgoja) značajno razlikuju u morfologiji krila u odnosu na laboratorijsku populaciju, a varijacije su primijećene na pet točaka (1, 7, 8, 9 i 12). Kao posljedicu ovih varijacija populacije štetnika iz integriranoga i ekološkoga uzgoja imale su izduženija i proširenija krila u odnosu na laboratorijski uzgojenu populaciju, koja je imala ovalni oblik krila. Značajne razlike primijećene su i u morfologiji krila populacija iz integriranoga u odnosu na ekološki uzgoj, u kojem su GM rezultati pokazali veću osjetljivost od genetskih i razdvojili tri različite skupine.

Genetskim analizama populacijama krumpirove zlatice utvrđena je genetska struktura bez značajne varijabilnosti. Ustanovljena je jedna panmiktička populacija ili genetski klaster koji karakterizira populacije krumpirove zlatice u Hrvatskoj. Rezultati GM analiza populacije krumpirove zlatice omogućili su nam pronaći morfološke promjene povezane s geografskim područjima Hrvatske, potvrdili su malu razliku između populacija odnosno fenotipsku plastičnost ove vrste. Rezultati su pokazali da jedinke krumpirove zlatice iz središnje Hrvatske imaju širi oblik krila dok iz sjeverne Hrvatske imaju izduženi oblik krila. Izduženija su krila su aerodinamičnija te nam ovi rezultati govore da su jedinke iz sjeverne Hrvatske najsposobnije za daleke letove i širenje na nova područja.

Glavni rezultati disertacije pokazali su da se kombinacijom genetskih (SNP) metoda i geometrijske morfometrije mogu detektirati promjene pomoću koji možemo razlikovati rezistentne i nerezistentne populacije. Provedenim istraživanjem utvrđene su iste karakteristike populacija genotipizacijom uzoraka primjenom SNP markera i korištenjem tehnika geometrijske morfometrije. Također, rezultati su pokazali da rezistentne populacije imaju različite oblike krila ovisno o tipu rezistentnosti (kukuruzna zlatica). Ovaj rezultat posebno je važan jer pokazuje da različiti okolišni uvjeti poput insekticidnih tretmana, različito djeluju na promjene u obliku krila. Kako je oblik krila pod utjecajem genetskih čimbenika, a svaka genetska promjena je rezultat mutacije, naši rezultati upućuju na promjene povezane s razvojem rezistentnosti.

Bez praćenja učinkovitosti pojedinih mjera zaštite te provođenja ranih mjera detekcije velika je opasnost da će se rezistentne populacije širiti te će njihovo suzbijanje biti otežano. Ovaj pristup nudi novi uvid u važno područje suzbijanja štetnika – o tome kako spriječiti ili odgoditi razvoj rezistentnosti i smanjiti njene negativne učinke. Praktična primjena istraživanja podrazumijeva implementaciju testiranih metoda (genetska SNPs analiza i geometrijska morfometrija) za brzu detekciju rezistentnosti. Rana detekcija rezistentnosti od iznimnoga je značaja za hrvatsku poljoprivredu i stručnjake koji se bave zaštitom bilja jer takve metode, odnosno takvi testovi, ne postoje. Istraživanje je rezultiralo podacima važnim na nacionalnoj i međunarodnoj razini. Ovim istraživanjem dokazana je učinkovitost obiju testiranih metoda u otkrivanju promjena koje bi mogle biti posljedica razvoja rezistentnosti, što u praksi omogućuje pravovremenu reakciju proizvođača s jedne strane i zakonodavstva s druge.

Ključne riječi: polimorfizam pojedinačnog nukleotida, geometrijska morfometrija, rezistentnost, mehanizmi rezistetnosti, genetska struktura, genetska varijabillnost, populacijska struktura, monitoring, mjere zaštite, antirezistetne strategije.

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List of abbreviation

SNP	Single nucleotide polymorphism
GM	Geometric morphometrics
WCR	Western corn rootworm
CPB	Colorado potato beetle
CM	Codling moth
STRUCTURE	Bayesian-based model of population structure
DAPC	Discriminant analysis of principal components
PCA	Principal component analysis
NJ	Neighborhood cluster analysis
DArT	Diversity Array Technology
IRM	Integrated resistance management

Publications included in the doctoral dissertation:

Published scientific papers arising from this PhD dissertation				
Scientific paper	**Base**	**Category**	**Quartile**	**Impact factor**
Mrganić, M., Bažok, R., Mikac, K.M., Benítez, H.A., Lemic, D. (2018). Two decades of invasive western corn rootworm population monitoring in Croatia. Insects 9(4): 160. DOI:10.3390/insects9040160	WoS	A1	Q1	2.769
Kadoić Balaško, M., Bažok, R., Mikac, K.M., Lemic, D., Pajač Živković, I. (2020). Pest management challenges and control practices in codling moth: a review. Insects 11(1): 38. DOI:10.3390/insects11010038	WoS	A1	Q1	2.769
Kadoić Balaško, M., Mikac, K.M., Bažok, R., Lemic, D. (2020). Modern techniques in Colorado Potato Beetle (*Leptinotarsa decemlineata* Say) control and resistance management: History review and future perspectives. Insects 11(9): 581. DOI:10.3390/insects11090581	WoS	A1	Q1	2.769
Kadoić Balaško, M., Mikac, K. M., Benítez, H. A., Bažok, R., & Lemic, D. (2021). Genetic and Morphological Approach for Western Corn Rootworm ResistancevManagement. Agriculture 11(7): 585. DOI: 10.3390/agriculture11070585	WoS	A1	Q1	2.925
Kadoić Balaško, M., Bažok, R., Mikac, K. M., Benítez, H. A., Suazo, M. J., Viana, J. P. G., Lemic, D., Živković, I. P. (2022). Population Genetic Structure and Geometric Morphology of Codling Moth Populations from Different Management Systems. Agronomy, 12(6): 1278. DOI: 10.3390/agronomy12061278	WoS	A1	Q1	3.949
Kadoić Balaško, M., Bažok, R., Mikac, K.M., Benítez, H.A., Correa, M.,	WoS	A1	Q1	3.949

Lemic, D. (2022). Assessing the population structure of Colorado potato beetle populations using genetic and geometric morphometric tools. Agronomy, 12(10): 2361. DOI: 10.3390/agronomy12102361	

Writing style: chapter 2 and subchapter 3.1 were written for publication in peer-reviewed journals, so they may have different writing styles. In some chapters I say “we” as the manuscripts contained coauthors. All coauthors understand these manuscripts form part of my PhD thesis.

Explanation of the connection between research hypotheses and published research papers	
Research hypotheses	Explanation of the connection
1. Pest resistance to insecticides is a result of genetic mutations in insects. 2. These mutations can be effectively detected at the population level and demonstrated as district changes in SNP variation among and between certain pest populations.	The Publication No.1 summarizes the research on WCR in Croatia from when it was first detected in 1995 until 2018. For more than two decades WCR adult population abundance and variability was monitored using traditional density monitoring. More recent genetic monitoring, and the newest morphometric monitoring of WCR populations is now used. Croatia now possesses a great deal of knowledge about the beetle's invasion process over time and space. Publication No. 2 summarizes information about the origin and biology of the CM, describes the mechanisms of resistance in this pest, and provides an overview of current research of resistant pest populations and genetic research both in Europe and globally. Publication No. 3 summarizes the literature on resistance development in CPB and on new approaches to the old CPB control problem. The possibility of using SNPs and GM methods is described as a way to go deeper into our understanding of resistance and how it influences genotypes and phenotypes. The research was conducted on populations resistant to different toxins (WCR), on populations from integrated and organic orchards (CM) and, for both pests, on a laboratory-bred population that had never been treated with insecticides and from field populations in continental Croatia. The SNPs have provided deeper insight into the molecular mechanisms of resistance and show that changes that can be related to resistance development can be detected; this finding confirmed **hypothesis 1**. For example, it was demonstrated that many point mutations are found in different genes, suggesting that these mechanisms can occur simultaneously, making it more difficult to understand which one is truly responsible for the resistance genotype. The main results for WCR and CM in Publications No. 4 and 5 show that the combination of genetic methods (SNP) and GM offer a possibility to reveal spatial differences among WCR and CM populations. For CPB, Publication No. 6 low genetic and phenotypic variability was found among CPB populations and the presence of a single panmictic population in the study area that is well adapted to different environmental conditions, suggesting high phenotypic plasticity.

	Therefore, **hypothesis 2** was affirmed for all three pests investigated. Geometric morphometric results showed that resistant populations have different wing shapes depending on the type of resistance. This result is particularly important because it shows that different toxins have different affects on changing wing shape. Since wing shape is affected by genetic factors and any change is the result of a mutation, we confirmed that geometric morphometrics can be used as a biomarker for resistance detection as part of a larger integrated resistance management strategy for WCR and CM. The estimates of genetic diversity, population structure, and genetic relatedness among CPB individuals provided information on the efficacy of control strategies so that recommendations can be made to improve the effectiveness of control programs. Based on the results, where adaptation of CPB populations was found, it is necessary to implement an area wide approach to future pest control management.

1. INTRODUCTION

Insect resistance to pesticides is a serious and growing problem in agricultural production systems. Insects have posed a constant threat to food supply since humans have become dependent on growing crops as their primary food source (Oberemok, 2015). According to Oerke (2006), the potential production loss from pests, if left uncontrolled, can range from 50% to 80% depending on the crop. The ability of insect pests to develop resistance to insecticides threatens global food security and the development of sustainable agricultural practices, especially when their rate of development outpaces the development of new control strategies (Chen and Schoville 2018; Gould et al., 2018).

The era of synthetic pesticides began about fifty years ago, and there was great enthusiasm that they could provide a lasting solution to the world's food and agricultural productivity problems (Oberemok, 2015). But then the first cases of pest resistance began to appear. According to the World Health Organization (WHO, 2012), resistance is defined as the ability of an insect to resist the effects of an insecticide by becoming resistant to its toxic effects through natural selection and mutations. Insect resistance to the synthetic insecticide DDT was documented in 1947, and since then resistance to new insecticides has been found in major pest organisms within 2-20 years of a chemical's release (Forgash, 1984). When a pest becomes resistant, the insecticide is used more frequently and greater concentrations until it eventually must be replaced as pest control declines (WHO, 2012). Pesticide resistance is becoming more common. Worldwide, more than 500 species of insects, mites, and spiders have developed some degree of pesticide resistance (IRAC, 2022).

Agricultural production in Croatia is conducted on 1, 500, 000 ha. On approximately 815, 000 ha of arable land and public and private gardens in Croatia (Croatian Bureau of Statistics, 2018), 250,000 ha of maize and 305,000 ha cereals (including wheat, barley, rice and oats) are sown. Although potatoes are not sown on large (10 ,000 ha), they remain a very important crop in agricultural production in Croatia. A little less than 32,000 ha of arable lands are orchards and apple is produced on 6, 500 ha. Production of the most important arable (maize and potato) and perennial crops (apple) in Croatia is threatened by many insect pests, of which the most important are the western corn rootworm (*Diabrotica virgifera virgifera* LeConte) (WCR), Colorado potato beetle (*Leptinotarsa decemlineata* Say) (CPB) and Codling moth (*Cydia pomonella* L.) (CM). These three pests have shown resistance to insecticides (CPB and CM) and to the management strategies (WCR) used to control them (Lemić et al., 2017; Bažok et al., 2021). Currently, CPB has developed resistance to 56 different compounds

belonging to all major insecticide classes and there are 306 cases of resistance reported worldwide. For CM there are 196 cases of resistance reported to 22 different active chemical ingredients. WCR has developed resistance to 13 active chemicals but what is more important is the need for the management of control strategies (e.g. crop rotation) (APRD, 2022)

Regular monitoring for insecticide resistance is essential to proactively prevent insecticide resistance from compromising control. Many authors agreed that only by monitoring, characterizing and predicting the appearance and spread of resistance we can use existing chemical tools in a sustainable manner (Foster, 2006; Liu, 2012). Currently most resistance monitoring is dependent on bioassays and the data is reported as percentage mortality and/or Knock Down (KD) effect (WHO, 2012). It is possible to use fixed dose concentrations or to conduct dose response assays. Additionally, resistance could be detected using biochemical assays which identify the activity of enzymes associated with insecticide resistance or by molecular assays that detect resistant alleles (IRAC, 2016). Each of the available methods has advantages and disadvantages. Bioassays are either not sensitive enough (if fixed concentrations are used) or require large number of insects for experimental trals, while biochemical and molecular methods are not available for all types of resistance detection and/or require the use of specialized and costly equipment (Corbel and N'Guessan, 2013).

Knowledge of evolutionary changes and the total genetic diversity of a pest population can provide useful information to understand the genetic patterns associated with each stage of pest resistance development so that management, including monitoring and control, can be tailored to suit the resistance of the pest in question (Sakai et al., 2001). Therefore, there is a need for validated methods of resistance detection in agricultural pests. Diversity Array Technology (DArT) is method for DNA polymorphism analysis which offers a low-cost high-throughput, robust system with minimal DNA sample requirements capable of providing comprehensive genome coverage (Jaccoud et al., 2001). DArTseq technology is a one-step procedure of SNP discovery and genotyping; it enables a substantial discovery of SNPs in a wide variety of non-model organisms and provides measures of genetic divergence and diversity within the major genetic groups (Nantoume et al., 2013). The use of SNPs, in non-model organisms has become an affordable and readily accessible means of generating important genomic data on species that otherwise would have been impossible to generate due to cost and expertise availability. Given the vast number of SNPs (thousands to millions) that are easily and affordably generated in a single sequencing run, SNPs have now surpassed microsatellites as the marker of choice when investgiating the population genetics of a no-model organisms (Xing et al., 2005). The use of SNPs to understand the population genetics

of WCR, CPB and CM on a deeper level can now be undertaken. The generation of genomic data, may be used to investigate the genome changes associated with the development of resistance, which is crucial for the implementation of agricultural, food biosecurity measures and integrated pest management strategies tailored for each pest.

Alongside genetic information it has been shown that metric properties of insects established by geometric morphometric techniques (i.e., shape analysis) are one of the first physical characters to change in an organism as they are under the influence of both environmental and genetic factors (Levine and Oloumi-Sadeghi, 1996; Bouyer et al., 2007). Recently, geometric morphometric (GM) has been used to study the genetic variability of different insect species (Lemic et al., 2016; Benitez et al., 2018; Pajač Živković et al., 2018; Lemic et al., 2020; Lemic et al., 2021). In addition to the use of geometric morphometrics as a monitoring tool, it will be possible to also gain important data about basic insect ecology. Specifically, wing or body shape and size can be used as population markers to detect differences between wild-type and resistant variants (Mikac et al., 2013; Mikac et al., 2019).

Genetic studies are an important tool for developing improved methods for detecting resistance, for studying resistance mechanisms, and for choosing approaches to resistance management. Morphometric methods have the benefit over molecular methods of being inexpensive, easy to use, and able to yield a lot of information quickly. However, numerous studies are in agreement that the combination of genetic markers and geometric morphometric methodgenerate more accurate data, as morphology can show clear differentiation patterns where molecular markers cannot detect population structure (Garnier et al., 2005; Camara et al., 2006; Ortego et al., 2011; Francuski et al., 2016; Henriques et al., 2020).

1.1. Hypothesis and aims of the thesis

1.1.1. Hypotheses

1. Pest resistance to insecticides is a result of genetic mutations in insects.

2. These mutations can be effectively detected at the population level and demonstrated as district changes in SNP variation among and between certain pest populations.

1.1.2. Specific aims

- ✓ Establishment of SNPs genotype for each individual in population and SNPs library for WCR, CPB and CM populations.

- ✓ Through analysis of total genomic variability detect the differences in WCR, CPB and CM population using SNPs.

- ✓ Determine the connection between genetic mutations and whether the detected differences between populations can be related to insect resistance status.

2. LITERATURE REVIEW

A detailed literature review centered on the three pests that form the basis of this thesis, western corn rootworm (WCR), codling moth (CM) and colorado potato beetle (CPB), are presented here and consist of three articles published in international peer-reviewed journals (subchapters 2.1. – 2.3). Each subchapter describes the most important information about the biology, pest status and resistance development in each pest. Also, the present work on developing new methods to maintain effective control using appropriate integrated resistance management (IRM) strategies for these economically important pests are also described.

Subchapter 2.1. was published in Insects, 9(4), 160 by Mrganić, M., Bažok, R., Mikac, K.M., Benítez, H.A. and Lemic, D. The paper summarizes the research on WCR in Croatia from when it was first detected in 1995 until 2018. For more than two decades WCR adult population abundance and variability was monitored. The publication details the traditional density monitoring conducted as well as more recent genetic monitoring, and the newest morphometric monitoring of WCR populations. As a result of the work reviewed and undertaken Croatia possesses a great deal of data and knowledge about WCR invasion processes over time and space.

Subchapter 2.2. was published in Insects, 11(1), 38 by Kadoić Balaško, M., Bažok, R., Mikac, K. M., Lemic, D., and Pajač Živković, I. The review summarizes the information about the origin and biology of the codling moth, describes the mechanisms of resistance in this pest, and provides an overview of current research of resistant pest populations and genetic research undertaken both in Europe and globally. Also, novel techniques for the detection of resistant variants and possibilities for future monitoring of resistance populations is described.

Subchapter 2.3. was published in Insects, 11(9), 581 by Kadoić Balaško, M., Mikac, K. M., Bažok, R., and Lemic, D. The publication summarizes the literature on resistance developments in CPB and on new approaches to the existing CPB control problem. The possibility of using single nucleotide polymorphisms and geometric morphometric methods is described as a way to deepen the understanding of resistance and how it influences genotypes and phenotypes of insects.

2.1. Publication No. 1

insects

MDPI

Review

Two Decades of Invasive Western Corn Rootworm Population Monitoring in Croatia

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Abstract: Western corn rootworm (WCR) is the worst pest of maize in the United States, and since its spread through Europe, WCR is now recognized as the most serious pest affecting maize production. After the beetle's first detection in Serbia in 1992, neighboring countries such as Croatia have established a national monitoring program. For more than two decades WCR adult population abundance and variability was monitored. With traditional density monitoring, more recent genetic monitoring, and the newest morphometric monitoring of WCR populations, Croatia possesses a great deal of knowledge about the beetle's invasion process over time and space. Croatia's position in Europe is unique as no other European nation has demonstrated such a detailed and complete understanding of an invasive insect. The combined use of traditional monitoring (attractant cards), which can be effectively used to predict population abundance, and modern monitoring procedures, such as population genetics and geometric morphometrics, has been effectively used to estimate inter- and intra-population variation. The combined application of traditional and modern monitoring techniques will enable more efficient control and management of WCR across Europe. This review summarizes the research on WCR in Croatia from when it was first detected in 1992 until 2018. An outline of future research needs is provided.

Keywords: western corn rootworm; population genetics; microsatellites; mitochondrial DNA; geometric morphometrics; Croatia; Europe

1. Introduction

Invasive Western corn rootworm (WCR)

Western corn rootworm (WCR) *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) overwinters in the egg stage in soil and emerges in spring from mid-May to early July [1,2]. The main damage to maize plants is caused by larval feeding on the roots, affecting key plant physiological

processes [3]. The resulting injury leads to stalk lodging and yield losses, which further leads to economic levels of damage to maize crops.

D. v. virgifera was first detected in Europe in Serbia during 1992 [4], but it is suspected that the WCR began its invasion of Europe ca. 1980; however, the pest was not officially recorded until 1992 [5,6]. Once introduced, *D. v. virgifera* started to spread across Europe. Five separate introductions of the WCR from North America into Europe are known to have occurred since 1998. WCR were introduced into Northeast Italy: Veneto in 1998, Pordenone in 2002, Udine in 2003 [7], Northwest Italy and Switzerland in 2000 [7], near Paris (France) in 2002 and 2004, and in 2003 at locations in Eastern France, Switzerland, Belgium, the United Kingdom, and the Netherlands [7]. Although the invasion history of WCR in Europe is now well known, the native populations of the Western European outbreaks are still unknown [7–9]. Given the sequence of outbreaks, Central Southern Europe (CSE) has generally been assumed as the source of most of the Western European populations [10]. However, each outbreak could have a source population from North America, CSE Europe, or some other Western European geographic locations [10].

The invasion of Europe by the WCR occurred in three phases since the 1980s. The first phase was the accidental introduction of WCR into Europe, which occurred ca. 1980–1992 [11]. The second phase was the establishment of WCR in countries surrounding the introduction location ca. 1995–2000 (Croatia, Bosnia and Herzegovina, Hungary and Romania) [11]. From 1995 until 2001, newly invaded fields were routinely identified in this part of Europe. The final phase of the invasion (2001–2018) was the dispersal phase, where WCR spread from Serbia to occupy 22 European countries spanning tens of thousands of hectares of maize fields [11]. In subsequent years from 2002 to 2011, WCR population densities have been relatively stable in all areas of maize production where their reproduction (an indicator of an established population) has been stable [8]. Evidently, established WCR have been spreading since their original introduction (ca. 1995), and as such, the more recent invasion phases of establishment and spread co-exist in Southern Europe [2].

During all stages, different monitoring techniques have been conducted in Croatia to detect, estimate and predict WCR population abundance and annual variations. In this review, we present traditional population metric surveys that were conducted in the first years of the WCR invasion in Croatia, and modern monitoring techniques, such as population genetics and geometric morphometrics, which were subsequently used to provide information on the variation within and among WCR populations. The monitoring techniques and procedures used in Croatia since the 1990s were implemented to inform management practices and contribute data to the effective integrated pest management (IPM) of WCR and other invasive pests in agricultural production.

2. Monitoring Trap Methods

Formal WCR monitoring in central European countries started in 1996. This initiative was undertaken by the International Working Group on *Ostrinia* and other maize pests (IWGO) as part of the International Organization for Biological and Integrated Control (IOBC) to organize and facilitate international collaboration. The first international meeting was held in Graz, Austria (20–21 March 1995) where the decision was made to start a monitoring program in countries at risk of WCR invasion and determine suitable control methods [11]. Soon after this meeting, the first formal survey and detection of WCR adults in Croatia [3,12–22] and in Hungary [23] were completed in 1995. Since then, IWGO has organized regular international conferences to report on the status of WCR and the associated research completed in Central and Eastern Europe. Due to these meetings and the associated reporting framework, WCR has become the only insect pest in the world whose monitoring and spread have proceeded in different areas and have been documented using the same methodologies. In the initial phase of WCR monitoring, cucurbitacin traps were implemented; however, pheromone traps developed

by Hungarian researchers were found to be suitable for the early detection of a pest population. The usefulness of the pheromone traps was quickly realized and as early as 1996, all the monitoring actions in all the invaded countries used pheromone traps.

However, yellow sticky traps have been used, especially when WCR population levels exceeded a threshold [15].

The results of research activities on WCR in Europe and the USA were presented during IWGO conferences [11]. Adult WCR monitoring by European countries allowed for the rapid detection and consequent understanding of WCR invasion processes since their first detection in Serbia [4,5,8]. The success of WCR monitoring and research in Europe resulted from the establishment of permanent monitoring sites in network partner countries, i.e., Serbia [24], Hungary [8], and Italy [25]. Permanent monitoring sites have allowed for the measurement of population fluctuations over the years. Between 1996 and 2006, the monitoring of WCR was regularly conducted in invaded and non-invaded areas in Croatia. The aims of the monitoring activities were to establish the rate of spread [14,18] and route [17] of WCR across Croatia, to evaluate the attractiveness of pheromone traps vs. yellow sticky traps [22,26–29], to document the flight dynamics of WCR adults [30], and WCR population changes over time [31]. This research was undertaken with the aim of assisting with their ongoing integrated management.

3. Spatial and Density Monitoring

Soon after WCR detection in Serbia, Maceljski and Igrc-Barčić [32] studied the biology and ecology of WCR and the potential for its establishment in Croatia. Preliminary studies on these areas showed that WCR would likely survive and develop wherever maize is grown in Europe [33]. WCR monitoring started in Croatia and in surrounding countries specifically for its detection and dispersion compliance [17,34–37]. During the monitoring period, four types of attractant traps were used: cucurbitacin traps, pheromone traps, Pherocon® AM (PhAM) non-baited yellow sticky traps (Treece, Salinas, CA, USA), and Multigard® (Sentry, Billings, MT, USA) non-baited yellow sticky traps. The first cucurbitacin trap designed for capturing *Diabrotica* spp. was constructed from amber plastic vials measuring 3 cm in diameter and 9 cm in length [38]. Pheromone traps are baited with synthetic sex pheromones and only catch males; they are highly sensitive tools for detection of occurrence and general monitoring. The sticky sheet is transparent and has a catch capacity of 3–400 beetles [39]. PhAM and Multigard® are yellow sticky surface traps used to monitor WCR. The color of the trap is visually attractive to the pests [40]. In the first years of monitoring, Multigard® yellow sticky traps were used, but in 2000, they were replaced with the PhAM trap. The switch was made to enable comparison with U.S. monitoring data [31].

During 1995, the first year of monitoring, 150 baits from USA with low attractant cucurbitacins, were placed in maize fields in Croatia. Cucurbitacin traps were really acting as a feeding arrestant, rather than attractant, because they are small tubes with dry plant material inside, and the plant material come from Cucurbita spp., rich in cucurbitacin. This compound is a feeding stimulant for WCR, so it keeps the beetles coming to the trap, but does not attract them [38]. As a result of this intensive monitoring process, one WCR specimen was caught in Bošnjaci near the border with Serbia, this was the first detection of WCR in Croatia [12]. Since 1996, the Department for Agricultural Zoology at the Faculty of Agriculture University of Zagreb, supported by the Croatian Ministry of Agriculture and Forestry, formally organized and undertook the WCR monitoring activities in Croatia [37]. After the first WCR detection in Europe, a pheromone lure was produced by European scientists and pheromone traps for monitoring purposes were designed. This trap was used in Croatia during the period between 1996 and 2006 [33]. Monitoring was conducted in seven Croatian counties during 1996, and in eight counties during 1997, 1998, and 1999. According to Igrc-Barčić and Dobrinčić [17], in 1996, the beetle spread 80 km to the west of the initially invaded sites and further infested 6000 km^2 of the maize production area;

in 1997, the beetle infested approximately 9000 km² of the maize production area. In 1998, movement of the WCR to the west was less than recorded in the previous three years. The only movement of the beetle was recorded along the river Sava. From 2000 to 2002, monitoring was conducted in 11 counties, this increased to 13 counties from 2003 to 2005, and in 2006, monitoring occurred in 11 counties. Each year, traps were set in maize fields (between 31 and 148 fields/year) situated in different areas of Croatia where the beetle could be found. Together with Pherocon® AM (PhAM) pheromone traps, non-baited yellow sticky traps (Treece, Salinas, CA, USA) or Multigard® (Scentry, Billings, MT, USA) non-baited yellow sticky traps were installed [31]. Economic damage levels in maize, resulting in an 85% reduction in yield, were observed in the Baranja region in Croatia during 2002, which is 200 km from Surčin, Serbia, the site where the WCR was first introduced into Europe [3]. During the 11 years of WCR monitoring in Croatia, it was possible to accurately predict the direction and intensity of the spread of WCR for the following year. From the data gathered, WCR spread at a rate of between 20 and 60 km/year in a westerly direction through Croatia, which acted as a corridor for the beetle's dispersal into the rest of Europe (Figure 1) [37].

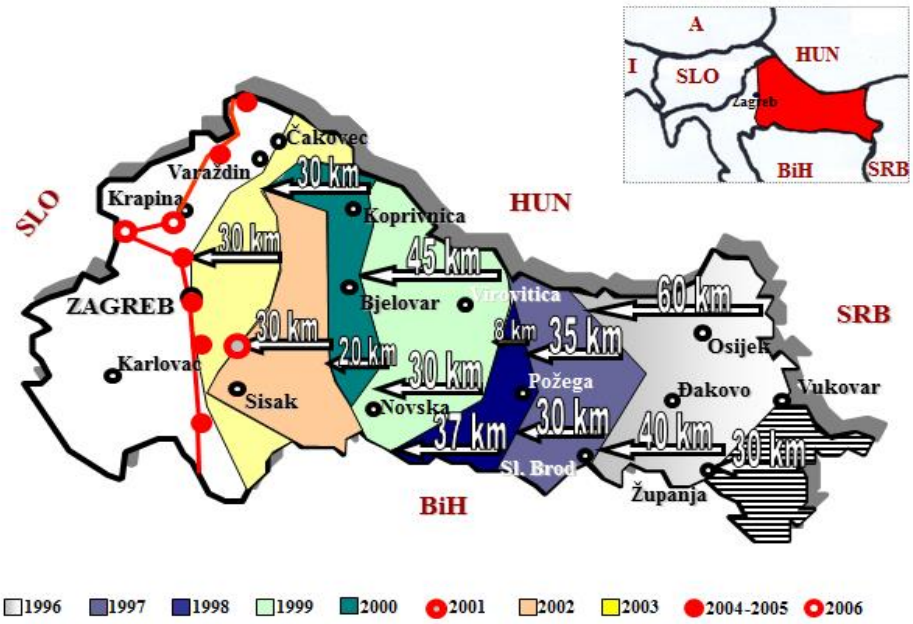

Figure 1. Distribution of western corn rootworm (WCR) in Croatia, established using spatial and density monitoring techniques.

Of all the traps evaluated, pheromone traps were most sensitive for early detection purposes. They were used not only to predict the line of spread, but also to describe the flight and population dynamics in a continuously sown maize field (a prelude to research on crop rotation as a mechanical control) [31]. Pheromone traps were also used to measure how far WCR adults would travel into neighboring fields for oviposition. WCR adults were monitored in continuous maize fields in 2003 and 2005 using Pherocon® AM non-baited yellow sticky traps [41]. Adult WCR population densities in 30 cornfields were determined weekly over a 74-day period each year (from the 24th to 35th week of the year) during 2006–2009 [42]. Adult population density was established in the 29th week of the year. At that time, the maize phenology stages varied from R65 to R67, according to the BBCH scale [43].

Pheromone trapping enabled efficient WCR occurrence and population abundance monitoring and the prediction of potential damage to maize crops during the following year [22,44]. According to Bažok

et al. [22], a potential substitute for the Pherocon®AM trap is the "whole plant count" method used in the first half of August. The Pherocon®AM trap/week capture corresponds well with the whole plant count method. Both methods can be used to estimate adult WCR population density. WCR larvae are present in the soil during the maize phenology stage from R18 to R34 according to the phenological growth stages and the BBCH maize identification keys [43]. Larval infestation was best predicted by maximal weekly capture; however, root damage was better predicted by the capture of adults in the 31st week of the previous year [45]. To predict plant lodging, three parameters were found to be equivalent in their predictive ability: maximal weekly capture; average daily capture; and the capture of adults in the 29th week of maize production [42]. Plant lodging was estimated in the 38th week of the year. At that time, the maize phenology stages varied from R83 to R97 according to the BBCH scale [43]. Larval emergence was predicted by the observed number of adults and eggs in the year preceding repeated maize sowing [2,45,46]. The highest density of Croatian WCR populations was recorded in 2003, when the average number of adults was n = 1275 and n = 177 on pheromone traps and yellow sticky traps, respectively. The relationship between the average number of adults captured per trap and climatic conditions (mean weekly temperature and rainfall) from weeks 25 to 35 of the year was estimated during 2007–2009. The average number of WCR per field was highest in years with higher amounts of rainfall and lower summer temperatures. Regression tree analyses showed that total rainfall was the best predictor of WCR population abundance [2]. The identification of the most important habitat parameters for WCR enabled predictions of infestation and potential levels of annual damage with the main purpose of informing farmers about the most efficient control strategies [45,46].

Traditional population surveys are important in WCR IPM, and can be effectively used to predict WCR population abundance [47]. Pheromone traps are more suitable for the monitoring and prediction of population increase, but for scouting purposes, yellow sticky traps are more a better option. Determining the factors that positively or negatively affect WCR population abundance in some regions is the starting point for the development of IPM strategies on a national and international scale.

4. Genetic Monitoring

In Croatia, the historical and contemporary population genetic structure of WCR was investigated from 1996 until 2009 [48–50]. This was the first study to use the temporal and spatial genetic structure to estimate the diversity, gene flow, invasion dynamics of WCR in Croatia and the influence of control practices on these population genetics parameters [51,52]. From the more than 1500 adult WCR investigated from 1996 to 2009, six microsatellite markers revealed that one large WCR population existed in Croatia in 1996 and in 2009. While the population changed over time, microsatellite markers revealed the persistence of a single large population.

Deciphering the temporal and spatial genetic structure of WCR has had important implications for the IPM of this invasive pest. By investigating WCR across Croatia over a 13-year period, it was possible to determine that in the absence of control (during 1996–2009), genetic diversity increased and minimal genetic structure remained, even to this day. Through crop rotation control practices, the WCR population should respond with a decrease in the genetic diversity of the populations/individuals under investigation as well as a noted increase in genetic structure. The genetic structure should then act to fragment or sub-structure and isolate populations geographically thus restricting gene flow. Ciosi et al. [9] found a pattern of isolation by distance, suggesting that the spreading population in Eastern Europe was split into genetically differentiated populations. Despite this, lower genetic diversity has not hampered the invasion and spread of WCR in Croatia, with 85,000 ha [15] of maize crops infested in 1996 compared with the 295,000 ha infested in 2007 [41]. A single panmictic population characterizes the overall population genetic structure of WCR in Croatia [50].

In addition to nuclear microsatellites, mitochondrial DNA markers have been used to monitor WCR population genetics on a microgeographic scale in Croatia [53]. This was the first study to formally conduct genetic monitoring of WCR through the use of multiple markers. Specifically, microsatellite markers were used to investigate the genetic variability and structure of the WCR collected in 1996, 2009, and 2011 from numerous locations across Croatia, Serbia, and the U.S. The study also reported bottleneck events and the location of the geographic source of WCR in Croatia (i.e., Serbia).

Ivkosic et al. [53] demonstrated that the seven U.S. WCR populations investigated maintained the greatest allelic diversity when compared to Croatian and Serbian WCR. In Europe, the largest number of alleles was found in locations near international airports (Rugvica, Croatia and Surčin, Serbia). The highest number of mtNDA haplotypes was observed in Croatia in 1996, soon after WCR was first recorded there. From 2009 to 2011, haplotype diversity declined, and Croatia and Serbia had one fixed haplotype. Furthermore, continuous maize cropping locations in the U.S. had one haplotype, whereas three haplotypes were found in soybean-maize crop-rotated locations. Minimal temporal genetic variability was found among the populations in Europe and the U.S.; a result previously demonstrated for the species only in the U.S. [54]. Bayesian cluster analysis revealed two genetic clusters that joined the WCR from Croatia and Serbia, but separated them from U.S. populations. These clusters showed that numerous U.S. individuals had both European and U.S. ancestry, which suggests the existence of bidirectional gene flow [55]. Bottlenecks were identified within all Croatian populations sampled in 1996 and 2011 and only two populations in 2009. Bottlenecks were not identified at all in Serbia from 1996 to 2011, or in the U.S. in 2011. As suspected, Serbia was revealed as the geographic source of WCR in Croatia. The temporal genetic monitoring conducted from 1996 until 2011 allowed a deeper understanding of the WCR genetics in Croatia, Serbia, and its original geographic region in the U.S.

More recently, the population genetics of WCR in Southern Europe during all invasive phases (introduction, establishment, and spread) were investigated [55]. Results from the study showed that during the first phase (introduction), the number of observed alleles was low (19–27; 45%) in Southern Europe compared to suspected source populations from the U.S. (Iowa or Illinois). Within a relatively short time period, the number of alleles present in Southern Europe approximately doubled. Of all known WCR alleles [54,56], 84% were found in locations in Southern Europe, 14 years after WCR was first introduced. During the second and third invasive phases (establishment and spread, respectively), the number of alleles in the population in Croatia had doubled compared with the other countries investigated in the study. However, this may have been due to the intensive monitoring program in Croatia during the study period [50]. The results confirmed the original finding that allelic richness during the introduction phase was low but consistent throughout all Southern European populations [55]. However, during the establishment and spread phases of the invasion process, allelic richness was higher for all Southern European populations. Croatian populations in the same period had significantly higher allelic diversities than any other European population investigated. These analyses revealed previously undiscovered alleles during the invasive phases of WCR in Europe. Specifically, two unique alleles were found in the introduction phase, whereas nine previously unrecorded alleles were found during the establishment and spread phases. The large number of unique alleles found in this study could reflect multiple and ongoing invasions in Southern European countries from different locations within Europe and the U.S. These results confirm that Serbia was the primary source of WCR to its neighboring countries (Croatia, Hungary, and some parts of Italy). The only exception to this was the WCR population in Venezia, Italy, which was formed after a second introduction from the U.S. [55].

A detailed population genetics investigation of the WCR invasion phases (introduction, establishment, and spread) conducted by Lemic et al. [55] revealed that the three phases often overlap and that these phases of invasion are still in progress in Europe. Extensive population genetic investigations of WCR in South Europe have revealed that low genetic variation exists among the

populations in Italy, Austria, Hungary, Slovenia, Croatia, and Serbia, and showed minimal genetic differences between populations and among regions [55,57].

For over a decade, population genetic monitoring has been used to inform the effective control and ongoing integrated management of invasive WCR in Croatia [58] and has proven useful in understanding WCR invasion in Croatia and other invaded countries. The results obtained from these studies are crucial to further understand WCR population dynamics during the major phases of its European invasion [57]. An investigation into the WCR's population genetic structure, gene flow, and dispersal patterns has helped to understand the impact this invasive species has had on global agriculture production and food resources.

5. Geometric Morphometric Monitoring

The expense and need for specialist skills associated with population genetics were the main reasons to search for additional non-genetic based techniques to monitor WCR. Geometric morphometrics (GM) were tested and deemed an existing novel use method to easily, cheaply, and quickly yield robust data. After almost two decades of traditional (distribution and abundance) and genetic monitoring of WCR populations in Croatia, geometric morphometric monitoring was used with the aim of assessing whether WCR wing shape and size were influenced by specific habitat parameters that could enable the discovery of a population biomarker [55].

In the application of the technique to understand invasion patterns in WCR, Mikac et al. [59] were the pioneer researchers to include GM in IPM research for WCR. These authors demonstrated discernable patterns in wing size and shape between resistant (crop rotation) and susceptible populations in the USA. Their research provided the foundation for and set the research agenda of GM use in WCR IPM research that has since followed [2,57,58,60–63].

Following Mikac et al. [59], Lemic et al. [57] and Benítez et al. [61] showed that GM could be used as a tool to examine wing shape differences influenced by environment. These authors tested their hypotheses in WCR populations principally from Croatia, where varying soil types are known to directly influence larval and adult WCR development [41]. Both Lemic et al. [57] and Benítez et al. [61] demonstrated that WCR wing shape changed according to major soil type classifications in Croatia. These results were novel for WCR and a need to further test these findings drove the research questions of subsequent similarly themed work.

For example, Lemic et al. [57] compared the hindwing shape and size between sexes of WCR from populations sampled in the U.S. and Europe. The populations investigated showed high levels of sex wing shape dimorphism [57]. Both in the U.S. and Europe, female WCR had more elongated wings. Since elongated wings are considered to be involved in migratory movement, this investigation provided morphological evidence that most migration in WCR (as well as invasive migrations) could be attributed to the females of this species. Female WCRs are also known to undertake migratory flights over relatively long distances. This was also discussed by Mikac et al. [59], who suggested that elongated wings were probably more aerodynamic and may be a useful invasive dispersal strategy for mated females. When investigating sexual dimorphism within a species, it is also important to examine whether allometry contributes to sexual dimorphism [62,64]. Allometry is the relationship between size and shape and is normally categorized as a percentage where shape is explained by size. Insect studies of allometry are normally related to the nutritional aspect to which development is directly related [65]. In addition to the described results, the presence of asymmetries in the WCR wings is a novel finding for coleopterans and is an important contribution to the ever-growing pool of data on the evolution of insect wings [61].

Morphological integration and modularity are another set of analyses that can be performed using GM tools to infer the developmental structure of morphology [66] and to answer questions about the

invasiveness of WCR. Benitez et al. [62] analyzed the relationship among landmarks in the hindwings of WCR to explain why their wing structure is composed of different modules. Surprisingly, the results showed an integrated behavior of the hindwings of WCR. These findings paved the way for future flight performance and biogeographical studies on how wing shape and size change across the native and newly invaded range of WCR in the U.S. and Europe [62].

Two years later, Mikac et al. [63] confirmed that GM tools were again useful to identify invasion processes (i.e., multiple WCR introductions into Europe) for the WCR and could be used as a special monitoring tool for this pest species. This research studied the hindwing size and shape variations within and among WCR populations over a larger geographic region in Southern Europe, spanning an area of 160,000 km^2. The data generated represent the greatest morphological investigation of an invasive species with global importance. The results allowed the WCR populations from Italy and those in Central and Southeastern Europe to be clearly separated [6,64], a result mirrored in Lemic et al. [55] who demonstrated the same result using population genetic markers. Additionally, the wing shape differences found using GM procedures followed an east to west direction of spread as described by Igrc-Barčić et al. [37]. Based on genetic [55] and now GM data [63], it was possible to conclude that the Italian WCR population had no link to the aforementioned populations and originated from a different and more recent introduction from the U.S. Notably, although the conclusion on genetic monitoring required two decades of WCR analysis [55], through the use of GM monitoring, valuable information on the invasion process was obtained from the analysis of WCR in a single time period (i.e., here in 2012).

Most recently, Mikac et al. [60] extended the use of hindwing size and shape differences to examine changes in WCR related to the development of resistance, specifically investigating possible differences among rotation resistant, *Bacillus thuringiensis* (*Bt*)-resistant, and non-resistant (or susceptible) populations in the U.S. In general, the hindwings of non-resistant beetles were significantly more elongated in shape and narrower in width (chord length) in comparison to beetles that were resistant to *Bt*-maize or crop rotation. Such differences may impact the dispersal or long-distance movement of resistant and susceptible WCR, as wing morphology is a critical element of an insect's dispersal capacity. Understanding which morphotype of the beetle is the superior flier and disperser has implications for the management of WCR via integrated resistance strategies. Overall implications from the GM work conducted to date suggest that GM can be used to monitor population changes related to the invasion process and could be used as a cheaper and more accessible population biomarker compared to expensive and specialized-use genetic markers when investigating biological invasions in species that have similar characteristics to WCR.

6. Future Work

In an effort to broaden our understanding of WCR invasion biology and the response to integrated management practices, genetic and phenotypic methods must be investigated. Currently, the use of single nucleotide polymorphisms (SNPs, pronounced 'snips') in non-model organisms has become an affordable and readily accessible means of generating important data on species that otherwise would have been impossible due to cost and expertise availability. For use in population genetics, SNPs have surpassed microsatellites as the marker of choice, and using them to understand the population genetics of WCR on a deeper level must be explored. The use of SNPs as population genetic marker in WCR has been attempted, though only a limited number of individuals (n = 12) were genotyped and the results were similar to those from microsatellites [67]. Given the latest technology in next generation sequencing and the now routine use of genotyping by sequencing SNPs, the potential for robust and plentiful population genomic data to understand WCR movement patterns on small and large geographic scales warrants investigation. Finally, future work on phenotypic aspects of WCR are needed to compliment any population genomic data that is generated. In particular, a greater understanding of WCR

intraspecific flight morphology is needed to better understand the fundamentals of WCR dispersal. Our findings on the changing WCR hindwing shape and size, according to resistance, has provided researchers and managers alike with important morphological information on resistant morphotypes on which monitoring can focus. A deeper understanding of WCR wing shape and flight morphology, aspect ratio, and flight efficiencies will assist with the management of the species. Such information is crucial for the implementation of biosecurity measures and integrated pest management strategies for the WCR globally.

List of Projects Related to WCR in Croatia

- 2017–2021: Monitoring of insect pest resistance: novel approach for detection, and effective resistance management strategies (MONPERES), Croatian science foundation (coordinator: R. Bažok)
- 2009: The landscape genetics of the invasive western corn rootworm in Croatia (Ministry of science, education and sport—Unity through knowledge fund—UKF)
- 2007–2013: The spatial distribution of economically important pests with the use of GIS (Ministry of science, education and sport, Croatia)
- 2005–2007: Developing IPM in maize through WCR risk management—FAO
- 2005–2006: Development of IPM for WCR in collaboration with Secondary agricultural schools-FAO
- 2003–2007: Integrated pest management for western corn rootworm in Central and Eastern Europe (FAO, GTF)
- 2002–2006: Biological control the base of ecologically acceptable plant protection (Ministry of science and technology, Croatia)
- 2002–2004: The possibility of the control of the Western corn rootworm with minimal input (Ministry of agriculture and forestry Croatia)
- 1998–2006: Monitoring of the western corn rootworm (Ministry of agriculture and forestry Croatia)
- 1998–2001: *Diabrotica virgifera virgifera* (Ministry of science and technology Croatia—young researcher project)
- 1997–2000: Management of Western corn rootworm in central Europe FAO/TCP

7. Conclusions

The thorough knowledge of the WCR invasion in Croatia is unique in Europe, as no other European nation has demonstrated such a detailed and complete understanding of an invasive insect till now. This review summarized the research on WCR in Croatia from 1992, when it was first detected, until 2018. It outlines the important work undertaken on multiple aspects of WCR biology, ecology, population genetics and morphometrics to inform integrated pest management strategies used for its effective control. Early stages of the research focused on the detection and monitoring of the beetle using traditional methods (yellow sticky traps etc.) and then progressed to genetic monitoring (microsatellites and mitochondrial DNA markers) of Croatian and wider European populations of WCR. The most recent research on WCR in Croatia has focused on the use of geometric morphometrics as a monitoring tool and population biomarker. Given the very detailed understanding of the biology, ecology and genetics of WCR that Croatia has, it is very well placed to effectively detect, monitor and control WCR within its borders.

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2.2. Publication No. 2

insects

MDPI

Review

Pest Management Challenges and Control Practices in Codling Moth: A Review

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Abstract: The codling moth, *Cydia pomonella* L., is a serious insect pest in pome fruit production worldwide with a preference for apple. The pest is known for having developed resistance to several chemical groups of insecticides, making its control difficult. The control and management of the codling moth is often hindered by a lack of understanding about its biology and ecology, including aspects of its population genetics. This review summarizes the information about the origin and biology of the codling moth, describes the mechanisms of resistance in this pest, and provides an overview of current research of resistant pest populations and genetic research both in Europe and globally. The main focus of this review is on non-pesticide control measures and anti-resistance strategies which help to reduce the number of chemical pesticides used and their residues on food and the local environment. Regular monitoring for insecticide resistance is essential for proactive management to mitigate potential insecticide resistance. Here we describe techniques for the detection of resistant variants and possibilities for monitoring resistance populations. Also, we present our present work on developing new methods to maintain effective control using appropriate integrated resistance management (IRM) strategies for this economically important perennial pest.

Keywords: codling moth; resistance mechanisms; genetics; control strategies; anti-resistance program; geometric morphometrics; SNPs

1. Introduction

Origin and Biology of the Codling Moth, Cydia pomonella

The codling moth (CM) (*Cydia pomonella* L.) is a key pest in most pome fruit orchards in Croatia and worldwide. This pest, besides apple, also is a pest of pear, walnut, quince and some stone fruits where it causes economic losses in fruit production [1]. Balachowsky and Mesnil [2] were the first to mention CM, and provided data on its origin and damages caused to fruit historically. In Croatia, according to Kovačević [3], CM has been present since ancient times. In North America, it is known that the pest was

introduced ca. 1750 [4]. CM was originally from Eurasia, most likely Kazakhstan, but interestingly it was not reported in China until 1953 [5]. Over the last two centuries it dispersed globally with the cultivation of apples and pears. Currently, CM is present in South America, South Africa, Australia and New Zealand [6]. CM occurs in almost every country where apples are grown, and it has achieved a nearly cosmopolitan distribution, being one of the most successful pest insect species known today [7].

CM adults are small (~10 mm in length). They can be distinguished from other moths associated with fruit trees by their dark brown wingtips that have shiny, coppery markings [8]. It overwinters as a fully grown larva within a thick, silken cocoon that can be found under loose scales of bark and in the soil or debris around tree bases [9]. The larvae pupate inside their cocoons in early spring when temperatures exceed 10 °C. Depending on ambient temperature, pupal development occurs within 7–30 days. For the development of adults, the sum of 100 degree-days measured from the 1st of January are required [10]; this value is usually attained at the end of April (i.e., northern hemisphere growing season). For one whole generation of CM, the sum of 610 degrees is required for the complete development of the insect, i.e., from eggs until the appearance of adult moths [10]. A second generation appears after ten days and its flight and egg laying lasts from mid-July to mid-August. Diapausing larvae overwinter in their hibernacula, pupate and then emerge the following spring [11].

The CM has adapted successfully to different habitats by forming various ecotypes, often designated by the term 'strains', which differ among each other in several morphological, developmental and physiological features [12]. On apples and pears, larvae penetrate fruit and bore into the core, leaving brown-colored holes in the fruit that are filled with frass (larval droppings) [8]. If chemical treatment is not used during production, CM can cause a decrease in apple harvest from 30% up to 50%. For apples, intensive production tolerates 1% of infested fruit. Producers, with various methods of fruit protection, try to lower that number below 0.5% [1,3].

Depending on the cultivation area and climatic conditions, the pest develops one to four generations/year. According to Neven [13,14], CM diapause can be facultative and depends on both photoperiod and temperatures. The overwintering generation emerges synchronously in the spring followed by one to two slightly overlapping emergence peaks later on in the season. The CM life cycle can be affected by temperature and day length, resulting in different emergence patterns. Pajač et al. [15] confirmed that there is a possibility that an additional (third) generation of the pest can develop in Croatia in years in which the sum of degree-days is higher than the average. CM abundance cannot be explained by any single ecological factor [16]. Following the dynamics and abundance of CM adults over a 10-year period (2000–2009) Pajač and Barić [17] observed marked differences in their population dynamics. Their research confirmed the earlier appearance of adults in the early season and associated longer flight times. Also, the total number of adults caught in pheromone traps increased as the maximum daily number of moths caught per trap also increased. As the climate has changed and higher daily and annual temperatures are recorded, it is thought that this has a resulting impact on the biology of this pest. It is this global phenomenon coupled with chemical-resistant CM biotypes that could be responsible for the longer flight period and observed overall increase in abundance of CM.

2. Insecticides Resistance

In apple orchards, 70% of insecticides used are to control CM [6]. CM control is achieved using various neuroactive products such as organophosphates, carbamates, synthetic pyrethroids, neonicotinoids, and insect growth regulators (IGR). The CM is a very plastic species and easily adapts to different climatic conditions including the development of resistance to various groups of synthetic insecticides in the USA and Europe [6,18–20]. According to May and Dobson [21], the spread of resistance in insect populations depends on multiple factors, including: the intensity of insecticide selection pressure, the migration ability of individuals, and the fitness costs linked with resistance. In

the CM, the first case of resistance recorded was to arsenates in 1928 in the USA [22]. Since then, new cases of resistance have been reported in almost all of the main apple-growing regions worldwide [18,23–25]. During the 1980s and 1990s CM control in Europe was achieved using broad spectrum insecticides (pyrethroids and organophosphates [OP]), however, the evolution of pesticide resistance efficacy for these insecticides diminished quickly [18,20,26,27]. Reyes et al. [28] states that insecticide resistance in CM in Europe was first detected ca. 1990 to diflubenzuron (in Italy and southeastern France); further pesticide control failures were observed in Switzerland and Spain. CM populations are now resistant to neonicotinoids including environmentally friendly avermectins [28]. Further, CM has developed resistance to azinphos-methyl and tebufenozide in post-diapausing larval stages, to OP [29] insecticides and more recently to insect growth regulators (IGRs). Resistance is mainly associated with the detoxification system's mixed-function oxidases (MFO), glutathione-S-transferases (GST) and esterases (EST) [18,28,30]. A kdr mutation in the voltage-dependent sodium channel is involved in resistance to pyrethroids [31] and an acetylcholinesterase (AChE) mutation has been identified in a laboratory strain selected for resistance to azinphos-methyl [32]. Evidently, the last 20 years' usage of chemical insecticides has modified the development of resistance [6]. An additional problem appeared in the mid-1990s with the development of cross-resistance due to the CM becoming resistant to several chemical groups of insecticides simultaneously [33].

Bosch et al. [34] determined the efficacy of new versus old insecticides against the CM in Spain. In their bioassays, they used 10 different active ingredients on twenty field populations of CM. Very high resistance ratios were detected for methoxyfenozide and lambda-cyhalothrin, while 50% of the populations were resistant or tolerant to thiacloprid. Tebufenozide showed very good efficacy in all the field trials. Even though CM showed resistance to chlorpyrifos-ethyl because of its widespread use, in this trial it was effective against CM populations. All other insecticides (indoxacarb, spinosad, chlorantraniliprole, emamectin, and spinetoram) provided high efficacy. These results showed that resistant CM populations in Spain can be controlled using new reduced-risk insecticides [34]. The newest and, at the same time, the first study of insecticide resistance and analysis about its resistance status in China showed insensitivity to chlorpyrifos-ethyl and carbaryl [35]. The first study of insecticide resistance in Greece showed reduced susceptibility to major groups of insecticides which were included in bioassays (azinphos-methyl, phosalone, deltamethrin, thiacloprid, fenoxycarb, tebufenozide, methoxyfenozide and diflubenzuron). But, also important, known target-site resistance mechanisms (kdr and modified AChE) were not detected [36].

Baculoviruses are insect pathogenic viruses that are widely used as biological control agents of insect pests in agriculture. One of the most important commercially used baculoviruses is the *Cydia pomonella* granulovirus (CpGV) [37]. For more than 30 years, commercial CpGV products have been successfully applied to control CM in organic and integrated fruit production. For all European CpGV products, the original Mexican isolate described by Tanada in 1964, CpGV-M, has been used [37]. According to Harison and Hoover [38], a granulovirus (GV) was identified from CM cadavers and found to be a type 2 GV that killed larvae in three to four days at higher concentrations. After promising field tests as a control measure in 1968 and 1977 [39,40], CpGV was developed into several control products in Europe and in North America. CpGV is used to control CM on over 100,000 ha of organic and conventional apple orchards in Europe [41,42]. Since 2005, resistance against the widely used isolate CpGV-M has been reported from different countries in Europe [41,43,44]. In a multination monitoring program, Schulze-Bopp and Jehle [45] identified that 70% of CM were resistant or partly resistant to CpGV across multiple orchards in Germany, Austria, Switzerland, Italy, and the Netherlands. The recent research by Sauer et al. [46] described autosomal and dominant inheritance of this resistance and demonstrated cross-resistance to different CpGV genome groups. The same authors report a CM field population with a new type of resistance, which appears to follow a highly complex inheritance in regards to different CpGV isolates [47]. In the European Union (EU) there are no strategic integrated

pest management (IPM) programs that solve the current confusion surrounding CM control and resistance. There is a need for new control tools and a fresh approach to CM control and management in the EU.

3. Present Strategies in Codling Moth Suppression

3.1. Mechanical Control

Because of resistance development in CM populations, there is a need for alternatives to insecticides and CpGV. In recent studies, special attention is given to insect exclusion netting systems in apple production. The first netting system was designed in France in 2005 and in 2008 it was introduced in Italy. In both countries, a high level of efficacy of nets was observed against CM, especially for the 'single-row' system, which the authors recommend because it was more efficient and more durable than the 'whole-orchard' version. Also, this method enables a significant reduction in pesticide use without any major risks for apple production [48]. Pajač Živković et al. [49] tested the effectiveness of insect exclusion netting systems in preventing the attack of CM on apple fruits in Croatia. The authors showed a significant reduction in CM catches and also fruit injury compared to the non-netted control. This is consistent with similar studies in which nets significantly reduced the number of CM catches [50,51]. Modifying the orchard microclimate and reducing the interception of light using netting systems could have a negative consequence on the organoleptic quality of apple fruit according to Baiamonte et al. [52]. While the netting system prevents the entry of insect pests, it also serves as a barrier to beneficial insects (e.g., ladybugs, true bugs and syrphid flies) which could negatively affect natural pest control services. [49]. Alaphilippe et al. [48] recommend, due to the cost and constraints of netting, that this method be used in areas where CM is difficult to control.

3.2. Chemical Control

Chemical control of CM is still the main method used in integrated pome fruit production [53]. According to the Insecticide Resistance Action Committee (IRAC) [54] for CM control in most countries, there are 11 modes of action (MoA) available on the market depending on the country. For CM, some insecticides affect the nervous system, or pest growth and development. Acetylcholinesterase inhibitors (carbamates and organophosphates), sodium channel modulators (pyrethroids), nicotinic acetylcholine receptor agonists (neonicotinoids), nicotinic acetylcholine receptor agonists allosteric modulators (spinosyns), chloride channel activators (avermectins), voltage-dependent sodium channel blockers (oxadiazines) and ryanodine receptor modulators (diamides) all affect the pest's nervous system; these insecticides are fast-acting [54]. Juvenile hormone mimics (phenoxyphenoxy-ethylcarbamate), chitin biosynthesis inhibitors—type 0 (benzonylureas) and ecdysone agonists (diacylhydrazines) all affect pest growth and development [54]. Insect development is controlled by juvenile hormones and ecdysone by directly perturbing cuticle formation/deposition or lipid biosynthesis. Such insect growth regulators are generally slow to moderately-slow acting [54].

From ca. the 1890s until today, insecticide groups and active substances used for CM suppression have been rapidly evolving. As can be seen from Table 1, chlorinated hydrocarbons, organophosphates, and carbamates were first used for the suppression of CM. Frequent applications of pyrethroids began in 1980 due to their lower toxicity to mammals and strong initial effect on insects. Although they are more environmentally friendly and can be applied in low doses per unit, area resistance has been observed. Microbial insecticides and insect growth regulators have been mostly used since the 1980s but after several years of application, resistance to them also occured. Since 2000 there have been a couple of new active compounds (i.e., chlorantraniliprole, spinetoram) that meet the requirements of integrated pest management (IPM) programs.

Table 1. Review of registered insecticides to suppress codling moth from 1890–current [54,55] and time of resistance development according to the Arthropod Pesticide Resistance Database [56].

Insecticide Group	MoA [54]	Insecticide/Active Substance	Use Period (Approximate)	Resistance Development (Year of First Report/Region)
Inorganic/others		Arsenate	1890s–1950s	1928/USA
		Nicotine	1960s	
Chlorinated hydrocarbons		DDT	Mid 1940s–1970s	1955/USA
		Thiodan/Endosulfan	1960s–1970s	1965/Syria
Organophosphates	1B	Diazinon	1950s–2000s	
		Phosalone	1960s–2000s	
		Azinphosmethyl	1950s–present	1991/USA
		Chlorpyrifos-ethyl	1960s–present	
		Chlorpyrifos-methyl	1960s–present	2011/France, Spain
		Methidation	1950s–1990s	
		Phosmet	1970s–present	1999/USA
		Mevinphos	Mid 1950s–mid 1990s	
		Methomyl	1970s–1990s	
		Oxamyl	Mid 1980s–1990s	
		Formetante hydrochloride	1970s–1990s	
Charbamates	1A	Carbaryl	1970s–present	2012/Spain
Pyrethroids	3A	Fenvalerate/ Esfenvalerate	1970s–present	
		Permethrin	1970s–present	
		Bifenthrin	1980s–present	
		Deltametrin	1970s–present	2001/China
		Flucythrinate	1980s–present	
		Lambda-cyhalotrin	1980s–present	2008/USA
		Gama-cyhalotrin	1980s–present	
		Tau-fluvalinate	1980s–present	
Microbial insecticides		Bacillus thuringiensis sub sp. kurstaki	1980s–present	
		Codling moth granulovirus (CpGV)	1980s–present	2007/Germany
Naturalites	5	Spinosad	1990s–present	
Insect growth regulators	15	Benzonylureas (diflubenzuron, hexaflumuron, flufenoxuron, triflumuron,	1970s–present	diflubenzuron/1988/USA triflumuron/1995/France teflubenzuron/1995/France

		lufenuron, teflubenzuron)		flufenoxuron/2011/Spain
	7B	Fenoxycarb	1980s–present	2007/Czechoslovakia
	18	Tebufenozide	1990s–present	1995/France
		Methoxyfenozide	1990s–present	2008/USA
	7B	Pyriproxyfen	2000–present	
Nicotinoids	4A	Acetamiprid	1990s–present	2010/USA
		Thiacloprid	2001–present	2011/Spain
		Thiamethoxam	2001–present	
Avermectins	6	Emamectin benzoate	2000–present	
Anthranilic diamide insecticides	28	Chlorantraniliprole	2007–present	
Spinosyns	5	Spinetoram	2011–present	

The classic model of CM suppression implies the intense application of aggressive chemical preparations, most commonly a wide spectrum of activity. Due to the altered biology of the CM (i.e., more generations/year) insecticides must be applied several times per season [57,58]. Some populations of CM have gained simultaneous resistance to several chemical subgroups of insecticides. In light of this and to delay resistance development, the rotation of compounds from different MoA groups ensures that repeated selection with compounds from any single MoA group is minimized. By rotation of insecticides across all available classes, selection pressure for the evolution of any type of resistance is minimized and the development of resistance will be delayed or prevented. The presence of kdr resistance renders pyrethroids less effective, whereas carbamates and organophosphates can still be used. In addition, the use of larvicides such as the organophosphate in conjunction with pyrethroids can support resistance management through rotation of MoA across different life stages. Effective long-term resistance management is important, but many factors have to be considered (including regional availability of insecticides). Currently, there are eight MoAs for CM control. In practice, it should not be difficult to implement rotation programs because there are enough active substances of insecticides in Europe that have mandated approval for CM. Alternatives to more persistent molecules are being developed [59,60]. For example, Bassi et al. [61] describe the development of a new compound, chlorantraniliprole, which belongs to a new class of selective insecticides. That makes chlorantraniliprole a valuable option for insecticide resistance management (IRM) strategies. Chlorantraniliprole is safe for key beneficial arthropods and honey bees, which renders it IPM compliant (i.e., excellent toxicity profile and use in low doses provide safety for consumers and agricultural workers). Nevertheless, there is a need for the improvement of alternative pest control methods, such as the application of microbial insecticides, mating disruptors or attract-and-kill methods. Production of high quality and healthy fruit that does not harm human health and the environment should continue to rely on an integrated production system where insecticide treatments must be applied responsibly and only when they are needed [62].

3.3. Biological Control

Biological control agents play a key role in most IPM strategies; these include entomopathogens, parasitoids and predators [63]. For augmentative biological control of CM, viruses such as granulovirus and entomopathogenic nematodes (EPNs) (*Steinernema carpocapsae*, *Steinernema feltiae*, *Heterohabditis* spp.) have been used as microbial agents [61].

The most widely used biopesticide is *Bacillus thuringiensis* (*Bt*) [64]. For controlling CM, *Bt* is very limited because of the improbability of ingesting a lethal dose of *Bt* toxin during feeding by neonate larvae [63]. On the other hand, granulovirus (GV) (Baculoviridae) is one of the most efficient and highly selective pathogens for suppression of CM. Its specificity for CM and safety to non-target organisms is documented by Lacey et al. [65]. It is one of the most virulent baculoviruses known. According to Laing and Jaques (1980) and Huber (1986), the LD_{50} for neonate larvae has been estimated at 1.2 to 17 granules/larva. The biggest disadvantage of CpGV is its sensitivity to solar radiation [66–68], and the need for frequent reapplication.

Parasitoids are insects whose larvae feed and develop within or on the bodies of other arthropods. Each parasitoid larva develops on a single individual and eventually kills that host [53]. Parasitoid wasps from the families Braconidae (*Ascogaster quadridentata* and *Microdes rufipes*), Ichneumonidae (*Mastrus ridibundus* and *Liotryphon caudatus*) and Trichogrammatidae (*Trichogramma* sp.) are the best known parasitoid species of CM. The parasitism of entomophagous wasps *M. ridibundus* and *A. quadradentata* has been successfully applied in CM control in some US states [63]. Species from Braconidae most commonly parasitize CM larvae, and Ichneumonidae parasitize CM larvae and adults and Trichogrammatidae parasitize eggs of Tortricidae moths. A reduction of 53%–84% of CM was achieved by the experimental release of two *Trichogramma* species (*T. dendrolimi* and *T. embryophagum*) in apple orchards in Germany [53]. An additional benefit of the release of parasitoids is the simultaneous control of other pest species in apple orchards. The beneficial organisms alone can play an effective role in IPM but in general, the effect on CM control in economically productive orchards is considered insufficient [69].

For biological control, the most promising EPN species for suppression of CM are from the families Steinernematidae and Heterorhabditidae [70]. Species from both families are obligatorily associated with symbiotic bacteria (*Xenorhabdis* spp. and *Photorhabdis* spp., respectively) which are known for quickly killing its host insect. The most promising results for CM control have been with *Steinernema feltiae* and *Steinernema carpocapsae* [71]. Cocooned overwintering CM larva is the life stage most practical to control using EPNs. That life stage occurs between late summer and early spring in cryptic habitats, such as underneath loose pieces of bark or in pruning wounds on trees [71]. Eliminating cocooned larvae would protect fruit from damage in the following growing season [72]. The main obstacles for successful CM control with EPNs are low fall temperatures and desiccation of the infective juvenile stage of EPNs before they have penetrated the host's cocoon.

Few studies exist on CM predators and biological antagonists. The largest group of CM predators are insects. Other important CM predators can be spiders, bats and birds [73–75]. In undisturbed habitats the eggs and neonate larvae of CM are most commonly preyed upon by small heteropteran insects, including: Anthocoridae, Miridae, *Phytocoris* sp., *Diaphnidia* sp., and *Deraeocoris* spp. Larger Carabidae and Dermaptera also play an important role [76]. The review of CM natural enemies and stages that are affected are summarized in Table 2.

Table 2. Review of codling moth natural enemies and life stage attacked [63].

Natural Enemies	Organism/Family	Family/Species	CM Life Stage Attacked
Entomopathogenic organisms	Virus	Granulovirus (CpGV)	Neonate larvae
	Bacteria	*Bacillus thurigiensis*	Neonate larvae
	Fungi	*Beauveria bassiana*	Cocooned overwintering larvae
	Nematodes	Steinernematidae	

		Heterorhabditidae	Cocooned overwintering larvae
Predators	Anthocoridae	*Orius insidiosus* *Anthocoris musculus*	Eggs and neonate larvae
	Miridae	*Hyaliodes harti* *Phytocoris* sp. *Diaphnidia* sp. *Blepharidopterus angulatus* *Deraeocoris* spp.	
	Reduviidae		Mature larvae
	Nabidae		
	Carabidae, Trogossitidae, Malachiidae, Staphylinidae, Cleridae, Cantharidae, Elateridae		Cocooned larvae
	Formicidae		Mature larvae
	Phlaeothripidae	*Haplothrips faurei* *Leptothrips mali*	Eggs
	Dermaptera	*Forficula auricularia*	
Parasitoids	Braconidae	*Ascogaster quadridentata*	Larvae
	Ichneumonidae	*Microdes rufipes* *Mastrus ridibundus* *Liotryphon caudatus*	Larvae and adults
		Pimpla turionellae	Pupae
	Trichogrammatidae	*Trichogramma* sp.	Eggs

Part of biological control is also ecological engineering, which includes the manipulation of farm habitats to be less favorable for arthropod pests and more attractive to beneficial insects [77]. To increase the activity of EPNs, ecological engineering encourages the use of environmental modification with mulches and irrigation [63]. Mulching is a strategy for conserving water and it is likely to become increasingly important for long-term sustainability in orchards [78]. In support of mulch, compared with bare ground, it may enhance CM control by providing cocooning sites for larvae, in a substrate that is easy to treat, maintains moisture and enhances nematode activity [72,79,80]. De Wall et al. [81] investigated the potential of using the EPN *Heterorhabditis zealandica* in combination with different mulch types (pine chips, wheat straw, pine wood shavings, blackwood and apple wood chips) to control diapausing CM. Their results showed that highest CM mortality was when they used pine wood shavings as mulch (88%) compared to pine chips, wheat straw, blackwood and apple wood chips (41%–88%). Importantly, their research showed that humidity had to be maintained above 95% for at least 3 days to ensure nematode survival.

3.4. Population Genetic Monitoring

Analysis of population genetic structure is a key aspect in understanding insect pest population dynamics in agriculture [82]. The development of effective pest management strategies relies on a multidisciplinary approach [83] and one component of this is knowledge of the population genetics of

the pest. Genetic structure and patterns of dispersal at the local and landscape scale are important for establishing a control strategy for insect pests [84]. Understanding the population genetics of CM invasions enables identification of the geographic origin, number of introduction events and the spread of the infestation [85]. According to Keil et al. [86] CM populations are composed of mobile and sedentary genotypes and this has direct consequences for the local observable population dynamics of the species as well as the implementation of new behavior-based pest management measures (e.g., mating disruption, attract-and-kill and SIT technique) [87]. The first attempt to elucidate the population genetic structure of CM on a global geographic scale (i.e., inter-continental) using allozymes was conducted by Pashley and Bush [88]. These authors showed that CM populations were not differentiated among countries investigated (F_{ST}: 0.05). Following this, Bues and Toubon [89] used the same approach to study populations in Switzerland and France. More recently, Timm et al. [90] and Thaler et al. [7] used amplified fragment length polymorphism (AFLP) markers to study the molecular phylogeny and genetic structure of CM where they found large differences among these populations (F_{ST}: 0.70). More recently, co-dominant microsatellite markers from CM were developed by Zhou et al. [91] who characterized 17 loci. An additional 24 microsatellite loci were characterized by Frank et al. [92], with these loci most frequently used in population genetic studies worldwide [6,15,82,84,93].

Franck et al. [6] used those markers to investigate the genetic structure of CM populations from 27 orchards from three continents (Europe, Asia and South America) to determine the dynamics of CM meta-populations and the impact that human activities had on these dynamics. Franck et al. [6] showed that populations of CM are structured by geographic distance on the intercontinental level. However, analyses of CM populations from treated and untreated orchards in Europe and South America (France and Chile) did not show significant genetic differentiation by country, but rather a pattern of minor influence of insecticide treatments on allelic richness. A similar comparison of CM genetic structure from treated versus untreated populations using microsatellite markers (following Franck et al. [6]) was conducted in Croatia [15]. Even though differences in genetic structure among populations were low and not statistically significant, untreated populations of CM had the highest average number of alleles and the largest number of unique alleles compared to treated populations. Overall, the study's findings suggested a possible reduction of allelic richness in treated populations due to the frequent application of insecticides. The authors have questioned whether these genetic changes may relate to the increase in reproductive abilities of CM and a change in its overall biology in Croatia [15].

Frank and Timm [82] also used microsatellite markers to study CM genetic structure and gene flow from organic versus treated apple orchards. They found low genetic variation between populations but significant partitioning of genetic variation within individuals. Chen and Dorn [93] used nine microsatellite markers to investigate genetic differentiation and the amount of gene flow between populations from orchards in Switzerland and laboratory populations. They noted significant genetic differentiation among populations from apple, apricot and walnut orchards and also between populations collected from orchards that were less than 10 km apart. These results are consistent with Timm et al. [90] and Thaler et al. [7] and provide significant evidence for CM population differentiation at small spatial scales, even within the same bio-region. Fuentes-Contreras et al. [94] found significant but weak genetic differentiation between populations across time and space comparisons. These authors found no significant correlation (r: −0.03; p: 0.56) between genetic distance and geographic distance of the studied populations and the lack of structure at a local scale with frequent adult movement between treated and untreated orchards. Also, their data highlights the importance of developing area-wide management programs for successful CM control. Men et al. [95] used eight microsatellite loci to infer the characteristics of genetic diversity and genetic structure of 12 CM populations collected from the main distribution regions (Xinjiang, Gansu and Heilongjiang Provinces) in China and compared them with one German and one Swiss population.

They found ascertained loss of genetic diversity and important structuring related to distribution, however no important correlation between genetic distance and geographic distance among populations (F_{ST}: 0.22091) was found. Voudouris et al. [96] used 11 microsatellite loci to analyze nine CM populations from Greece and six from France for comparison. Results from Bayesian clustering and genetic distance analyses separated CM populations in two genetic clusters. In agreement with previous published studies F_{ST} values showed low genetic differentiation among populations (Greek populations F_{ST}: 0.009 and F_{ST}: 0.0150 French populations).

Dispersal of fertilized females is important because it directly affects the effectiveness of pest control programs. Margaritopoulos et al. [97] used the mark-release-recapture (MRR) method on male and female individuals from two laboratory and one wild CM populations. Kinship analysis was made on 303 genotyped individuals (11 microsatellite loci) from two contiguous apple orchards to see the dispersal patterns in the Greek CM populations. The collected data confirm the view of the sedentary nature of CM and indicate that genotypes able to migrate at long distances are not present in the studied area. The information obtained could be fundamental for determining the dynamics and genetics of the pest populations and for developing efficient management programs. Results about the dispersal pattern of codling moths might have practical applications in mating disruption or mass trapping pest control programs.

3.5. Area-Wide Integrated Pest Management

The 5-year CAMP (CM Area-Wide Management Program) was the first of the area-wide programs initiated by the US Department of Agriculture [98]. Demonstration of this was initiated in 1995 in a multi-institutional program created through the collaboration of university and government researchers in Washington, Oregon and California. The goal of this program was to implement, assess, research and educate industry users about promising new IPM technologies. CAMP was highly successful in fueling the rapid adoption of a new paradigm in orchard pest management that resulted in significant reduction in fruit injury using nearly 80% less broad-spectrum insecticides [95].

IPM is based on environmentally and toxicological acceptable treatments. Using pheromones, attract-and-kill methods and mating disruption results in a promising way of controlling CM. According to Witzgall et al. [99], orchard treatments with up to 100 g of synthetic pheromone per hectare effectively control CM populations over the entire growing season. The disadvantage of these techniques is that females are not affected [100].

After Roelofs et al. [101] identified the main pheromone components for CM attraction (i.e., E8, E10-dodecadienol (codlemone)), pheromone traps started to be a useful tool for insect detection and monitoring and later for its suppression. Mating disruption is based on tactics to employ synthetic sex pheromones that interfere with the ability of males in finding female moths and as a control strategy it shows considerable promise. Currently, it is used to suppress CM populations in over 160,000 ha of apple and pear orchards worldwide [99]. The first commercially available pheromone dispenser for control of CM was Isomate-C®, which became available in the USA in 1991 [55]. Monitoring of CM in orchards treated with sex pheromone mating disruption (MD) has become widely adopted and is very important for its effective management [99]. Traps used for monitoring are baited with the sex pheromone (E, E)-8,10-dodecadien-1-ol (codlemone) that attracts males [102] and ethyl (E, Z)-2,4-decadieonate, a pear-derived kairomone, to attract both sexes of CM [103]. The combination of pear ester with codlemone (PH-PE) in a lure is effective for monitoring both sexes of codling moth in sex pheromone-treated orchards. Monitoring females, instead of only male CM, has certain benefits, like egg density and timing of egg hatch. A number of studies have used pear ester's attractiveness for both male and female CM to develop alternative approaches to further enhance the catch of female moths [104–106]. Using pear ester with acetic acid (AA) can increase moth catches, especially of females [107]. The

co-emission of acetic acid improves the capture performance of pear ester in clear traps to levels equivalent to the PH-PE lure when used in orchards treated with sex pheromone dispensers [108]. The effectiveness of this mating disruption as a technique depends on numerous factors (shape, size, isolation and environment of orchards) as well as the starting density of the CM population itself. In order for mating disruption to be successful there is a need for low CM population levels and a reliable monitoring system [109]. Mating disruption for CM began in the US in 1995 in large contiguous apple blocks (400 ha) and small private orchards [110]. According to Witzgall et al. [99] and Casado et al. [111], Europe also does not lag far behind in its application of this technique. In Croatia, this method is not widely used, although the first field trials in 1999 and 2000 [112] were promising and did reduce the number of insecticides being used during those growing seasons. Barić and Pajač Živković [113] showed that the highest protection efficacy was achieved with 92.65% control in the standard part of the orchard, and the efficacy of mating disruption was 67.65% and 73.53%. Although the authors concluded that this method of control was not economically justifiable given the high cost (approx. 150 €/ha) of protection and first-class fruit losses. However, their results also confirmed that the mating disruption method must be combined with the application of two insecticide treatments to increase the efficacy and profitability of apple production. Miller and Gut [114] agree that pest control by mating disruption is an important and growing industry. This combined control of CM is more ecologically oriented and also meets the toxicological minimum requirements of the food suppliers and the food retail chain. They propose some key economic and policy questions that will require the collective efforts of scientists and society as a whole if the benefits of mating disruption are to be maximized. There is still a lot of work to be done to optimize the role of mating disruption as one of the components of modern integrated pest management.

Mass trapping, as one of the first mating control strategies, can significantly reduce CM damage levels. However, several intensive field studies have shown that it is not effective enough for CM control because of the low damage thresholds (no more than 1%–2% of the crop) required in commercial apple growing. Since adequate control cannot be achieved by using only mass trapping, there is a need for combining it with other control measures [115]. Another problem is the cost and practical difficulties of deploying sufficient trapping stations. If droplets containing sex pheromones and a fast-acting insecticide are used instead of traps [116], then the costs can be substantially reduced. The potential strength of the approach is that males have been removed from the system, stopping their ability to find a mate.

The attract-and-kill method, in its technically simplest form is the attractant applied as a 'tank-mix' with an insecticide. This method uses the same attractants as mass trapping but in an envelope impregnated with an insecticide on the outside. This technology has shown efficacy in the control of several important lepidopteran pests including pink bollworm, *Pectinophora gossypiella* (Saunders), light brown apple moth, *Epiphyas postvittana* (Walker), and CM [117]. In both systems, mass trapping and attract-and-kill, chemicals are utilized only when the population increases considerably [118].

For AW-IPM the integration of sterile insects is a very effective and environmentally friendly control tactic that can be combined with other control practices and offers great potential [119,120]. Sterile insect technique (SIT) is non-destructive to the environment, does not affect non-target organisms, and can easily be integrated with other biological control methods such as parasitoids, predators and pathogens [121]. The technique has gained traction in the last few decades [122,123]. SIT is an autocidal pest control technique that controls pests with a form of birth control [121]. The target pest species is mass-reared, sterilized through the use of gamma radiation and then released in the target area in high numbers. After release, sterile males will locate and mate with wild females and transfer the infertile sperm thus reducing the wild population. Another method of sterilization is genetic manipulation or sexing strains, where lethal mutations are incorporated into sperm [121]. The SIT, together with mating disruption, granulosis virus and EPNs, are the options that offer great potential as cost-effective additions to accessible management techniques for AW-IPM approaches.

In Table 3, a review of changes in the suppression of CM through the last two decades and factors that affect the current scenario in comparison to the year 2000 is shown. Reduction of chemical control measures due to EU regulations and food chain pressures, increased adoption of semiochemicals for mating disruption, and microbial insecticides contributed to the suppression of CM. Improved investigation tools for resistance detection and confirmatory assays have contributed to the decrease of field resistance issues and better knowledge of resistance.

Table 3. Changes in codling moth control from 2000 until now (modified according to IRAC [54]).

	2000	2012	2017
No. of MoA available for codling moth control *	8	10	11
No. of individual insecticides available **	High	Decreasing	Fewer
Use of semiochemicals (mating disruption)	Minor	Moderate	Increasing
Microbial insecticides	Minor	Moderate	Moderate
Biological control	Minor	Minor	Minor
Regulatory pressure	Low	High	Decreasing
Food chain pressure	Low	High	Decreasing
Field resistance issues **/***	Moderate	Decreasing	Low
Resistance knowledge and investigation tools	Moderate	Increasing	High

* According to IRAC Mode of Action (MoA) classification, four MoA were introduced from 1997–2000, and two during 2007–2010. ** Number of individual insecticides available is decreasing every year. The criteria introduced in the revision of EU Directive 91/414 may concern a significant number of available insecticides, with an impact on sustainable control options. *** Dependent on the implementation of the other factors. The assumption is that sustainable insecticide use will continue to be possible and implemented. In this respect, increased use of non-chemical tools will play a key role.

4. Resistance Management Strategies

The most effective strategy to combat insecticide resistance is to do everything possible to prevent it from occurring in the first place. To this end, crop specialists recommend insect resistance management (IRM) programs as one part of a larger (IPM) approach covering three basic components: monitoring pest complexes in the field for changes in population density, focusing on economic injury levels and integrating multiple control strategies. IRM is the scientific approach of managing pests long term and preventing or delaying pest evolution towards pesticide resistance and minimizing the negative impacts of resistance on agriculture [124]. The basic strategy for IRM is to incorporate as many different control strategies as possible for particular pests including the use of synthetic insecticides, biological insecticides, beneficial insects (predators/parasitoids), cultural practices, transgenic plants (where allowed), crop rotation, pest-resistant crop varieties, and chemical attractants or deterrents. The establishment of an anti-resistance program in perennial crops is slightly more difficult than in arable crops where crop rotation is possible. If non-chemical methods provide satisfactory pest control, preference should be given to them over chemical methods. Key insect pests of apple and grape such as CM and grapevine moths are effectively controlled via mating disruption. In Switzerland, mating disruption is in use in 50% of the apple orchards and 60% of vineyards, and this has enabled a reduction of synthetic pesticide use by two thirds [125].

Insecticides, if necessary, must be selected with care and their impact on future pest populations considered. Broad-spectrum insecticides should always be avoided when a more specific insecticide will suffice. Even cultural practices, such as irrigation for destroying overwintering stages (e.g., cotton

bollworm, *Helicoverpa armigera*) of pests can play a role in managing resistance [126]. When insecticide is applied it should be timed correctly and for the best efficacy, it should target the most vulnerable life stage of the insect pest. It is important to mix and apply insecticides carefully. With the increasing problem of resistance, there is no space for error in terms of insecticide dose, timing, coverage, etc.

Reducing doses, application frequency, and resorting to the partial application of pesticides contribute to the IPM goal of reducing or minimizing risks to human health and the environment. Regular monitoring for insecticide resistance is essential to react proactively to prevent insecticide resistance from compromising control [127].

Before applying any CM control action, it is necessary to monitor CM occurrence and early infestation of apples. Pheromone traps are used in orchards to determine the present amount of adult male moths. For estimating the potential infestation risk of the second generation, it is recommended to examine 1000 young apples in June for damage or the presence of CM [128]. Spray thresholds are also based on the number of moths in the pheromone traps or on infestation rates detected in the harvest of the current or last season. For apples, the economic threshold for the CM is 1% of infested fruit [55].

Figure 1 shows recommendations for effective CM control and resistance management based on current knowledge: I. to monitor; II. application of ecotoxicological favorable protection measures like mating disruption (when CM population levels are low); III. application of chemical control measurements (if necessary); and IV. control of overwintering stages by applying biological agents (e.g., CpGV, nematodes) to reduce the late summer and fall CM population in order to minimize the population in the following growing season. It is an effective example of how resistance management should work in orchards (Figure 1).

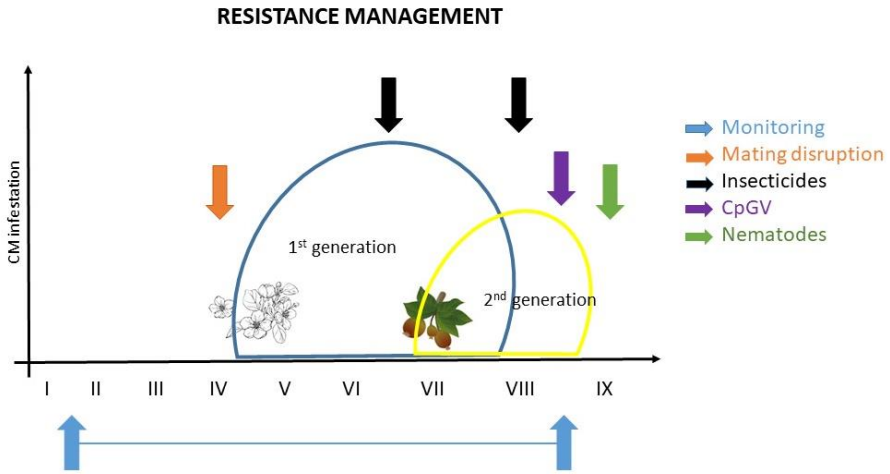

Figure 1. Example of resistance management for codling moth; the ideal control is a combination of different measures (modified by Martina Kadoić Balaško).

5. Perspectives in Codling Moth Resistance Detection

Reliable data on resistance are essential to successful resistance management. Bioassay is a method used for evaluating the status of resistance in insect populations. Effective resistance management relies on sound information about the extent and intensity of resistance problems [128]. There are several different bioassay methods to monitor for CM resistance, such as diagnosing metabolic resistance using differential enzymatic activity between life-stages within the same population. The analysis of the enzymatic activity (MFO, GST, EST) in a CM population is a key element for resistance evaluation [54]. In the last decade, large-scale monitoring for field resistance mostly relied on topical application to diapausing codling moth larvae. Recent studies have confirmed their validity for IGRs but questioned

their reliability for the prediction of field resistance with some neurotoxic insecticides [54]. Bioassay of the target-stage includes resistance monitoring done on the target instar. For larvicidal products, ingestion bioassays on neonate larvae (F1 or F2 of the feral population), IRAC method no. 017, normally provide a more reliable indication of the field situation than topical application to diapausing larvae [54].

So far, the only approved method for CM sensitivity monitoring is IRAC method 017 [54]. This method is specifically recommended by the IRAC Diamide Working Group for evaluating the susceptibility status of diamide insecticides (IRAC MoA 28). Also, it is suitable for the following insecticide classes (IRAC MoA class): organophosphate (1B), pyrethroid (3A), neonicotinoids (4A), spinosyn (5), avermectin (6), juvenile hormone mimics (7A), fenoxycarb (7B), benzyl urea (15), diacylhydrazine (18), indoxacarb (22A), metaflumizone (22B), and pyridalyl (un) [54]. According to this method, the first step is to collect a representative sample of insects from a field. These may be larvae, pupae or adults for rearing to the appropriate stage from which an F1 population for testing can be reared. A minimum of 100 larvae or diapausing pupae should be collected for each population to be tested, to establish a breeding colony of at least 50 adults. When we have enough CM larvae for the bioassay, the second step is to prepare an accurate dilution of the test compound from the identified commercial product. Six evenly spaced rates allowing a clear dose-response are recommended [54]. For this method, a single neonate (less than 24 h old) of CM larvae should be used. In the case of diamide insecticides, organophosphates (1B), pyrethroids (3A), neonicotinoids (4A), spinosyns (5), avermectins (6), indoxacarb (22A), metaflumizone (22B) and pyridalyl (un), a final assessment of larval mortalities (dead and live) is made after 96 h. For juvenile hormone mimics (7A), fenoxycarb (7B), benzyl urea (15) and diacylhydrazine (18), a 120-h assessment period should be used. Also, larvae should go through full molt before the mortality assessment [54]. The number of dead larvae and moribund larvae (seriously affected larvae which are unable to make coordinated movement and cannot return to an upright position when turned upon their backs with a seeking pin or fine-pointed forceps) are to be summed and considered as dead. Results should be expressed as percentage mortalities, correcting for "untreated" (control) mortalities using Abbott's formula [54].

Through innovation it is possible to establish reliable strategies for detecting resistant CM populations. Of most importance is the timely detection of resistant populations in order to suppress them and prevent further spread of resistance. For this purpose, exploration of existing tools, though with novel use as monitoring tools, is warranted (i.e., geometric morphometrics and population genomics).

Geometric morphometrics (GM) offers a powerful method for studying intraspecific variation or ecotypes and it has been shown to be a useful bio-monitoring tool [129]. It is known that metric properties (wing shape and size) are the first morphological characters to change as influenced by environmental and genetic factors [130,131]. This therefore makes them an ideal technique to detect and monitor population variation and resistant variants in the field [132,133]. Furthermore, the use of GM generates important new data on basic insect biology and ecology.

Recently, wing or body shape and size has been used as a population bio-marker to detect: differences between susceptible and resistant variants [134]; population changes related to invasion [135]; and morphological differences in resistant versus non-resistant populations and rotation versus *Bt*- resistant strains of western corn rootworm [136]. GM was tested as an existing method, though novel in its application, for morphological differences in field-insect pest populations versus laboratory populations and integrated versus ecological populations in Croatia. That is, Pajač Živković et al. [137] revealed two noticeable wing shape morphotypes in *Drosophila suzukii* (i.e., vein configuration) between grape and strawberry crops. Different IPM practices in agro-ecosystems generate different degrees of disturbance in insect communities, as shown by Benitez et al. [138] where shape variation and

fluctuating asymmetry levels were estimated by applying GM methods to the beetle *Pterostichus melas melas.*

Specifically, for CM, Khaghaninia et al. [139] used GM methods as tools to show significant differences in CM fore and hindwings as a function of season (overwintered vs. summer), geographic location and sex. Also, Pajač Živković et al. [140] investigated the relationship between different pest management types and CM morphology using GM. The authors detected population changes related to different types of apple production. The aforementioned publications provide compelling evidence for the use of GM as a population bio-marker when applied to CM and other insect pest monitoring.

Recent enhancements with the speed, cost and accuracy of next generation sequencing are revolutionizing the discovery of single nucleotide polymorphisms (SNPs) and field of population genomics. SNPs are increasingly being employed as the marker of choice in the molecular ecology toolkit in non-model organisms. SNPs are attractive markers for many reasons [141,142], including: the availability of high numbers of annotated markers; low-scoring error rates; relative ease of calibration among laboratories compared to length-based markers; and the associated ability to assemble combined temporal and spatial data sets from multiple laboratories.

SNPs are single base substitutions found at a single genomic locus. Although they have lower allelic diversity and provide less statistical power to discriminate unique genotypes, they have a denser and uniform distribution within genomes which makes them very useful for population genetic studies. In recent times, SNPs have become an affordable and readily accessible means of generating a lot of data quickly for non-model species [143]. Genotyping of SNPs has potentially far-reaching applications in insect population genomics. SNP detection has facilitated association mapping studies in many insect species including: *Drosophila melanogaster* [144], *D. v. virgifera* [145], *Aedes aegypti* [146], *Glossina fuscipes* [147], *Diatraea saccharalis* [148], *Phaulacridium vittatum* [149] and other insects in which specific nucleotides are statistically associated with complex phenotypic traits. Detailed genomic data could provide an answer about genetically conditioned resistance development in insects. By combining genetic and GM population monitoring, it may be possible to identify the addition or deletion of alleles and different haplotypes, and the genetic and morphometric patterns which have developed under the selective pressure of control.

6. Conclusions

CM is the most harmful insect species of the Tortricidae family that causes economic damage to apple production worldwide. The suppression of this pest in the past relied on intensive insecticide application(s) which ultimately led to the development of resistance and caused a decrease in population of beneficial species which were once the only natural regulators of pest populations in apple farming. One of the basic goals of integrated production is growing high quality and healthy fruits that contain minimal residues of pesticides; such production is safer for human health and the environment. To achieve this goal, environmentally friendly area-wide IPM strategies must be established. This involves the use of pheromones and kairomones (attract-and-kill methods and mating disruption) and sterile males (SIT technique) which combined with the use of natural enemies (mainly viruses and nematodes) serve as good alternatives to chemicals. Also, recent advancements in the use of mechanical protection measures against CM (insect-proof nets) have shown very promising results in field trials. All available control measures against CM should be used in combination and there should be an informed and systematic strategy for their use. Effective IRM strategies should involve all available tools for pest control (e.g., natural enemies, biotechnical tools, alternative insecticides) and make a concerted effort to trial and use existing technologies, though with novel applications (e.g., GM for monitoring population phenotypic changes and SNPs for monitoring population genetic changes) for their monitoring, therefore fulfilling the best practice resistance management strategy discussed here.

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2.3. Publication No. 3

insects

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Review

Modern Techniques in Colorado Potato Beetle (*Leptinotarsa decemlineata* Say) Control and Resistance Management: History Review and Future Perspectives

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Simple Summary: The Colorado potato beetle (CPB) is one of the most important potato pest worldwide. It is native to U.S. but during the 20th century it has dispersed through Europe, Asia and western China. It continues to expand in an east and southeast direction. Damages are caused by larvae and adults. Their feeding on potato plant leaves can cause complete defoliation and lead to a large yield loss. After the long period of using only chemical control measures, the emergence of resistance increased and some new and different methods come to the fore. The main focus of this review is on new approaches to the old CPB control problem. We describe the use of *Bacillus thuringiensis* and RNA interference (RNAi) as possible solutions for the future in CPB management. RNAi has proven successful in controlling many pests and shows great potential for CPB control. Better understanding of the mechanisms that affect efficiency will enable the development of this technology and boost potential of RNAi to become part of integrated plant protection in the future. We described also the possibility of using single nucleotide polymorphisms (SNPs) as a way to go deeper into our understanding of resistance and how it influences genotypes.

Abstract: Colorado potato beetle, CPB (*Leptinotarsa decemlineata* Say), is one of the most important pests of the potato globally. Larvae and adults can cause complete defoliation of potato plant leaves and can lead to a large yield loss. The insect has been successfully suppressed by insecticides; however, over time, has developed resistance to insecticides from various chemical groups, and its once successful control has diminished. The number of available active chemical control substances is decreasing with the process of testing, and registering new products on the market are time-consuming and expensive, with the possibility of resistance ever present. All of these concerns have led to the search for new methods to control CPB and efficient tools to assist with the detection of resistant variants and monitoring of resistant populations. Current strategies that may aid in slowing resistance include gene silencing by RNA interference (RNAi). RNAi, besides providing an efficient tool for gene functional studies, represents a safe, efficient, and eco-friendly strategy for CPB control. Genetically modified (GM) crops that produce the toxins of *Bacillus thuringiensis* (*Bt*) have many advantages over agro-technical, mechanical, biological, and chemical measures. However, pest resistance that may occur and

public acceptance of GM modified food crops are the main problems associated with *Bt* crops. Recent developments in the speed, cost, and accuracy of next generation sequencing are revolutionizing the discovery of single nucleotide polymorphisms (SNPs) and field of population genomics. There is a need for effective resistance monitoring programs that are capable of the early detection of resistance and successful implementation of integrated resistance management (IRM). The main focus of this review is on new technologies for CPB control (RNAi) and tools (SNPs) for detection of resistant CPB populations.

Keywords: Colorado potato beetle; resistance problem; control strategies; GM potato; RNAi; SNPs

1. Introduction

Colorado Potato Beetle—a Global Pest of Potato production

Potato (*Solanum tuberosum* L.) is an especially important crop worldwide. According to Food and Agriculture Organization of the United Nations (FAO STAT) [1], it is the fourth most important food crop, following wheat, rice, and maize. More than 1 billion people consume potatoes as a staple, and the crop plays an increasingly important role in future global food security. At a global scale, approximately 20 million hectares are planted with an average yield of 17 tons/hectare resulting in 370 million tons valued annually at approximately US $50 billion [1]. Without crop protection, about 75% of attainable potato production would be lost to pests [2]. Oerke [3] estimated quantitative losses of potato due to insect pests to be around 34% annually.

The Colorado potato beetle, CPB *(Leptinotarsa decemlineata* Say) is the main insect pest of potato plants [4]. According to Weber [5], its current distribution covers about 16 million km^2 in North America, Europe, and Asia. It was first observed in the U.S. in 1811 by Thomas Nuttall [6]. The first serious damage to the potato in the U.S. was observed in 1874 in Colorado [7]. In the first several years after appearing, the CPB turned out to be a very devastating potato pest [8]. In Europe, the first CPB population was discovered in Germany in 1877, but it was successfully eradicated at that time. However, in 1922, CPB population was established in France [9], and by the end of 20th century, it spread across Europe (Figure 1), Asia, and western China. CPB continues to expand in an east and southeast direction [5]. Cong et al. [10] reports that CPB has been found in provinces in Northeast China; hence, we can say that China has become the frontier for the global CPB spread.

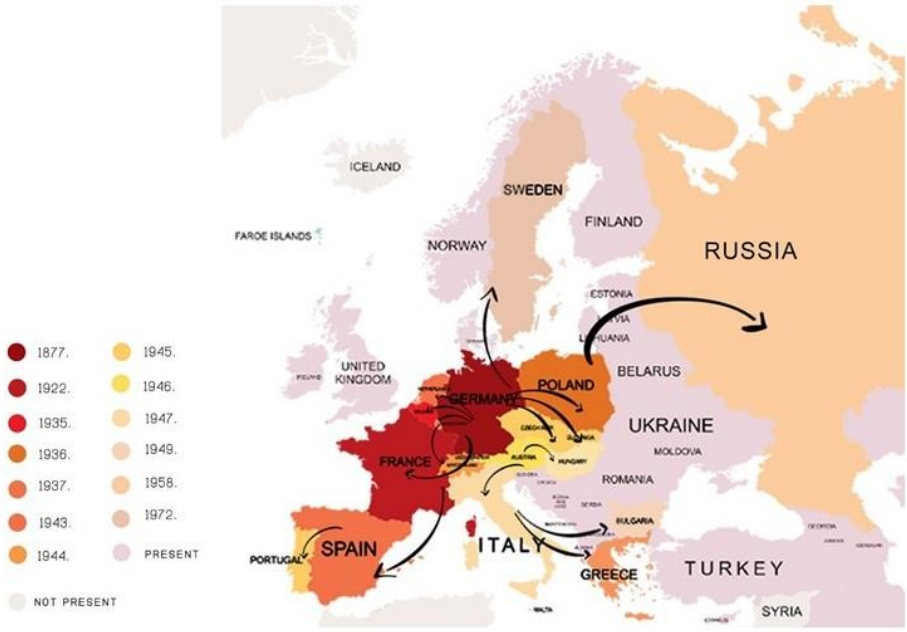

Figure 1. Spread of the Colorado potato beetle over Europe during the 20th century.

Damage to potato plant leaves caused by the CPB adults and larvae appears as holes of varying sizes, usually starting around the margins. The leaf blades are eaten, often leaving a skeleton of veins and petioles behind. This can result in defoliation. A single CPB during its larval stage can consume 40 cm^2 of potato leaves [11]. Then, when the plant has been defoliated, adult CPB feed on stems and exposed tubers [6]. Defoliation of potato plants by the CPB can completely destroy potato crops and significantly decrease tuber production [12,13]. Control of this pest has proved very challenging because of its highly destructive feeding habits and its ability to adapt to a range of environment stresses [14] that would otherwise suppress other Chrysomelidae pests [15].

Current CPB management and control practices include biological control, cultural practices, and chemical treatments [9,14]. Overwhelmingly, historical and contemporary CPB control strategies have relied upon insecticides [16]. Gauthier et al. [17] stated that CPB has been credited with being largely responsible for creating the modern insecticide industry. Even though the use of insecticides resulted in a drastic reduction of CPB populations, resistance development against the active substances resulted. It is now well documented that CPB have developed resistance to most registered insecticides [18–22]. Currently, CPB has developed resistance to 56 different compounds (Figure 2) belonging to all major insecticide classes [23].

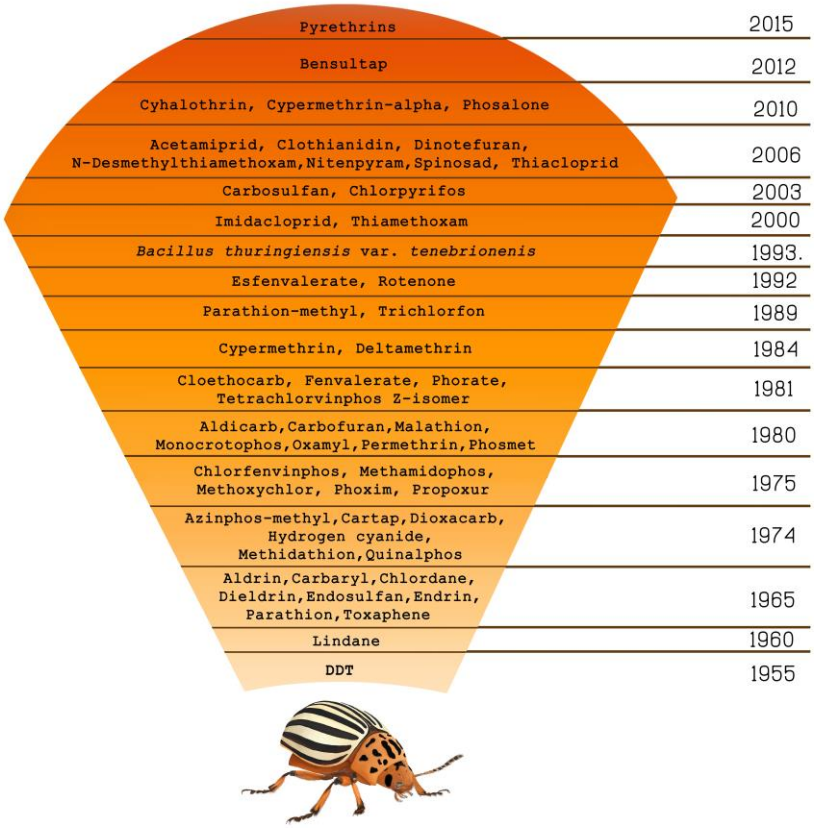

Figure 2. Timeline of resistance development in Colorado potato beetle.

Given that CPB has developed resistance to all major classes of chemical insecticides, other control solutions are required. One such possible solution is genetically modified (GM) crops. In the worldwide cultivation of GM crops, cotton and maize varieties are most represented [2]. *Bacillus thuringiensis* (*Bt*), maize expressing crystalline (Cry) toxin (Cry3Bb1) that specifically targets the western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, (Coleoptera; Chrysomelidae) has increased rapidly since commercialization in 2003 [24]. Currently, a number of genetically modified *Bt* crop cultivars are widely used by farmers as alternatives to chemical insecticides for control of economically important insect pests globally (United States, Canada; India, China, Brazil, Argentina, South Africa) [2]. In 2016, the total area cultivated with GM crops globally was estimated as 185 million hectares [25].

There are no genetically modified potatoes in production in the European Union (EU), but through breeding programs commercial seed companies are working on mitigating the resistance of potato varieties to late blight, caused by the fungus *Phytophthora infestans* (Peronosporales; Peronosporaceae). There are five major potato-breeding companies in Europe: Kweekbedrijf Smeenge-Research, Solana, HZPC, Nijs Potatoes, and Meijer Potato [26]. Potato breeding is considerably time consuming as it takes between eight to 15 years to develop and introduce new varieties to market [26]. On the EU market, there are no commercial cultivars of potato for human consumption that show a strong level of resistance towards the CPB [27]; the cultivar Dakota Diamond has shown some level of host resistance however [28].

While genetically modified potato is not mandated in production systems globally [2], and breeding programs are yet to develop resistant cultivars it is nevertheless important to evaluate current knowledge on and modern approaches to CPB control and resistant management.

2. *Bacillus thuringiensis* (*Bt*) in the Fight to Control Colorado Potato Beetle

Current integrated crop management strategies for potato cultivation include combination of cultural practices, biological control, and chemical treatments [14]. As a result of CPB resistance to insecticides, and various health and environment concerns connected with pesticides, there is an increasing public demand for the reduction of pesticide use [29]. *Bacillus thuringiensis* (Bt) strains have been used as foliar sprays against various pests [30]. *Cry* proteins are the primarily active components of *Bt*-based microbial insecticides, which have been used as foliar sprays in agriculture for several decades [31]. *Bacillus thuringiensis* var. *tenebrionis* (B. t. t.) produces a parasporal crystal protein, Cry3A, which is displaying insecticidal properties towards CPB. This protein is characterized by its high unit activity and specificity for certain coleopteran insect pests including CPB [31]. The advantage of *Bt* insecticides is that they are generally not harmful to humans, non-target wildlife, or beneficial arthropods. The unique mode of action and selectivity make *Bt* an important alternative to conventional chemical insecticides in many integrated pest management (IPM) programs. However, the use of *Bt* sprays provides only limited plant protection as the toxins are photosensitive and degrade quickly compared to most other chemical insecticides [32]. Moreover, the use of *Bt* sprays for pest control raises concerns about the potential for accelerated resistance development to *Bt* [33,34].

Bt-derived Cry genes are also widely used to generate transgenic plants resistant to insects [35]. The first genetically modified potato cultivars, expressing the Cry3A toxin, were introduced in 1995 [36]. One of the first experiments occurred in which the Cry3A protein was inserted into potato plants by Perlak et al. [31]. By the insertion of a Cry3A gene, Russet Burbank potato plants were genetically improved to resist insect attack and damage (Figure 3). Results showed that the damage by all insect stages in the laboratory and also at multiple field locations was significantly reduced. Further analyses showed that GM-potatoes were the same quality in terms of agronomic characteristics including taste in comparison with the standard or non-GM Russet Burbank potatoes. The GM variety for human food was commercially available in the USA from 1996 until 2001, and during that time, ensured good control against the CPB [16]. However, because of complications connected with planting GM potatoes, new insecticide compounds, and rejection of the public, GM potato did not sustain long on the market. "Amflora", is currently the only GM potato variety grown commercially and it is approved only for industrial use and animal feed [2].

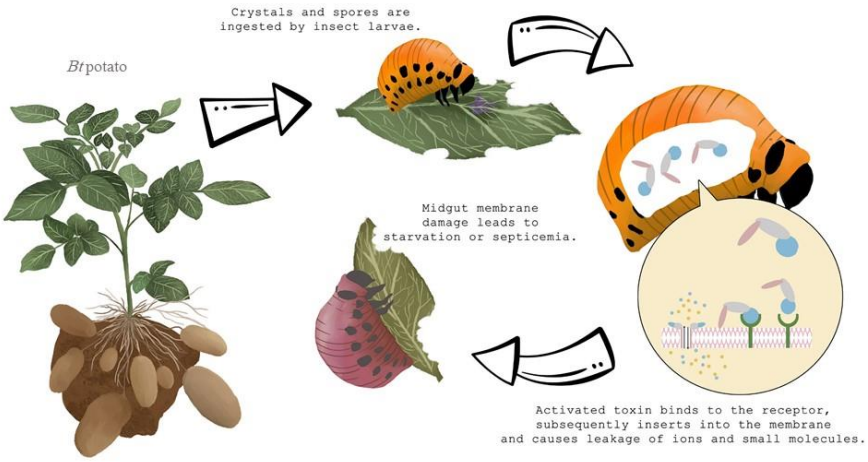

Figure 3. How *Bacillus thuringiensis* (*Bt)* toxin affects Colorado potato beetle larvae.

2.1. Bt Potato Development

Modified Cry3Aa1 gene has been used to enhance protection of the Russet Burbank potato variety against the CPB [31,37]. Another Cry3 gene, Cry3Ca1, was found to be effective against CPB and was engineered for enhanced insecticidal activity [38] as well as Cry genes for Cry1 [39] and Cry3Bb1 [40].

Reed et al. [41] carried out a two-year field study to evaluate the efficacy of *Bt* potatoes (NewLeaf™, which expresses the insecticidal protein Cry3A) and conventional insecticide spray programs against CPB and their impact on non-target arthropods in a potato agro-ecosystem. There were six control regimes used in the experiment. Data generated showed that NewLeaf™ potato plants had greater efficiency in suppressing populations of CPB in comparison with early- and mid-season applications of systemic insecticides (phorate and disulfoton), bi-weekly applications of permethrin and weekly sprays of a microbial *Bt*-based formulation containing Cry3Aa. Importantly, the experiment showed that there was no significant difference on the abundance of beneficial predators or secondary potato pests among conventional potato plants not treated with any insecticides, the effective control of CPB by NewLeaf™ potato plants or weekly sprays of a *Bt*-based formulation. These findings are not surprising because the Cry3Aa protein is highly selective in its activity, affecting only Coleoptera (such as CPB) in the family Chrysomelidae [42]. Transgenic *Bt* potato and *Bt*-based microbial formulations are compatible with the development of integrated pest management (IPM). However, re-introduction of GM potatoes awaits changes in consumer preferences [16].

2.2. Why Bt Potato Did Not Sustain on Market

Resistance problems in the U.S. in the early 1990s reached critical levels [9] and growers in some potato-producing regions completely exhausted their chemical control options. In 1995, Monsanto introduced the NewLeaf™ potato variety to market, which was their first genetically modified crop. The use of NewLeaf™ potatoes led to a significant reduction in pesticide use and cost savings for growers [43]. However, there were concerns with NewLeaf™ potatoes. That is, CPB may also develop resistance

to the *Bt* endotoxin because of its constant presence in the transgenic crop. Resistance to *Bt* toxins can emerge in CPB under high levels of *Bt* endotoxin stress [44].

Hoy [45] developed resistance management strategy, which include five main steps to avoid resistance development to the Cry3A protein. This strategy includes combining and switching varieties of potato during the planting operation. All potato growers needed to plant non-transformed potatoes along planting NewLeaf™ potatoes to reduce the potential for development of resistance. This was a complication that many potato growers were not used to and one of the factors against planting NewLeaf™. One more factor that worked against market adoption was the introduction of a new class of insecticides. A brief period of relief in areas where the beetles had developed resistance to other chemicals came with the use of neonicotinoid insecticides in 1995 [46]. The neonicotinoid imidacloprid was introduced at about the same time as NewLeaf™, and offered an effective conventional pesticide alternative to producers struggling to control beetles that were becoming resistant to other insecticides [47]. However, CPB gained resistance to imidacloprid very quickly and the first cases of resistance were reported from commercial potato farms in several U.S. States in 2000s [48–51].

When the NewLeaf™ potato became interesting to the media and the public debate about the risks and benefits of biotechnology started, potato growers, and retailers had to come up with an idea about how to respond to any potential controversy. This resulted in a strategy to separate potatoes in an effort to allow customers the 'choice' between GM and non-GM potatoes. However, problems arose in this strategy because GM testing protocols and segregation techniques were not well-developed [46]. Finally, growers realized that the NewLeaf™ potato was not adding value to their business, also the signals from market became less certain and many decided they could not afford the risk of planting NewLeaf™ potatoes. Many growers turned their attention and hope to the new active substances on the market. After the 1999 season, potato acreage planting declined rapidly and in response to market demands, Monsanto discontinued the sale of NewLeaf™ seed in 2001 [46]. CPB did not develop resistance to NewLeaf™ potatoes; however, because of the problems discussed, production and cultivation did not continue [46].

3. Sources of Host-Plant Resistance

There remains a market need for potato varieties resistant to the CPB due to resistance problems, restrictions on the registration and use of plant protection products in the EU, and the fact that the number of active substances in the insecticides market is declining. Spooner and Bamberg [52] suggested host-plant resistance as one of the practical and long-term solutions for controlling CPB. Two natural insect host plant resistance mechanisms in potatoes are leptine glycoalkaloids and glandular trichomes. Balbyshev and Lorenzen [53] found that one *Solanum* spp. hybrid responded to egg masses of the CPB with a hypersensitive necrotic zone that subsequently disintegrated around the border and detached from the leaf. Their results showed detachment of CPB eggs with subsequent deposition on the ground and this can be considered a new mechanism in host-plant resistance. Lorenzen et al. [54] described a new source of host-plant resistance to the CPB in a tetraploid potato. Their resistant genotypes included low levels of leptines I and II. Results after four days showed delayed development of neonate larva and inhibited larval weight gain by 75%, relative to larval development and weight gain on susceptible genotypes. According to several authors, leptines are effective natural mechanisms of potato resistance against CPB [55]. Coombs et al. [55] combined natural leptine glycoalkaloids and glandular trichomes and engineered *Bt* Cry3A host plant mechanisms as a possibility to prevent the resistance development to *Bt* endotoxin. Their study was the first report combining natural and engineered anti-resistance management options in potato and showed promising results for effective management of CPB.

For the development of CPB resistant potato varieties, natural variation of wild potato relatives can be used as source of resistance. Materials and tools to develop CPB resistant potato varieties through

classical breeding programs and GM approaches are available and should be used to make potato production more sustainable [14]. The use of natural variation could avoid the problems with public relations and regulatory issues connected to GM crops, which is still present in many countries especially in the EU [16].

4. New Approaches to Colorado Potato Beetle Management

4.1. RNA Interference (RNAi)

RNAi is a gene silencing technology that uses double stranded RNA (dsRNA) to hinder the normal gene function directly against a specific gene sequence or promoter region of messenger RNA (mRNA) [56]. RNAi is a robust tool for the suppression of CPB gene expression and to study their biological function [57]. When dsRNA is ingested by insects, the transcript of target insect gene is silenced through RNAi pathway. Silencing of certain genes may cause insect growth or developmental defects, morbidity, or mortality [58]. The most important advantage of RNAi technology is that it acts on a specific insect species, because it targets a specific gene [59], and by altering the target genes, it is possible to completely avoid resistance development. RNAi in insects has three pathways: small interfering RNA (siRNA), microRNA (miRNA), and piwi-interacting RNA (piRNA) [60]. These pathways involve different proteins and play different roles in insects. This gene silencing strategy functions well in many coleopteran insects [61]. Analysis of the gut transcriptome indicates that CPB possesses all of the RNAi-related genes, providing a genetic basis for triggering RNAi in this pest [62]. The availability of the CPB transcriptome [63] will be very helpful in this respect. Duplications of some genes involved in the RNAi pathway might explain why CPB is more sensitive to dsRNA than other insects [64].

4.2. RNAi in Colorado Potato Beetle Control Management

Zhu et al. [65] investigated the potential of feeding dsRNA expressed in bacteria or synthesized in vitro to CPB to control their populations. Feeding RNAi successfully triggered the silencing of five target genes tested (*actin, vATPase A, B, E, Sec23, and COPβ*). These genes were related to cellular physiological processes and silencing them can impede growth and induce mortality. This study is the first example of an effective RNAi response in insects after feeding dsRNA produced in bacteria. Zhu et al.'s [65] results suggest that the efficient induction of RNAi using bacteria to deliver dsRNA is a possible method for the management of CPB. This could be also a promising bioassay approach for genome-wide screens to identify effective target genes for use as novel RNAi-based insecticides [65]. Numerous studies demonstrated successful knockdown of target genes in dsRNA fed CPB (Table 1). Zhou et al. [66] showed feeding bacterially expressed AdoHcy hydro-lase (*SAHase*) dsRNA to CPB decreased SAHase and Krüppel homolog 1 gene (*Kr-h1*) mRNA levels, reduced juvenile hormone (*JH*) titer, and that can cause the death of larvae, and pupae, and blocked adult emergence. Another very important study in CPB showed that feeding ryanodine receptor (*RyR*) dsRNA reduced *RyR* mRNA levels in the larvae and adults, and caused a decrease in chlorantraniliprole-induced mortality confirming that *RyR* is the target site for this insecticide [67]. The xenobiotic transcription factor Cap 'n' collar isoform C (*CncC*), regulates the expression of multiple cytochrome P450 genes, and plays crucial roles in CPB insecticide resistance. The suppression of *CncC* by RNAi reduced imidacloprid resistance of CPB [68]. Feeding dsRNA method has been used to knockdown expression of the gene coding for P450 enzyme Shade (*shd*). A reduction in the hydroxylation of ecdysone caused delay in development and death of CPB larvae and pupae [69]. Ochoa-Campuzano et al. [70] in their study identified prohibitin, an essential protein for CPB viability, as Cry3Aa binding protein. Combination of feeding prohibitin dsRNA and treatment with Cry3Aa enhanced Cry3Aa toxin induced mortality by threefold and the time to kill was reduced. Results showed

100% mortality in five days. Although the molecular mechanisms of synergism between prohibitin RNAi and Cry3Aa toxin application are not known yet, this study proposes an interesting method of combining RNAi with toxins derived from microbes and other sources to improve the efficacy of RNAi in pest control.

In Wan et al. [71] the authors investigated two dsRNAs (dsLdp5cdh1 and dsLdp5cdh2) that were bacterially expressed and fed to CPB adults. The result showed significant decrease in CPB Ldalt mRNA abundance, flight speed, flight duration, and flight distance, and also caused adult mortality. CPB adults are proficient fliers and flight, is their primary mode of dispersal. Wan et al. [72] in their study showed that if we know that proline is the main energy source for CPB flight knocking down the Pyrroline-5-carboxylate dehydrogenase (*P5CDh*) gene can weaken flight competence, and increase adult mortality. Flight in CPB is also connected with alanine aminotransferase (*alt*). Hussain et al. [73] focused on the suppressed transcripts level of highly expressive Ecdysone receptor (*EcR*) gene of CPB using plant-mediated RNAi approach. Bioassays of transgenic plants showed 20–80% mortality of CPB instars. Larvae feeding on transgenic potato plants showed halted metamorphosis, lower body weight, and larvae were not able to shift to their next instar. These results are very encouraging to control CPB, a notorious potato pest by using an alternative, effective, and reliable non-chemical method of population control and suppression. The dsRNA targeting CPB genes could be expressed in potato plants to control this pest.

Table 1. Review of target genes for RNA interference (RNAi)-based Colorado potato beetle control (modified from He et al. [57]).

Target Gene	Annotation	Reference
VATPase, A, B, E	Vacuolar ATP synthase subunit	[61] [65]
Sec23	Protein transport protein *sec23*	[65]
COPβ	Coatomer β-subunit	[65]
Actin	β-Actin	[65]
Prohibitin	Prohibitin protein	[70]
SAHase	S-adenosyl-L-homocysteine hydrolase	[66]
FTZ-F1	Nuclear receptor type transcription factor that responses to 20-hydroxyecdysone	[74]
shd	Ecdysone 20-monooxygenase	[69]
NAT1	Nutrient amino acid transporter	[75]
Actin	β-Actin	[76]
JHEH	Juvenile hormone epoxide hydrolase	[77]
alt	Alanine aminotransferase	[71]
p5cdh	Δ1-pyrroline-5-carboxylate dehydrogenase	[72]
HR3	Nuclear receptor that early-late responses to 20-Hydroxyecdysone	[78]
UAP	Uridine diphosphate N-acetylglucosamine pyrophosphorylase	[79]
ChS	Chitin synthase	[80]
TPS and TREs	Trehalose biosynthesis and degradation	[81]
E75	Ecdysone-induced protein 75	[82]
JHAMT	Juvenile hormone acid methyltransferase	[83]

ILP2	Putative insulin-like peptide	[84]
HR4	ecdysteroidogenesis and mediates 20-hydroxyecdysone signaling during larval-pupal metamorphosis	[85]
CncC	Xenobiotic transcription factor	[69]
EcR	Ecdysone receptor	[73]
Mesh	gut-membrane-associated protein	[86]

Previous attempts at introducing transgenic potato plants to control CPB were not highly successful [87]. Petek at al. [86] in their study designed dsRNA to silence the CPB mesh gene (*MESH*). They did laboratory-feeding trials to assess impacts on beetle survival and development and also a field trial to compare dsRNA sprayed potato with a spinosad-based insecticide. Results showed that dsMESH ingestion consistently and significantly impaired larval growth and decreased larval survival in laboratory feeding experiments. Results of the field trial showed that dsMESH was as effective in controlling CPB larvae as a commercial spinosad insecticide, only its activity was slower. Most recently, Gui et al. [88] used the CRIPR/Cas9 system mutagenesis studies in the CPB for the first time. The CRISPR/Cas system is an efficient genome editing technology. First results from Gui et al. [88] showed low efficiency, but this methodology could possibly lead to the development of better and environmentally friendly CPB management strategy.

4.3. RNAi Based Products in Wide Use

There are three possible methods for mass-production of dsRNA for pest control: (1) expression of dsRNA in plants using transgenic technologies; (2) chemical synthesis of dsRNA in factories; and (3) production of dsRNA in microorganisms (Figure 4). Zhang et al. [76] used dsRNA targeted against the Actin-Like Protein (*ACT*) gene to produce CPB resistant potato plants. The ACT gene encodes the essential cytoskeletal protein *b-actin*. Using transgenic plants that produced the dsRNA in the chloroplast genome, Zhang et al. [76] were able to show that the resulting RNAi caused 100% mortality of CPB in five days. Hence, for CPB control chloroplast transformation is a reliable and efficient delivery method [76]. Although plant-incorporated protectants (transgenic plants) are the most cost-effective way of using RNAi-based pesticide technology, their public acceptance is challenging, especially in the EU. Another possibility, again using genetically modified organisms, is the usage of transformed insect symbionts [89] or viruses expressing pesticidal RNA molecules [90]. Thus, dsRNAs application by non-transformative strategies, i.e., through spray-induced gene silencing, is currently a more realistic option of controlling CPB [91]. Petek at al. [86] showed in laboratory trials as well as in the field that spraying with insecticidal dsRNA is a highly efficient strategy for managing CPB. Future research will have to focus on formulations to improve dsRNA stability and cellular uptake. Efficiency, safety, and possible undesirable effects of dsRNA on non-target organisms is an important though understudied topic [92].

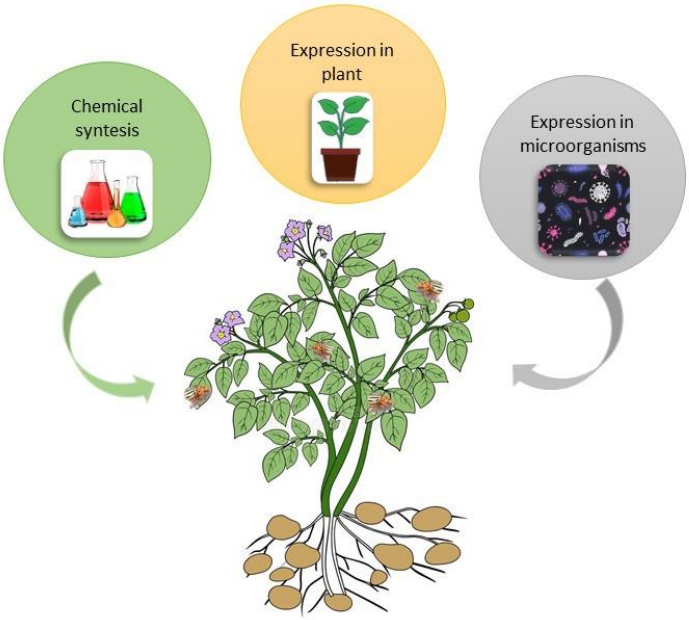

Figure 4. Possible methods for producing double stranded RNA (dsRNA) for pest control.

Although in the beginnings of development, RNAi technology shows great potential for application in the control of various insect pests [62]. Several difficulties still have to be overcome before the full potential in insect pest control can be exploited [76,93,94]. Prior to its exploitation for insect pest control, it is important to document the potential limiting factors, like immune reaction and fitness cost, RNAi efficiency and dsRNA degradation, and virus-encoded suppressor of RNAi factors within the development of the RNAi-based pest control strategy. Additional challenges including the lack of feasible dsRNA delivery methods in practice, low efficiency in pest control capacity, and evolution of resistance to RNAi have largely constrained the appliance of RNAi in practice. Substantial research remains to be done before the application of RNAi in field conditions becomes an effective and cost-effective protection measure. The biggest challenge will be public acceptance. The genomes of many insects, including economically important pests, are sequenced and made available publicly to better understand RNAi processes and identify new target genes. One of the most important factors is the way in which RNA molecules are introduced into insect cells. In the future RNAi could become part of integrated plant protection measures.

5. Genetic Tools in Colorado Potato Beetle Management

In addition to new and effective suppression measures for CPB, there is a need for effective resistance monitoring tools that are capable of the early detection of resistance and will allow implementation of insect resistance management (IRM) strategies. Clark et al. [95] were the first to combine three DNA based genotyping techniques for the detection of mutations associated with insecticide resistance in CPB populations. They compared bi-directional PCR amplification of specific allele (bi-PASA), single-stranded conformational polymorphism (SSCP), and minisequencing to detect mutations associated with azinphos-methyl and permethrin insecticides. These authors stated that the methods could enable the precise monitoring of the resistant and susceptible allele frequencies in field population of CPB. Udalov and Benkovskaya [96] in their review summarize the population studies of CPB. Moreover, their work shows that molecular genetic methods can be used to assess the nonspecific resistance of the CPB to insecticides.

Genetic studies of CPB started with the work of Grapputo et al. [97]. They investigated the population structure and genetic variability of North American and European populations of CPBs using mtDNA and Amplified Fragment Length Polymorphism (AFLP) markers. Understanding gene flow is particularly important for CPB management given that insecticide resistance is widespread in this species. Kumar et al. [63] subjected European CPB adult and larval transcriptome samples to 454-FLX massively parallel DNA sequencing to characterize a basal set of genes from this species. Their results offer new insights into insecticide-resistance-associated genes in this species and provides a foundation for comparative studies with other species of insects. Knowledge of evolutionary changes and the total genetic diversity of a pest population can provide useful information to understand the genetic patterns associated with each stage of the pest resistance development so that management, including monitoring and control, can be tailored to suit the resistance of the pest in question [98].

5.1. Single Nucleotide Polymorphism (SNPs) as Prospective Tool in CPB Resistance Management

SNPs are single base substitutions found at a single genomic locus. They are very useful for population genetic studies because of their dense and uniform distribution within genomes (Figure 5). Recently, SNPs have become an affordable and readily accessible means of generating a lot of data quickly for non-model species [99]. SNP detection has facilitated association-mapping studies in many insect species including: *Drosophila melanogaster* Meigen, 1830 [100], *D. v. virgifera* [101], *Aedes aegypti* Linnaeus, 1762 [102], *Glossina fuscipes* Wiedemann, 1830 [103], *Diatraea saccharalis* Fabricius, 1794 [104], *Phaulacridium vittatum* Sjöstedt, 1920 [105]. Schoville et al. [64] identified 1.34 million biallelic single nucleotide polymorphisms (SNPs) from pooled RNAseq datasets in CPB from Long Island. Their result showed that CPB when compared with vertebrates (e.g., ~1 per kb in humans, or ~1 per 500 bp in chickens) and other beetles (1 in 168 bp for *Dendroctonus ponderosae* Hopkins, 1902 and 1 in 176 bp for *Onthophagus taurus* Schreber, 1759) has an exceptionally high rate of polymorphism (1 variable site for every 22 base pairs of coding DNA). Given the vast number of SNPs (thousands to millions) that are easily and affordably generated in a single sequencing run, they have surpassed microsatellites as the marker of choice when understanding the population genetics of a species [106]. Genotyping of SNPs has potentially far-reaching applications in insect population genomic studies and other insects in which specific nucleotides are statistically associated with complex phenotypic traits [107].

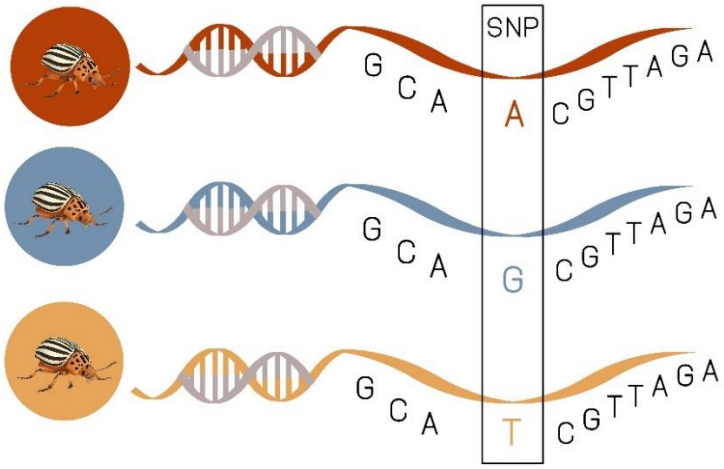

Figure 5. Example for single nucleotide polymorphisms (SNPs), single changes in the genetic code.

Diversity Array Technology (DArT) is method for DNA polymorphism analysis, which offers a low-cost high-throughput, robust system with minimal DNA sample requirement capable of providing comprehensive genome coverage [108]. DArTseq technology is a united one-step procedure of SNP discovery and genotyping; it enables a substantial discovery of SNPs in a wide variety of non-model organisms and provides a measure of genetic divergence and diversity within the major genetic groups [109]. The use of SNPs, in non-model organisms has become an affordable and readily accessible means of generating important data on species that otherwise would have been impossible due to cost and expertise availability [99,106]. Detailed genomic data could provide an answer about genetically conditioned resistance development in insects. The use of SNPs to understand the population genetics of CPB populations on a deeper level can be explored. Such data, which investigate genome changes associated with the development of resistance, is crucial for the implementation of agricultural, food biosecurity measures and integrated pest management strategies. Through genotyping of SNPs, an understanding of the genomic structure, population differentiation, gene flow, dispersal, and adaptive potential of CPB populations will be possible. The goal of effective and economically feasible resistance management remains impossible largely without efficient and cheap diagnostic procedures for separating susceptible and resistant genotypes [95]. Using SNPs, detection and monitoring of resistant and non-resistant variants of CPB can be performed in a novel application of this genetic marker.

6. Conclusions

CPB is the most harmful insect of potato that causes great economic damage to potato production worldwide. The suppression of CPB in the past relied on intensive insecticide applications, which ultimately led to the development of resistance. Now, when the number of available insecticides is decreasing, especially in the EU, we need to think about new possibilities and solutions to CPB control. Using SNPs, it should be possible to detect genetic differentiation correlated with resistance development in CPB. This would allow quick detection and monitoring of resistant variants as the first step towards the implementation of anti-resistant strategies and sustainable use of pesticide against CPB. RNAi has proven successful in controlling pests and based on research to date, shows great potential for CPB control. Better understanding of the mechanisms that affect efficiency will enable the development of this technology and boost potential of RNAi to become part of integrated plant protection in the future. Although there are barriers to overcome, the newly introduced technologies and approaches can be used to solve the problem of CPB control and resistance development.

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3. RESULTS AND DISCUSSION

3.1. Review of published scientific papers

A results part consists of three articles published in international peer-reviewed journals and is presented in subchapters 3.1.1 – 3.1.3. Each subchapter describes the results of genetic and geometric morphometrics analyzes and main findings on genetic structure and morphological traits of three investigated pests.

Subchapter 3.1.1. was published in Agriculture, 11(7), 585 by Kadoić Balaško, M., Mikac, K. M., Benítez, H. A., Bažok, R., and Lemic, D. The paper describes a possibility that combining genetic and geometric morphometrics could be a reliable technique that can be used to reveal differences among western corn rootworm (WCR) populations. Results showed that geometric morphometrics can be used as a biomarker for resistance detection as part of a larger integrated resistance management strategy for western corn rootworm.

Subchapter 3.1.2. was published in Agronomy, 12(6), 1278 by Kadoić Balaško, M., Mikac, K. M., Benítez, H. A., Suazo, M. J., Viana, J. P. G., Lemic, D., and Pajač Živković, I. The paper describes a possibility to find a reliable pattern of differences in Codling moth (CM) populations related to the type of apple control method. Here SNP markers did not show enough power to detect changes among CM populations. However, geometric morphometrics showed higher sensitivity for detecting population changes associated with different types of apple production and proved to be a reliable, accurate, and cost effective technique.

Subchapter 3.1.3. was published in Agronomy, 12(10), 2361 by Kadoić Balaško, M., Bažok, R., Mikac, K.M., Benítez, H.A., Correa, M., Lemic, D. This study is the first attempt to investigate the population structure of Colorado potato beetle (CPB) in Croatia. In this paper SNPs and GM techniques provided us with data about the population structure of the CPB population. Low genetic and phenotypic variability of CPB populations was detected and the presence of a single panmictic CPB population in the study area well adapted to different environmental conditions indicating high phenotypic plasticity.

3.1.1. Publication No. 4

agriculture

MDPI

Article

Genetic and Morphological Approach for Western Corn Rootworm Resistance Management

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Abstract: The western corn rootworm (WCR), is one of the most serious pests of maize in the United States. In this study, we aimed to find a reliable pattern of difference related to resistance type using population genetic and geometric morphometric approaches. To perform a detailed population genetic analysis of the whole genome, we used single nucleotide polymorphisms (SNPs) markers. For the morphometric analyses, hindwings of the resistant and non-resistant WCR populations from the US were used. Genetic results showed that there were some differences among the resistant US populations. The low value of pairwise FST = 0.0181 estimated suggests a lack of genetic differentiation and structuring among the putative populations genotyped. However, STRUCTURE analysis revealed three genetic clusters. Heterozygosity estimates (HO and HE) over all loci and populations were very similar. There was no exact pattern, and resistance could be found throughout the whole genome. The geometric morphometric results confirmed the genetic results, with the different genetic populations showing similar wing shape. Our results also confirmed that the hindwings of WCR carry valuable genetic information. This study highlights the ability of geometric morphometrics to capture genetic patterns and provides a reliable and low-cost alternative for preliminary estimation of population structure. The combined use of SNPs and geometric morphometrics to detect resistant variants is a novel approach where morphological traits can provide additional information about underlying population genetics, and morphology can retain useful information about genetic structure. Additionally, it offers new insights into an important and ongoing area of pest management on how to prevent or delay pest evolution towards resistant populations, minimizing the negative impacts of resistance.

Keywords: *Diabrotica virgifera virgifera*; Bt toxins; resistance; geometric morphometrics; SNPs

1. Introduction

Maize (Zea mays L.) is one of the most important crops worldwide. About 200 million hectares is planted, with an average yield of 22 tons/hectare, resulting in 1150 million tons of maize harvested worldwide [1]. The western corn rootworm (WCR*) Diabrotica virgifera virgifera* is the worst pest in the United States and a major alien invasive pest in Europe [2,3]. The main damage caused by WCR to maize

plants is by its larval stage that feeds on corn roots, which affects important physiological processes of the plant. The resulting damage leads to stalk lodging and yield losses, which in turn leads to economic damage to crops [4].

Suppression with chemical insecticides is an important management tool for this pest [5], but WCR has rapidly developed resistance to the insecticides used for control [6]. The first noted case of resistance to insecticides was to cyclodiene insecticides (aldrin and heptachlor) in 1959 in Nebraska [7,8]. So far, WCR has evolved resistance to organophosphates (methyl parathion), carbamates (carbaryl) [6,9], and pyrethroids (bifenthrin and tefluthrin) [10,11]. In addition to insecticides, WCR has developed resistance to crop rotation [12–14] and to the Bt toxin in genetically modified maize [15]. Crop rotation remains the most effective control tactic against WCR. However, resistance to crop rotation has been documented in Illinois and other neighboring states [12]. Spencer et al. [16] observed that some of the WCR populations in northern Indiana and east central Illinois feed on soya bean foliage and flowers, as well as lay eggs in soya bean fields. This behavioral change in the WCR populations in the eastern Corn Belt has eliminated the effectiveness of crop rotation as a rootworm management option. As a consequence, the use of soil and foliar insecticides for WCR has increased to protect corn following soya bean. It was estimated that each year WCR costs US farmers at least USD 1 billion through yield losses and treatment costs [17], but after adaptation to crop rotation, these losses are estimated to be higher [18]. Transgenic maize expressing Bacillus thuringiensis (Bt) was introduced in 2003 in the United States [15]. However, resistance to maize expressing Cry3Bb1 was reported in Iowa in 2009 [19]. Afterwards, resistance to Cry3Bb1 was detected in fields throughout Iowa [20,21] but also in WCR populations found in Illinois, Nebraska, and Minnesota [22–24]. Selected rootworm populations developed resistance to the toxins Cry34/Cry35Ab1, Cry3Bb1, and mCry3A under laboratory and greenhouse conditions [25–28]. Cross-resistance was found in WCR field populations between the Cry3Bb1, mCry3A, and eCry3.1Ab toxins [21–23,29]. WCR populations evolved resistance to all four currently available Bt toxins (Cry3Bb1, mCry3A, eCry3.1Ab, and Cry34/35Ab1) [19,23,29–31], and consequently, the challenge of managing has become more difficult.

Resistance is a dynamic phenomenon, meaning that mechanisms already known can change over time. Ongoing monitoring is essential to determine whether management recommendations remain valid or need to be revised in light of changing circumstances or newly acquired knowledge [32]. WCR resistance to insecticides and management strategies is a serious and growing problem in maize production, and before it becomes an even more widespread and major problem, there is a need to explore and implement novel methods (such as single nucleotide polymorphisms and geometric morphometrics) for the early detection of resistance or adaptation that causes WCR resistance.

Population genetic markers can be used to provide genetic data for WCR that is useful when investigating changes in genetic structure and differentiation [3,33,34]. Different types of molecular markers (allozymes, mtDNA sequencing, AFLPs, microsatellites, and SNPs) have already been used in North American WCR populations. The result showed high genetic diversity and a general lack of population structure across the US Corn Belt [35–37].

Several studies on WCR resistance mechanisms have been performed [38–40]. Coates et al. [41] attempted the use of SNPs as population genetic markers in WCR in the US and showed that both markers (microsatellites and SNPs) gave similar results. This does not suggest that SNPs are less effective at separating genetic variation in the species, but it is likely a result of low numbers of SNPs and low genome coverage because the authors used 12 biallelic loci among 190 individuals. Wang et al. [40] found that cylcodiene resistance is correlated with SNPs in the gamma-aminobutyric acid (GABA) receptor. Flagel et al. [42] used SNPs to identify candidate gene families for insecticide resistance and to understand how population processes have shaped variation in WCR populations. Their WCR transcriptome assembly included several gene families that have been implicated in insecticide resistance in other species and that have provided a foundation for future re- search. Flagel et al. [43]

discovered and validated genetic markers in WCR associated with resistance to the Bt toxin Cry3Bb1. They found that the inheritance of Cry3Bb1 resistance is associated with a single autosomal linkage group and is almost completely recessive. Niu et al. [44] found that SNP markers identified in a single autosomal linkage group (LG8, 115–135 cm) were correlated with resistance to Cry3Bb1 in field populations of WCR. Although the linkage of these genes to Cry3Bb1 resistance was strong, the causal gene for Cry3Bb1 resistance was not confirmed and remains to be reported.

Geometric morphometrics (GM) (i.e., phenotype size and shape analysis) is a technique that can be used to show hindwing shape and size differences among rootworm populations [45]. By analyzing wing size and shape, it is possible to reveal the invasive adaptation of the adults' traits to different environmental influences. Numerous studies have been performed on the WCR hindwings using geometric morphometry [46–49]. Mikac et al. [46] provided preliminary evidence of wing shape and size differences in WCR from rotated versus continuous maize. Most recently, Mikac et al. [45] determined morphological differences in wing shape in populations adapted to crop rotation and Bt maize compared with a non-resistant WCR population. This study showed evidence of differential wing shape in relation to resistance development and highlights the importance of wing size and shape as a reliable, inexpensive, yet effective biomarker for resistance detection in corn rootworm. The research of Mikac et al. [45] looked at the Bt-resistant individuals as a whole, so it is necessary to extend their research to each Bt toxin separately. A deeper understanding of maize rootworm wing shape and flight morphology, wing geometry, aspect ratio, and flight efficiencies will help identify which resistant phenotypes are most likely to invade geographic areas where they are not yet present.

According to Bouyer et al. [50], changes in an organism's genotype takes much longer to manifest than in its phenotype, thus making geometric morphometrics a much more useful tool than genetics for detecting changes in populations in the short term. That suggests morphology can retain useful information on genetic structure and has the benefit over molecular methods of being inexpensive, easy to use, and able to yield a lot of information quickly. However, resistance cannot be fully understood without genetic data. Genetic studies are an important tool for developing improved methods for detecting resistance, for studying resistance mechanisms, and for choosing approaches to resistance management [51]. Several studies suggest that results are more accurate when both methods are combined. Morphological traits can provide additional information about underlying population genetics, and morphology can retain useful information about genetic structure [52–56].

This is the first study that combines both genetic and geometric morphometric techniques on the same WCR populations and same individuals. The aim of this study was to define genetic variables between known phenotypes and to explore phenotypic markers related to changes in the genome. We hypothesized that by combining genetic and morphological markers, it would be possible to determine and predict resistance to Bt toxins and crop rotation in the field.

2. Materials and Methods

2.1. Sample Collection

All WCR individuals used in this research were populations from the US. The same individuals were used both for the genetic and morphometric analysis. WCR individuals were collected from South Dakota in the fields containing transgenic corn. Individuals adapted to crop rotation from Illinois were collected in fields with documented resistance. Non-resistant (susceptible) adults were obtained from the NCARL laboratory. The non- resistant laboratory population was originally collected in 1987 near the town of Trent, South Dakota, in Moody County. It has been in continuous rearing since that time without mixing with other collections. It is approximately one generation per year. The original beetles were selected in cornfields or on the edge of cornfields and the adult beetles were returned to the

laboratory. The non-resistant colony is reared in soil on maize roots and the adult beetles are fed on an artificial diet. Attempts are being made to keep the rearing protocol "field-like" to keep it "wild" (Chad Nielson personal communication). According to Mikac et al. [45], there are minimal differences between rotation-resistant laboratory and field-collected populations, suggesting that the rearing system was not the main reason for the differences observed in their study. Therefore, we excluded the possibility that different conditions (field, laboratory rearing) may contribute to differences in wing shapes and sizes. Individuals were placed in 95% ethanol pending genetic and morphometric analysis. WCR individuals used in this research were adapted to crop rotation, were non-resistant, and were collected from Bt corn expressing different toxins (Cry3Bb1, Cry34/35Ab1, Cry3Bb1, and Cry34/35Ab1) (Table 1).

Table 1. Number of WCR individuals used for geometric morphometric and SNPs analyses. n = sample size.

Western corn rootworm populations	Geometric Morphometric Wings (n)	Males/ Females	Adult Single Nucleotide Polymorphisms Genotyped (n)	Males/ Females
Cry3Bb1	433	184/252	7	2/5
Cry3Bb1_Cry34/35Ab1	86	27/59	5	3/2
Cry34/35Ab1	91	32/59	6	3/3
Adapted to crop rotation	31	14/17	4	1/3
Non-resistant	134	66/68	7	4/3

2.2. DNA Extraction and SNPs Genotyping

Before DNA extraction, hindwings from all individuals were removed for morphometric analysis. DNA was then extracted from the whole-body tissue of 29 adult WCR. DNA extractions were performed using the Qiagen DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol.

The DNA concentration for all samples was measured using spectrophotometer (BioSpec-nano Micro volume) and adjusted to 50 ng/μL prior to SNPs genotyping by Diversity Arrays Technology (DArT) [57,58]. After quality control, 29 samples were sent for genotyping. Genotyping was undertaken by Diversity Array Technology Pty Ltd. (DArT, Canberra, Australia) using the extracted WCR DNA. This method is based on methyl filtration and next-generation sequencing platforms [58]. The data we received were filtered for minor allele frequency (MAF) lower than 0.1 and also for missing data higher than 10%. Quality of SNP markers was determined by the parameters "reproducibility" and "call rate" [59]. Remaining SNPs were used for further analysis of genetic diversity and population structure.

2.3. Geometric Morphometric Sample Preparation

The adult WCRs (see Table 1) were investigated using geometric morphometric pro- cedures and analyses based on hindwing venation undertaken. In total, 775 hindwings of WCR were analyzed. Left and right hindwings were removed from each individual and slide-mounted using the fixing agent Euparal (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) based on standard methods [60]. Slide-mounted wings were photographed using a Canon PowerShot A640 digital camera (10-megapixel) on a trinocular mount of a Zeiss Stemi 2000-C Leica stereo-microscope and saved in JPEG format using the Carl Zeiss AxioVision Rel. 4.6. (Carl Zeiss Microscopy GmbH, München, Germany). Fourteen type 1 landmarks defined by vein junctions or vein terminations were used (Figure 1.) [47–49,61].

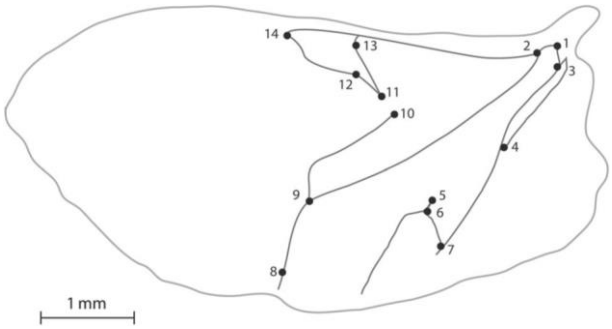

Figure 1. Representation of the 14 morphological landmarks identified on the hindwings of western corn rootworm [61].

2.4. Data Analysis

2.4.1. Genetic Data

All population genetic data analyses were undertaken using the coding environment in R using the R packages adegenet v2.1.3 [62] and dartR v1.1.11 [63]. In the first instance, the SNP dataset was subject to a filtering process using dartR to remove potentially erroneous SNPs. Monomorphic SNPs were excluded followed by the removal of SNPs with a reproducibility of <95%, a call rate of <90% (i.e., SNPs which have 10% missing genotypes or greater), and secondaries.

Pairwise FST, estimated as θ [64], was calculated between the five putative populations (Cry3Bb1, Cry34/35Ab1Ab1, Cry3B1_Cry34/35Ab1Ab1, adapted to crop rotation, and non-resistant), along with observed (Ho) and expected (He) heterozygosity. Departure from Hardy–Weinberg equilibrium (HWE) was tested for each population using the function gl.report.hwe as implemented in the R package dartR [63], which includes Bonferroni correction for multiple testing. Using the function gl.basic.stats in dartR, overall basic population genetics statistics per locus, such as the observed (HO) heterozygosity, (FIS) inbreeding co-efficient per locus, and FST corrected for the number of individuals, was undertaken. To summarize genetic similarity among populations, gl.tree.nj in dartR was used.

The Bayesian model-based clustering algorithm implemented in the STRUCTURE v 2.3.4 [65] Evanno method was employed to determine the genetic structure of the WCR populations investigated. Genetic clusters (K-values) ranged between 1 and 6 (1 more population than the total number of populations for the complete data set), and a series of 10 replicate runs for each prior value of K were analyzed. The parameter set for each run consisted of a burn-in of 10,000 iterations followed by 100,000 Markov chain Monte Carlo iterations based on the admixture model of ancestry with the correlated allele frequency model and the default parameters in STRUCTURE. The most suitable value of K was calculated using the ΔK method as used in Structure Harvester web version 0.6.94 [66], where the highest ΔK value was indicative of the number of genetic clusters.

The marker-based kinship matrix (K) was calculated with the same genotypes using the VanRaden method [67] and then used to create a clustering heat map of the association mapping panel in the GAPIT [68].

2.4.2. Geometric Morphometrics

Each of fourteen previously established landmarks [48] for the WCR were digitized using the software program tpsDIG v.2.16 [69], for which x, y coordinates were generated to investigate hindwing shape. Statistical analyses were performed using MorphoJ version 1.06d [70]. Landmark coordinates were determined, and shape information was extracted using a full Procrustes fit [70]. Principal

component analysis (PCA) was used to visualize hindwing shape variation in relation to the development of resistance [71]. PCA was based on the covariance matrix of individual hindwing shape. To visualize the average change in Bt-resistant strains, a covariance matrix of the average data (for all specimens, regardless of sex) was created. A PCA of the averaged data was used to better visualize shape morphology [72]. To compare morphological relationships between Bt-resistant and non-resistant populations, a canonical analysis of variance (CVA) was performed in order to calculate the morphological relationship between groups using the Mahalanobis and Procrustes distances. Mahalanobis and Procrustes morphological distances were calculated and reported with their respective p-values after a permutation test (10,000 runs). Finally, a multivariate regression of shape versus centroid size was performed to confirm whether size had an allometric effect [73].

3. Results

3.1. Genetic Data

3.1.1. Population Diversity Metrics

From the 29 WCR genotyped, 25,304 SNPs were detected. The 90% call rate filter then removed 13,852 SNPs from the data set. Following this, the minor allele frequency filter, SNPs with frequencies <1%, hence removed another 3555 SNPs. Filtering for monomorphs, secondaries, and reproducibility set at 95% removed 772 SNPs. For final analyses, 7125 SNPs were used.

The overall population estimate was applied, and moderate observed heterozygosity (HO) was observed across all loci, with an estimated value of HO = 0.325. Moderate genetic diversity, estimated by expected heterozygosity (HE), was observed with an estimated value of HE = 0.302. Moderate inbreeding was observed (FIS = 0.121). There were no significant deviations from HWE for all loci. The low overall value of the genetic structure (FST = 0.0181) estimated for the five populations suggested a lack of genetic differentiation amongst them as a whole.

Heterozygosity estimates (HO and HE) over all loci and populations were very similar. The average HO per population ranged from 0.315 (non-resistant) to 0.338 (Cry3Bb1_Cry34/ 35Ab1), while average HE ranged from 0.315 (Cry34/35Ab1) to 0.349 (Cry3Bb1_Cry34/35Ab1) (Table 2). Moderate levels of genetic diversity across all populations were therefore suggested.

Table 2. Expected heterozygosity (He) and observed heterozygosity (Ho) values for western corn rootworm populations over all loci.

	No. of Individuals	No. of Loci	Ho	He
Cry3Bb1	7	6487	0.3203	0.3296
Adapted to crop rotation	4	6610	0.3352	0.3464
Cry34/35Ab1	6	6247	0.3165	0.3158
Cry3Bb1_Cry34/35Ab1	5	6562	0.3380	0.3494
Non-resistant	7	6261	0.3149	0.3170

Distribution of heterozygous WCR genotypes and SNP markers revealed moderate values of heterozygosity in 25 individuals out of 28, with heterozygosity <0.35 (Figure 2).

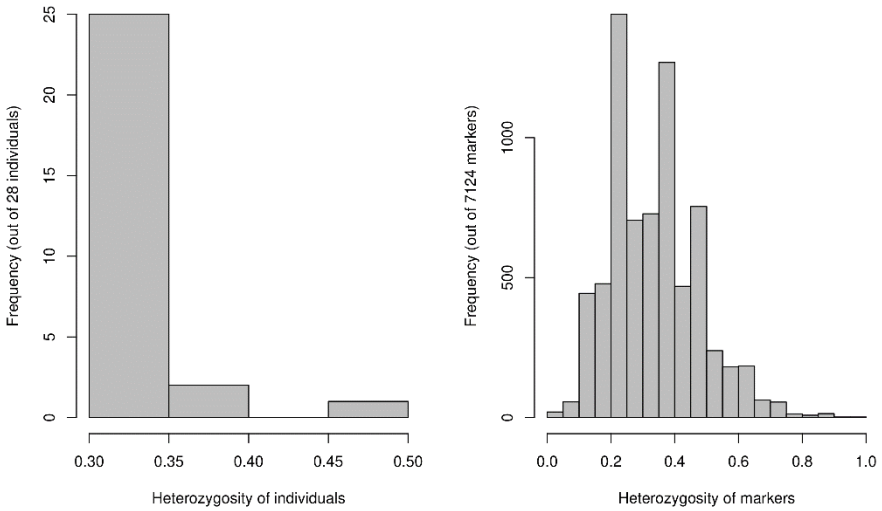

Figure 2. Frequency of heterozygous genotypes and heterozygosity of 7125 SNP markers.

In contrast, pairwise genetic structure does however show differentiation between pairwise population comparisons (Table 3). Pairwise FST θ estimates ranged from 0.0021 (non-resistant population versus Cry3Bb1 resistant population) to 0.0531 (Cry34/35Ab1 resistant population versus Cry3Bb1_Cry34/35Ab1 resistant population). Cry34/35Ab1 and Cry3Bb1_Cry34/35Ab1 populations showed the greatest enetic differentiation with respect to all other populations.

Table 3. Population pairwise estimates of fixation index (F_{ST}).

	Cry3Bb1	**Rotation Resistant**	**Cry34/35**	**Cry3Bb1_ Cry34/35**
Cry3Bb1				
Rotation resistant	0.0028			
Cry34/35	0.0250	0.0242		
Cry3Bb1_Cry34/35	0.0238	0.0333	0.0531	
Non-resistant	0.0021	0.0110	0.0206	0.0286

3.1.2. Genetic Structure

STRUCTURE analysis revealed ΔK = 3 was the most likely number of clusters or populations present within the sampled US WCR individuals (Figure 3). Beetles were assigned to three clusters in consultation with results from STRUCTURE (Figure 4). Along with the results of the kinship analysis with the genetic clustering, a heat map of kinship matrix for evaluating the genetic differences among WCR genotypes was generated. Kin- ship coefficients between pairs of WCR genotypes varied very little on a scale of - 1 to 1. However, the kinship matrix obtained from DArTseq SNP markers resulted in three distinct groups (Figure 5).

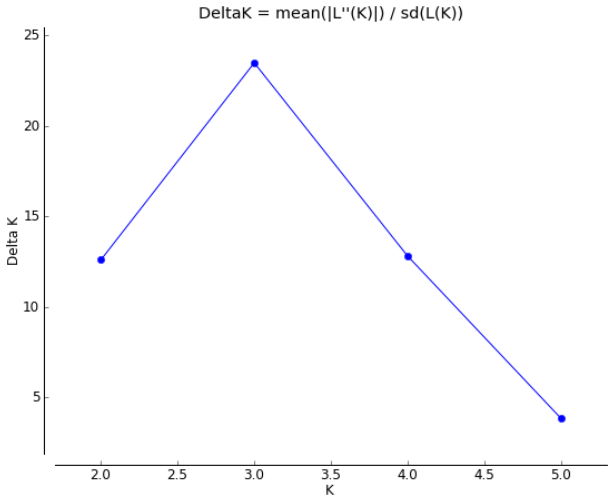

Figure 3. Results from Structure Harvester analysis to reveal the most likely value of K based on STRUCTURE results.

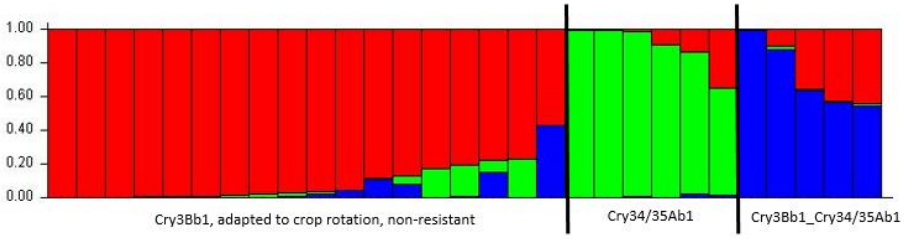

Figure 4. Determination of the optimal value of K = 3 and population structure of 29 WCR genotypes using DArTseq SNP markers.

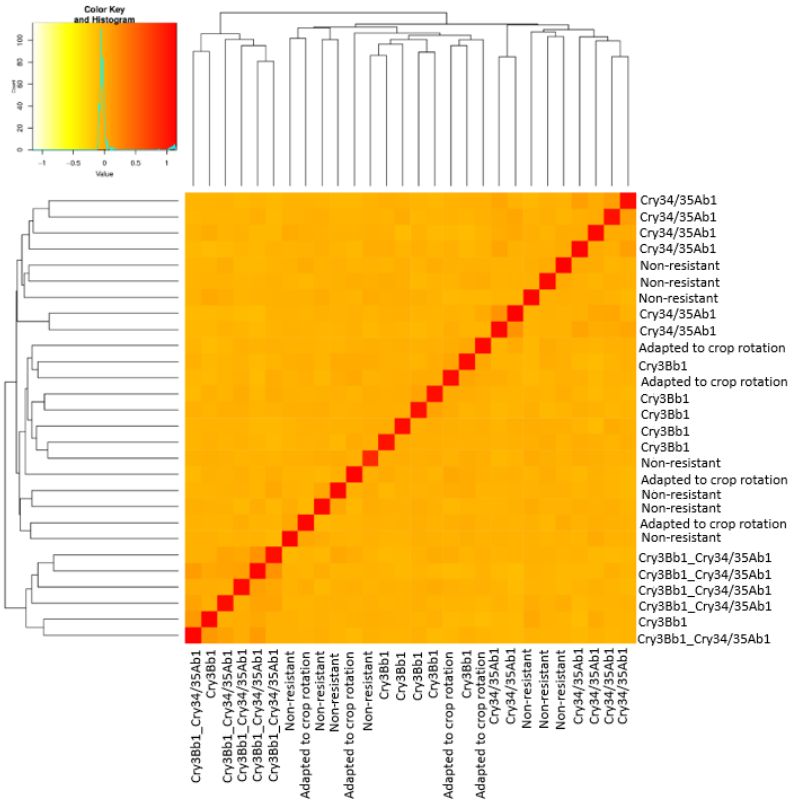

Figure 5. Heat map plot of kinship matrix using average linkage clustering based on SNP markers depicts the existence of three different groups among WCR genotype.

Further analysis of genetic structure using neighbor-joining (NJ) cluster analysis differentiated WCR genotypes into tree clusters (Figure 6). Cluster I was the largest, and it comprised 18 genotypes that included non-resistant individuals, Cry34/35 and Cry3Bb1 resistant. Cluster II contained individuals with combined Bt toxins Cry3Bb1 and Cry34/35 toxin, and Cluster III contained individuals adapted to crop rotation.

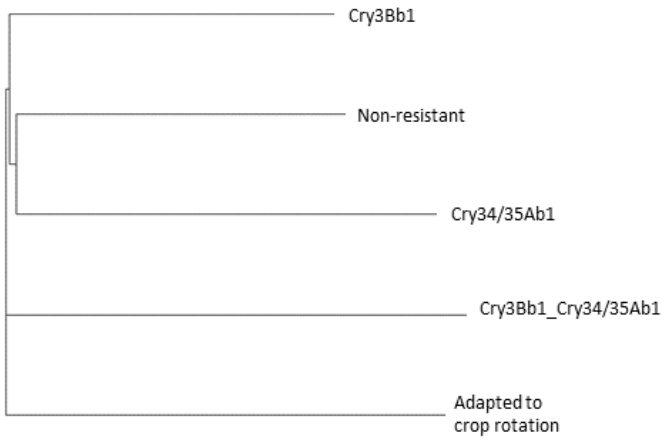

Figure 6. The neighbor-joining cluster analysis using DArTseq SNP markers for grouping 29 WCR genotypes.

3.2. Geometric Morphometrics

To avoid measurement error in our results, we calculated a Procrustes ANOVA show- ing that the mean square for individual variation exceeds the measurement error for wing shape (MS centroid size individuals: 0.000002 < 0.000107 MS centroid size error; and 7.0284 106 MS shape individuals <7.428 105 MS shape error), so we can retain the fol- lowing results. A multivariate regression analysis was performed before all the subsequent statistical analyses, discarding any allometric effect on the data (% predicted: 0.8033%).

The PCA of the hindwing shape showed an accumulation of the shape variation in a very few number of dimensions. The first three PCs accounted for 51.246% (PC1 = 21.12%; PC2 = 17.18%; PC3 = 12.93%) of the total shape variation and provided an approximation of the total amount of hindwing shape variation. After averaging the shape variation between the different populations, the population with Cry34/35Ab1 toxin was localized at the left of the PCA closer to the wing shape phenotype of the Cry3Bb1 but far away from the resistant and non-resistant populations where the latter was similar to the population of the combination Cry3Bb1_Cry34/35 (Figure 7).

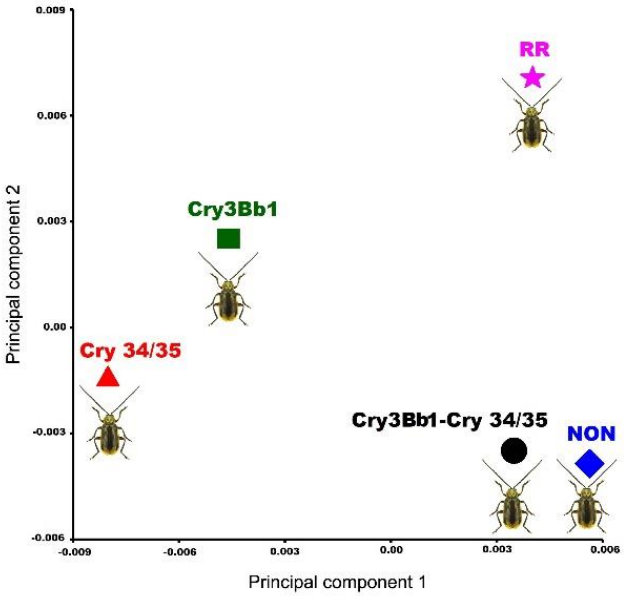

Figure 7. Principal component analysis of the hindwing average shape between different populations: resistant to the toxins, adapted to crop rotation, and non-resistant *Diabrotica virgifera virgifera*. Color and sign code: red triangle: Cry34/35Ab1 resistant population; green square: CryBb1 resistant population; pink star: population adapted to crop rotation (RR); black circle: CryBb1—Cry34/35Ab1 resistant population; and blue rhomboid (NON): non-resistant population.

Procrustes ANOVA showed clear significant differences between the hindwings size and shape between populations (Table 4).

In order to graphically visualize the differences, the CVA maximized the variance between groups, finding similar results with the genetic type in which the population of Cry34/35Ab1 separated from the non-resistant populations (Figure 8). Finally, significant differences (using the different morphometric distances) were found between populations after a permutation was run (Table 5).

Table 4. Procrustes ANOVA for both centroid size and wing shape of *Diabrotica virgifera virgifera,* Sums squares (SS) and mean squares (MS) are in units of Procrustes distances (dimensionless).

Centroid size							
Effect	SS	MS	df	F	P (param.)		
Toxins	1135911.475839	283977.869	4	21.6	<0.0001		
Individual	3431958.659351	13149.26689	261	45.74	<0.0001		
Residual	56921.18152	287.480715	198				
Shape							
Effect	SS	MS	df	F	P (param.)	Pillai tr.	P (param.)
Toxins	0.03076466	0.0003204652	96	4.7	<.0001	1.12	<.0001
Individual	0.42691601	6.81539E-05	6264	2.36	<.0001	17.64	<.0001
Residual	0.13725163	2.88829E-05	4752				

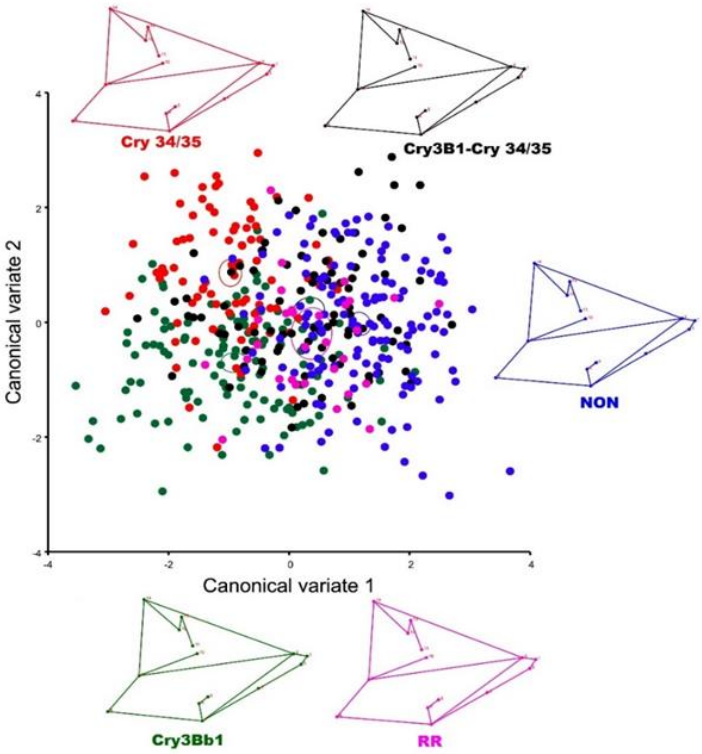

Figure 8. Canonical variate analysis of the hindwing shape between different populations resistant to the toxins: adapted to crop rotation and non-resistant population in *Diabrotica virgifera virgifera*. Color and sign code: red Cry34/35Ab1 resistant population; green CryBb1 resistant population; pink population adapted to crop rotation (RR); black CryBb1-Cry34/35Ab1 resistant population; and blue (NON): non-resistant population.

Table 5. Mahalanobis and Procrustes distances between groups obtained from canonical variate analysis. *: $p < 0.05$; **: $p < 0.001$.*

Mahalanobis Distances			
Effects	Cry 34/35	Cry3Bb1	NON
Cry3B1_Cry 34/35	1.8022**		
Cry3Bb1	1.5633**	1.7142**	
NON	2.3832**	1.3276**	2.2068**
RR	2.305**	1.6339**	1.9881**
Procrustes Distances			
	Cry 34/35	Cry3Bb1	NON
Cry3B1_Cry 34/35	0.0135**		
Cry3Bb1	0.0107**	0.0124**	
NON	0.0155**	0.0069*	0.013**
RR	0.0154**	0.0118*	0.0132**

4. Discussion

In this research we aimed to find a reliable pattern of differences related to resistance type using genetic and geometric morphometric analyses. For population structure analysis, we used DArTseq SNP markers. One of the questions we were interested in was whether resistant WCR populations differ at the genetic level. We found no significant evidence of high genetic diversity in any of the assumed populations. However, the estimated values were congruent with moderate genetic diversity across the genotyped beetles. The STRUCTURE revealed three genetic clusters. This classification was also supported by the VanRaden kinship algorithm, where Cry3Bb1_Cry34/35Ab1 individuals and Cry34/35Ab1 were separated from Cry3Bb1 adapted to crop rotation and non-resistant individuals, although some non-resistant individuals mixed between Cry34/35Ab1, which could be due to the normal evolutionary process. The fact that Cry3Bb1 non-resistant and adapted to crop rotation populations are mixed suggests that they are genetically similar (Figure 4). The neighbor-joining tree separated the individuals adapted to crop rotation, which is to be expected given that the first evolved resistance (not including insecticides) was to crop rotation [12]. Afterwards, all other resistance evolved, and we can see that clearly in this result. The fact that the non-resistant population is not separated could be due to an evolutionary process, as we mentioned earlier.

High-throughput sequencing has provided deeper insight into the molecular mechanisms of resistance [74]. It allowed us to find that many point mutations are found in different genes, suggesting that these mechanisms can occur simultaneously, making it more difficult to understand which one is really responsible for the resistance phenotype [75,76]. In our research, we focused on resistant populations, and we determined that there was some variability between them, but there was no exact pattern. Recent molecular studies show us that different sets of genes are involved in resistance [76–79], which makes it unlikely that universal markers of resistance can be developed to accurately determine the likelihood of a population becoming resistant to a particular compound [75,77,79]. A different number of genes may be involved in resistance, and individuals within a population exhibit different evolutionary patterns of resistance evolution. Therefore, resistance can be found throughout the whole genome, but it is not conditioned by the differences. However, certain shifts could be a warning that some changes in the genome have occurred. Through estimates of genetic diversity, population structuring, and genetic relatedness between individuals, information on the effectiveness of control strategies can be obtained, and recommendations to improve the efficacy of control programs may be possible.

The actual sample size of each site does not need to be large when using SNPs. SNP markers provide the power, not the sample size, as SNPs have genome-wide coverage and there end up being many thousands of SNPs by the time genotyping is complete [80]. The paper by Trask et al. [81] states, "Given that each SNP marker has an individual evolutionary history, we calculated that the most complete and unbiased representation of genetic diversity present in the individual can be achieved by including at least 10 individuals in the discovery sample set to ensure the discovery of both common and rare polymorphisms." The second paper by Li et al. [82], who also worked with beetles from the order Coleoptera, found that "a minimum sample size of 3–8 individuals is sufficient to dissect the population architecture of the harlequin ladybird, Harmonia axyridis, a biological control agent and invasive alien species." They also estimated the optimal sample size for accurately estimating genetic diversity within and between populations of Harmonia axyridis. They determined that six individuals are the minimum sample size required.

Wing morphology (size and shape) is the most important trait of an insect's dispersal capacity. For this reason, the integration of different techniques to understand the plasticity and variation of this trait is vital to understanding how they adapt to new environments and to coordinating strategic planning ahead of possible new invasions [3]. Different types of wing morphotypes have been studied to determine the dispersal capabilities of flying insects [83–85]. Le et al. [86] found that narrowed wings in beetles are more efficient for flapping low-level flights. Additionally, for D. v. virgifera, wing shape has been identified as a very good trait to measure in different agronomic studies, including studies of life history (sexual dimorphism) and interspecific and intraspecific shape variation [47–49], and wing shape has also been a useful variable when combined with other monitoring tools (genetics (e.g., microsatellites) and traditional traps (e.g., pheromones)) [3].

Mikac et al. [46] showed that beetles adapted to crop rotation had broader wings (cf. susceptible beetle). Mikac et al. [45] expanded the use of differences in hindwing size and shape to examine changes in WCR associated with the development of resistance, specifically to examine potential differences between (Bt)-resistant, non-resistant (or susceptible), and adapted to crop rotation populations in the US. In general, the hindwings of non-resistant beetles were significantly more elongated in shape and narrower in width (chord length) compared with beetles resistant to Bt maize or crop rotation. This result was confirmed by our study. Mikac et al. (2019) did not separate the Bt-resistant populations in their study, but considered them as one population. Therefore, in our study, we separated all Bt-resistant populations to see the differences between them. Cry3Bb1_Cry34/35Ab1 individuals had the broader shape and a more robust wing with an expansion of landmark 14 and a contraction of landmark 9. Cry3Bb1 individuals had the narrower wings, while individuals resistant to Cry34/35Ab1 had similar but smaller wings, distinguished by the expansion of landmarks 3 and 4. The more stable and elongated wing shape was that of the population adapted to crop rotation, in which there was an extension to landmarks 1 and 2 to the left and an elongation to landmark 9 to the right. The non-resistant population is also slightly wider than the population of Cry3Bb1-Cry34/35Ab1, with the movement of landmarks 14 and 2 also slightly to the right and the wider shape that is also produced by the movement of landmark 7 to the upper left. Elongated wings are more aerodynamic and are considered to be involved in migratory movement [46]. Mikac et al. [46] also suggested that this could be a useful invasive dispersal strategy for mated females. In our research, individuals adapted to crop rotation had more stable and elongated wings, suggesting that these individuals could fly long distances. Such differences may impact upon the dispersal or long-distance movement of resistant and non-resistant beetles. Understanding which beetle morphotype is the superior flyer and spreader has implications for managing WCR through integrated resistance strategies. These findings confirmed GM as a reliable technique for resistance detection. In this study, we aimed to confirm the results from SNPs markers with GM. We found that geometric morphometric tools could provide important clues to differentiate resistant and non-resistant populations. One of the principal results was the similarity of the hindwing shape variation

between the population after the STRUCTURE analysis, where using both monitoring techniques showed that the more differentiated population was the resistant Cry34/35Ab1.

Here we describe a possibility that combining genetic and geometric morphometrics could be a reliable technique that can be used to reveal differences among WCR populations. Hence, geometric morphometrics can be used as a biomarker for resistance detection as part of a larger integrated resistance management strategy for western corn rootworm.

In Croatia, WCR have been investigated in detail (traditional monitoring, genetic monitoring, and GM monitoring), and knowledge about dispersal and adaptive abilities of these invasive insects is well known [3,47,87,88]. Our future work will focus on populations collected in intensive maize-growing areas in Croatia, where WCR populations have become established since their introduction 30 years ago. We will use the comparative techniques presented in this paper to determine whether Croatian populations are potentially resistant and which US WCR population was the source population for Croatia and Europe. This knowledge would help to detect resistant individuals that might invade geographical areas where they are not yet present (e.g., beetles adapted to crop rotation invading Europe where such variants are not present). Such information is very important for biosecurity measures, resistance management, and future control strategies for this pest worldwide.

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Conflicts of Interest: The authors declare no conflict of interest.

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Population Genetic Structure and Geometric Morphology of Codling Moth Populations from Different Management Systems

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Abstract: Codling moth (CM), *Cydia pomonella* L., is an important pest of apples worldwide. CM resistance to insecticides is a serious problem in apple production. For effective management and control, monitoring of resistant CM populations is absolutely necessary. Therefore, in this study, we investigated whether it is possible to find a reliable pattern of differences in CM populations related to the type of apple control method. The genetic results showed low estimated value of the pairwise fixation index, F_{ST} = 0.021, which indicates a lack of genetic differentiation and structuring between the genotyped populations. Different approaches were used to analyze the genetic structure of codling moth populations: Bayesian-based model of population structure (STRUCTURE), principal component analysis (PCA), and discriminant analysis of principal components (DAPC). STRUCTURE grouped the CM genotypes into two distinct clusters, and the results of PCA were consistent with this. The DAPC revealed three distinct groups. However, the results showed that population genetic differentiation between organic and integrated orchards was not significant. To confirm the genetic results, the forewing morphology of the same CM individuals

was examined using geometric morphometric techniques based on the venation patterns of 18 landmarks. The geometric results showed higher sensitivity and separated three distinct groups. Geometric morphometrics was shown to be a more sensitive method to detect variability in genotypes due to pest control management. This study shows the possibility of using a novel method for a strategic integrated pest management (IPM) program for CM that is lacking in Europe.

Keywords: *Cydia pomonella;* single nucleotide polymorphism; geometric morphometrics; genetic structure; monitoring test

1. Introduction

Codling moth (CM) (*Cydia pomonella* L.) is a serious pest of apple production in Croatia and globally [1–4]. Apples are grown on about 4.7 million hectares of land, with an average yield of 18 tons/hectare, corresponding to a global quantity of 87 million tons of apples/year [5]. The larvae of CM cause the greatest damage to apple production. Larvae eat fruit flesh and seeds, and produce holes in the fruit full of larval feces called "larval droppings" [6]. Without the use of chemical control, the larvae can affect a 30–50% decline in an apple crop during the growing season [7]. Chemical treatments are the main method of controlling CM in integrated apple production [8]. Seventy percent of CM pest control is dependent on insecticides [9]. CM is a plastic species that has successfully adapted to different habitats and has also developed resistance to different groups of synthetic insecticides [10,11]. The first documented case of resistance was in 1928 in the United States against arsenates [12]. In Europe, the first case of resistance to diflubenzuron was documented in 1990 in southeastern France and Italy [13]. Ever since, more events of resistance have been progressively reported in almost all major apple-growing regions [10,14–16].

CM populations are now resistant to 22 different active chemical compounds, and 193 cases of resistance have been recorded [17]. The use of chemical insecticides in the last 30 years has altered the development of resistance [18–24]. An additional problem occurred during the 1990s regarding cross-resistance development, as CM simultaneously became resistant to numerous groups of pesticides [25,26]. Since 2005, resistance to the widely used isolate CpGV-M has also been reported in several European countries [27–32].

CM resistance to insecticides is an increasing problem in apple production. Reliable data on resistance are necessary for successful resistance management. In order to keep management recommendations, it is important to continue the monitoring processes in light of changing conditions or new data gained [23]. Resistant populations need to be continuously studied to suppress the further spread of resistance. Hence, there is a need for new control tools and a new approach to CM management.

A multidisciplinary approach is imperative to developing effective pest management strategies. One component of this is understanding the population dynamics of insect pests and their genetic structure [33]. To define a proper integrated pest management strategy for CM and other insects, understanding the population genetic structure and dispersal patterns of species and population is required at the field and landscape scales [34].

Several molecular markers (AFLPs, microsatellites, allozymes, among others) have been used to study modification in the structure of CM populations [3,9,15,26,34–40]. Franck et al. [3] studied CM populations from treated and untreated orchards in Europe and South America (France and Chile) and reported that there was no significant genetic differentiation by country but found that insecticide

treatment had some effect on allelic richness. Pajač et al. [26] used microsatellite markers to compare the genetic structure of treated and untreated populations CM in Croatia. The authors demonstrated that differences in genetic structure between populations were low; however, natural populations of CM had the most average number of alleles and the highest number of unique alleles compared with the handled populations. Frank and Timm [39] also used microsatellite markers to study CM genetic structure and gene flow in biologically and chemically treated apple orchards. These authors discovered less genetic variation between populations but significant genetic variation within individuals. Chen and Dorn [40] used microsatellite markers to examine genetic differentiation and the extent of gene flow among eight field populations. They found significant genetic differentiation between populations even when they were less than 10 km apart. These results are consistent with those of Timm et al. [38], Thaler et al. [9], and Duan et al. [41] and provide evidence for CM population differentiation at small spatial scales, even within the same bioregion. Men et al. [42] first investigated the genetic diversity and structure of the CM population in China from 12 apple orchards. They used eight microsatellite loci and observed sequential loss of genetic diversity and significant structuring associated with dispersal. Li et al. [43] confirmed Men et al.'s [42] results and found that the genetic diversity of populations from northeastern China was similar to that of native CM populations in Europe. Kuyulu and Genç [44] found low genetic differentiation among nine CM populations in Turkey, and Basoalto et al. [45] found low genetic differentiation among 34 populations (F_{ST} = 0.03) in Chile. Cichón et al. [46] used 13 microsatellite markers for 22 locations in Chile and Argentina and found significant genetic differentiation among populations (FST = 0.085).

Analyzing the geometric characteristics of the morphology (geometric morphometric tools) is a demonstrated monitoring tool for studying inter and intraspecific variation and is a useful tool to show forewing shape and size differences among codling moth populations [47]. It is well known that metric traits (wing shape and size) are the first morphological traits to change under the influence of environmental and genetic factors [48,49]. Over the last 20 years, geometric morphometric (GM) has been used to study the genetic variability of different insect species [50–55]. In CM populations, GM methods have been used to reveal differences between CM forewings and hindwings as a function of the season (overwintering vs. summer), geographical location, and sex [56]. Pajač Živković et al. [57] investigated the relationship between integrated and organic CM populations using GM, but on a limited number of moths. Nevertheless, the authors discovered population changes associated with different types of apple production.

GM, which uses phenotypic size and shape analysis, is a technique that can be used to reveal differences in forewing shape and size among populations of CM. Similar to single nucleotide polymorphisms (SNPs), which are genetic biomarkers, GM can be used as a phenotypic biomarker. Combining genetic and morphometric monitoring has been used to study other pest insects with success [58]. Moreover, studies suggest that the data generated are more precise when both methods are used in combination [50,59–62].

Here, we report on the combined use of genetic and geometric morphometric techniques to determine differences in field populations of CM related to the type of apple control method. The hypothesis of this study was that by combining genetic and morphological markers, it would be possible to identify CM populations based on control management to help improve the ongoing surveillance of CM populations. Through innovation and the use of novel methods (such as single nucleotide polymorphisms and geometric morphometrics), it may be possible to develop reliable strategies for monitoring CM populations in the field.

2. Materials and Methods

2.1. Collection Sites and Sampling

Adult male CM individuals were collected across 2 years (2017 and 2018) from mid-April to early September in apple orchards in continental (northern and eastern) Croatia (Figure 1) using funnel traps Csalomon® VARL (Plant Protection Institute, Budapest, Hungary) with the pheromone lure with rubber. Nine populations were collected from organic orchards (Garešnica, Veliko Polje, Vukovar, Donje Orešje, Jazbina, Šašinovec, Kravarići Barbarski, Beloslavec, and Zagreb) and nine populations from orchards with integrated pest management (IPM) practices (Veliki Zdenci, Dugo Selo Lukačko, Zdenci, Tovarnik, Lovas, Velika Mlaka, Čehovec, Kloštar Ivanić, and Obreška). A total of 18 field populations and 1 laboratory-reared sample (insecticide-free) were studied (Table 1). Laboratory-reared susceptible populations were obtained from the Entomos AG part of Andermatt Holding AG (Le Lieu, Switzerland).

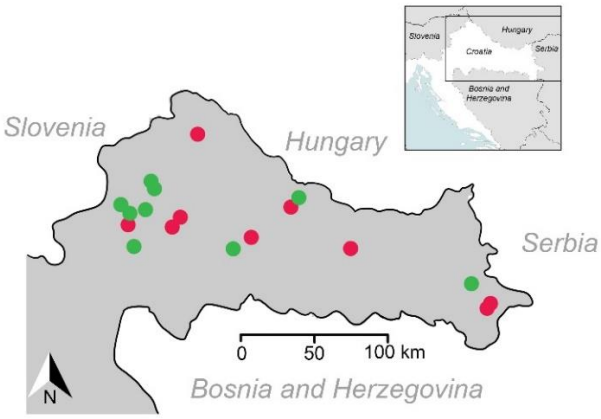

Figure 1. Sampling sites of *Cydia pomonella* in Croatian orchards: red, integrated orchard; green, organic orchard.

Table 1. Number of CM individuals used for geometric morphometric and SNPs analyses: n, sample size.

Codling Moth Population	Adults Single Nucleotide Polymorphism Genotyped (*n*)	Geometric Morphometric Wings (*n*)
Organic orchards	44	44
Integrated orchards	44	24
Laboratory population	6	99

The selected orchards represent typical apple farming in Croatia, and trees were 15–20 years old. According to the EU standard directive, pest management in integrated orchards includes pest monitoring and threshold-based applications [63]. The IPM orchard was systematically treated with different insecticides. The insecticides used in the orchards of IPM were: chlorpyrifos-ethyl (organophosphate insecticides), alpha-cypermethrin, deltamethrin (pyrethroids), lufenuron, methoxyfenozide (insect growth regulators), thiacloprid, acetamiprid (neonicotinoids), emamectin benzoate (avermectins), and chlorantraniliprole (diamides). The insecticides were applied 10 to 15 times during the growing season by spray treatments. The resistance of European populations to pesticides that are used in orchards in commercial apple production has been confirmed by Reyes et al. [13,64]. The

populations collected in the organic orchards were not treated with chemicals and were mainly controlled by maintaining high functional biodiversity (assemblages of beneficial insects). No mating disruption, *Cydia pomonella* granulovirus (CpGV), nematodes, entomopathogenic fungi, or nets were used in the organic orchards. In this research, all CM populations were collected in Croatia. We used the same populations for the genetic and morphometric analyses.

2.2. DNA Extraction and SNPs Genotyping

A total of 94 *C. pomonella* males were sampled in this study. DNA was extracted from the whole-body tissue using the Qiagen DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. The forewings from all individuals were removed and preserved for morphometric analysis. DNA quality and concentration were determined using a spectrophotometer (BioSpec-nano Micro-volume). After quality control, the samples were sent for commercial genotyping at Diversity Array Technology Pty Ltd. (DArT, Canberra, Australia) [65].

2.3. Geometric Morphometric Sample Preparation

The genotyped CM adults were also examined using GM techniques, and analyses based on forewing veins were performed. In total, 363 CM forewings were analyzed. Eighteen landmarks were digitized and defined by vein junctions (Figure 2) or vein terminations following the protocol of Pajač Živković et al. [57].

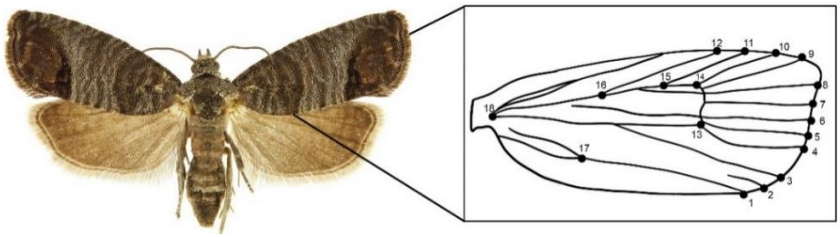

Figure 2. Position of 18 landmarks type 1 on a Codling moth forewing (adapted with permission from Ref. [57]. 2019, Pajač Živković, I.).

2.4. Data Analysis

2.4.1. SNP Quality Control

Genetic data were analyzed using the packages adegenet v2.1.5. [66], SNPrelate v1.6.4. [67], and dartR v1.9.1.1. [68] developed for the R Environment for Statistical Computing [69]. The SNP data set was subject to a filtering process to remove potentially erroneous SNPs. We used the following criteria: call rate <90% (i.e., SNPs that had 10% missing genotypes or greater) were removed from the data set, SNPs with reproducibility <95% were excluded, minor allele frequencies (MAF) >0.01, and monomorphic SNPs and secondaries were excluded. The following estimates of the parameters of genetic diversity were calculated for each population using the package SNPRelate: number of different alleles (A), number of private alleles (P), observed heterozygosity (Ho), and expected heterozygosity (He).

2.4.2. Population Genetics Analyses

Pairwise F_{ST} were calculated between CM populations (i.e., organic, integrated, and laboratory populations) using the gl.fst.pop command in dartR package. Deviation from the Hardy–Weinberg

equilibrium (HWE) was estimated for each population using the gl.report.hwe command as implemented in the R package dartR [68]. Using the function gl.basic.stats in dartR, we estimated the overall basic population genetics statistics per locus, such as the observed (H_O) heterozygosity, (F_{IS}) inbreeding coefficient per locus, and F_{ST} corrected for the number of individuals.

The Bayesian approach implemented in STRUCTURE v 2.3.4 [70] was used to find the probable number of genetic clusters. Genetic clusters (K) were set between 1 and 20 (one more than the total number of populations for the complete data set), and a series of 10 replicate runs for each prior value of K was analyzed. This analysis was comprised of independent runs consisting of a burn-in of 10,000 iterations followed by 100,000 Markov chain Monte Carlo iterations. Default parameters in STRUCTURE were set with an admixture model of ancestry and the correlated allele frequency model assumed. The number of genetic clusters was calculated using the ΔK method in Structure Harvester software [71].

Further analysis of population structures was conducted using the discriminant analysis of principal components (DAPC) implemented in the R package "adegenet" [66]. Principal component analysis (PCA) was performed to determine genetic similarities and dissimilarities present within the data set using the package "SNPrelate" [67]. Discriminant analysis of principal component (DAPC) was also employed to find the population structures.

2.4.3. Geometric Morphometrics

The established 18 landmarks for the CM [57] were digitized using tpsDIG v.2.16 [72]. Statistical analyses were performed using a coding environment in R using geomorph 4.0 R package [73] and package gmShiny [74]. Landmark coordinates were determined, and shape information was extracted using a full Procrustes fit [75]. Principal component analysis (PCA) was used to visualize forewing shape variations in relation to the pest management practice [76]. PCA was based on the covariance matrix of individual forewing shapes. To visualize the average change in populations from integrated and organic orchards, a covariance matrix of the average data was created [77]. It is important to state that PCA was performed to determine the overall variability among the studied populations, where the percentage of variation between axes (PCs) represents the different dimensions of the shape space. To detect statistical differences between organic and integrated wing shape differences, we performed a Procrustes ANOVA. Finally, to confirm whether size had an allometric effect, a multivariate regression of shape versus centroid size was performed [78].

3. Results

3.1. Genetic Dana

3.1.1. Population Diversity Metrics

An initial set of 57,392 SNPs were detected in the 94 genotyped CM samples. However, 52,513 SNPs were removed during the quality control steps (reproducibility, discarding monomorphic markers, call rate, minor allele frequencies, and removing secondaries). For final analyses, 4879 SNPs were retained.

Values of genetic diversity obtained across all loci were: low observed heterozygosity (Ho):0.130 and low genetic diversity estimated by expected heterozygosity (He):0.159, a moderate observed inbreeding coefficient (F_{IS} = 0.221), and a low overall value of the genetic structure (F_{ST} = 0.021) estimated for the three types of populations. The average Ho per population ranged from 0.104 (laboratory) to 0.147 (organic), while the average He ranged from 0.118 (laboratory) to 0.180 (organic and laboratory) (Table 2). Across all populations, we found a low level of genetic diversity.

Table 2. Detailed allelic diversity estimates of *Cydia pomonella.*

Population	n	A	*p*	Ho	He
Integrated	44	9010	1443	0.139	0.180
Organic	44	9163	1931	0.147	0.180
Laboratory	6	6746	187	0.104	0.118
Overall	94	24919	3561	0.130	0.159

n,number of samples; A, number of different alleles; *p*, number of private alleles; Ho, observed heterozygosity; He, expected heterozygosity.

Moderate genetic differentiation was found between the laboratory and field populations. No differentiation was found between the two field-sampled populations. Population pairwise estimates of F_{ST} between the integrated and organic populations were 0.001, integrated vs. laboratory was 0.140, and organic vs. laboratory 0.135.

3.1.2. Genetic Structure

The PCA shows strong patterns of structure between the laboratory and field populations (Figure 3).

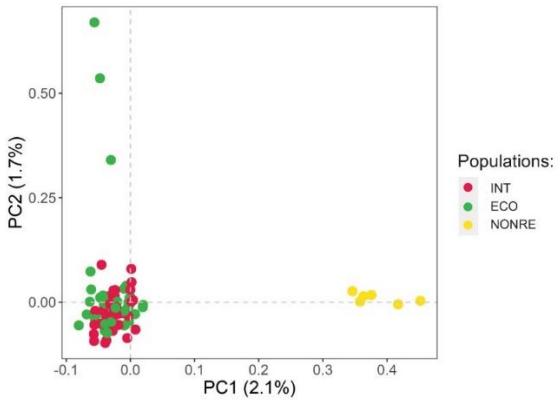

Figure 3. Principal component analysis (PCA) based on 4879 SNPs. Color and sign code: red, populations from integrated orchards (INT); green, populations from organic orchards (ECO); yellow, laboratory population (NONRE).

STRUCTURE analysis indicated $\Delta K = 2$ as the most likely number of clusters or populations present within the sampled CM individuals (Figure 4). Results from STRUCTURE assigned moths to two clusters (Figure 5).

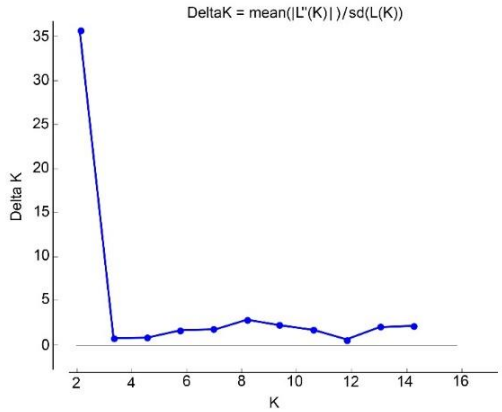

Figure 4. Results from Structure Harvester analysis reveal the most likely value of K based on STRUCTURE results.

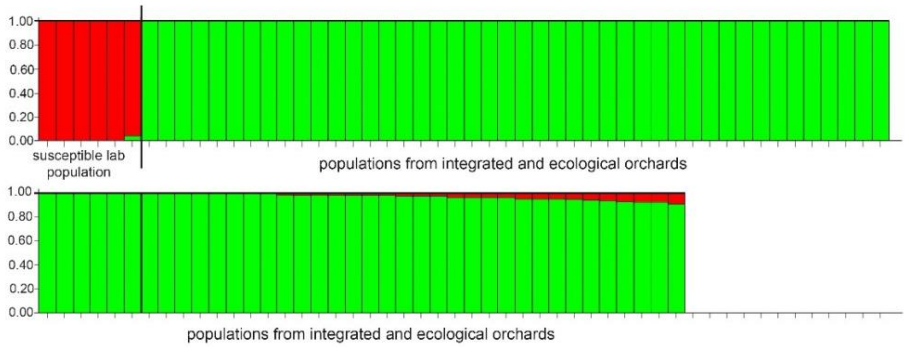

Figure 5. STRUCTURE analysis of 94 CM genotypes using SNP markers.

The DAPC showed the patterns of genetic structure in CM (Figure 6). The genotypes were grouped into three clusters (i.e., laboratory population, organic orchards, and integrated field orchards).

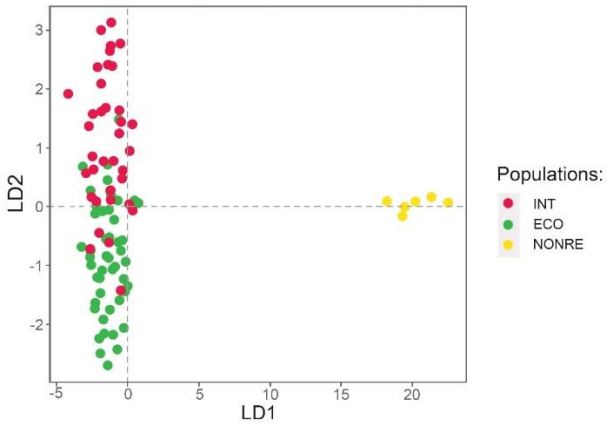

Figure 6. Discriminant analysis of principal components (DAPC) based on 4879 SNPs. Color and sign code: red, populations from integrated orchards (INT); green, populations from organic orchards (ECO); yellow, laboratory population (NONRE).

3.2. Geometric Morphometrics

A Procrustes ANOVA showed highly significant differences between organic and integrated populations (F: 8.68, $p < 0.001$, Figure 7). After incorporating the laboratory population into the analysis, the Procrustes ANOVA also showed highly significant differences between the three analyses groups (F: 8.24, $p < 0.001$, Figure 7).

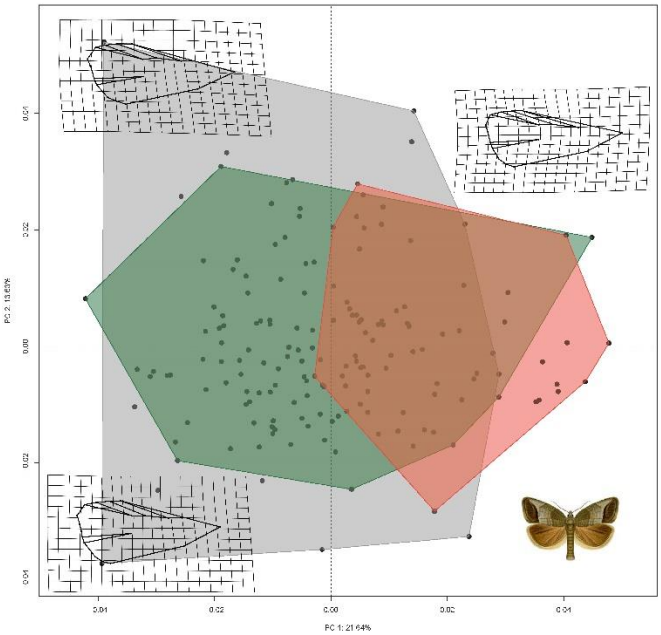

Figure 7. Principal component analysis of the average forewing shape among different populations from integrated orchard, organic orchard, and laboratory populations of *Cydia pomonella*: red, integrated orchard; green, organic orchard; gray, laboratory population.

Most of the total shape variation (21.6%) was explained by the PC1, while the PC2 explained 13.6% of the total shape variation.

Principal variation was noted in landmarks 16, 17, and 18 at the left extreme of the wing, where expansion and contraction of the wing occur during flight (Figure 8). These results can be explained by the management practice (organic vs. integrated cultivation) and may indicate that there is variability in the genotype due to pest control management.

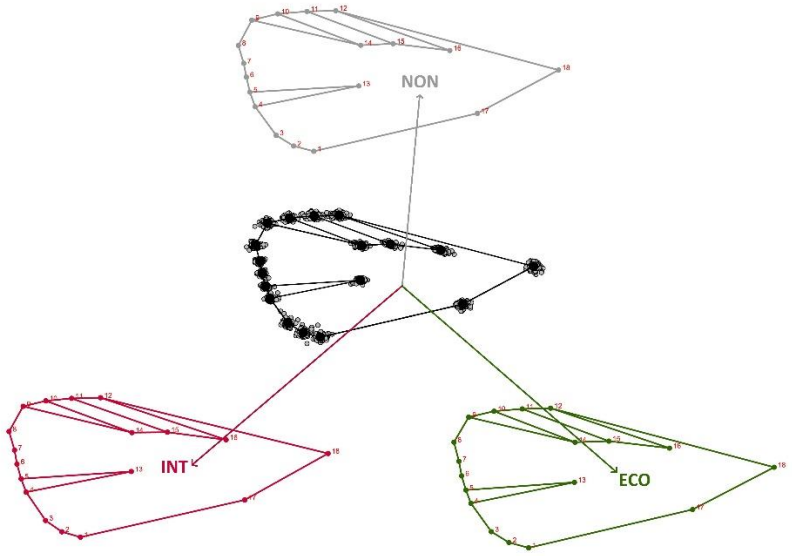

Figure 8. Average wing shape between different orchard populations. The middle wing represents the overall shape with the different averaged populations: red, integrated orchard (INT); green, organic orchard (ECO); gray: laboratory population (NON).

A multivariate regression did not show differences in wing size among the different populations. Therefore, a correction for allometry was not needed. Finally, the results from GM showed that populations from organic orchards are phenotypically similar to the laboratory population than to those from the integrated orchards.

4. Discussion

The aim of integrated production is to promote and care for human health by the production of high-quality fruits without residuals of pesticides. Environmentally friendly and area-wide IPM strategies must be developed to accomplish this aim. Suppressing and preventing the further spread of resistance is a prerequisite for successful and sustainable apple production in Europe. We monitored field CM populations to detect differences related to the type of apple control method and to identify specific biotypes. Our genetic results showed low levels of genetic diversity in the populations investigated in Croatia as well as the laboratory population. Those results are in accordance with the results from Pajač et al. [57]. The output revealed two genetic clusters, which were confirmed by PCA analysis, namely, the laboratory population and the integrated and organic populations (which were combined). However, the DAPC analysis showed three groups: organic orchards, integrated orchards, and the laboratory population (Figure 6). This result can be explained by the basic difference between PCA and DAPC analyses. PCA aims to summarize the overall variability among individuals, which includes both the divergence between groups (i.e., structured genetic variability) and the variation occurring within groups; that is why it is not appropriate to obtain a clear picture of between-population variation. On the other hand, DAPC attempts to summarize the genetic differentiation between groups while overlooking within-group variation and providing better population structure. In DAPC, data are first transformed using PCA, and, subsequently, clusters are identified using discriminant analysis (DA) [79].

However, the detected changes associated with different control methods in this study were very small, and this needs further investigation. In previous studies, markers such as microsatellites were

unable to show differences in the population genetic structure of CM populations in Croatia [80] or elsewhere in Europe [3]. Nevertheless, these authors did note the suspected influence of insecticide treatment on CM allelic richness.

High-throughput sequencing can provide us with deeper insight into the molecular mechanisms of resistance [81]. Thanks to a denser and more uniform distribution within genomes and a large number of SNPs (thousands to millions), we can generate a large amount of information in a single sequencing run, which is less time-consuming and less expensive than previous markers. In addition, SNP markers provide us with broader genome coverage and higher quality data than microsatellites or mtDNA [82]. However, resistance occurrence is dynamic, and resistance mechanisms can change over time. Resistance constantly occurs in insect populations and can even develop within a season [83]. Resistance depends on the number of treatments, the number of generations an insect can produce, and the treated organism itself [83]. Belinato and Martins [84] stated that "insecticide resistance is an adaptive trait in which a set of genes are favorably selected to maintain the insect alive and able to reproduce under an environment exposed to pesticides." It is known that different gene groups are involved in resistance [85]. This makes it difficult to determine and predict which populations will become resistant and when [86,87]. Some argue that it is, therefore, more effective to use morphometric markers to identify minor (and recent) genetic changes than to use genetic markers to identify major changes in the genome [49,50].

The metric properties of organisms, in our work, the wing morphology of CM, were the first morphological characters to change as influenced by environmental and genetic factors [48,49]. GM methods are used to study the smaller changes in population structure [77,88,89], and that is why GM can be used to detect and describe the changes in phenotype that occur under the influence of the genotype.

In our study, using GM methods, we differentiated integrated from organic CM populations based on wing shape. Populations from the organic orchards significantly differed in wing shape incomparison with integrated CM populations. Our data showed that the CM organic population was morphologically similar to the susceptive laboratory population, which had a differing wing shape in comparison with the integrated population. Individuals from the organic orchards had expansion and contraction of the forewing in landmarks 16, 17, and 18, making the wings more elongated and narrower. These results are consistent with that of Pajač Živković et al. [57], who found the same pattern of CM forewings from organic orchards in Croatia. Elongated wings are more aerodynamic and are an important trait needed for the migratory movement of insects (e.g., western corn rootworm) [90].

Mikac et al. [91] suggested that such phenotypic differences in wing shape and size have implications for dispersal and long-distance movement of resistant and nonresistant insects, as wing morphology is a crucial element in an insect's dispersal ability [92]. A study by Pajač Živković et al. [57] was the first to demonstrate significant differences in wing shape of lepidopterans in relation to resistance. In their study, CM populations from organic orchards showed the least wing deformation and were, therefore, reported to be the better fliers and dispersers compared with CM from integrated populations, which were found to be inferior fliers. According to our results, individuals from organic orchards were also found to be better fliers, which means that they are likely responsible for the expansion of the population. Intense selection pressure exerted by decades of pesticide use to control the species has altered the structural integrity of CM wings, making them less efficient at dispersal. This result suggests that the development of resistance could affect the fitness of the organism itself. That is, when the organism becomes resistant, it simultaneously loses other biological traits [84]. Despite the fact that resistant individuals are less capable of long flights, they still represent a pool of new genes, which means that they can transfer the resistance to their offspring. This research should also be conducted on CM females to confirm whether resistance equally affects both sexes since females are responsible for population expansion and enlargement in CM [26]. According to Schumacher et al. [93], some individuals are able to disperse over several kilometers in the field; even distances of up to 11 km have been reported. According to several studies on CM and insecticide resistance, larger females are more

resistant than smaller males [21,34,94] and, therefore, it is likely that this sex and morphotype combination is responsible for spreading resistant alleles throughout apple production areas. In this scenario, it does not matter if resistant males remain in a given area because it is the females that ultimately transfer the resistant genes to new areas via dispersal and offspring. According to Foster [95] and Liu [96], only by monitoring, characterizing, and predicting the occurrence and spread of resistance can we hope to use existing chemical agents in a sustainable manner. Therefore, it is very important to find effective monitoring tools that can serve as reliable biomarkers to detect changes and specific biotypes.

5. Conclusions

Our study has shown that geometric morphometrics is a reliable, accurate, and cost-effective technique for detecting population changes associated with different types of apple production. However, in our study, SNP markers did not show enough power to detect changes among CM populations. Further investigations that include biotests for detecting resistant populations could provide us with more results related to the detection and monitoring of resistant variants. Early detection of resistance will enable the implementation of insect resistance management (IRM) strategies and, thus, contribute to the implementation of antiresistance strategies for CM.

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Assessing the population structure of Colorado potato beetle populations in Croatia using genetic and geometric morphometric tools

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Abstract: The Colorado potato beetle (CPB, *Leptinotarsa decemlineata* Say) is one of the most successful invasive species worldwide. It has been present in Croatia since 1947, where it has caused significant damage to potato plants and developed resistance to several insecticides. Our study is the first attempt to investigate the population structure of CPBs in Croatia. SNP and GM techniques provided us with data about the population structure of the CPB population. A Bayesian model-based clustering algorithm implemented in STRUCTURE, principal component analysis (PCA), and discriminant analysis of principal components (DAPC) were used to analyze the genetic structure of CPBs. For the morphometric analysis, the hindwing shape of the same CPB individuals was examined using wing venation patterns. We detected the low genetic and phenotypic variabilities of CPB populations and the presence of a single panmictic population in the study area, well adapted to different environmental conditions, indicating high phenotypic plasticity. Due to such exceptional adaptation of the CPB population, it is necessary to implement an area-wide approach in future pest control management.

Keywords: *Leptinotarsa decemlineata*; invasive species; population structure; genetic variability; phenotypic plasticity

1. Introduction

Knowledge of insect pests' invasion pathways, genetic differentiation, and dispersal routes is very important for the accurate application of control measures. The Colorado potato beetle (CPB, *Leptinotarsa decemlineata* Say) has been the most damaging pest of potato plants since its introduction to Europe in 1922 [1]. In Croatia, the pest was first discovered in 1947 near Zaprešić (central Croatia) and is now widespread throughout Croatia, except for a few islands [2]. The larvae and adults of CPBs can cause the complete defoliation of potato crops by feeding on leaves and stems [3]. If not controlled, the pest can severely destroy all the potatoes, resulting in total crop loss [4]. For over 80 years, CPBs have been successfully controlled with insecticides [5]. According to Gauthier et al. [6], CPBs played a major role in the emergence of the modern pesticide industry, as hundreds of chemicals were tested against them. To date, more than 300 cases of resistance to 56 insecticides have been reported worldwide [7].

The CPB is also one of the most important invasive pest species worldwide [8]. It has a complicated and diverse life history and a remarkable ability to adapt to toxins by developing resistance [4]. The high phenotypic plasticity may be one of the reasons why CPBs constantly develop resistance to all control measures that have been used against them, demonstrating their remarkable adaptability [9]. Phenotypic plasticity is the ability of an organism to change its genotype under the influence of various environmental factors and to establish and maintain a population in a given area [10–14]. High phenotypic plasticity is one of the most critical characteristics of invasive species, and it has profound evolutionary implications [15,16]. According to Cingel et al. [17], high resistance developing ability, together with phenotypic plasticity, makes this insect "indestructible".

Information on the genetic structure of CPB populations is important for future sustainable control and management strategies [18–21]. The genetic study of this pest began with the work of Grapputo et al. [22]. They investigated the population structure and genetic variability of CPB populations using mtDNA and amplified fragment length polymorphism (AFLP) markers. Various molecular markers (isozymes, RAPD, RFLP, microsatellites, mtDNA) have been used to study the genetic differentiation and invasion process of CPBs [22–32]. Microsatellite markers have been found to be very useful in the study of invasive species [33,34]. Microsatellite markers for CPBs were developed by Grapputo in 2006 and have been used in several studies to investigate the invasive pathway of CPBs [29,31,32,35]. Recently, Crossley et al. [36] and Schoville et al. [37] used single nucleotide polymorphisms to study the CPB genome. Diversity array technology (DArT) is a method for DNA polymorphism analysis; it is a low-cost, robust, high-throughput system with minimal DNA sample requirements that provides comprehensive coverage of the genome [38]. DArTseq technology is a unified one-step method for SNP discovery and genotyping; it enables the comprehensive discovery of SNPs in a variety of non-model organisms and provides a measure of genetic divergence and diversity within major genetic groups [39]. Therefore, this method has become an affordable and accessible means to generate important data on species that would otherwise have been impossible due to the cost and availability of expertise.

In addition to genetic markers, the variability of insect populations can also be studied using geometric morphometric (GM) methods [40–42]. The first morphological traits to change under the influence of environmental and genetic factors are the metric traits (wing shape and size) [43,44]. That is why geometric morphometric (GM) method has been used to study the genetic variability and plasticity of different insect species [45–50] over the last several years. By analyzing wing size and shape, it is possible to reveal the invasive adaptation of the adults' traits to different environmental influences. GM methods can also be used as a monitoring technique for detecting resistant insect populations and as a precursor for effective integrated pest management strategies [51–53]. GM methods are relatively simple, easy to apply, and require minimal financial investment, expert guidance, and equipment [54].

In this study, we use single nucleotide polymorphism markers and geometric morphometric methods to estimate genomic and phenotypic variations in CPB populations. This is the first study where these methods are combined to evaluate the genetic and phenotypic variations of CPBs. Our approach aims to use this data to describe the overall CPB population and to improve pest management strategies in order to delay resistance development.

2. Materials and Methods

2.1. Sampling and DNA Extraction

A total of 15 putative CPB populations were sampled in this study (Table 1). Populations were collected from the main potato-growing areas in continental Croatia (Figure 1). Adult CPB individuals were collected by hand from infested potato plants during the growing seasons in the years 2017, 2018, and 2019. All samples were stored in labeled plastic cups in 95% ethanol at 4 °C. Genomic DNA was extracted from the thorax of 82 CPB individuals, and total genomic DNA was isolated using the Qiagen DNEasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. DNA quality and concentration were determined using a spectrophotometer (BioSpec–nano Micro–volume) and agarose gel electrophoresis (1% with GelGreen Nulceid Acid Stain–Biotium). Extracted DNA was sent to Diversity Array Technology, Australia, for sequencing and genotyping using DArTseq TM genotyping technology [55].

Table 1. The sample information of Colorado potato beetle populations in Croatia.

Region	Population	Location	Lat.	Long.	n	C_T
North Croatia	SVAM	Sv. Martin na Muri	46°31′	16°21′	6	2018
	CEHO	Čehovec	46°21′	16°37′	5	2019
	VIDO	Vidovec	46°17′	16°14′	5	2017
	LUDB	Ludbreg	46°15′	16°36′	5	2018
	BEDN	Bednjs	46°13′	15°58′	5	2018
Central Croatia	MLAD	Mladine	46°02′	16°32′	6	2017
	STAR	Starigrad	46°08′	16°49′	5	2017
	DURD	Đurđevac	46°02′	17°04′	6	2017
	NVIR	Novo Virje	46°05′	17°09′	6	2018
	DRAG	Dragičevci	45°47′	16°34′	5	2019
East Croatia	PASI	Pašijan	45°38′	16°56′	6	2017
	GARE	Garešnica	45°34′	16°56′	4	2017
	HERC	Hercegovac	45°39′	17°00′	6	2017
	ZDEN	Zdenci	45°34′	17°57′	5	2018
	DMEL	Donji Meljani	45°43′	17°37′	5	2017

lat. = sampling latitude; long. = sampling longitude; n = sample size; C_T = collecting time.

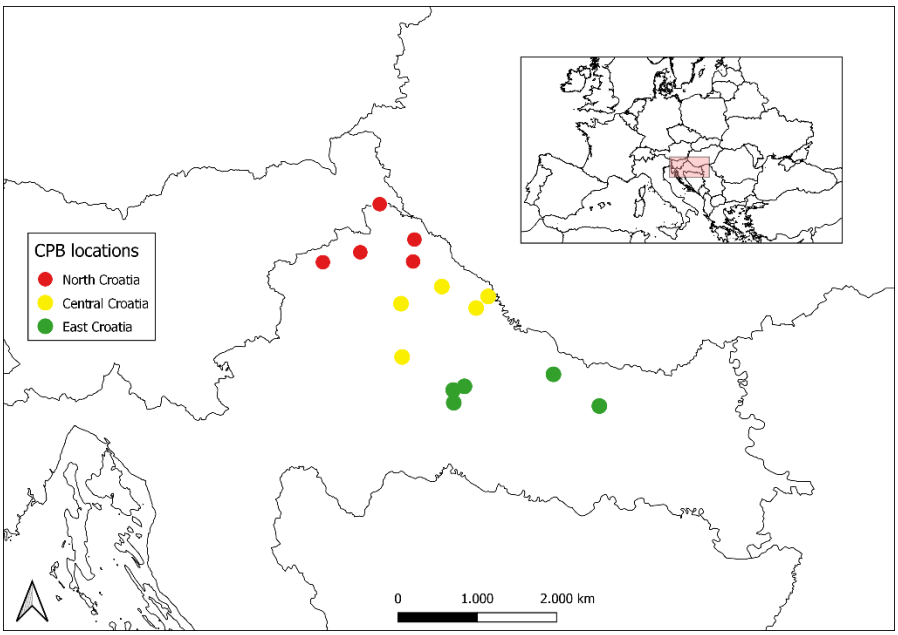


Figure 1. Sampling sites of the Colorado potato beetle in continental Croatia**.**

2.2. Genetic Analyses

Data received from DArT were first subjected to a filtering process using the dartR package [56] in R software [57]. Data were filtered using the following criteria: call rate <90% (i.e., removing all SNPs that have 10% missing genotypes or greater); reproducibility <95%; minor allele frequencies (MAF) >0.01; all monomorphic SNPs and fragments containing more than one SNP were removed from the data set.

The SNPRelate package [58] was used to estimate the parameters of genetic variability for each population number of different alleles (A); the number of private alleles (P); observed heterozygosity (H_O); expected heterozygosity (H_E). Using the filtered data set, pairwise F_{ST} was calculated between CPB populations using the gl.fst.pop command in the dartR package. To determine the overall basic population genetics statistics (observed heterozygosity (HO), inbreeding coefficient per locus (F_{IS}), and F_{ST} corrected for the number of individuals per locus), the function gl.basic.stats in dartR was used. An analysis of molecular variance (AMOVA) was performed to estimate the variance components and their significance levels of genetic variation within and among populations using GenALEx version 6.5 [59].

In order to observe the genetic relationships between populations, principal component analysis (PCA) was carried out using the package "dartR" [56]. For further analysis of the genetic structure of CPB populations, discriminant analysis of principal components (DAPC) was implemented in the R package "adegenet" [60].

The Bayesian model-based clustering algorithm implemented in STRUCTURE v.2.3.4, the Evanno method [61], was employed to determine the genetic structure of the CPB populations investigated. The genetic clusters (K-values) ranged between 1 and 16 (one more population than the total number of populations for the complete data set), and a series of 10 replicate runs for each prior value of K was analyzed. The parameter set for each run consisted of a burn-in of 10,000 iterations, followed by 100,000 Markov chain Monte Carlo iterations based on the admixture model of ancestry with the correlated allele frequency model and the default parameters in STRUCTURE. The most suitable value of K was calculated using the DK method, as used in STRUCTURE Harvester web version 0.6.94 [62], where the highest DK value is indicative of the number of genetic clusters.

Mantel tests were conducted to test for correlations between genetic distance and geographic distance; these analyses were conducted using the *vegan* package in R [63].

2.3. Geometric Morphometric Analyses

The hindwings of the CPB individuals were removed prior to DNA isolation to allow the same populations to be used for both genetic and morphometric analyses. To perform the geometric morphometric analyses, we divided the CPB data into three geographical locations—central, east, and north Croatian—in which the left and right hindwings were removed from each individual and slide–mounted using the fixing agent Euparal for the analyses; 258 left slide–mounted wings were photographed using a Canon PowerShot A640 digital camera (10–megapixel) on a trinocular mount of a Zeiss Stemi 2000–C Leica stereo–microscope and saved in JPEG format using Carl Zeiss AxioVision Rel. 4.6. (Carl Zeiss Microscopy GmbH, München, Germany). Sixteen landmarks on the wing vein junctions or vein terminations (Figure 2) were digitized using the software TPS Dig2 v2.16 [64].

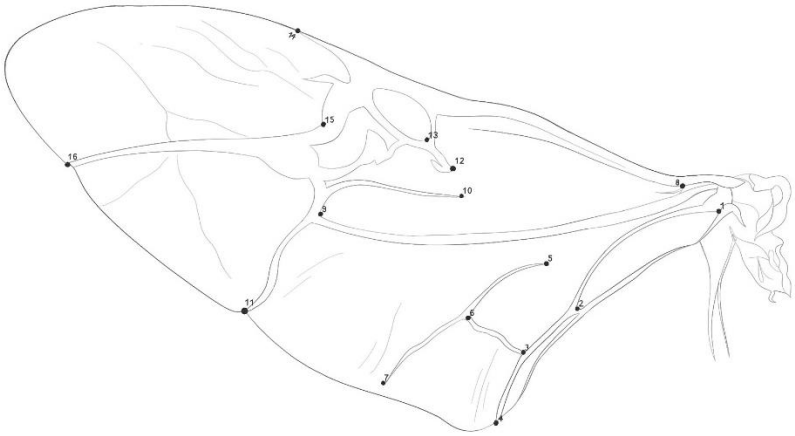


Figure 2. Colorado potato beetle hindwing schematic with sixteen type-one digitized landmarks.

Landmark coordinates were determined and shape information extracted using Procrustes superimposition analysis [65], which superimposes the landmark configurations of all the individuals analyzed, fitting them to a unit centroid size and removing mathematical information from the rotation and translation of all configurations. Principal component analysis (PCA) was performed using a covariance matrix of the individual shapes to simulate the shape space. In order to identify the principal wing changes, an average shape covariance matrix was performed, and the individual mean shapes were extracted (central, east, and north). In order to identify if there was any influence of size on shape (allometry) between populations, a multivariate regression using centroid size as an independent variable and shape as a dependent value was performed. Finally, to organize the data and maximize the disparity from the variance of each geographic group, canonical variate analysis (CVA) was performed, including on a sterile population, and the scatterplot was superposed with the mean shape by all geographical zones.

3. Results

3.1. Genetic Variability

A total of 22 772 SNPs were obtained from 82 CPB individuals that were genotyped. After the filtering process (90% call rate, the minor allele frequency filter, SNPs with frequencies <1%, reproducibility set at 95%) and removing monomorphs and secondaries, 7681 SNPs were used for the final analyses.

Heterozygosity (H_O and H_E) was estimated for all loci, and the results showed that CPB populations from different regions of Croatia were very similar (Table 2). The average H_O ranged from 0.251 (north Croatia) to 0.258 (central Croatia), while the average H_E ranged from 0.320 (north Croatia) to 0.326 (east Croatia). There were no observed differences between populations from different regions. F_{IS} was used to check the degree of inbreeding within populations, ranging from 0.201 (central Croatia) to 0.218 (east Croatia). Therefore, low levels of genetic variability across all populations are suggested.

Table 2. Genetic variability of Colorado potato beetles from different geographical regions in Croatia.

Region	n	A	P	Ho	He	F_{IS}
North Croatia	27	12478	120	0.251	0.320	0.216
Central Croatia	28	12539	193	0.258	0.323	0.201
East Croatia	27	12503	150	0.255	0.326	0.218

n = Number of samples; A = number of different alleles; P = number of private alleles; Ho = observed heterozygosity; He = expected heterozygosity; F_{IS} = inbreeding coefficient.

3.2. Population Relationship

Pairwise F_{ST} values were calculated to reveal the genetic relationships between the CPB populations (Figure 3). The result showed that the genetic differentiation between populations was very low. The F_{ST} values ranged from 0.05 (SVAM–LUDB) to 0.08 (MLAD–PASI) (Figure 3). The Mantel test was used to check the isolation by distance among populations. The result showed a low correlation between genetic and geographic distance, which was expected, considering that for isolation by distance, we would expect a high FST, indicating that the genetic differentiation would have been increased due to the distance. AMOVA revealed significant differences in F_{ST} values between pairwise populations in the study ($F_{15,224} = 2.31$; $p < 0.05$) (Table 3). There was no evidence to rule out the presence of a single large population of CPBs in continental Croatia.

Table 3. Analysis of molecular variance (AMOVA) using 7681 SNPs of the genetic variation among and within CPB populations.

Source of Variation	*SS*	*df*	*MS*	*F*	*p*-Value	*F crit*
Between Groups	0.008679583	15	0.000579	2.311803	0.004461	1.711235
Within Groups	0.056066667	224	0.00025			
Total	0.06474625	239				

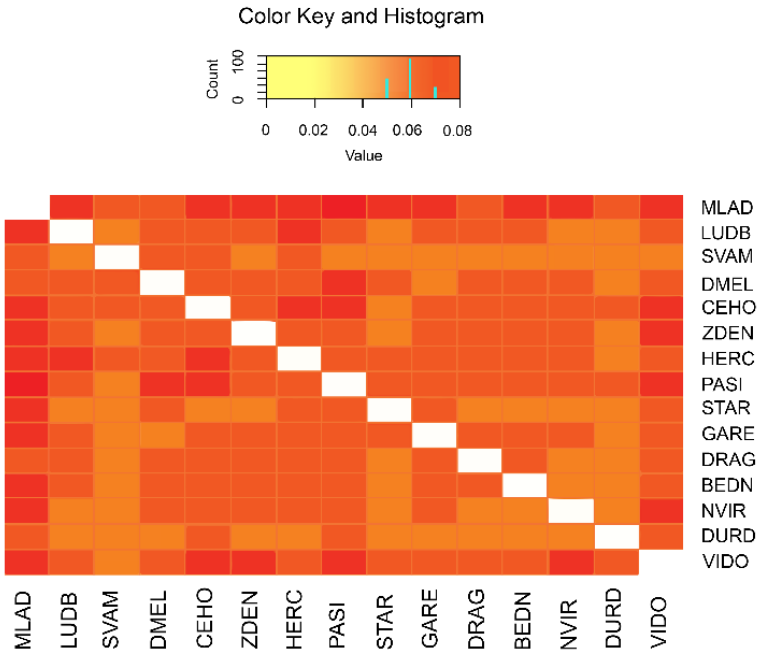
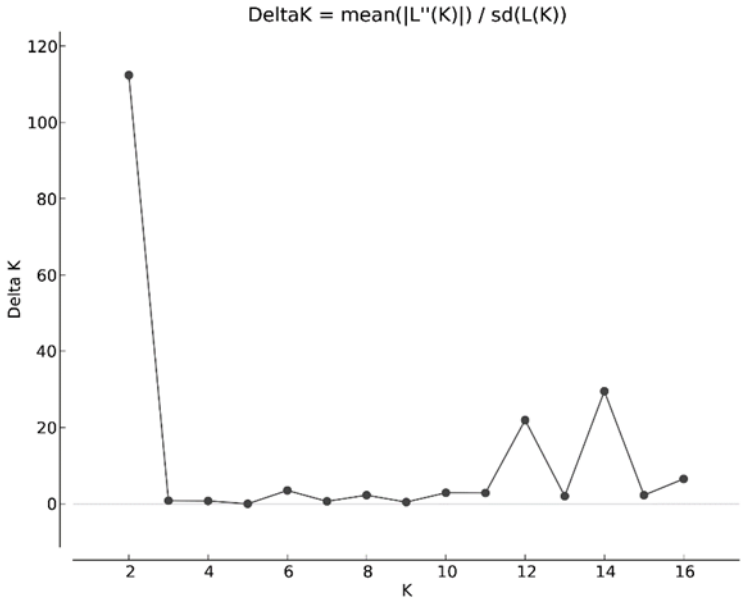
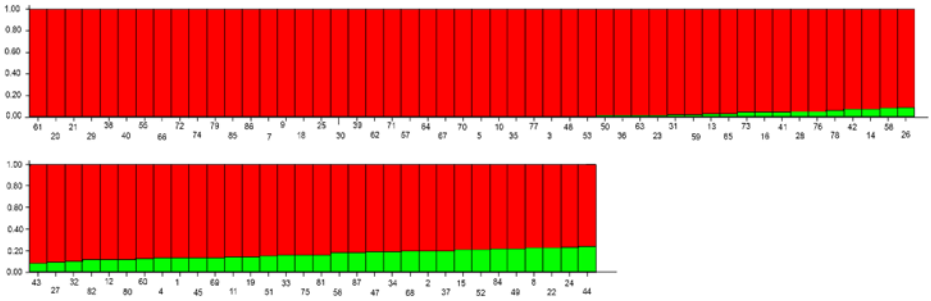


Figure 3. The range of the fixation index (F_{ST}) between Colorado potato beetle populations in Croatia.

The Bayesian approach of clustering by Evanno's method demonstrated a clear peak at K = 2 (Figure 4a), indicating that two groups were distributed across the CPB populations. A complete admixture of populations was observed in the STRUCTURE plot (Figure 4b). PCA was conducted to examine the structure of CPB populations in Croatia. The PCA analysis showed genetic similarities within the data set and confirmed a single large CPB population in Croatia (Figure 5). DAPC showed the same pattern of genetic structure in the CPB populations (Figure 6). We used PCA and DAPC (different approaches) to see if there were any differences in our results. DAPC attempts to summarize the genetic differentiation between groups while ignoring the variation within groups and provides a better population structure. In DAPC, the data are first transformed using PCA, and then clusters are identified using discriminant analysis (DA) [66]. PCA aims to summarize the total variability between individuals, which includes both the divergence between groups (i.e., structured genetic variability) and the variation within groups; therefore, it is not always suitable for obtaining a clear picture of variation between populations. However, the results were complementary.



(a)



(b)

Figure 4. (a). Results from the STRUCTURE Harvester analysis, revealing the most likely value of K based on STRUCTURE results; **(b).** determination of the optimal value of K and population structure of CPB genotypes using DArTseq SNP markers.

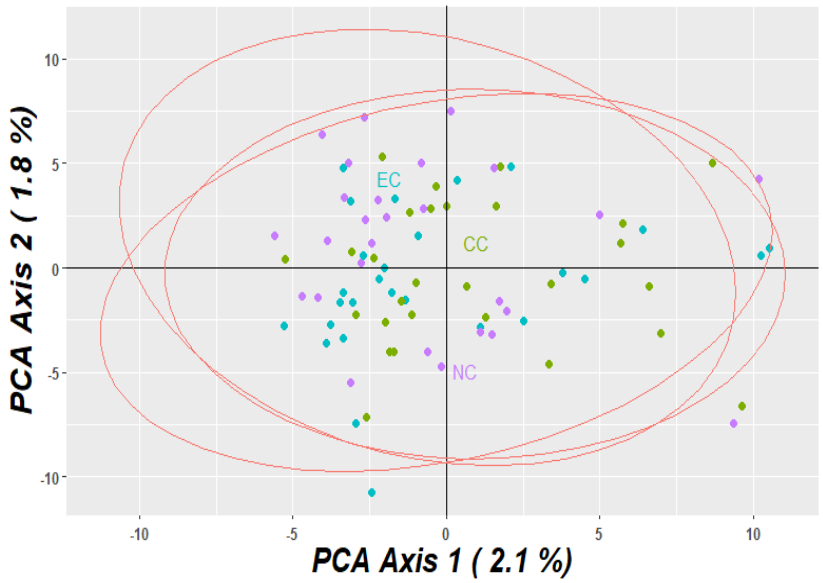


Figure 5. Principal component analysis (PCA) based on 7681 SNPs. CC: central Croatia, NC: north Croatia, and EC: east Croatia.

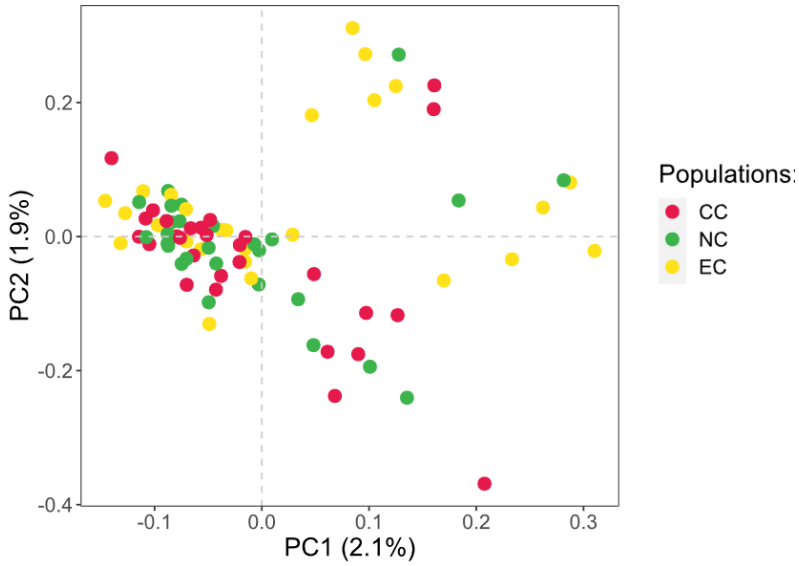


Figure 6. Discriminant analysis of principal components (DAPC) based on 7681 SNPs. CC: central Croatia, NC: north Croatia, and EC: east Croatia.

3.3. Geometric Morphometrics Results

PCA showed a shape space where the first three dimensions accounted for 41.2% of the shape variation (PC1: 16.1%, PC2: 13.01%, PC3:12.3%). The average shape found that the individuals from north Croatia (NC) had a more elongated wing shape than those from east (EC) and central Croatia (CC), where the displacement to the extreme left and right of landmarks 4 and 16 is noted. On the other hand, the CPBs from central Croatia had slight movements of landmarks 2, 13, and 14 and showed a broader phenotype. CPBs from east Croatia also showed wider wings but with a contraction of landmarks 1 and 8. Multivariate regression showed a low but significant relationship between shape and centroid size

(CS) (r^2: 0.033; $p < 0.001$, after 10,000 iterations). This was most noted in the differences in CS between central and northern Croatian populations, where the CS in the CC population was found to be smaller than in the NC population (Figure 7). The CVA between groups showed three principal clusters where the maximum variation of geographical zones was grouped. CV1 explains the hindwing variation between CC and NC populations; the hindwing shape for the EC population is explained by CV2 (Figure 8).

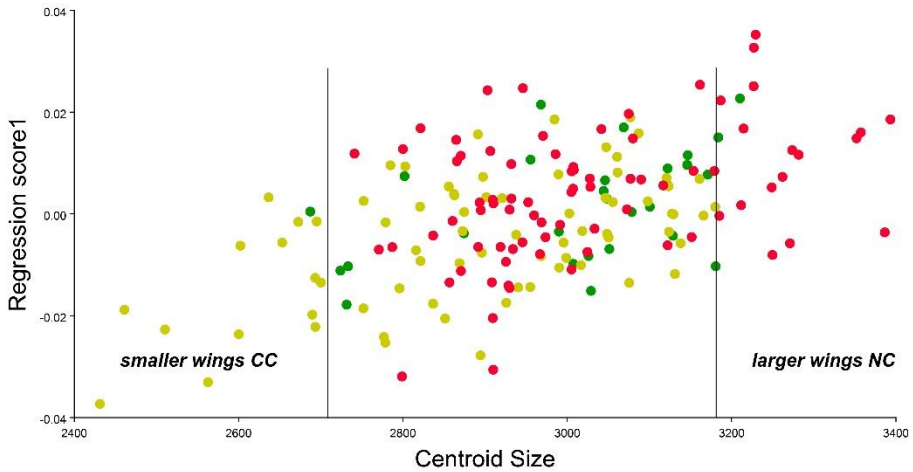


Figure 7. Multivariate regression of shape as a dependent variable vs. centroid size as an independent variable of Colorado potato beetle hindwing. CC: yellow, central Croatia; NC: red, north Croatia; EC: green, east Croatia.

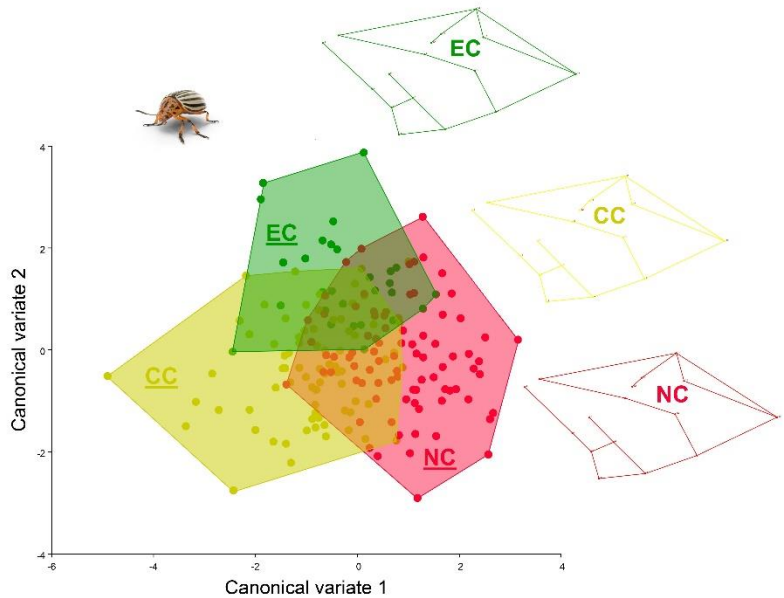


Figure 8. Canonical variate analysis of the Colorado potato beetle hindwing shape between populations from different regions of Croatia. CC: yellow, central Croatia; NC: red, north Croatia; EC: green, east Croatia.

4. Discussion

The CPB is considered an invasive species, and it has been present in Croatia for more than sixty years [2]. During this time, the CPB has adapted to a wide range of solanaceous plants, agroecological climatic conditions, and control measures [2].

In this study, we investigate the CPB populations using SNP and GM techniques. SNP and GM techniques have provided us with data about the population structure of the CPB population in Croatia. We detected the low genetic variability of CPB populations in Croatia and the presence of a single panmictic population in the study area. The GM method allowed us to find morphological changes associated with the geographical areas of Croatia; GM also confirmed a low difference while demonstrating phenotypic plasticity in this species. Results showed that we have one single CPB population in continental Croatia that is well established and well adapted.

The low genetic and morphological variability detected among the CPBs can be explained, according to Bouyer et al. [44], by genotype stability, which is reflected in a stable phenotype. The different approaches we used in this study (SRUCTURE, PCA, and DAPC) gave the same results.

Data on potato production in Croatia date back to 1991, and, according to FAO [67], the area under potato production has decreased from year to year (1992—60,758 ha; 2019—9390 ha). The structure of potato cultivation has also changed because, in the 1990s, potatoes were grown on a large scale on homesteads near settlements, and during that time, the availability of food for CPBs was much better. This information is very important because it can be assumed that CPBs were forced to search for new potato fields and move to new cropping areas. Today, potatoes are grown in fields that are often quite far apart, likely resulting in the need for longer flights to find food. Our results show the Wahlund effect, which can be defined as the excess of homozygotes or the deficit in heterozygotes observed in a sample of individuals obtained from a structured population, even when the local populations are randomly mating [68]. This can explain why once isolated subpopulations in a subdivided population have a deficiency of heterozygotes relative to that expected with random mating. Additionally, CPB populations experienced an increased gene flow resulting from their ability to fly more than 100 km when there are favorable wind and weather conditions and colonize new fields accordingly [4].

Grapputo et al. [28] examined the US and European CPB populations using AFLP markers and found a significant reduction in genetic variability in the European populations. This reduction often occurs in populations of invasive species due to bottlenecks and founder effects during the invasion that can lead to a decline in genetic variability [69]. Using mtDNA, Grapputo et al. [22] found that reduced genetic variability indicates a founder effect in Europe. These results agree with the studies of Yang et al. [32] and Özkan Koca et al. [35], where they used microsatellite markers to investigate the genetic structure, diversity, and invasion routes of CPBs. Their results showed low levels of genetic variation in CPB populations in Turkey [35] and China [32]. Conversely, Mikac et al. [70] suggested that geometric morphometric techniques can be used to detect population changes related to invasions and could, therefore, serve as a cheaper and more accessible alternative marker. Karsten et al. [71] combined the use of GM and population genetics to identify the genetic variability between populations in South Africa in a fly pest *Ceratitis rosa*, finding lower phenotypic diversity in contrast to higher genetic variability. Our results find the contrary result because of the lower genetic variability between populations, which were contrasted by wing shape adaptation to geographical zones in Croatia. A few studies have confirmed that the combination of genetic markers and geometric morphometrics results gives more accurate results, as morphology can show clear differentiation patterns where molecular markers cannot detect population structure [72–76].

Several studies have found that wing shape is very important for the migratory movement and dispersal strategy of insect species [52,70,75–77]. According to Voss and Ferro [78], there are three different types of flight in CPBs with different characteristics: short-distance flight, diapause flight, and long-distance flight. Long-distance or migratory flight is most important for the dispersal of the species and the colonization of new areas. For an insect to be capable of long flights, it must have aerodynamic wings, and according to Mikac et al. [75], this is an individual with an elongated wing shape. Our results showed that CPBs from central Croatia had a broader wing shape with slight movements of landmarks 2, 13, and 14, while CPBs from eastern Croatia had a broader wing shape with contraction of landmarks 1 and 8. Individuals from northern Croatia had a more elongated wing shape, with landmarks 4 and 16 extending to the left and right. Therefore, we can assume that CPB individuals from the north, with elongated wings, are capable of long-distance flight and could easily migrate to other parts of continental Croatia.

In a large panmictic population, such as the one found in Croatia, there is a high probability of genetic variants that provide high fitness under new conditions as well as the occurrence of new adaptive random mutations. Since CPBs can have multiple generations per year, there is a possibility that these genetic variants will quickly succumb to natural selection and lead to the expansion of adapted populations [17].

Similar findings for other Chrysomelidae pests have been described by Lemic et al. [79]. Their research revealed one large population of western corn rootworm (WCR). Knowledge of the genetic structure of WCR in Croatia has had important implications for the integrated pest management (IPM) of this invasive pest. This research showed that genetic variability increased and minimal genetic structure was maintained when the invasive pest was not controlled.

Therefore, information on the presence of a panmictic CPB population is very important for future IPM strategies and resistance control in the potato-growing areas in Croatia. An area-wide approach (AW) has been shown to be very helpful in reducing insecticide use [80]; in combination with other control measures, it also offers great potential for reducing damage levels [81]. Area-wide crop rotation has been shown to be very useful in keeping pest damage below the threshold [82]. Under AW treatments, populations are unable to exchange genetic material and spread resistance genes [83]. The AW approach could be used for successful CPB control and to keep the resistant population under control.

Our study confirms that CPB can adapt exceptionally to different conditions, indicating high phenotypic plasticity. The high phenotypic plasticity of CPB populations is a response to the high adaptability of this organism to different factors, which is characteristic of their invasiveness and their ability to rapidly adapt their genotype to environmental changes. Considering the high adaptability to different agro-ecological conditions (phenotypic plasticity) and the invasiveness of CPBs, it is expected that CPB populations will also adapt to new insecticides and control measures in the future. Thus, this type of combined CPB monitoring (SNPs and GM) increases our knowledge of this very important pest and represents valuable knowledge needed for the implementation of different management practices.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, M.K.B., D.L. and K.M.M.; methodology, M.K.B., D.L. K.M.M. AND H.A.B; software, M.K.B and M.C..; validation, M.K.B., D.L., R.B. and K.M.M.; formal analysis, M.K.B. and H.A.B.; investigation, M.K.B., R.B. and M.C.; resources, R.B.; data curation, M.K.B and H.A.B.; writing—original draft preparation, M.K.B; writing—review and editing, D.L., K.M.M, H.A.B.

and R.B.; visualization, M.K.B, H.A.B. and M.C.; supervision, R.B. K.M.M. and D.L.; project administration, R.B.; funding acquisition, R.B. All authors have read and agreed to the published version of the manuscript."

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3.2. General discussion

Well-established genetic and geometric morphometric analyses were used to study genomic structure, population differentiation, gene flow and dispersal of WCR, CM, and CPB populations in Croatia. These three insect pests have shown resistance to insecticides (CPB and CM) and to the strategies used to control them (WCR). Therefore, the focus of this dissertation was to establish effective resistance monitoring programs and early detection of resistance using these methods that would allow timely implementation of insecticide resistance management (IRM) strategies. This is the first study to combine the use of SNPs and GM methods to investigate reliable patterns of differences associated with resistance in three of the most important pests in Croatian agriculture. In addition, this dissertation was the first time that the population genetics of CPB populations were investigated in Croatia.

Analysis of the genetic structure of populations is an important aspect of understanding the population dynamics of insect pests in agriculture (Franck and Timm, 2010). The development of effective pest management strategies relies on a multidisciplinary approach (Blommers, 1994), and one component of this is knowledge on the population genetics of the pest in question. Population genetic structure and dispersal patterns at local and landscape scales are important in determining a control strategy for insect pests (Fuentes-Contreras et al., 2008). Understanding the invasion genetics of WCR, CM, and CPB allows identification of geographic origin, number of introduction events, and spread of infestations (Roderick, 1996). Further to the use of population genetics, Mikac et al. (2016) advocated for the additional or alternative use of geometric morphometric methods that sometimes can be used to detect population changes related to invasions, where genetic markers have failed to do so. The authors argue that GM could therefore serve as a cheaper and more accessible alternative population biomarker to the use of population genetics. Indeed, several authors now advocate for the combined use of GM and genetic methods to achieve more accurate data on insect invasions and to investigate resulting biological changes sustained by these populations. That is, morphological traits can provide additional information about underlying population genetics, and morphology can retain useful information about genetic structure (Garnier et al., 2005; Camara et al., 2006; Ortego et al., 2011; Francuski et al., 2016; Henriques et al., 2020).

3.2.1. Genetic analyses

The fact that the non-resistant and rotation-adapted Cry3Bb1 populations were mixed suggests that they are genetically similar. The neighboring joining tree separated the rotation-

adapted individuals, which is to be expected since the first resistance developed (without insecticides) was to crop rotation (Levine and Oloumi-Sadeghi, 1996). After that, all other resistances developed, which is clearly reflected in this result. The fact that the non-resistant population did not segregate could be due to an evolutionary process. In the case of WCR, high-throughput sequencing has provided deeper insights into the molecular mechanisms of resistance (Torres et al., 2018). For example, we have found that many point mutations are found in different genes, suggesting that these mechanisms can occur simultaneously, making it more difficult to understand which of them is truly responsible for the resistance phenotype (Saavedra-Rodriguez et al., 2012; Faucon et al., 2015). Several studies have been conducted on WCR using SNPs (Coates et al., 2009; Wang et al., 2013; Flagel et al., 2014; Niu et al., 2020), and all agree that resistance is a dynamic phenomenon, meaning that already known mechanisms can change over time. Therefore, there is an ongoing need to study and monitor resistance. In our research with WCR, we have focused on resistant populations and found that there is some variability among them, but no exact pattern. Recent molecular studies show that different sets of genes are involved in resistance (Saavedra-Rodriguez et al., 2008; Faucon et al., 2015; Faucon et al., 2017; Grigoriaki et al., 2017) making it unlikely that universal resistance markers can be developed to accurately determine the likelihood of a population becoming resistant to a particular compound (Saavedra-Rodriguez et al., 2008; Savedra-Rodriguez et al., 2012; Faucon et al., 2017). A different number of genes may be involved in resistance, and individuals within a population exhibit different evolutionary patterns of resistance development. Therefore, resistance may be found throughout the genome, but it is not conditioned by the differences. Estimates of genetic diversity, population structuring, and genetic relatedness among individuals can provide information on the effectiveness of control strategies and recommendations for improving the effectiveness of control programs (Publication No. 4).

For CM, field populations were studied to determine differences associated with the type of apple control and to identify specific biotypes. CM populations were collected in Croatia in organic orchards and in orchards with integrated pest management (IPM) practices, and a susceptible population was obtained from a laboratory in Switzerland (Publication No. 5). Our genetic results showed low genetic diversity and low genetic differentiation (F_{ST}=0.021). These results are in agreement with the results of Pajač et al. (2011). The results of STRUCTURE showed two genetic clusters confirmed by PCA analysis, namely the laboratory population and the integrated and ecological populations (which were combined). However, the DAPC analysis showed three groups: organic orchards, integrated orchards and the laboratory population. This result can be explained by the fundamental difference between PCA and DAPC analyzes. PCA

aims to summarize the total variability between individuals, which includes both divergence between groups (i.e., structured genetic variability) and variation within groups; therefore, it is not suitable for obtaining a clear picture of variation between populations. DAPC, on the other hand, attempts to summarize genetic differentiation between groups, overlooking variation within groups and providing a better population structure. In DAPC, data are first transformed using PCA and then clusters are identified using discriminant analysis (DA) (Jombart et al., 2010). However, the observed changes associated with the different control methods in this study were very small, and further investigation is needed. Frank and Timm (2010) used microsatellite markers to investigate the genetic structure and gene flow of CM in organic and treated apple orchards. They found little genetic variation among populations but significant partitioning of genetic variation within individuals. In previous studies in Croatia (Pajač et al., 2012) or elsewhere in Europe (Franck et al., 2007; Voudouris et al., 2012), markers such as microsatellites failed to reveal differences in genetic structure among populations of CM. Nevertheless, these authors noted the suspected influence of insecticide treatment on allelic richness of CM.

Subchapter 3.1.3. is the first publication on the population genetics of CPB populations in Croatia using SNPs (Publication No. 6). Low genetic variability of CPB populations were detected in Croatia and the presence of a single panmictic population in the study area was detailed. Data on potato production in Croatia date back to 1991, and according to FAO (2020) the area under potato production has decreased from year to year (1992: 60 758 ha; 2019: 9 390 ha). The structure of potato cultivation in Croatia has also changed. Where in the 1990s potatoes were grown on a large scale across many locations in Croatia, currently potatoes are grown more disperately and as such CPB likely need to undertake longer flights to find suitable oviposition and feeding sites. Our results show the Wahlund effect, which can be defined as the excess of homozygotes or the deficit in heterozygotes observed in a sample of individuals obtained from a structured population, even when the local populations are randomly mating (Garnier-Géré and Chikhi, 2013). This can explain why once isolated subpopulations in a subdivided population have a deficiency of heterozygotes relative to that expected with random mating. Also, CPB populations experienced increased gene flow, which results from their ability to fly more than 100 kilometers when there are favorable wind and weather conditions and colonize new fields accordingly (Alyokhin, 2009). Grapputo et al. (2006) examined US and European CPB populations using AFLP markers and found a significant reduction in genetic diversity in European populations. This reduction often occurs in populations of invasive species due to bottlenecks and founder effects during invasion that lead to a decline in genetic

diversity (Puillandre et al., 2008). Using mtDNA, Grapputo et al. (2005) found that reduced genetic variability indicates a founder effect in Europe. These results agree with the studies of Yang et al. (2020) and Özkan Koca et al. (2021) who showed low levels of genetic variation in CPB populations in China and Turkey respectively. In large panmictic population, such as are found in Croatia, there is a high probability of genetic variants that provide higher fitness under new conditions, as well as the occurrence of new adaptive random mutations. Since CPB can have multiple generations per year, there is a possibility that these genetic variants will quickly succumb to natural selection and lead to the expansion of adapted populations (Cingel et al., 2016). Similar findings for other Chrysomelidae pest have been described by Lemic et al. (2015). Their research revealed one large population of western corn rootworm (WCR). Knowledge of the genetic structure of WCR in Croatia has had important implications for integrated pest management (IPM) of this invasive pest. Their research showed that genetic diversity increased and minimal genetic structure was maintained when an invasive pest was not controlled.

One of the most important advantages of using SNPs is that the actual sample size of each site does not need to be large. Trask et al. (2011) states, “given that each SNP marker has an individual evolutionary history, we calculated that the most complete and unbiased representation of genetic diversity present in the individual can be achieved by including at least 10 individuals in the discovery sample set to ensure the discovery of both common and rare polymorphisms.” Further Li et al. (2020), who worked with beetles from the order Coleoptera, found that “a minimum sample size of 3–8 individuals is sufficient to dissect the population architecture of the harlequin ladybird, *Harmonia axyridis*, a biological control agent and invasive alien species.” They also estimated the optimal sample size for accurately estimating genetic diversity within and between populations of *H. axyridis.* They determined that six individuals are the minimum sample size required.

Results from this dissertation showed that high-throughput sequencing can provide a deeper insight into the molecular mechanisms of resistance (Torres et al., 2018). Thanks to a denser and more uniform distribution within genomes and a large number of SNPs (thousands to millions), we can generate a large amount of information in a single sequencing run, which is less time-consuming and less expensive compared to microsatellite and other molecular markers. In addition, SNP markers provide broader genome coverage and higher quantity data compared to studies that use microsatellites or mtDNA (Morin et al., 2004). However, resistance occurrence is dynamic, and resistance mechanisms can change over time. Resistance constantly occurs in insect populations and can even develop within in months,

rather than years (Denholm et al., 2002). Resistance depends on the number of treatments, the number of generations an insect can reproduce in and the treated organism itself (Denholm et al., 2002). Belinato and Martins (2016) stated that “insecticide resistance is an adaptive trait in which a set of genes are favorably selected to maintain the insect alive and able to reproduce under an environment exposed to pesticides.” It is known that different gene groups are involved in resistance (Grigoriaki et al., 2017). This makes it difficult to determine and predict which populations will become resistant and when (Saavedra-Rodriguez et al., 2008; Faucon et al., 2015).

Some argue that it is, therefore, more effective to use morphometric markers to identify minor (and recent) genetic changes than to use genetic markers to identify major changes in the genome (Bouyer et al., 2007; Camara et al., 2006). That suggests morphology can retain useful information on genetic structure and has the benefit over molecular methods of being inexpensive, easy to use, and able to yield a lot of information quickly. However, resistance cannot be fully understood without genetic data. Genetic studies are an important tool for developing improved methods for detecting resistance, for studying resistance mechanisms, and for choosing approaches to resistance management (Roush et al., 1990). In this dissertation, we aimed to confirm the results from SNPs markers using GM.

3.2.2. Geometric morphometric analyses

WCR individuals from Cry3Bb1_Cry34/35Ab1 population had the broader shape and a more robust wing with an expansion of landmark 14 and a contraction of landmark 9. Cry3Bb1 individuals had the narrower wings, while individuals’ resistant to Cry34/35Ab1 had similar but smaller wings, distinguished by the expansion of landmarks 3 and 4. The more stable and elongated wing shape was that of the population adapted to crop rotation, in which there was an extension to landmarks 1 and 2 to the left and an elongation to landmark 9 to the right. The non-resistant population is also slightly wider than the population of Cry3Bb1-Cry34/35Ab1, with the movement of landmarks 14 and 2 also slightly to the right and the wider shape that is also produced by the movement of landmark 7 to the upper left (Publication No.4). This result is in accordance with Mikac et al. (2013) where they showed that beetles adapted to crop rotation had broader wings (cf. susceptible beetle). Mikac et al. (2019) expanded the use of differences in hindwing size and shape to examine changes in WCR associated with the development of resistance, where the hindwings of non-resistant beetles were significantly more elongated in shape and narrower in width (chord length) compared with beetle’s resistant

to Bt maize or crop rotation. In our research, individuals adapted to crop rotation had more stable and elongated wings, suggesting that these individuals could fly long distances.

CM results showed differentiation between integrated and organic CM populations based on wing shape (Publication No .5). Populations from the organic orchards differed significantly in wing shape in comparison with integrated CM populations. Our data showed that the CM organic population was morphologically similar to the susceptive laboratory population, which had a differing wing shape in comparison with the integrated population. Individuals from the organic orchards had expansion and contraction of the forewing in landmarks 16, 17, and 18, making the wings more elongated and narrower. These results are consistent with that of Pajač Živković et al. (2019), who found the same pattern of CM forewings from organic orchards in Croatia. Pajač Živković et al. (2019) was the first to demonstrate significant differences in wing shape in lepidopterans in relation to resistance. In their study, CM populations from organic orchards showed the least wing deformation and were, therefore, reported to be the better fliers and dispersers compared with CM from integrated populations, which were found to be inferior fliers. According to our results, individuals from organic orchards were also found to be better fliers, which means that they are likely responsible for the expansion of the population. Intense selection pressure exerted by decades of pesticide use to control the species has altered the structural integrity of CM wings, making them less efficient at dispersal. This result suggests that the development of resistance could affect the fitness of the organism itself. That is, when the organism becomes resistant, it simultaneously loses other biological traits (Belinato and Martins, 2016). Despite the fact that resistant individuals are less capable of long flights, they still represent a pool of genes, which means that they can transfer resistance to their offspring. This research should also be conducted on CM females to confirm whether resistance equally affects both sexes since females are responsible for population expansion and enlargement in CM (Pajač et al., 2012). According to Schumacher et al. (1997), some individuals are able to disperse over several kilometers in the field; and despite being poor fliers, even distances of up to 11 km have been reported. According to several studies on CM and insecticide resistance, larger females are more resistant than smaller males (Varela et al., 1993; Fuentes-Contreras et al., 2008; Reyes et al., 2015) and, therefore, it is likely that this sex and morphotype combination is responsible for spreading resistant alleles throughout apple production areas. Under this scenario, it does not matter if resistant males remain in a given area because it is the females that ultimately transfer the resistant genes to new areas via dispersal and generation of offspring.

In this dissertation for the first time CPB populations were examined using GM techniques (Publication No. 6). GM method allowed us to find morphological changes associated with geographical areas of Croatia, and confirmed a low difference while demonstrating phenotypic plasticity in this species. Our results showed that CPB from central Croatia had a broader wing shape with slight movements of landmarks 2, 13, and 14, while CPB from eastern Croatia had a broader wing shape with contraction of landmarks 1 and 8. Individuals from northern Croatia had a more elongated wing shape with landmarks 4 and 16 found to be expanding. Therefore, CPB individuals from the north with elongated wings are capable of long-distance flight and could easily migrate to other parts of continental Croatia. According to Voss and Ferro (1990), there are three different types of flight in CPB with different characteristics: short-distance flight, diapause flight, and long-distance flight. Long-distance or migratory flight is most important for the dispersal of the species and the colonization of new areas. For an insect to be capable of long flights, it must have aerodynamic wings, and according to Mikac et al. (2013), this is an individual with an elongated wing shape.

Several studies have found that wing shape is very important for migratory movement and dispersal strategy of insect species (Mikac et al., 2013; Lemic et al., 2014; Mikac et al., 2016; Pajač Živković et al., 2019). Mikac et al. (2019) suggested that such phenotypic differences in wing shape and size have implications for dispersal and long-distance movement of resistant and non-resistant insects, as wing morphology is a crucial element in an insect's dispersal ability (DeVries et al., 2010). Understanding which morphotype is the superior flyer and spreader has implications for managing WCR, CM and CPB through integrated resistance strategies. Elongated wings are considered to be involved in migratory movement (Mikac et al., 2013). For this reason, the integration of different techniques to understand the plasticity and variation of this trait is vital to understanding how they adapt to new environments and to coordinating strategic planning ahead of possible new invasion fronts (Lemic et al., 2015). Different types of wing morphotypes have been studied to determine the dispersal capabilities of flying insects (Denno et al., 2001; Guerra et al., 2011; Sanzana et al., 2013). Le et al. (2013) found that narrowed wings are more efficient for flapping low-level flights. Additionally, for WCR, wing shape has been identified as a good trait to measure in different agronomic studies, including studies of life history (sexual dimorphism) and interspecific and intraspecific shape variation (Lemic et al., 2014; Benítez et al., 2014; Mikac et al., 2016), and wing shape has also been a useful variable when combined with other monitoring tools (genetics (e.g., microsatellites) and traditional traps (e.g., pheromones)) (Lemic et al., 2015).

The main results of this thesis for WCR and CM show that the combination of genetic (SNP) method and geometric morphometrics can effectively detect changes related with resistance development. The research was tested on populations that were resistant to various toxins (WCR), populations from integrated and organic orchards (CM) and for both pests on a laboratory-grown population that had never been treated with insecticides. The research results demonstrated the same populations by genotyping samples with SNP markers and using geometric morphometrics techniques. The results showed that resistant populations have different wing shapes depending on the type of resistance. GM tools can provide important clues for distinguishing between resistant and non-resistant populations. The change has been detected, however what is causing the change needs further investigation using different methods and analyses.

Collectively the results together show that resistance is a dynamic phenomenon and only by monitoring, characterizing, and predicting the occurrence and spread of resistance can we hope to use existing chemical agents in a sustainable manner (Foster, 2011; Liu, 2012). Therefore, this dissertation is one step forward in finding effective monitoring tools that can serve as reliable biomarkers to detect changes and specific biotypes.

Practical application of this research involves implementation of the tested methods (genetic SNP analysis and geometric morphometrics) for rapid detection of resistance. Early detection of resistance is extremely important for agriculture and professionals involved in plant protection, as such methods/tests currently do not exist. The result of this research is data that is important at the national and international level. The research has proven the effectiveness of the two tested methods in the early detection of resistance, which in practice allows timely response of the producer on the one hand and legislation on the other. Without monitoring production status and implementing early detection measures, there is a risk that resistant populations will spread and their suppression will become even more difficult. The combined use of SNPs and geometric morphometrics to detect resistant populations is a novel approach where morphological traits can provide additional information about population genetics and morphology can provide useful information about genetic structure. This approach offers new insights into an important area of pest management, namely how to prevent or delay the development of resistance and how to reduce the negative impact of resistance. This combined approach could be applied on a much larger scale to other pests where resistance has been identified (sugar beet weevil, sugar flea beetle, pollen beetle) or where resistance development is suspected in certain populations. The research findings could be incorporated into the

Integrated Farming Guidelines as a recommendation for all future activities and protective measures against the development of resistance in modern food production.

In this research, I found the change, but what causes the change needs to be further investigated using different methods and analyses. Future research should focus on association studies to find out what is really causing the change. Genome-wide association studies (GWAS) could be a good tool for deeper exploration of the insect genome and deeper insights into resistance evolution.

4. CONCLUSIONS

Based on the research conducted, the following conclusions can be made:

1. For WCR, the results showed that resistant populations have different wing shapes depending on the type of resistance. I found that geometric morphometric tools can provide important clues for distinguishing between resistant and non-resistant populations. One of the most important results was the similarity of hindwing shape variation between populations after STRUCTURE analysis, where the use of both monitoring techniques showed that the resistant Cry34/35Ab1 population was the more differentiated. Therefore, geometric morphometrics can be used as a biomarker for resistance detection as part of a larger integrated resistance management strategy for western corn rootworm.

2. For CM, the results showed that the genetic differentiation of the population between organic and integrated orchards was not significant. On the other hand, geometric morphometrics proved to be a more sensitive method for detecting genotype variability due to pest management. This study demonstrates the possibility of using a novel method for a strategic integrated pest management program (IPM) for CM.

3. The results for WCR and CM are particularly important because they show that different toxins and management strategies have different effects on wing shape change. Since wing shape is affected by genetic factors and any change is the result of a mutation, our results are evidence that resistance to a particular toxin is the result of mutations in different genes.

4. For Colorado potato beetle, we could not demonstrate the differences based on resistance status, but our results confirmed that CPB can adapt exceptionally well to different conditions, indicating high phenotypic plasticity. This type of combined CPB monitoring (SNPs and GM) has increased our knowledge of this very important pest in Croatia and represents valuable knowledge needed for the implementation of various management practices. Information on the presence of a panmictic CPB population is very important for future IPM strategies and resistance control in Croatian potato growing areas.

5. Finally, the results proved that the two methods studied can be effectively used to asses the early emergence of resistance to the most important pests in agricultural production in Croatia. Early detection of resistance is extremely important for Croatian agriculture and professionals involved in plant protection, as currently there are no such methods/testing. In practice, these methods could enable a timely response by producers on the one hand and legislation on the other. Also, it would be very useful to carry out more research like this on other pests that have developed resistance or for which there is a risk of developing resistance.

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Autobiography

Martina Kadoić Balaško, MSc, was born in Zagreb on September 23, 1992. In 2011 she enrolled in Plant protection undergraduate studies and then in graduate studies of Phytomedicine at the University of Zagreb Faculty of Agriculture. During her studies, she actively participated in IPA project and as a result, one scientific paper was published. In 2016, she received a semester scholarship to study at the University of Natural Resources and Applied Life Sciences (BOKU) in Vienna and a one-year scholarship from the Faculty of Agriculture Foundation for her academic success. She graduated with the highest honors in 2017. (*Summa cum laude*).

In 2017, she was employed at the Faculty of Agriculture, Department of Agriculutral Zoology as an apprentice. In 2018 she was employed as a PhD student on the Young Researchers' Career Development Project Training of New Doctoral Students (DOK-01-2018) funded by the Croatian Science Foundation and she started her postgraduate studies at the Faculty of Agriculture.

Her scientific interests include the application of integrated and biological pest management, entomology, population genetics, geometric morphometry, and innovative methods in plant protection. During her studies she participatet in several scientific trainings and mobilitys (i) University of Wollongong, Australia (2022) (ii) University of Novi Sad Faculty of Agriculture learning new methods Electrical penetration graph (2022); (iii) University of Novi Sad Faculty of Agriculture CASEE Summer school „Ecosystem Services within an Agricultural Landscape Institute of Plant Protection (2021); (iv) Workshop "Bioinformatic school in Transcriptomics" organized by the Mediterranean Institute for Life Research (MedILS) (2021); (v) Getting to know the syntax of the R language and its application in statistical and graphical data analysis, University of Zagreb University Computing Center (2021).

Collaboration with foreign and domestic scientists resulted in the publication of a one book chapter, 12 scientific papers indexed in group a1, 7 papers indexed in group a2, and three papers from group a3. As an author or co-author, she participated in 16 international and seven national scientific and professional conferences with a total of 39 presentations. She actively participated in the implementation of three scientific research projects (IPA, MP, HRZZ,) one professional project (Syngenta) and one teaching project (ERASMUS+).

She is a member of the Croatian Plant Protection Society, Croatian Entomological Society, International Organization for Biological Pest Control (IOBC) and Royal Entomology Society. She is one of the leaders of the Entomology Group, an extracurricular activity in which she mentors up to 10 students per year and has made significant contributions (Rector's Award 2018, work with gifted children in the "Panda" project and participation in the European Researchers' Night). She is a member of the Editorial Board of the scientific journal Entomologia Croatica. She regularly and actively participates in all promotional activities of the Faculty.

She is the recipient of numerous awards and recognitions: Best young resercher award by University of Zagreb Faculty of Agriculture (’22); Faculty of Agriculture Foundation Annual Award ('21); Award for the best scientific article by a doctoral student in the academic year 2019/2020 (’20); University of Zagreb Faculty of Agriculture. Commendation for contribution to faculty recognition and enhancement of student extracurricular activities (’19); "Milan Maceljski" award for the excellent success and scientific activity during study by Croatian plant protection society (’16), and several more awards for best presentation and poster at international conferences.