Dalmatian pyrethrum (Tanacetum cinerariifolium /Trevir./Sch. Bip.) population diversity based on pyrethrin content and microsatellite marker analysis

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Sveučilište u Zagrebu

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DECLARATION OF ORIGINALITY

I, **Filip Varga**, declare that I have composed solely by myself the thesis titled:

DALMATIAN PYRETHRUM (*Tanacetum cinerariifolium* /Trevir./ Sch. Bip.) POPULATION DIVERSITY BASED ON PYRETHRIN CONTENT AND MICROSATELLITE MARKER ANALYSIS

With my signature I confirm that:

- I am the sole author of this thesis;
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- I am familiar with the provisions of the Code of Ethics of the University of Zagreb (Article 19).

Zagreb, April 19th, 2021

_____
PhD Candidate signature

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Supervisor information

Assoc. Prof. Martina Grdiša was born on 29 December 1978 in Zagreb. On 18 March 2004 she graduated from the University of Zagreb, Faculty of Agriculture. Since 1 March 2006 she has been employed at the Faculty of Agriculture, Department of Seed Science and Technology. She is registered in the Register of Researchers at the Ministry of Science and Education under registration number 283245. On June 10, 2011 she defended her dissertation entitled 'Morphological, chemical and genetic diversity of Dalmatian pyrethrum (*Tanacetum cinerariifolium* /Trevir./ Sch. Bip.)'.

She was elected Senior assistant professor in July 2011, Research associate on January 25, 2013, Senior research associate on April 11, 2014, Assistant professor on July 15, 2015, and Scientific advisor on June 13, 2019. On January 13, she was elected to the title Associate Professor. Martina Grdiša has been actively involved in teaching at the University of Zagreb, Faculty of Agriculture since 2008. She is the coordinator of the courses 'Pest Repelling and Insecticide Plants' and 'Production and Processing of Medicinal and Aromatic Crops' and the collaborator in the courses 'Plant Propagation', 'Medicinal, Aromatic and Honey Plants', 'Seed Science and Technology of Field Crops', 'Plant Genetic Resources Conservation', Molecular Biodiversity and Evolution', 'Plant propagation of Energy Crops' and 'Research Methods in Plant Propagation'.

Martina Grdiša conducted scientific training at Biotechnical Faculty at the University of Ljubljana, the Faculty of Science in Zagreb and the Faculty of Chemical Engineering and Technology in Zagreb, where she learned numerous analytical methods and techniques, including molecular (DNA isolation, molecular markers AFLP and SSR) as well as chemical (HPLC, ultrasound extraction, MSPD). She is a member of three international (ISHS, EUCARPIA and AMAPSEEC) and one national scientific society (HBoD). Her research interests relate to genetic and biochemical diversity and conservation of plant genetic resources, with a particular focus on natural populations of medicinal and aromatic plants. The research work focuses in particular on the plant species Dalmatian pyrethrum (*Tanacetum cinerariifolium* /Trevir./ Sch. Bip.), the analyzes of which at the morphological, chemical and genetic levels were the subject of her PhD thesis. Martina Grdiša actively participates in the management of the Collection of Plant Genetic Sources of Medicinal and Aromatic Plants, which is as a part of National Collection of Plant Genetic Sources of the Republic of Croatia. She regularly contributes to the maintenance of the collection, description, characterization and evaluation of the collected accessions, their regeneration, as well as in the maintenance of Croatian Plant Genetic Resources Database, in the establishment of which she participated.

She has published eighteen (18) a1 papers, twelve (12) a2 scientific papers and five (5) a3 papers and participated as an author or co-author in 38 international and national scientific conferences. She has reviewed papers submitted for publication in several reputed journals (Industrial Crops and Products, Journal of Essential Oil-Bearing Plants, Natural Products Research, etc.). She is the leader of the scientific project financed by Croatian Science Foundation entitled 'Genetic basis of insecticidal potential of Dalmatian pyrethrum (*Tanacetum cinerariifolium* /Trevir./ Sch. Bip.)', and actively participated in the implementation of eight (8) national and six (6) international scientific projects. She is a member of the Center of Excellence for Biodiversity and Molecular Plant Breeding (Crop-BioDiv), WP08- Dalmatian Pyrethrum / Sage. From 2007 to 2015 Martina Grdiša was Junior Editor, and from 2016 to 2020 Scientific Editor for Plant Sciences of the scientific journal Agriculturae Conspectus Scientificus (ACS).

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Summary

Dalmatian pyrethrum (*Tanacetum cinerariifolium* /Trevir./ Sch. Bip.) is an endemic species of the eastern Adriatic from the family Asteraceae. The plant produces pyrethrin, a secondary metabolite that has shown to be an effective natural insecticide with little or no adverse effects on humans.

To assess biochemical and genetic diversity, samples were collected from 10 natural populations of Dalmatian pyrethrum along the Adriatic coast and islands representing the species' distribution range.

A matrix solid phase dispersion extraction (MSPD) method was used for the extraction of pyrethrin compounds from the dried flower heads of Dalmatian pyrethrum. High performance liquid chromatography with diode array detector was carried out for the determination and quantification of the six pyrethrin compounds and total pyrethrin content. The total pyrethrin content in the samples ranged from 0.10% to 1.35% of the dry flower weight and the pyrethrin I/pyrethrin II ratio ranged from 0.21 to 5.88. Four chemotypes of different pyrethrin extract quality were determined using multivariate statistical methods.

A total of 17 microsatellite markers were developed using Next Generation Sequencing (NGS), of which two were classified as highly polymorphic (*PIC* > 0.70) and eight as moderately polymorphic (*PIC* > 0.44). The presence of null alleles was detected at four loci.

Genetic diversity analysis of 10 Dalmatian pyrethrum populations was performed using 12 microsatellite markers. Evidence of bottleneck events was identified only in the Biokovo population. Analysis of molecular variance showed that the majority of the genetic diversity can be explained by differences between individuals within populations (87.03%). Bayesian analysis of population structure revealed the existence of two gene pools. Spatial analysis revealed that only a small proportion of genetic differentiation can be attributed to isolation by distance (0.7%), while 40.6% of genetic differentiation can be attributed to isolation by environmental distance.

The results of this research will contribute to the development of future breeding programs and hopefully commercial varieties of *T. cinerariifolium* to revive Dalmatian pyrethrum production in Croatia and the region. Furthermore, these results will help to improve conservation strategies for the species.

Keywords: Dalmatian pyrethrum, *Tanacetum cinerariifolium* (Trevir.) Sch. Bip., pyrethrin content and composition, natural insecticide, MSPD, SSR, NGS, plant genetic resources

Populacijska raznolikost dalmatinskog buhača (*Tanacetum cinerariifolium* /Trevir./ Sch. Bip.) na temelju analize sadržaja piretrina i mikrosatelitnih biljega

Dalmatinski buhač (*Tanacetum cinerariifolium* /Trevir./ Sch. Bip.) endemska je biljna vrsta istočnog Jadrana iz porodice Asteraceae. Biljke dalmatinskog buhača sintetiziraju piretrine, sekundarne metabolite koji su dokazano učinkoviti u suzbijanju velikog broja štetnika, bez štetnog utjecaja na ljude.

U svrhu procjene biokemijske i genetske raznolikosti prikupljeni su uzorci 10 prirodnih populacija dalmatinskog buhača duž obale Jadrana, predstavljajući područje prirodnog rasprostiranja ove vrste u Hrvatskoj.

Metoda ekstrakcije disperzijom matrice u čvrstoj fazi (MSPD) primijenjena je za ekstrakciju piretrinskih spojeva iz suhih cvatnih glavica dalmatinskog buhača, a za determinaciju i kvantifikaciju piretrinskih spojeva korištena je tekućinska kromatografija visoke djelotvornosti (HPLC). Ukupni sadržaj piretrina u uzorcima kretao se od 0,10 % do 1,35 % mase suhog cvijeta, dok se omjer piretrin I/piretrin II kretao od 0,21 do 5,88. Upotrebom multivarijatnih statističkih metoda utvrđena su četiri kemotipa različite kvalitete piretrinskog ekstrakta.

Ukupno 17 mikrosatelitnih biljega je razvijeno koristeći sekvenciranje nove generacije (NGS), od kojih su dva klasificirana kao visoko polimorfni biljezi (*PIC* > 0,70), a osam kao umjereno polimorfni biljezi (*PIC* > 0,44). Prisutnost nul-alela utvrđena je kod četiri mikrosatelitna biljega.

Analiza genetske raznolikosti 10 populacija dalmatinskog buhača provedena je na temelju 12 mikrosatelitnih biljega. Učinak genetskog uskog grla utvrđen je samo kod populacije s Biokova. Analiza molekularne varijance pokazala je da se većina genetske raznolikosti može objasniti razlikama između jedinki unutar populacija (87,03 %). Bayesovska analiza populacijske strukture otkrila je postojanje dva genska skupa u analiziranim populacijama. Prostorne analize pokazale su da se mali udio genetske diferencijacije može objasniti izolacijom uslijed udaljenosti (0,7 %), dok se čak 40,6 % genetske diferencijacije može objasniti izolacijom uslijed ekološke udaljenosti.

Rezultati ovog istraživanja doprinijet će razvoju budućih programa oplemenjivanja i stvaranju komercijalnih kultivara dalmatinskog buhača u nastojanju da se revitalizira njegova proizvodnja u Hrvatskoj i regiji. Nadalje, ovi rezultati pomoći će u unaprjeđenju strategija očuvanja ove vrste.

Ključne riječi: dalmatinski buhač, *Tanacetum cinerariifolium* (Trevir.) Sch. Bip., sastav i sadržaj piretrina, prirodni insekticid, MSPD, SSR, NGS, biljni genetski izvori

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List of abbreviations

ADH - alcohol dehydrogenase
AFLP - amplified fragment length polymorphism
ALDH - aldehyde dehydrogenase
AMOVA - analysis of molecular variance
ANOVA - univariate analysis of variance
A-T – adenine-thymine
CA - cluster analysis
CCC - cubic clustering criterion
CDP - - chrysanthemyl diphosphate
CoA - coenzyme A
CPC - centrifugal partition chromatography
DDT - dichlorodiphenyltrichloroethane
DMAPP - dimethylallyl diphosphate
DPSA - distance based on the proportion of shared alleles
EST SSR - expressed sequence tag SSR
FISH - fluorescence in situ hybridization
GC - gas chromatography
G-C - guanine-cytosine
GC-FID - gas chromatography with flame ionization detector
GC-MS - gas chromatography–mass spectrometry
HPLC - High performance liquid chromatography
HPLC-DAD- HPLC with diode array detector
HSCCC - high-speed counter-current chromatography
IBD - isolation by distance
IBED – isolation by environmental distance
IPP - isopentenyl pyrophosphate
LD50 - lethal dose, 50%
MCMC - Monte Carlo Markov Chain
MEKC - micellar electrokinetic chromatography
MEP - 2-C-methyl-D-erythritol 4-phosphate
MSPD - matrix-solid phase dispersion
NGS - next generation sequencing
NJ - neighbor-joining
NP-HPLC - normal-phase HPLC
OPDA - 12-oxo-phytodienoic acid
PCA - principal component analysis
PCR - polymerase chain reaction
PIC – polymorphism information content
PI/PII - pyrethrin I and pyrethrin II ratio
PSF - pseudo-F
PVP – polyvinylpyrrolidone
QTL – quantitative trait loci
QuEChERS - quick, easy, cheap, effective, rugged, safe
RSLDE - rapid solid–liquid dynamic extraction
SFC-FID - supercritical fluid chromatography with flame ionization detection
SFE - supercritical fluid extraction
SOX - Soxhlet or Soxtec
SSR - simple sequence repeats
TcADH2 - *T. cinerariifolium* alcohol dehydrogenase 2
TcALDH1 - *T. cinerariifolium* aldehyde dehydrogenase 1
TcCCMT - 10-carboxychrysanthemic acid 10-methyltransferase
TcCDS - chrysanthemyl diphosphate synthase
TcCHH - chrysanthemol 10-hydroxylase
TcGLIP - *T. cinerariifolium* GDSL lipase
TcJMH - jasmone hydroxylase
TcPYS - pyrethrolone synthase
TE - Tris-EDTA
TPM - two-phase model
UAE - ultrasound-assisted extraction
UPGMA - unweighted pair group method with arithmetic mean

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1. INTRODUCTION

Dalmatian pyrethrum (*Tanacetum cinerariifolium* /Trevir./ Sch. Bip.) is a species from the family Asteraceae, endemic to the eastern Adriatic coast. Its distribution spans across coastal parts of Croatia (and islands), southern parts of Bosnia and Herzegovina, Montenegro, and Albania (Euro+Med, 2006; Nikolić, 2015). This species inhabits dry grasslands with shallow rocky soils (Grdiša et al., 2009), and is easily recognized during the flowering stage by its flower heads, composed of white petal-like ray florets on the margins and yellow disc florets in the center of the receptacle (Bhat, 1995).

The active ingredient pyrethrin, one of the most commercially exploited botanical insecticides, is concentrated in light brown achenes formed after pollination. Pyrethrin is composed from six compounds (pyrethrin I and II, cinerin I and II, jasmolin I and II) out of which pyrethrin I and II are found in the highest concentrations (Casida, 1973). According to various authors pyrethrin content of natural populations ranges from 0.36 to 1.3% with an average of 0.86% (Ambrožič Dolinšek et al., 2007; Grdiša et al., 2013). For comparison, in commercial varieties from Australia pyrethrin content of up to 2.5% is reported (Morris et al., 2006). As insecticide, pyrethrin has a knock-down effect based on disruption of the insect's nervous system functionality, followed by paralysis and death of the insect (Davies et al., 2007).

The 1850s mark the start of commercial cultivation of pyrethrum in coastal parts of Dalmatia and islands. The region became the leading producer of pyrethrum up until the World War I. By then, production was introduced to other parts of the world, primarily to Japan and Kenya. The production spread further to other countries, while production in Croatia (at that time part of Yugoslavia) further decreased and eventually stopped additionally impacted by the discovery and widespread usage of DDT (Ožanić, 1955). Largest producers today are concentrated in two regions, East Africa (Tanzania, Rwanda, and Kenya) and Australia (Tasmania) (FAO, 2019).

Numerous factors play a role in selection of pyrethrin extraction method such as time needed for the extraction, toxicity of the solvents, equipment needed for the extraction as well as complexity of the method. Additional factors influence the pyrethrin content in the extract such as harvest time, and handling of the samples before extraction process (Romdhane and Gourdon, 2002). Several extraction methods were utilized to this day in pyrethrin extraction. Soxhlet or Soxtec (SOX) method has been used in the earliest studies of pyrethrin content (Otterbach and Wenclawiak, 1999; Kiriamiti et al., 2003). Other, most commonly used method is Ultrasound extraction (Babić et al., 2012; Grdiša et al., 2013). Maceration, as the simples

and one of the oldest extraction techniques has also been applied in pyrethrin extraction (Gallo et al., 2017). Recently, Matrix-solid phase dispersion extraction (MSPD) method was also optimized for pyrethrin extraction showing certain advantages compared to other extraction methods such as significantly shorter extraction time, no expensive equipment required, lower consumption of toxic solvent and lower extraction temperature (Biošić et al., 2020).

Microsatellites are parts of the DNA composed of tandem repeating motifs, otherwise known as simple sequence repeats (SSR). They are often used as molecular markers in population genetics due to their high degree of polymorphism (Vaz Patto et al., 2004; Belaj et al., 2007). The genome of Dalmatian pyrethrum is estimated to be 7.1 Gb, much larger than other species in Asteraceae family (Yamashiro et al., 2019). The presence of highly repetitive sequences presents a challenge in development of SSR markers for this species. The only available study on population genetics and structure of natural Dalmatian pyrethrum populations using amplified fragment-length polymorphisms (AFLP) shows great genetic diversity across the geographical range of the species (Grdiša et al., 2014). Microsatellite markers are more informative due to the fact they are codominant (differentiation of homozygotes and heterozygotes), are locus specific and are more reproducible than AFLPs (Vieira et al., 2016). The emergence of Next Generation Sequencing (NGS) has enabled the efficient generation of a large amount of genome sequence data from which microsatellite primers can be identified and reduced the cost and time needed to develop microsatellite markers significantly (Purwoko et al., 2019).

1.1. Research hypotheses and objectives

Hypotheses

Intra- and interpopulation diversity of six pyrethrin compounds exists in natural populations of Dalmatian pyrethrum. Next generation sequencing can be utilized in microsatellite development for Dalmatian pyrethrum. Natural populations of Dalmatian pyrethrum can be separated into two geographically separated gene pools.

Aims of the research

1. To determine content and composition of pyrethrin components in samples of Dalmatian pyrethrum using optimized MSPD;
2. To develop microsatellite markers for Dalmatian pyrethrum using NGS data;
3. To analyze genetic diversity of Dalmatian pyrethrum based on data obtained by developed microsatellite markers.

2. OVERVIEW OF FORMER RESEARCH

2.1. Dalmatian pyrethrum

2.1.1. Taxonomy and morphology

Dalmatian pyrethrum (*Tanacetum cinerariifolium* /Trevir./ Sch. Bip.) is a plant species in the composite family (Asteraceae), genus *Tanacetum* L. The family Asteraceae is one of the largest and most diverse families of flowering plants (Angiospermae) in the world with ≈ 1600 genera and 24,000 species (Funk et al., 2009). The genus *Tanacetum* consists of 150-200 species distributed in Europe, North America, North Africa and temperate parts of Asia (Abad et al., 1995). Five species of the genus are part of the Croatian flora: *Tanacetum cinerariifolium* /Trevir./ Sch. Bip. (Dalmatian pyrethrum), *Tanacetum corymbosum* (L.) Sch. Bip. (corymbflower tansy), *Tanacetum macrophyllum* (Waldst. et Kit.) Sch. Bip. (tansy), *Tanacetum parthenium* (L.) Sch. Bip. (feverfew), and *Tanacetum vulgare* L. (common tansy) (Nikolić, 2020). *T. cinerariifolium* has two homotypic synonyms reflecting historical changes in the taxonomy of the species: *Chrysanthemum cinerariifolium* (Trevir.) Vis. and *Pyrethrum cinerariifolium* Trevir. (Nikolić, 2015). In Croatia the species is known by various common names depending on the region: ‘buhač’, ‘buhar’, ‘buharika’, ‘bujar’, ‘buvač’, ‘buvijak’, ‘divji pelin’, ‘koren studeni’, ‘matrikolda’, ‘matrikovo zelje’, ‘osjenač’, etc. (Šugar, 2008).

Dalmatian pyrethrum is a perennial herbaceous species that reaches a height of 100 cm, measured as the length of the central stem ending with the inflorescence from the ground (Figure 1a). The plant has a deep (30-35 cm) and well-branched out root system. The primary height growth of the plant begins at germination and it is common for a plant to have several secondary stems in addition to the central stem. The stems are stiff, erect, and covered with gray-green hairs. The leaves are oblong, pinnately lobed, and doubly notched and are located at the base of the stem, forming a herbaceous shrub (Bhat, 1995). The inflorescence of Dalmatian pyrethrum is 3-5 cm in diameter and consists of two types of flowers, the marginal white petal-like ray florets, and yellow disk florets in the center, which are perched on a slightly convex receptacle (Figure 1b). The ray florets are female while the disk florets are hermaphrodite (have both male and female reproductive organs) and consist of a calyx, yellow tubular corolla composed of five petals and five stamens and is epigynous. The ovary is

pentagonal with a single ovule. The pollen grains are trinucleate which is characteristic of the Asteraceae family (Figure 1c). Flowering begins a month after the first buds appear, usually in May, and lasts until late June with cross-pollination encouraged by the flowering rhythm. Insects from the order Hymenoptera are the main pollinators of Dalmatian pyrethrum (Brewer, 1968). The fruits are beige-brown, cylindrical ribbed (5-7 ribs) achenes (Figure 1d) (Bojnanský and Fargašová, 2007).

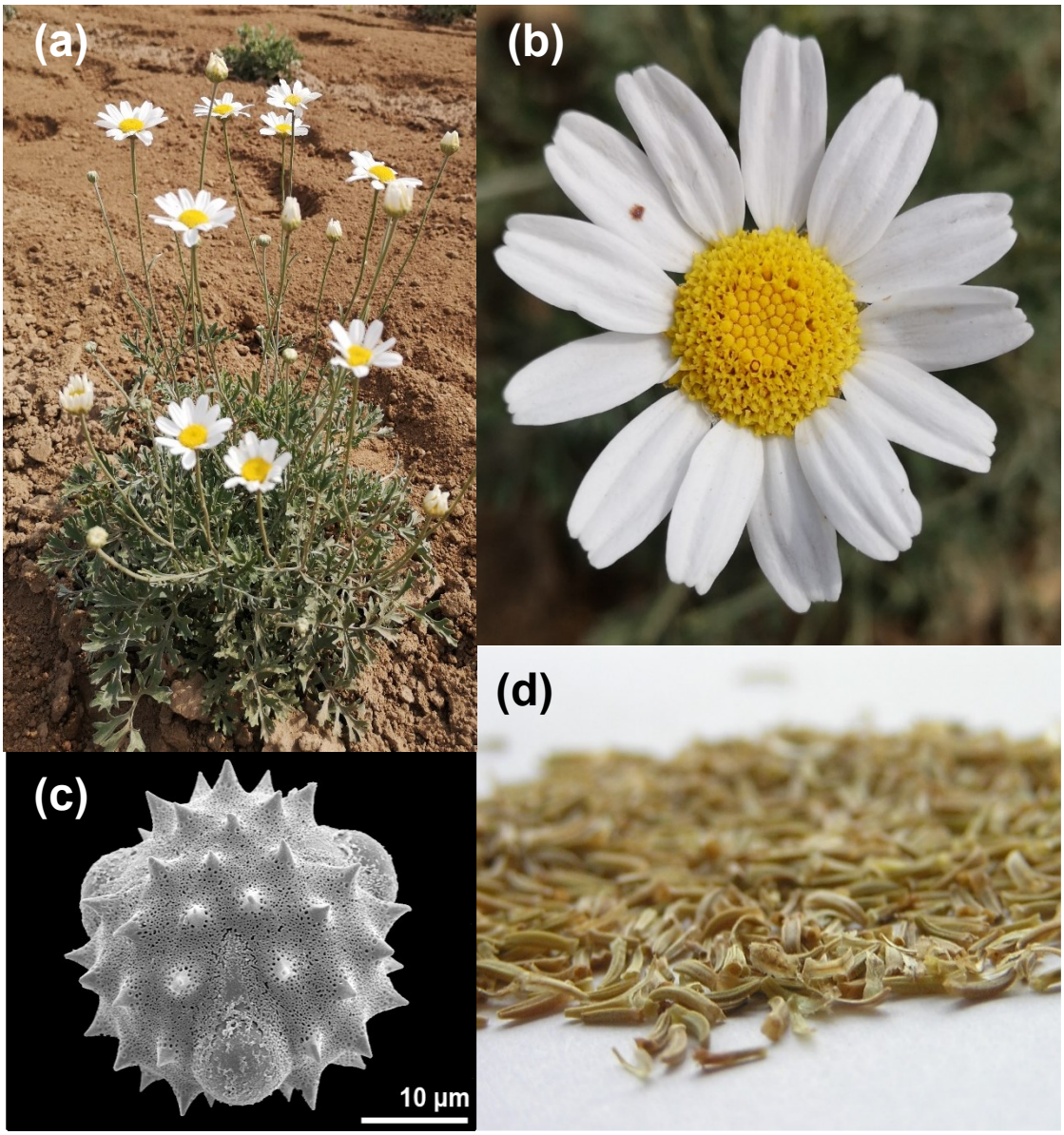


Figure 1. Morphological characterstics of Dalmatian pyrethrum: plant habitus (a), inflorescence with two distinct floret types (b), pollen grain under scanning electron microscope (Halbritter and Weis, 2016) (c), achenes (d).

2.1.2. Geographical distribution and ecology

The distribution of Dalmatian pyrethrum is restricted to the eastern coast of the Adriatic, making the species endemic. Most of its range is in Croatia, and includes the coastal region and islands, but the species also occurs in southern parts of Bosnia and Herzegovina and in the coastal regions of Montenegro and Albania, as shown in Figure 2 (Euro+Med, 2006). It grows mainly at altitudes up to 200 m above sea level, with some exceptions such as the mountainous regions of Dalmatia, where it can be found at altitudes of 500 m above sea level and higher (Nikolić, 2015). Populations of Dalmatian pyrethrum are found most northerly on the islands of the Kvarner Bay (Krk, Cres and Lošinj) and Cape Kamenjak (Istrian Peninsula). The species also occurs on Mount Velebit, along the Dalmatian coast and on islands (Ugljan,

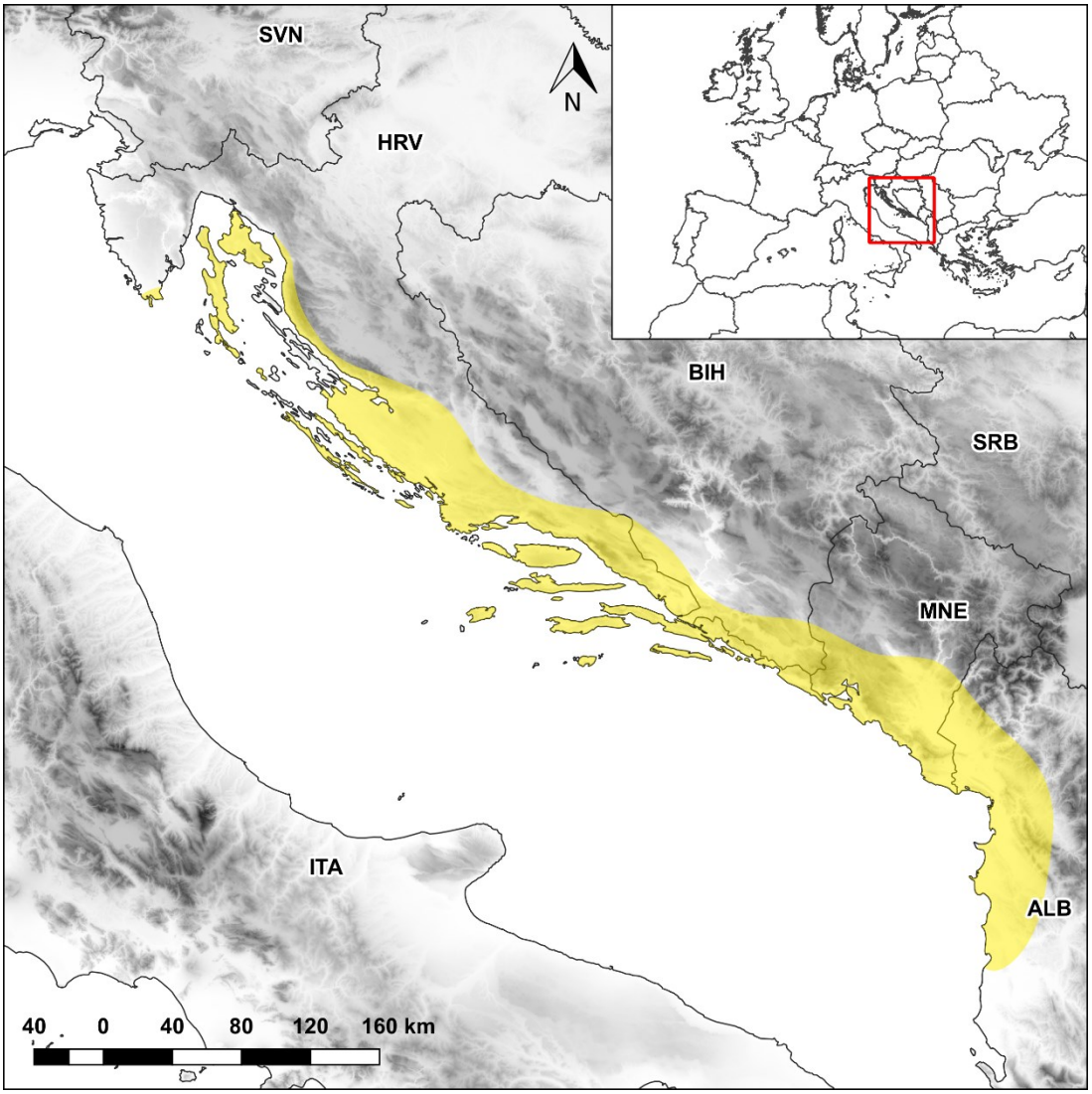


Figure 2. Distribution of *T. cinerariifolium*.

Pašman, Dugi otok, Čiovo, Šolta, Brač, Hvar, Biševo, Vis, Korčula, Lastovo, Mljet), as well as on Mount Biokovo (Nikolić et al., 2015).

Dalmatian pyrethrum is a thermophilic species growing in extremely degraded habitats such as sub- and epimediterranean dry grasslands, rocky pastures, calcareous rocks, garigues and olive groves. The species prefers shallow, rocky, and sandy soils as well as high amount of sunlight and shelter from the harsh winds (Nikolić, 2015). Urbanization and habitat conversion in the coastal region of Croatia threaten this species, therefore the Dalmatian pyrethrum is nationally protected by the Croatian Regulation as Strictly Protected Wild Taxa (Official Gazette, 2013).

2.1.3. Pyrethrum cultivation history

A long tradition of using dried and ground flowers of Dalmatian pyrethrum in households and agriculture in Croatia. According to some sources, the first person to recognize the insecticidal potential of pyrethrum was a pharmacist from Dubrovnik, Antun Drobac (1810-1882). After that, the demand for Dalmatian pyrethrum grew drastically, and the exploitation of wild plants could not satisfy this demand. Around 1845, the beginnings of pyrethrum cultivation around Dubrovnik are recorded (Bakarić, 2005). The harvested flowers were dried in the sun and stored in dry bags or barrels (Figure 3) and later ground and used for mosquito and lice control in humans and animals (Ožanić, 1955; Glynne-Jones, 2001). The production of pyrethrum in Dalmatia reached its maximum in the period 1910-1930, during which time it was grown on an area of 2100 ha for the most part around Šibenik, Split, Hvar, Dubrovnik and Zadar (Ožanić, 1930). After 1930, production in Dalmatia declined significantly, and with the discovery and widespread use of synthetic insecticide DDT (dichlorodiphenyltrichloroethane), it came to a complete halt. In the 1960s, some attempts to revive production were made, but without success (Bakarić, 2005).

Shortly after the start of production in Dalmatia, pyrethrum was introduced in other countries, first in Japan in 1885 (Ueyama, 2017), then in Italy, Kenya and Tanzania, and later in some South American countries like Brazil and Ecuador and Australia (Gullickson, 1995). Japan rapidly became the world's leading producer and held this position until the start of World War II (Ueyama, 2017). Kenya, where pyrethrum was introduced in 1928, immediately began developing breeding programs for the species and became the world's leading producer by

1945 (Thorpe, 1940). The breeding programs developed in each producing country were aimed at producing clones with high pyrethrin content (Contant, 1963; Bhat and Menary, 1986).

The chemical structure of pyrethrin compounds was discovered gradually. First, in 1924 the chemical structure of pyrethrin I and pyrethrin II was discovered. In 1945, the chemical structure of cinerin I and cinerin II was discovered, and finally in 1966, the chemical structure of jasmolin I and jasmolin II. This led to researches aimed at modifying natural pyrethrins and subsequently to the discovery and commercialization of synthetic pyrethrins – pyrethroids (Matsuo, 2019). The global market has witnessed a significant decline in pyrethrum production due to the widespread use of synthetic pyrethroids. In recent years, pyrethroids have been shown to be toxic to humans and persist in the environment, leading to an increase in the popularity of pyrethrins and consequently increased cultivation of Dalmatian pyrethrum (FAO, 2019; Ramchandra et al., 2019). In 2019, the largest pyrethrum producers were Tanzania (7347 t), Rwanda (2993 t) and Papua New Guinea (1264 t), but there is also active production in Kenya, Ecuador, Australia (mainly in Tasmania) and China, as shown in Figure 4 (FAO, 2019).

Figure 3. Pyrethrum flowers drying in the sun (highlighted in yellow) on the Cres boardwalk in 1919 (Image source: Zvonimir Vlatković).

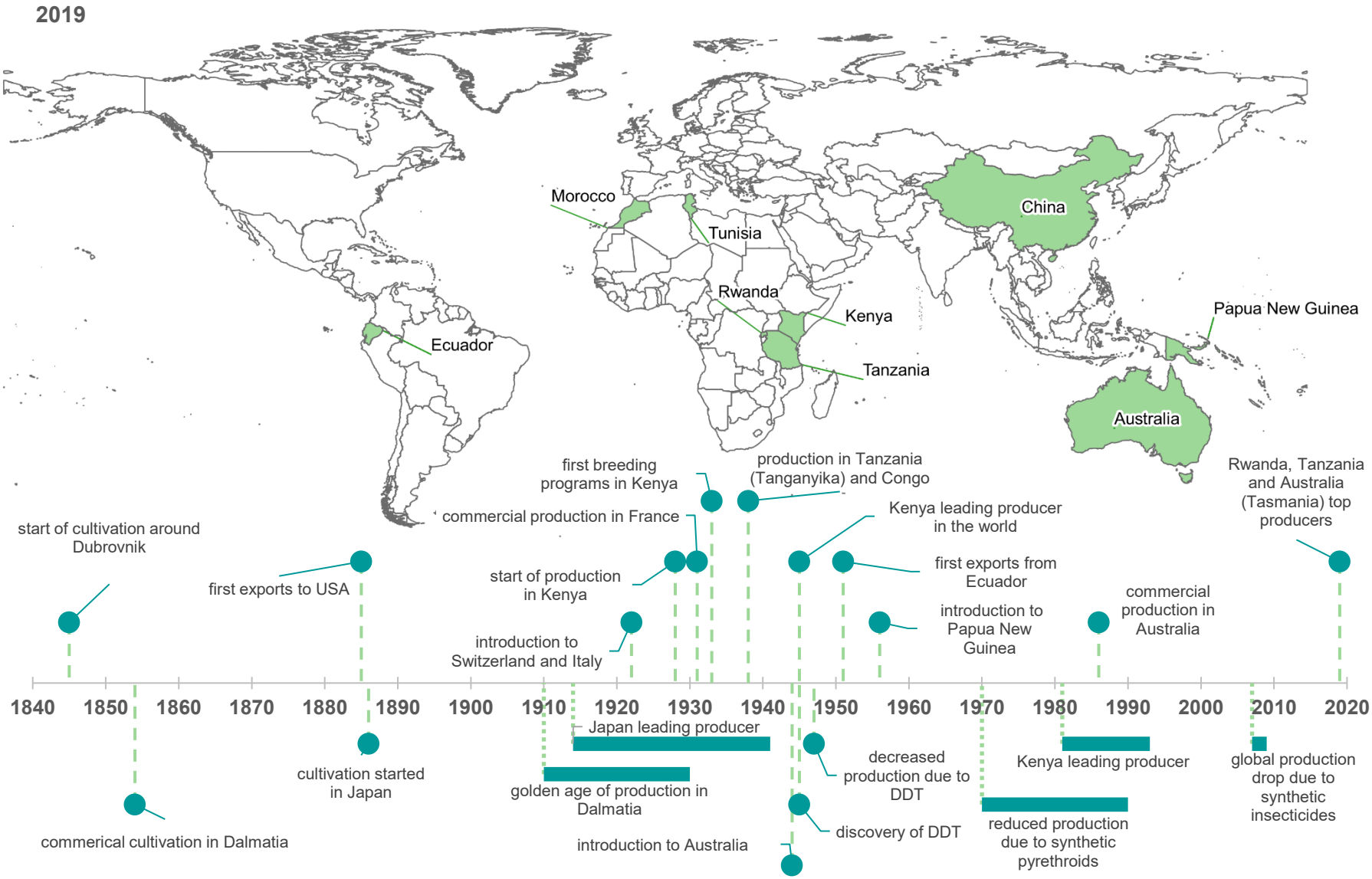


Figure 4. Leading producers of Dalmatian pyrethrum today and historical timeline of pyrethrum production

2.2. Pyrethrins

2.2.1. Chemical structure and biosynthesis

Pyrethrin is a natural mixture of six compounds from two groups, pyrethrins I and pyrethrins II, secondary metabolites found in Dalmatian pyrethrum. Pyrethrins I (cinerin I, jasmolin I and pyrethrin I) are esters of chrysanthemic acid and the alcohols cinerolone, jasmolone and pyrethrolone, respectively. Pyrethrins II (cinerin II, jasmolin II and pyrethrin II) are esters of pyrethric acid and the alcohols cinerolone, jasmolone and pyrethrolone, respectively (Casida, 1973; Crombie, 1995; Essig and Zhao, 2001b). Pyrethrolone, jasmolone and cinerolone contain a cyclopentenone ring, whereas chrysanthemic and pyrethric acids are monoterpenes with a cyclopropane ring (differing only by a methylated carboxyl group). The alcohols and acids that form pyrethrin compounds are synthesized in different pathways.

The synthesis of the acidic components of the pyrethrin compounds is carried out from D-glucose via two different synthetic pathways, mevalonate and non-mevalonate. While in the first pathway the intermediate is mevalonic acid, in the second pathway the intermediate is 2-C-methyl-D-erythritol 4-phosphate (MEP). Of these two pathways, the non-mevalonate pathway is the dominant one (Matsuda et al., 2005; Khan et al., 2017;). The outputs of both pathways are dimethylallyl diphosphate (DMAPP) and isopentenyl pyrophosphate (IPP). Dimethylallyl diphosphate enters two successive reactions catalyzed by chrysanthemyl diphosphate synthase (TcCDS). The product of the first reaction is chrysanthemyl diphosphate (CDP), and the second reaction is chrysanthemol (Rivera et al., 2001; Yang et al., 2014). Chrysanthemol is then converted to chrysanthemic acid by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). The synthesis of pyrethric acid from chrysanthemol involves six steps catalyzed by four different enzymes (chrysanthemol 10-hydroxylase – TcCHH), *T. cinerariifolium* alcohol dehydrogenase 2 – TcADH2), *T. cinerariifolium* aldehyde dehydrogenase 1 – TcALDH1), and 10-carboxychrysanthemic acid 10-methyltransferase - TcCCMT) (Xu et al., 2019).

The alcohol components of pyrethrum compounds are synthesized from linolenic acid via 12-oxo-phytodienoic acid (OPDA) and *cis*-jasmon via the octadecanoid pathway (Matsuda et al., 2005; Khan et al., 2017; Matsui et al., 2020). *Cis*-jasmone is converted to jasmolone by jasmone hydroxylase (TcJMH) and jasmolone is converted to pyrethrolone by pyrethrolone

synthase (TcPYS) (Li et al., 2018). The enzymes and reactions by which cinerolone is synthesized are not know yet, although it is speculated that cinerolone is also synthesized from jasmolone (Li et al., 2019).

In the final step of pyrethrin biosynthesis, chrysanthemic and pyrethric acids are first activated by coenzyme A (CoA) to form chrysanthemoyl-CoA and pyrethroyl-CoA, respectively, and then esterified with the alcohols by *T. cinerariifolium* GDSL lipase (TcGLIP) and GDSL lipase-like protein (Kikuta et al., 2012). The complete biosynthetic pathway and chemical structures of final products (pyrethrins I and II) are shown in Figure 5.

It was found that all currently known enzymes involved in pyrethrin biosynthesis and their intermediates are localized in trichomes of the ovaries. From there, the intermediates are transported to the pericarp of the ovaries, where the final steps of pyrethrin synthesis, esterification, occur (Rivera et al., 2001; Ramirez et al., 2012; Li et al., 2018, 2019; Xu et al., 2019). The intercellular spaces of the pericarp surrounding the embryo are the accumulation zones of pyrethrins. As the seed matures, it takes up the pyrethins to use as a defense mechanism, since it cannot yet produce pyrethrins itself (Ramirez et al., 2012).

At the cellular level, the majority of the biosynthesis occurs in plastids and their role in regulating biosynthesis in seedlings and flowers has been confirmed (Matsuda et al., 2005; Khan et al., 2017), while some steps in pyrethrum biosynthesis were observed on the endoplasmatic reticulum (Li et al., 2018).

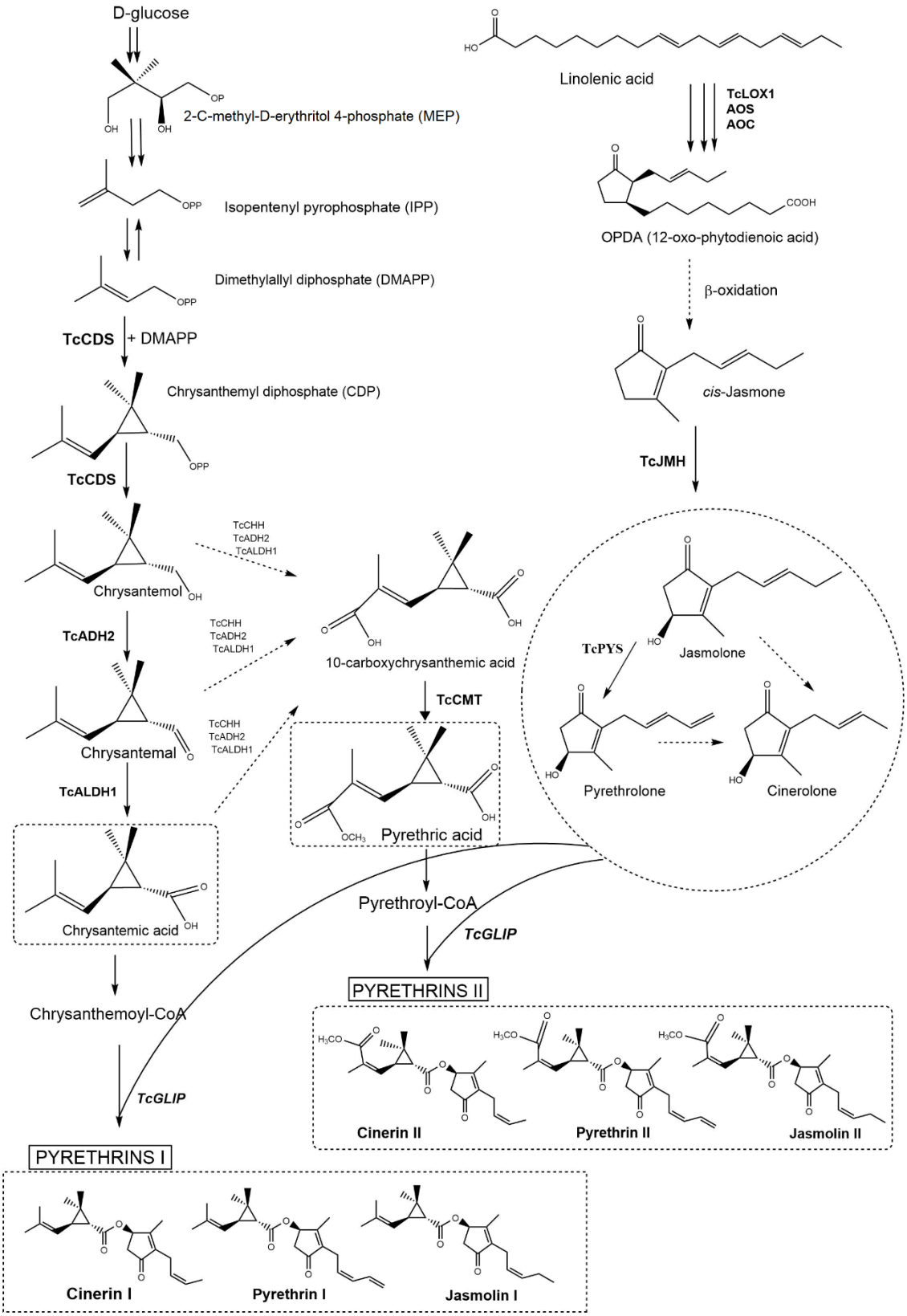


Figure 5. Biosynthesis of pyrethrin compounds.

2.2.2. Pyrethrin content and composition

Pyrethrins are concentrated in the flower heads of *T. cinerariifolium*, more specifically in the achenes (Head, 1966). Mature plants organs such as leaves also contain pyrethrins, but in much lower concentrations than the flower heads (Zito et al., 1983; Ambrožič Dolinšek et al., 2007). To date, several studies have been conducted on pyrethrin content and composition in natural populations of Dalmatian pyrethrum, most of them reporting a total pyrethrin content of ≈ 1.2% of dry flower weight (Ambrožič Dolinšek et al., 2007; Ban et al., 2010; Babić et al., 2012). The most comprehensive study to date on pyrethrin content in natural populations found similar results with total pyrethrin content ranging from 0.36 to 1.3% of dried flower weight based on 25 populations collected along the Croatian coast (Grdiša et al., 2013). In another study, high total pyrethrin content (1.58% of dry flower weight) was observed in natural population from the island of Ugljan (Rončević et al., 2014).

The total pyrethrin content in samples of cultivated plants of *T. cinerariifolium* from different regions of the world varies. Some studies from Italy (Marongiu et al., 2009; Baldino et al., 2017; Gallo et al., 2017), Kenya (Kiriamiti et al., 2003), and India (Nagar et al., 2015) report similar total pyrethrin contents in samples of cultivated plants as in natural populations. In commercial cultivars from Tasmania, total pyrethrin content reaches up to 2.5% of dry flower weight (Morris et al., 2006), while in the USA clones with total pyrethrin content above 3.0% have been recorded (Hitmi et al., 2000). Factors that influence the pyrethrin content include developmental stage of the plants, plant type, climate/cultivation region (Glynne-Jones, 2001; Ambrožič Dolinšek et al., 2007) and extraction method (Pan et al., 1995; Kiriamiti et al., 2003).

Pyrethrin I and pyrethrin II are the most abundant out of the six pyrethrin compounds. In natural populations of *T. cinerariifolium* the composition of pyrethrin compounds expressed as percentage of total pyrethrin content is: cinerin I (6.0%), cinerin II (5.2%), jasmolin I (3.5%), jasmolin II (2.6%), pyrethrin I (51.7%), pyrethrin II (30.4%). Due to high biochemical diversity based on pyrethrin composition five chemotypes were proposed. Chemotype A, characterized by highest pyrethrin I content and pyrethrin I and pyrethrin II ratio (PI/PII)) was determined as the chemotype with greatest potential for future breeding programs (Grdiša et al., 2013).

The PI/PII ratio is another important factor that determines the quality of the pyrethrum extract besides the total pyrethrum content. It has been reported that the PI/PII ratio changes with the developmental stage of the pyrethrum flower (Pattenden, 1970; Maciver, 1995).

The correlation between certain morphological traits, especially floral characteristics, and pyrethrin content is well established in previous studies. Flower size (Pandita and Bhat, 1986),

flower yield (Bhat and Menary, 1986), dry matter content of flowers (Parlevliet, 1974) are some of the traits correlated with pyrethrin content. The stage of the plant development, maturity of flowers and duration of flowering period affect the pyrethrin yield in flowers (Suraweera et al., 2017a). Plant height and shrub diameter, traits related to resistance to lodging, have also been shown to influence pyrethrin content (Parlevliet and Contant, 1970; Ban et al., 2010).

The flowers of Dalmatian pyrethrum open one month after the first buds become visible. The process of flower development can be divided into eight or ten stages or phases depending on the author. (Head, 1973; Ikahu and Ngugi, 1989). The flowers are mature when 2⁄3 of the disc florets are open, which is considered the harvesting stage in most studies. The highest pyrethrin content was found in this stage (Ambrožič Dolinšek et al., 2007). Different climatic conditions may indirectly influence total pyrethrin content through flower yield (Wandahwa et al., 1996; Glynne-Jones, 2001).

Pyrethrin content is also strongly influenced by genetic factors. Selection and breeding for higher pyrethrin content showed great success in India, with pyrethrin content increasing by 46% after one round of selection (Bhat et al., 1985). Dalmatian pyrethrum is a diploid species with 2n = 18 (MacDonald, 1995), but triploids (2n = 27) and tetraploids (2n = 36) have been produced by artificial hybridization and colchicine treatment (Bhat, 1995). Flowers of autotetraploid breeding lines also showed higher pyrethrin content compared to diploid breeding lines (Zhang et al., 2008).

Dalmatian pyrethrum is considered a drought tolerant species, however, several morphological traits show significant increase under irrigation such as flower, achene and trichome size (Suraweera et al., 2017b). In addition, Li et al. (2011) showed that there is a vulnerability to drought stress at early plant development stages (seed germination and early seedling growth), which can be mitigated by seed hydropriming.

Several postharvest factors may have a negative effect on pyrethrin content, particularly drying temperature and drying time (Atkinson et al., 2004). Pyrethrin degradation was observed at 60 °C, while drying at 50 °C had no effect on pyrethrin content (Beckley, 1952; Ngugi and Ikahu, 1990). The choice of extraction method also affects the total pyrethrin content (Nagar et al., 2015; Grdiša et al., 2020), as does the solvent used in pyrethrin extraction (Ban et al., 2010; Gallo et al., 2017).

2.2.3. Biological activity of pyrethrins

2.2.3.1. Mode of action

Pyrethrins disrupt the nervous system of insects by inhibiting the function of sodium channels in the same way as some other synthetic insecticides (DDT and pyrethroids). When they enter the insect's nervous system, the insect initially enters a state of hyperactivity, followed by a rapid *knock-down* effect and paralysis within minutes and death (Sawicki and Thain, 1962; Camougis, 1973; Davies et al., 2007). Other plant insecticides are relatively slow acting compared to pyrethrins (Isman, 2008).

The broad spectrum of pyrethrin's insecticidal activity is the result of synergistic action of all six active pyrethrin compounds, showing higher efficiency (LD_{50} of 0.11 µg/fly of 25% pyrethrin extract) compared to individual pyrethrin compounds (Sheppard and Swedlund, 2000). Of the six compounds that make up the pyrethrins, pyrethrin I and pyrethrin II are the most active. While pyrethrin I alone is more lethal to insects and acts more rapidly, pyrethrin II plays an important role in the rapid *knock-down* effect that lasts for several hours (Sawicki et al., 1962; Sawicki and Thain, 1962). In combination, pyrethrin I and pyrethrin II have a large insecticidal activity on a wide range of insect species (Casida and Quistad, 1995), which further increases with an increased PI/PII ratio (Maciver, 1995).

2.2.3.2. Target species and resistance potential

Pyrethrins have a wide range of uses as an insecticide both in households (Kennedy and Hamilton, 1995) and in agriculture (Silcox and Roth, 1995). In addition, it also acts as an insect repellent at low concentrations (Kennedy and Hamilton, 1995). The use of powder from dried and ground flowers of Dalmatian pyrethrum has a long tradition in the control of body lice (Phthiraptera) and fleas (Siphonaptera) in both humans and animals with good results (Gerberg, 1995; Glynne-Jones, 2001; Meister, 2016). Pyrethrin-based sprays have been used to control various mosquito species (*Aedes* spp., *Anopheles* spp., *Culex* spp.), helping to reduce malaria transmission (Boyce et al., 2007; Duchon et al., 2009).

Several studies determined insecticidal activity of pyrethrins against agricultural pests from the orders Coleoptera (Kalinović et al., 2011; Liu et al., 2014; Marchand et al., 2018), Hemiptera (Hitmi et al., 2000; Bigirimana et al., 2018), Homoptera (Chermenskaya et al., 2010;

Kalaitzaki et al., 2015), Lepidoptera (Hitmi et al., 2000; Akhtar et al., 2008), Thysanoptera (Silcox and Roth, 1995; Yang et al., 2012) and others. Furthermore, the efficacy of pyrethrins against mites – Acarina (Silcox and Roth, 1995; Chermenskaya et al., 2010) and ticks – Ixodida (Pajnik et al., 2017) was determined. In addition to arthropodicidal properties, pyrethrins exhibit antiprotozoal activity, namely against *Plasmodium falciparum* Welch and *Trypanosoma brucei rhodesiense* Stephens & Fantham (Hata et al., 2011), as well as some antifungal activity (Ramirez et al., 2012).

The mode of action of pyrethrins is similar to that of synthetic insecticides such as DDT and pyrethroids, which increases the risk of cross-resistance development to pyrethrins due to the widespread use of these synthetic insecticides (Busvine, 1951). Nevertheless, resistance to pyrethrins has been reported in a small number of studies showing previously developed resistance to other insecticides in tested populations (Marcombe et al., 2009; Estep et al., 2017; Yang et al., 2020). It appears that the complex structure of pyrethrum extract somewhat hinders insect adaptation to this insecticide despite its long history of use (Duchon et al., 2009).

2.2.3.1. Toxicity to non-target species and environmental impact

The effect of pyrethrins on non-target species has been investigated in several studies. Most notably, Boyce et al. (2007) examined the effect of aerial spraying with pyrethrins on non-target arthropod species. Larger arthropod species such as dragonflies, spiders, and butterflies were not affected by spraying, while a measurable effect was observed on smaller species from 25 families. The effect of pyrethrins on honey bees (*Apis mellifera* L.), the most important pollinator, was also tested and no significant effects were observed (Boyce et al., 2007; Oliveira et al., 2019a). Consequently, pyrethrins were classified as relatively non-toxic to honey bees and can be used in agriculture (Sanford, 2011). The toxicity of pyrethrins to aquatic invertebrates has been proven under laboratory conditions (Camougis, 1973), fish (Pillmore, 1973) and amphibians (Oliveira et al., 2019b).

In mammals, pyrethrins are rapidly metabolized to inactive forms that are excreted from the body (WHO, 2000). In rats, pyrethrins have shown acute neurotoxic effects, while chronic toxicity has targeted the liver of experimental animals. Although no developmental abnormalities occurred in the offspring after mothers were exposed to toxic doses of pyrethrins, significant reduction in body mass, anemia, alterations in kidney and liver function were observed. Based on these findings, pyrethrins were classified as moderately hazardous (WHO,

2010). There are a few reports of respiratory and dermatological complaints in humans (Glynne-Jones, 2001), however, agricultural use at recommended levels does not affect the safety of farmers and consumers (Hernández-Moreno et al., 2013). Furthermore, a study investigating a possible connection between pyrethrins and allergic contact dermatitis in humans found that allergies in subjects were caused by sesquiterpene lactones contained in low-purity pyrethrum extracts, and not by pyrethrins themselves (Osimitz et al., 2006). In addition, a genotoxic effect of pyrethins on human cell cultures of lymphocytes (Azab et al., 2017) and epithelium (Tisch et al., 2005) has been observed.

Pyrethrins are rapidly biodegraded upon contact with sunlight, water, or air. Due to a relatively short half-life (≈ two hours), they are not accumulated in food chains and groundwater (Gunasekara, 2004). When applied at recommended insecticidal rates, no residues were detected in samples of aquatic sediment (Woudneh and Oros, 2006) and fish tissue (Rawn et al., 2010). In addition, pyrethrin residues were detected at extremely low levels (order of 10^{-3} ppm) in soil and water samples one month after treatment with pyrethrins at recommended rates (Antonious et al., 1997). Pyrethrins are now a leading insecticide in organic agriculture with little to no negative environmental impact (Casida and Quistad, 1995).

2.2.4. Pyrethrin extraction and determination methods

2.2.4.1. Extraction methods

Several different extraction methods have been used in pyrethrin extraction from Dalmatian pyrethrum to date (Table 1). Many of them compared different extraction methods or tested different extraction conditions affecting the pyrethrin yield in the extract.

The simplest and oldest extraction method, maceration, proved to be one of the cheapest, but requiring larger amounts of solvent, being time consuming and also the most inefficient method of pyrethrin extraction (Baldino et al., 2017; Gallo et al., 2017). Percolation was found to be a slightly more efficient method, but with the same disadvantages as maceration in terms of time and amount of solvent required (Nagar et al., 2015).

The Soxhlet or Soxtec method (SOX), used in the earliest studies of pyrethrin content in Dalmatian pyrethrum, proved to be a simple and efficient method, but still requires a large amount of solvent, is time consuming, and also requires a higher extraction temperature that

may accelerate the degradation of pyrethrins in the sample (Otterbach and Wenclawiak, 1999; Ban et al., 2010; Nagar et al., 2015).

Modern extraction methods reduce the health risk and toxic waste associated with organic solvents used in traditional extraction methods and are more environmentally friendly. One such method is ultrasound-assisted extraction (UAE) which requires less solvent and is efficient even with ethanol (Babić et al., 2012; Rončević et al., 2014). UAE combines high pressure and high temperature to increase the solubility of pyrehrins in the solvent used. The method was optimized for the extraction of pyrethrins considering the type and amount of solvent, temperature and duration of extraction, and sample size (Babić et al., 2012). When compared, no significant differences were found between SOX and UAE, but UAE seems to be more reproducible (Otterbach and Wenclawiak, 1999; Ban et al., 2010).

Supercritical fluid extraction (SFE) is an extraction method based on the use of CO_2 as a supercritical solvent (the supercritical state of CO_2 is reached at pressure of 7 MPa and a temperature above 31 °C), which eliminates the need for organic solvents (Pan et al., 1995). In addition, CO_2 can be completely removed and recycled, making this method the most environmentally friendly. The disadvantage is the need for expensive equipment and high pressure, which increases the cost of the method. Another advantage of this method is the shorter extraction time compared to methods such as UAE and SOX, which reduces possible degradation of pyrethrins during longer extraction processes (Otterbach and Wenclawiak, 1999).

Since pyrethrins start to degrade at temperatures above 60 °C and the optimal temperature for their extraction is between 20 and 40 °C, the development of extraction methods that can be carried out at room temperature and are environmentally friendly is more than welcome (Baldino et al., 2017). One of these methods is rapid solid–liquid dynamic extraction (RSLDE), which is based on cyclic pressure changes of the solvent in contact with the solid sample. This method has low energy cost and better performance compared to maceration (Gallo et al., 2017).

Most recently, the matrix-solid phase dispersion extraction (MSPD) method was optimized for the extraction of six pyrethrin compounds (Biošić et al., 2020). The method is based on disruption and dispersion of the sample by mixing it with solid support material (sorbent), which is then eluted with solvent, as seen in Figure 6 (Barker, 2007). The advantages of this method is shorter extraction time, no need for expensive equipment, and extraction at room temperature using the same solvent volume and amount of sample as in UAE (Babić et al., 2012; Grdiša et al., 2013).

In addition, different extraction methods have been used in the extraction of pyrethrin residues from various foods such as fruits and vegetables, including homogenization (Peruga et al., 2013), shaking with solvent (Caboni et al., 2005) and QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) (Anastassiades et al., 2003).

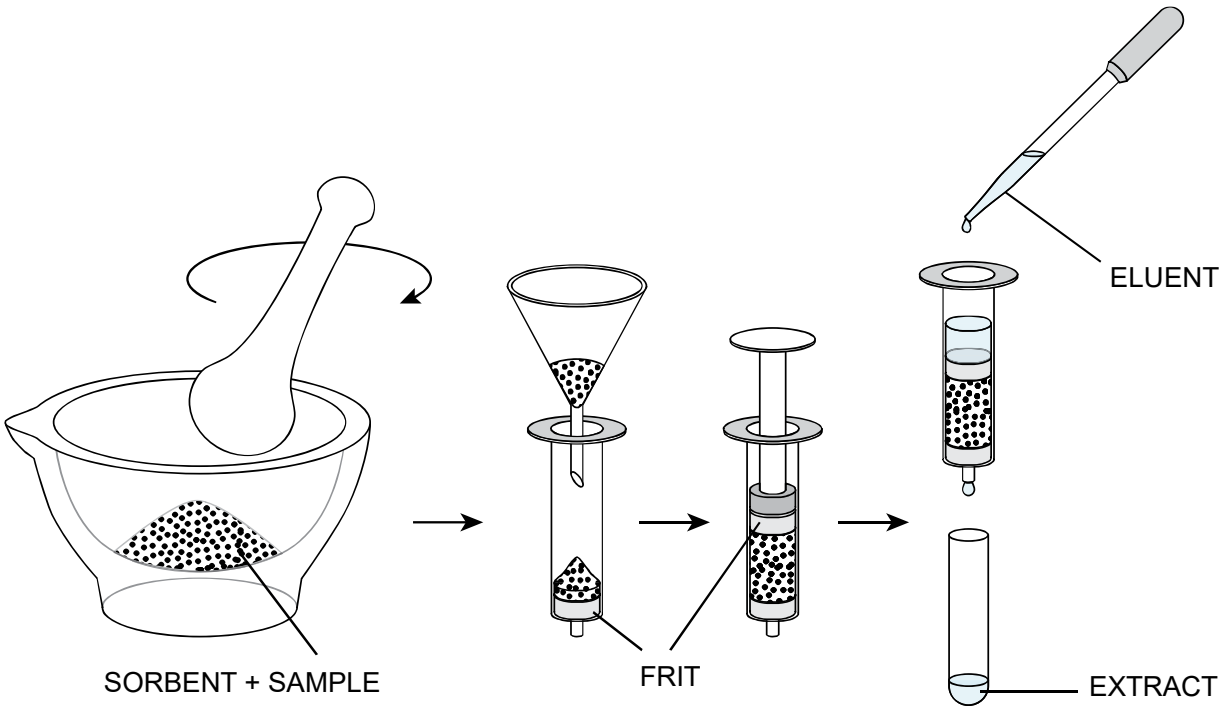


Figure 6. Basic steps in Matrix solid phase dispresion extraction method (Author: Mario Primorac).

Table 1. Comparison of the different extraction methods applied for the analysis of pyrethrin content in the dried flowers

Extraction method	Complexity	Research	Pyrethrum source	Sample size	Solvent used	Conditions applied	Pyrethrin content
Maceration	low	Pan et al., 1995	n/a	50 g	hexane (200 mL)	20 °C / 300 min stirrer	1.1%
		Ambrožič Dolinšek et al., 2007	Croatia	n/a	petroleum ether	40-60 °C	1.1%
		Gallo et al., 2017	Italy	50 g	ethanol (500 mL)	20 °C / 4 days	1.14%
		Baldino et al., 2017	Italy	n/a	petroleum ether	20 °C / 1 day	0.9%
Percolation	low	Morris et al., 2006	Tasmania	0.2 g	hexane (9.8 mL)	n/a	2.39%
		Nagar et al., 2015	India	10 g	acetonitrile (100 mL)	20 °C / 300 min	0.62%
Soxhlet	low	Otterbach and Wenclawiak, 1999	Germany	1 g	isopropanol (50 mL)	60 min	0.91-1.04%
		Kiriamiti et al., 2003	Kenya	n/a	hexane	n/a	1.14%
		Ban et al., 2010	Croatia	1 g	petroleum ether	135 °C / 80 min	1.1%
		Nagar et al., 2015	India	10 g	acetonitrile (100 mL)	8h	1.08%
Ultrasound extraction	moderate	Kasaj et al., 1999	Albania	1 g	hexane (3x 10 mL)	30 °C / 150 bar / 120 min	0.93%
		Otterbach and Wenclawiak, 1999	Germany	1 g	isopropanol (10 mL)	60 min	0.94-1.13%
		Ban et al., 2010	Croatia	1 g	ethanol (15 mL)	30 °C / 150 rpm / 60 min	1.21%
		Babić et al., 2012	Croatia	0.25 g	acetone (5 mL)	50 °C / 60 min	1-1.2%
		Grdiša et al., 2013	Croatia	0.25 g	acetone (5 mL)	50 °C / 60 min	0.36 - 1.3%
		Rončević et al., 2014	Croatia	3 g	ethanol (10 mL)	60 min	1.58%
		Nagar et al., 2015	India	10 g	acetonitrile (100 mL)	30 °C / 120 min	0.92%
Supercritical fluid extraction	high, more suitable for industrial scale	Pan et al., 1995	n/a	200 g	CO_2	40 °C / 80 bar	1.95%
		Otterbach and Wenclawiak, 1999	Germany	1 g	CO_2	100 °C / 300 bar / 40 min	0.78-0.94%
		Kiriamiti et al., 2003	Kenya	50 g	CO_2	20 °C and 40 °C / 100 bar	0.9%
		Marongiu et al., 2009	Italy	n/a	CO_2	40 °C / 300 bar	1.1%
		Baldino et al., 2017	Italy	200 g	CO_2	40 °C / 90 bar / 80 min	1.34%
Rapid solid-liquid dynamic extraction	low	Gallo et al., 2017	Italy	50 g	hexane	20 °C / 6-9 bar / 120 - 210 min	1.27%
Matrix-solid phase extraction	low, simultaneous extraction and clean-up	Biošić et al., 2020	Croatia	0.25 g	ethyl-acetate (5 mL)	20 °C / 5 min	0.45-0.59%

2.2.4.2. Determination methods

In earlier studies, before the development of reliable, precise, and accurate analytical methods, pyrethrin content was reported as ‘total pyrethrins’ or ‘total pyrethrins I’ and ‘total pyrethrins II’ because six pyrethrin compounds are similar in structure, making them difficult to separate. However, to date, different types of chromatographic methods have been used to separate and quantify pyrethrins in pyrethrum extract (Jeran et al., 2020).

Gas chromatography (GC) is generally faster, but the high temperatures generated at the capillary column convert pyrethrins to isopyrethrins, making analysis difficult. GC with flame ionization detector (GC-FID) has been used to quantify pyrethrins (Moorman and Nguyen, 1997) and gas chromatography–mass spectrometry (GC-MS) has been used to identify the structures of six pyrethrin compounds (Pan et al., 1995; Baldino et al., 2017).

High performance liquid chromatography (HPLC), on the other hand, has been shown to be more accurate in the determination of pyrethrin compounds without interference in the chromatograms due to thermal degradation (Wang et al., 1997). Normal-phase high-performance liquid chromatography (NP-HPLC), the first HPLC method used in pyrethrin quantification, had certain drawbacks such as the lack of baseline separation for all pyrethrin compounds and the longer run time (Bushway, 1985; McEldowney and Menary, 1988). An improved analytical NP-HPLC method was later developed for routine analytical analysis in the pyrethrum production industry (Essig and Zhao, 2001a) and has also been used in scientific research (Kiriamiti et al., 2003). In most studies, reversed-phase high-performance liquid chromatography (RP-HPLC) was used for pyrethrin quantification (Wang et al., 1997; Kasaj et al., 1999; Ban et al., 2010; Grdiša et al., 2013; Rončević et al., 2014).

Other chromatographic methods used in pyrethrin quantification include supercritical fluid chromatography with flame ionization detection (SFC-FID) (Wenclawiak et al., 1998), micellar electrokinetic chromatography (MEKC) (Henry et al., 1999), centrifugal partition chromatography (CPC) (Wong and Glinski, 2017), and high-speed counter-current chromatography (HSCCC) (Lu et al., 2020).

2.3. Molecular research of *T. cinerariifolium*

While there is a wealth of research on the morphology and pyrethrin content of Dalmatian pyrethrum, much of it for breeding purposes, far fewer molecular studies have been conducted with *T. cinerariifolium* as the focus. One of the earliest studies attempted to elucidate the relationship between the degree of ploidy and certain morphological traits of Dalmatian pyrethrum in order to easily distinguish diploids from triploids based on morphology (Ottaro, 1977). More recent studies have mainly focused on the identification of candidate genes involved in the biosynthesis of pyrethrin compounds, such as the chrysanthemyl diphosphate synthase gene (Rivera et al., 2001; Liu et al., 2012; Tang et al., 2012; Sultana et al., 2015) and genes involved in the MEP pathway (Khan et al., 2017). A study focused on the variation of tandem repeats in subtelomeres between individuals of this species showed high polymorphism of subtelomeres based on detailed FISH (fluorescence *in situ* hybridization) analysis (Mlinarec et al., 2019). A draft genome of the species was also constructed (7.1 Gb), revealing some genes possibly encoding enzymes specific for pyrethrin biosynthesis (Yamashiro et al., 2019).

To date, only one study has been conducted that focused on the genetic diversity and structure of natural populations. In this comprehensive study (20 populations sampled along the Croatian Adriatic coast), based on amplified fragment length polymorphism (AFLP) markers, researchers discovered high gene diversity and number of private alleles in northern Adriatic populations, which gradually decreased towards the south, most likely due to historical overexploitation (Grdiša et al., 2014).

3. MATERIALS AND METHODS

3.1. Ecogeographical survey and seed sampling

Seed samples of Dalmatian pyrethrum were collected from 10 locations in Croatia during the summer periods of 2016 and 2017 (Figure 7). Sampling sites were evenly distributed across the species' distribution range in Croatia, considering both mainland and island populations, different habitat types and altitudes (Figure 8).

Figure 7. Seed sampling of Dalmatian pyrethrum in August of 2017 on island of Cres

All specimens collected were preserved and are kept in the Collection of Medicinal and Aromatic Plants at the Department of Seed Science and Technology of the University of Zagreb Faculty of Agriculture. Voucher specimens were also deposited in the Herbarium of the Faculty of Agriculture. A detailed description of each population, including the sampling sites, geographic coordinates, accession numbers assigned to specimens in Croatian Plant Genetic

Resources Database (https://cpgrd.hapih.hr), and herbarium IDs assigned to herbarium specimens in the Herbarium of the Faculty of Agriculture are presented in Table 2.

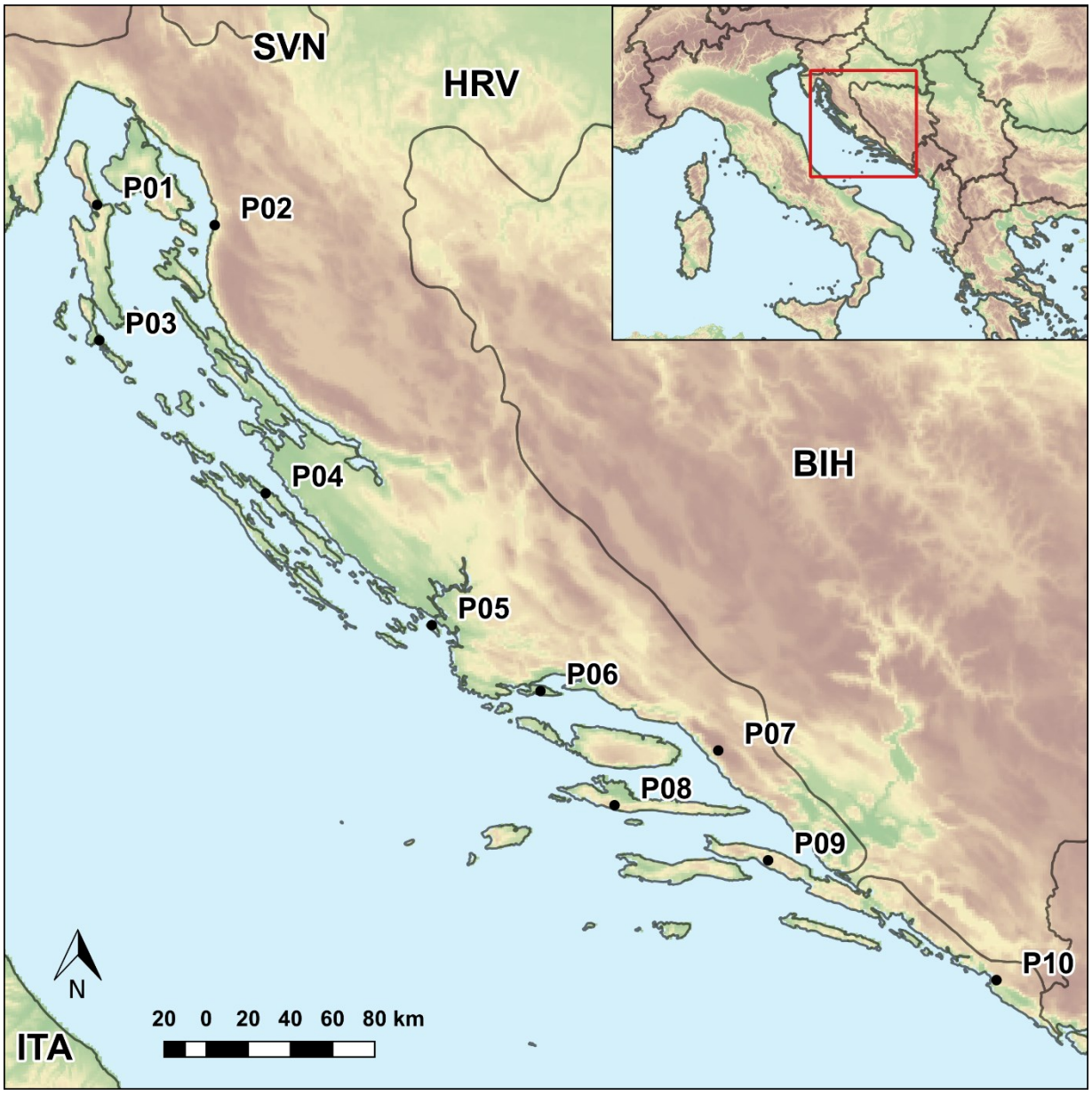


Figure 8. Map of sampled populations of *Tanacetum cinerariifolium*. Detailed description of populations is given in Table 2.

Table 2. Detailed description of Dalmatian pyrethrum sampling sites

Accession Number[a]	Herbarium ID[b]	Population ID	Location	Latitude[c]	Longitude
MAP02797	ZAGR 47780	P01	Cres	4984718	335244
MAP02799	ZAGR 47775	P02	Senj	4977030	375067
MAP02814	ZAGR 47681	P03	Mali Lošinj	4939164	335134
MAP02800	ZAGR 47680	P04	Ugljan	4886124	390659
MAP02813	ZAGR 47779	P05	Zlarin	4840339	447073
MAP02807	ZAGR 47778	P06	Čiovo	4817558	484365
MAP02809	ZAGR 47777	P07	Biokovo	4797236	545876
MAP02806	ZAGR 47776	P08	Hvar	4778173	509987
MAP02803	ZAGR 47774	P09	Pelješac	4759390	563466
MAP02769	ZAGR 47773	P10	Konavle	4718925	643500

[a]Accession number from The Collection of Medicinal and Aromatic Plants, as available at the CPGRD (https://cpgrd.hapih.hr); [b]ID number assigned to herbarium specimens in the Herbarium of the Faculty of Agriculture (http://herbarium.agr.hr); [c]Latitude and longitude are expressed in HTRS96 coordinate reference system

3.2. Field trial and sampling

Seedlings of Dalmatian pyrethrum were grown in the greenhouse of the Department of Seed Science and Technology of the University of Zagreb Faculty of Agriculture (Figure 9a). In October 2017, the seedlings were planted at the experimental field station of the Institute for Adriatic Crops and Karst Reclamation in Kaštel Stari near Split (Figure 9b). The field experiment was laid out in the Randomized Complete Block Design. Each population was represented by 20 plants divided into two replicates, 200 plants in total. Individuals in each population were planted with spacing of 0.5 m, and with one meter between populations and replicates.

Young leaves from all 200 individuals in the field trial were sampled for molecular analyses and stored in resealable plastic bags containing silica gel (Figure 9c). The following year (June 2018), flower heads were sampled from all 200 individuals in the field experiment at the flowering stage (Figure 9d), when 3/4 of the disc florets were opened (Head, 1966; Grdiša et al., 2013). Flower heads were air dried to 10-12% moisture content and stored in sealed jars kept in a dark and cool storage room to minimize pyrethrin decomposition.

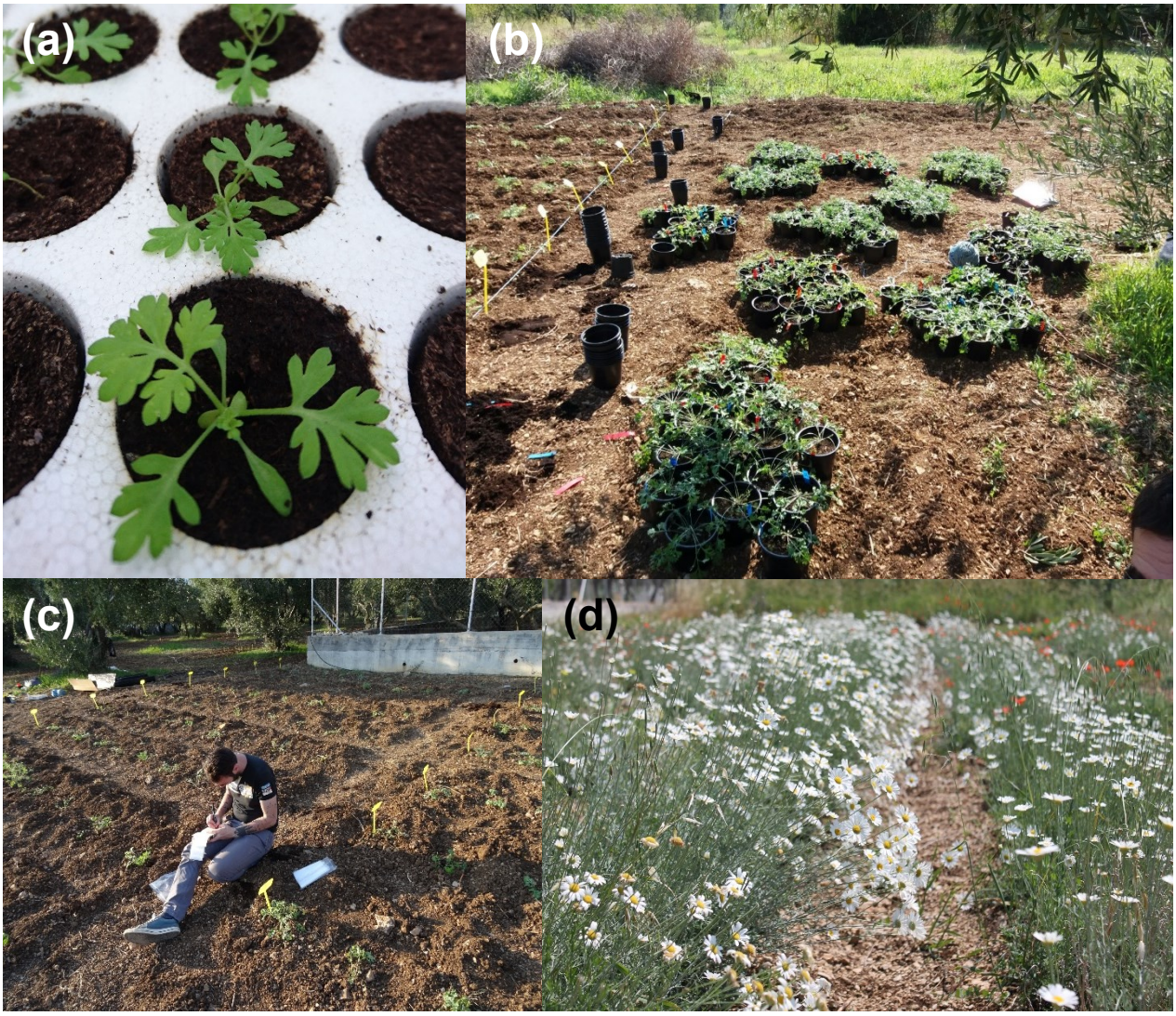


Figure 9. Steps in establishment of the field experiment: seedlings grown in greenhouse in Maksimir, Zagreb (a), setting up the field experiment in Kaštel Stari (b), sampling of plant material for molecular analyses (c), experimental field in full bloom prior to flower head sampling (d).

3.3. Chemical analyses

Extraction and quantification of pyrethrins were performed at the Department of Analytical Chemistry (Faculty of Chemical Engineering and Technology, University of Zagreb). The organic solvents used (acetone, acetonitrile, ethyl acetate, methanol, *n*-hexane) were of HPLC grade (Kemika, Zagreb, Croatia). The ultrapure water (MilliQ) used for the mobile phase was prepared using the Millipore Simplicity UV system (Millipore Corporation, Billerica, Massachusetts, USA). The standard pyrethrin mixture, Pestanal and 4'-methoxyflavanone,

which was used as an internal standard, were both purchased from Sigma-Aldrich (Steinheim, Germany). The mass fractions of pyrethrin compounds in Pestanal were: 2.57% for cinerin I, 2.06% for cinerin II, 1.73% for jasmolin I, 1.26% for jasmolin II, 26.09% for pyrethrin I and 15.97% for pyrethrin II. Florisil, polypropylene columns and polypropylene frits were purchased from Agilent (Santa Clara, California, USA).

3.3.1. Extraction of pyrethrins

Samples of flower heads were pulverized immediately before extraction using Microton MB 550 (Kinematica AG, Lucerne, Switzerland) (Figure 10). Pyrethrins were extracted from flower head samples using the recently optimized Matrix solid-phase dispersion method (Biošić et al., 2020). Florisil used as sorbent was activated at 160 °C, washed first with *n*-hexane and then with methanol, and air-dried. Powdered pyrethrum flower heads (0.25 g) were mixed with florisil (0.5 g) and anhydrous sodium sulfate (0.4 g), used as dehydrating agent, in a glass mortar with a glass pestle to completely disrupt and disperse the sample. The homogeneous mixture was then transferred to a polypropylene column with a polypropylene frit positioned at the bottom. The mixture was covered with the second frit and compressed to avoid unwanted channels. The columns were then placed on the SPE vacuum manifold (Visiprep™24, Supelco) with the flow set to 1 mL min^{-1} (Figure 11). The mixture was eluted with 5 mL of acetone-ethyl acetate 1:1 (*v/v*). The eluates were then evaporated at 40 °C by rotary evaporation and redissolved in 900 µL acetonitrile-water 1:1 (*v/v*) and 100 µL 4'-methoxyflavanone as internal standard.

Figure 10. Microton MB 550 used for pulverizing flower heads of Dalamatian pyrethrum.

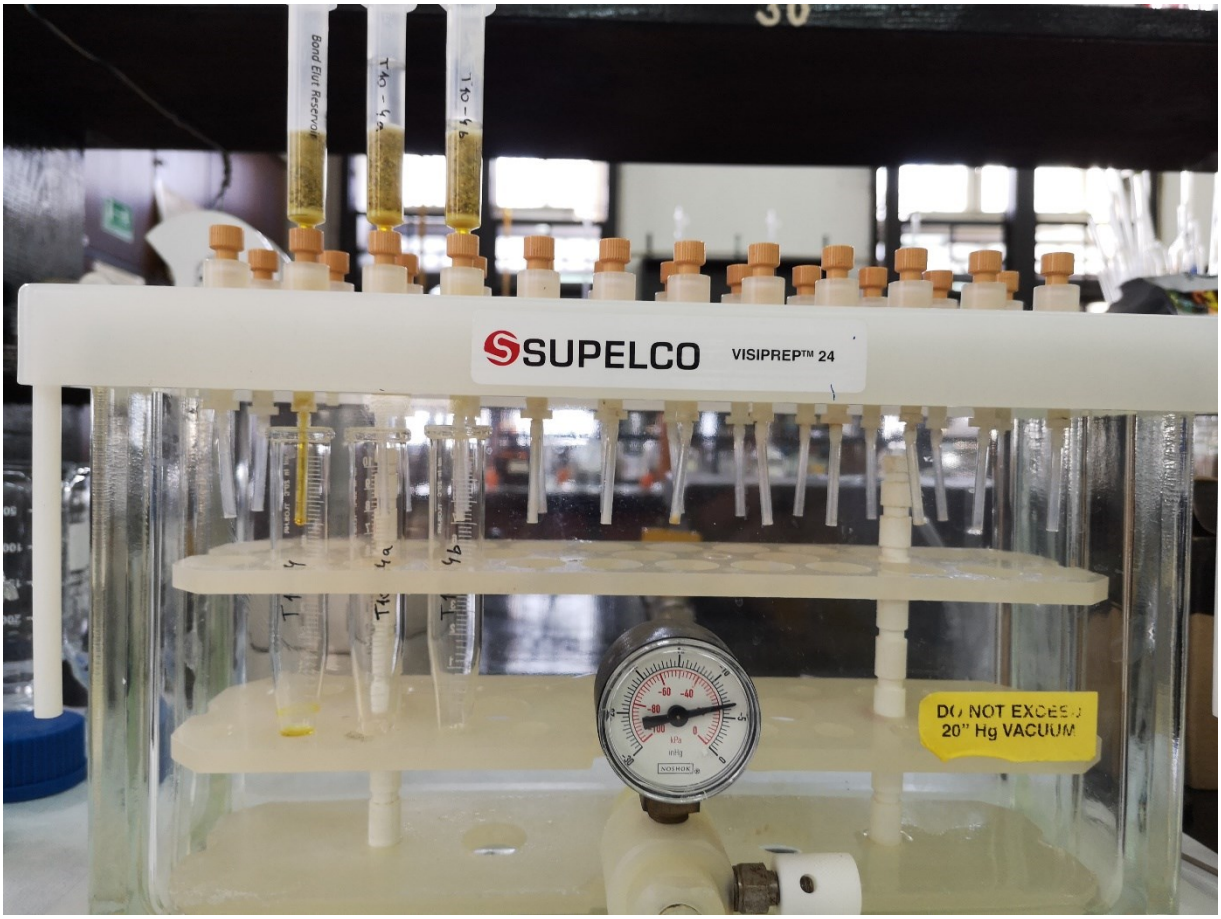


Figure 11. Extraction of pyrethrins using the SPE vacuum manifold.

3.3.1. Chromatographic analysis

High performance liquid chromatography with diode array detector (HPLC-DAD) was performed to determine and quantify the six pyrethrin compounds and total pyrethrin content using Varian ProStar 500 system (Walnut Creek, California, USA) which consists of ProStar 410 autosampler, ProStar 230 tertiary pump system and ProStar 330 diode array detector (Figure 12). A Luna C18 column 250 x 4.6 mm with a particle size of 5 µm (Phenomenex, California, USA) was used to separate the pyrethrin compounds. MilliQ water containing 0.1% formic acid (eluent A) and acetonitrile containing 0.1% formic acid (eluent B) were used as the mobile phase. A gradient elution was adopted with a constant flow rate of 1.4 mL min^{-1} (Table 3).

Table 3. Elution gradient used in HPLC

Time/minutes	Eluent A	Eluent B
0-15	40%	60%
15-25	decrease 20%	increase 80%
25-45	20%	80%
45-50	increase 40%	decrease 60%

Figure 12. Varian ProStar 500 HPLC system used in quantification of extracted pyrethrins.

All samples were injected three times and the injection volume was 10 µL. Calibration was performed using internal standard method. A stock solution of Pestanal in acetonitrile (5 mg mL^{-1}) was prepared and further diluted to prepare working solutions with gradient concentrations (4, 3, 2, 1, 0.75, 0.5 and 0.25 mg mL^{-1}). To each of the solutions, 0.1 mL of internal standard 4'-methoxyflavanone was added. The prepared solutions were stored in the dark at 4 °C until use. Pyrethrin compounds were monitored by their absorbance at 225 nm. Chromatographic data were collected using Varian ProStar 360 Star Chromatography WorkStation version 5.5. For each sample, the area of the chromatographic peak (A) and retention time (t_R) were compared to those recorded in the stock solution to identify each pyrethrin compound.

3.4. Development of microsatellite markers for *T. cinerariifolium*

DNA isolation and molecular analyses were performed in the Laboratory of Genetic Diversity, Phylogeny and Molecular Systematics of Plants at the Faculty of Science, University of Zagreb.

3.4.1. DNA isolation for NGS

For next generation sequencing (NGS), a single plant of Dalmatian pyrethrum (from the seed sample of P08 Hvar) was grown in the Botanical Garden of the Faculty of Science, University of Zagreb. The DNA for NGS was isolated using the OmniPrep™ for Plant kit, following the standard protocol with minor modifications:

1. A sample of fresh plant tissue (100 mg) was frozen in liquid nitrogen and ground to a fine powder using the TissueLyser II (Qiagen®).
2. Finely ground plant tissue was added to a microcentrifuge tube containing 500 µL Genomic Lysis Buffer to which 1% 2-mercaptoethanol and 1% polyvinylpyrrolidone (PVP) were added.
3. 5 µL proteinase K solution was added to the mixture to digest difficult-to-handle tissue and improve yield.
4. The sample was incubated at 65 °C for 10 minutes, inverting periodically.
5. 200 µL of chloroform - isoamyl alcohol mixture (24:1) was added after cooling to room temperature and mixed by inverting the tube several times.
6. The sample was centrifuged at 14,000 x g for 10 minutes and the upper phase was carefully removed to a clean microcentrifuge tube.
7. Steps 5 and 6 were then repeated.
8. 50 µL of the DNA Stripping Solution was added to the sample and inverted several times to mix. The sample was then incubated at 60 °C for 5-10 minutes.
9. 100 µL of Precipitation Solution was added to the sample to remove polysaccharides and mixed by vortexing at maximum speed for 20 seconds, after which a white precipitate formed.
10. The sample was centrifuged at 14,000 x g for 5 minutes.
11. The supernatant was transferred to a clean tube and 2 µL of Mussel Glycogen was added as DNA carrier to increase DNA recovery. The genomic DNA was precipitated using 500 µL isopropanol and inverting the tube 10 times.

12. The DNA floating in the solution was collected using a micropipette with a hooked tip and transferred to a new tube filled with 70% ethanol. The tube was inverted several times to wash the DNA.
13. The DNA was collected again and transferred to a new empty tube. The tube was inverted on a clean, absorbent surface for 15 minutes to allow excess ethanol to drain.
14. 50 µL of Tris-EDTA (TE) buffer was added to the formed pellet and incubated for 15 minutes to rehydrate it, after which 1 µL of LongLife™ RNaseA was added.
15. The DNA was stored at -20 °C until use.

The concentration and purity of the isolated DNA was measured using the NanoPhotometer P300 spectrophotometer (Implen, Munich, Germany) and Qubit™ Fluorometer (Invitrogen, Carlsbad, USA).

3.4.2. DNA isolation for testing microsatellite loci

Total genomic DNA of samples for testing the microsatellite loci and molecular analyses was isolated from 25 mg of silica-dried plant leaf tissue using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich®, Steinheim, Germany) as follows:

1. 25 mg of dried plant tissue was frozen in liquid nitrogen and ground to a fine powder using the TissueLyser II (Qiagen®).
2. 350 µL of Lysis Solution A and 50 µL of Lysis Solution B were added to the tube and thoroughly mixed using a vortexer (GVLab- Gilson®). The mixture was incubated at 65 °C for 10 minutes, inverting occasionally to dissolve the precipitate.
3. 130 µL of Precipitation Solution was added to the mixture, mixed completely with the vortexer and, placed on ice for 5 minutes. The sample was centrifuged at 12,000 x g for 5 minutes to pellet the cell debris, proteins, and polysaccharides.
4. 400 µL of the supernatant was pipetted onto a GenElute Filtration column and centrifuged for 5 minutes to remove any cell debris that was not removed in the previous step.
5. 700 µL of Binding Solution was added directly to the flow-through liquid from the previous step and thoroughly mixed by inversion.
6. 500 µL of Column Preparation Solution was added to a prepared binding column and centrifuged at 12,000 x g for 1 minute.

7. 700 µL of the mixture from step five was pipetted onto the column prepared in the previous step and centrifuged at 12,000 x g for 1 minute. The flow-through liquid was discarded and the remaining lysate from step five was applied to the column. Centrifugation was repeated, after which the flow-through liquid and collection tube were discarded.
8. The binding column was placed in a fresh collection tube and 500 µL of the diluted Wash solution (Wash Solution concentrate with ethanol added) was applied. The tube was centrifuged at 12,000 x g for 1 minute. The flow-through liquid was discarded.
9. 500 µL of additional diluted Wash Solution was applied to the column and centrifuged at 12,000 x g for three minutes to dry the column. The collection tube containing the flow-through liquid was discarded and the binding column was placed in a new collection tube and left open for five minutes.
10. 100 µL of Elution Solution prewarmed to 65 °C was pipetted into the column, left closed for five minutes and then centrifuged at 12,000 x g for 1 minute. The column was discarded and the eluate containing pure genomic DNA was stored at -20 °C until use.

The concentration and purity of the isolated DNA was measured using the NanoPhotometer P300 spectrophotometer (Implen, Munich, Germany).

3.4.3. Development of microsatellite primers

The sequencing library was prepared by random fragmentation of the DNA sample, followed by 5' and 3' adapter ligation using the TruSeq DNA PCR Free kit (Illumina®, San Diego, California, USA). Fragmentation was verified using the High Sensitivity DNA kit on the Agilent 2100 Bioanalyzer (Agilent Technologies®, Santa Clara, California, USA). The library was sequenced using the Illumina NovaSeq6000 sequencer at Macrogen Europe®.

The FastQC tool (Andrews, 2010) was used to check the quality of the raw sequences and the possible presence of the sequence adapters. Two approaches were used in the development of microsatellite markers for *T. cinerariifolium*, (1) based on contigs from *de novo* assembly and (2) based on contigs from overlapping paired-end reads data.

The first approach was based on *de novo* assembly of 20% of the total sequencing data subsampled using the reformat.sh converter, part of the BBMap/BBTools package (Bushnell et al., 2017). *De-novo* assembly was performed using the *De-Novo* Assembly algorithm of the

CLC Genomics Server ver.20.0.2 (Qiagen Bioinformatics) with default parameters and high similarity fraction (0.95) and mapping option ON.

In the second approach, evidence of contiguity from paired end reads were used. The complete dataset was searched for pairs with overlapping evidences using the bbmerge tool from the BBMap/BBTools package, which were then deduplicated using the dedupe tool from the BBMap/BBTools package (Bushnell et al., 2017). The obtained contigs were re-mapped with the full set of sequencing data using the Map Reads to Contigs tool from CLC Genomics Server to obtain coverage information. Screening for possible repeat associated annotations of sequences was performed using RepeatMasker ver. 4.1.0 against combined Dfam 3.1 and RepBase (20170127) element databases (Smit et al., 2015).

Sequences obtained from both approaches were combined. To find positions of microsatellite repeats in the contigs, the MISA tool script (Thiel et al., 2003) was used with search parameters for motif length of 2-6 nucleotides and with defined minimum microsatellite repeat lengths (2-8, 3-6, 4-5, 5-4, 6-4). The sequences were further checked against the draft genome of *T. cinerariifolium* (Yamashiro et al., 2019) for a set of occurrences using the locally installed BLAST 2.10.1+ executable (Zhang et al., 2000).

The list of sequences was filtered for further *in vitro* testing using the following selection criteria: (1) loci with compound microsatellite repeats were removed, (2) sequences with transposon occurrences were removed, (3) reads mapped with multi-mapping occurrences were removed, (4) sequences with an average coverage of raw reads close to the sequencing coverage and a length of more than 130 nucleotides were considered, (5) sequences with one or two occurrences in the draft genome of *T. cinerariifolium* were considered, (6) dinucleotide and trinucleotide motifs with longer repeats were preferred, and (7) sequences with guanine-cytosine (G-C) content closer to the G-C-content of the genome (43 +/-10 %) were preferred. To locate transposon occurrences in the sequences RepeatMasker (Smit et al., 2015) was used. Sequences were checked against the draft genome using the locally installed BLAST 2.10.1+ executable (Zhang et al., 2000). Sequences that met the listed requirements were used for primer design using Primer3 (Koressaar and Remm, 2007; Untergasser et al., 2012) with the parameter ‘one primer for each seq’ to obtain a single primer pair for each locus.

3.4.4. Microsatellite marker testing

The designed microsatellite primers were first tested on five samples from different populations (P01 Cres, P02 Senj, P05 Zlarin, P07 Biokovo and P10 Konavle). Polymorphic microsatellite markers with high amplification rate were further tested on all 20 DNA samples from population P08 Hvar using a tailed primer protocol (Schuelke, 2000). The 20 µl of reaction mix contained 2 pmol of tailed forward SSR primer, 8 pmol of reverse SSR primer, 8 pmol of FAM labeled M13 primer (5'-TGTAAAACGACGGCCAGT-3), 1 x polymerase chain reaction (PCR) buffer, 4 pmol of each dNTP, 0.5 U Taq™ HS DNA polymerase (Takara® Bio Inc., Shiga, Japan), and 5 ng template DNA. A PCR protocol with an initial touchdown cycle (94 °C for 5 minutes; 5 cycles of 45 seconds at 94 °C, 30 seconds at 60 °C, which was lowered by 1 °C in each cycle, and 90 seconds at 72 °C; 25 cycles of 45 seconds at 94 °C, 30 seconds at 55 °C, and 90 seconds at 72 °C; and 8-minutes extension step at 72 °C) was used (Radosavljević et al., 2011). Fluorescently labeled PCR products were detected on an ABI 3730XL (Applied Biosystems®, Foster City, California, USA) by the service Fragment Analysis (Macrogen Europe®). Allele sizes of PCR products were estimated using GeneMapper 4.0 software (Applied Biosystems®, Foster City, California, USA).

3.5. Molecular analyses

Isolation of DNA from samples of 10 natural populations of *T. cinerariifolium* (200 in total) was performed following the same protocol used to isolate DNA for testing newly developed microsatellite loci. The analysis based on 12 developed microsatellite markers was performed using the same PCR protocol used for testing the microsatellite loci (described in Chapter 3.4.4).

3.6. Statistical analyses

3.6.1. Statistical analyses of chemical data

3.6.1.1. Descriptive statistics, analysis of variance and correlations

Univariate analysis of variance (ANOVA) for six pyrethrin compounds, total pyrethrin content, and pyrethrin I/pyrethrin II ratio was performed in SAS Studio 3.8 (SAS Institute, Cary North Carolina, USA) using the PROC MIXED procedure (SAS Institute, 2004). Post-hoc comparisons of population means were performed using Tukey's studentized range test at $P < 0.05$. Before performing the analysis, variables were normalized using the arcsine transformation:

$$y = \arcsin(\sqrt{x})$$

Pearson correlation coefficients were calculated for six pyrethrin components and total pyrethrin content using the PROC CORR procedure in SAS. Coefficient of variability was calculated for each pyrethrin compound and total pyrethrin content for all populations in the sample:

$$CV\ (\%) = \frac{StDev}{Mean} \times 100$$

3.6.1.2. Principal component analysis

Principal component analysis (PCA) based on six pyrethrin components was performed using the PROC PRINCOMP procedure in SAS. The cumulative proportion of variance explained, and the eigenvalues of the principal components were used to determine the number of principal components using the Kaiser criterion and the Scree plot (Cattell, 1966). The biplot was constructed, with individuals represented by points and pyrethrin compounds represented as vectors.

Based on the scores of the first three principal components, Euclidean distances between populations were calculated representing biochemical distance between sampled populations in PAST v. 3 (Hammer et al., 2001).

3.6.1.3. Cluster analysis and analysis of variance

The scores of the first two principal components were used in cluster analysis (CA). The unweighted pair group method with arithmetic mean (UPGMA) was applied using the PROC CLUSTER procedure in SAS. To determine the optimal number of clusters, the Cubic Clustering Criterion (*CCC*) statistic and the pseudo-F (*PSF*) statistic were calculated. Based on the cluster analysis results, individuals were classified into distinct chemotypes.

Again, ANOVA was performed for six pyrethrin compounds, total pyrethrin content and pyrethrin I/pyrethrin II ratio in SAS using the PROC MIXED procedure. Post-hoc comparisons of chemotype means were performed using Tukey's studentized range test at $P < 0.05$. Before performing the analysis, variables were normalized using the arcsine transformation.

3.6.1.4. Spatio-ecological analyses

Based on the geographic coordinates of the sampled populations, the geographic distance matrix was calculated using the distGeo function from the geosphere package (Karney, 2013) in RStudio v. 1.1.456 (RStudio Team, 2016). The Mantel test was used to examine the relationship between the geographic distance matrix (ln km) and the biochemical distance matrix. Significance was estimated based on 10,000 permutations in NTSys software v. 2.10s (Rohlf, 2000).

Environmental conditions of the sampling sites were described by 25 spatio-ecological variables (Table 4). Eleven temperature and 8 precipitation related variables were calculated from data from Croatian Meterological and Hydrological Service (https://meteo.hr) following O'Donnell and Ignizio (2012). Monthly solar radiation data were obtained from the WorldClim database (www.worldclim.org) and average annual solar radiation was calculated. Three soil variables were obtained from the SoilGrids database (https://soilgrids.org), altitude was extracted from EU-DEM v1.1 (https://land.copernicus.eu) and distance to the shoreline was calculated for all sampled locations. Extraction of data from spatial layers and calculation of distance from coastline were performed using QGIS v3.10.7 (QGIS, 2020). Pearson correlation coefficients were calculated between six pyrethrin components and total pyrethrin content and 25 spatio-ecological variables using the PROC CORR procedure in SAS

Principal component analysis (PCA) based on 25 spatio-ecological variables was performed using the PROC PRINCOMP procedure in SAS. The cumulative proportion of explained variance and eigenvalues of principal components were used to determine the

number of principal components using Kaiser criterion and Scree plot (Cattell, 1966). The biplot was constructed with populations represented as points and variables represented as vectors.

Based on the scores of the first four principal components, Euclidean distances between populations were calculated, representing the ecological distance between sampled populations in PAST v. 3 (Hammer et al., 2001).

The Mantel test was used to examine the relationship between the matrix of ecological distance and the matrix of biochemical distance. Significance was estimated based on 10,000 permutations in NTSys software v. 2.10s (Rohlf, 2000).

Table 4. Ranges of ecological variables based on Dalmatian pyrethrum sampling locations

Variable	Description	Unit	Min	Max	Average
BIO1	Annual Mean Temperature	ºC	8.14	17.55	15.48
BIO2	Mean Diurnal Range (Mean of monthly (max temp - min temp))	ºC	5.57	9.72	7.68
BIO3	Isothermality (BIO2/BIO7) (x 100)	ºC	24.30	32.71	29.27
BIO4	Temperature Seasonality (standard deviation *100)	ºC	587.93	671.93	636.37
BIO5	Maximum Temperature of Warmest Month	ºC	22.02	32.60	30.44
BIO6	Minimum Temperature of Coldest Month	ºC	-0.92	7.30	4.34
BIO7	Temperature Annual Range (BIO5-BIO6)	ºC	22.94	29.70	26.10
BIO8	Mean Temperature of Wettest Quarter	ºC	4.18	18.20	13.99
BIO9	Mean Temperature of Driest Quarter	ºC	-0.16	25.30	20.31
BIO10	Mean Temperature of Warmest Quarter	ºC	16.44	25.30	23.90
BIO11	Mean Temperature of Coldest Quarter	ºC	-1.47	9.13	6.55
BIO12	Annual Precipitation	mm	649.50	1681.40	1066.11
BIO13	Precipitation of Wettest Month	mm	124.00	255.40	186.41
BIO14	Precipitation of Driest Month	mm	0.00	3.20	1.04
BIO15	Precipitation Seasonality (Coefficient of Variation)	mm	54.94	78.00	67.06
BIO16	Precipitation of Wettest Quarter	mm	239.10	561.30	406.62
BIO17	Precipitation of Driest Quarter	mm	72.80	209.00	117.22
BIO18	Precipitation of Warmest Quarter	mm	72.80	268.40	147.33
BIO19	Precipitation of Coldest Quarter	mm	96.50	270.60	179.17
RAD	Average annual Solar radiation	kJ m^{-2} day^{-1}	12879.25	14372.58	13800.18
CESOL	Average Cation exchange capacity of soil	cmolc kg^{-1}	20	26.29	22.19
CLYPPT	Average Clay content (0-2 µm) mass fraction	%	29.43	37.43	34.63
ORCDRC	Average Soil organic carbon content (fine earth fraction)	g kg^{-1}	26.14	49	33.83
ALT	Altitude	m	17.35	1377.66	341.98
COAST	Distance to Shoreline	m	68.49	4344.55	1355.74

3.6.2. Statistical analyses used in the testing of developed microsatellite markers

For each microsatellite locus, the average number of alleles per locus (N_a), observed (H_E) and expected (H_O) heterozygosity, the probability of deviations from Hardy-Weinberg equilibrium, and the inbreeding coefficient (F_{IS}) were calculated using GENEPOP v. 4.4 (Rousset, 2008). Sequential Bonferroni corrections (Holm, 1979) were applied when multiple statistical tests were performed in SAS. For each locus, the frequency of null alleles (F_{null}) was calculated, and loci were analyzed for scoring errors and allelic dropout using MicroChecker v. 2.2.3 (van Oosterhout et al., 2004). Polymorphic information content (*PIC*) and the probability of identity (*PI*) were calculated using Cervus v. 3.0.7 (Kalinowski et al., 2007).

3.6.3. Statistical analyses of molecular data

3.6.3.1. Within-population diversity

For each of the 12 microsatellite markers used, the number of alleles and polymorphism information content – *PIC* (Botstein et al., 1980) were calculated using Cervus v. 3.0.7 (Kalinowski et al., 2007). Problems associated with scoring errors due to stuttering, major allele dropout, and the presence of null alleles were assessed using Micro-Checker v. 2.2.3 (van Oosterhout et al., 2004). Null allele frequencies were estimated using the expectation-maximization algorithm (Dempster et al., 1977) implemented in FreeNA software (Chapuis and Estoup, 2007).

For each population the following genetic parameters were calculated using Genepop v. 4.7 (Rousset, 2008): observed heterozygosity (H_O), expected heterozygosity (H_E), and inbreeding coefficient (F_{IS}). Potential deviations from Hardy-Weinberg equilibrium were also examined in Genepop, with significance of P-values adjusted by sequential Bonferroni corrections for multiple tests (Rice, 1989) using SAS. Allelic richness (N_{ar}) and number of private alleles (N_{pr}) of each population were estimated using FSTAT v. 2.9.4 (Goudet, 2002).

To test evidence for recent bottleneck events, a two-phase model (TPM) was run assuming 100% multistep changes and a variance of 0.36 with BOTTLENECK v. 1.2.002

(Cornuet and Luikart, 1996; Piry et al., 1999). The significance of the Wilcoxon sign-rank test for excess heterozygosity was determined after 10,000 replications.

3.6.3.2. Genetic differentiation and structure

Cavalli-Sforza's chord distances (Cavalli-Sforza and Edwards, 1967) between all population pairs were calculated and used in cluster analysis using the neighbor-joining (NJ) method (Saitou and Nei, 1987) with 1,000 bootstraps (Felsenstein, 1985) based on 12 microsatellite loci. The above calculations were performed using the GENDIST, NEIGHBOR, SEQBOOT and CONSENSE programs of the PHYLIP ver. 3.6b software package (Felsenstein, 1993).

In addition, the distance based on the proportion of shared alleles (DPSA) (Bowcock et al., 1994) between all pairs of individuals based on 12 microsatellite loci was calculated using MICROSAT (Minch et al., 1997). Cluster analysis using the neighbor-joining (NJ) method with 1,000 bootstraps was performed with the programs NEIGHBOR and CONSENSE (PHYLIP package).

Factorial correspondence analysis (FCA) was performed to visually represent the genetic relationship among natural populations of Dalmatian pyrethrum and to determine possible admixture among populations using the 'AFC sur populations' module of GENETIX v 4.05 software (Belkhir et al., 2004).

Analysis of molecular variance (AMOVA) by partitioning total microsatellite diversity between and within populations and testing variance components by randomization test with10,000 permutations was performed in Arlequin software v. 3.5 (Excoffier et al., 1992). Additionally, Φ_{ST} values between all pairs of populations were calculated with estimation of their significance.

Genetic structure was inferred using a model-based clustering method implemented in Structure v. 2.3.4 (Pritchard et al., 2000). Thirty runs per cluster (K) ranging from 1 to 11 were performed on the Isabella computer cluster at the University of Zagreb, University Computing Centre (SRCE). The runs consisted of a burn-in period of a burn-in period of 200,000 steps followed by 1,000,000 Monte Carlo Markov Chain (MCMC) replicates assuming an admixture model and correlated allele frequencies. The most likely number of clusters (K) was selected by calculating average estimates of the likelihood of the data [$ln\ P(X|K)$] and ΔK values (Evanno

et al., 2005) for each K in Structure Harvester v. 0.6.94 (Earl and von Holdt, 2012). The *ΔK* value was calculated according to the formula:

$$\Delta K = \frac{L_{K-1} + L_{K+1} - 2 \times L_K}{sd_K}$$

where L_K – average posterior probability [*ln P(X|K)*] for the number K of clusters based on multiple runs*;* L_{K-1} - average posterior probability [*ln P(X|K)*] for the number K-1 of clusters; L_{K+1} - average posterior probability *[ln P(X|K)*] for *K*+1 number of clusters; sd_K – standard deviation of posterior probabilities for K number of clusters. Runs were clustered and averaged using StructureSelector (Li et al., 2018). For each individual, the proportion of the genome originating from different clusters (*Q*) was estimated. Individuals with more than 75% of their genome originating from a single gene pool (cluster) were considered to belong to that gene pool, while individuals with membership probabilities of less than 75% for all gene pools were considered to be of 'mixed origin' (Matsuoka et al., 2002).

3.6.3.3. Spatial genetics

Pairwise F_{ST} values were calculated between population pairs and their respective P-values were estimated after 10,000 permutations using FSTAT v. 2.9.4 (Goudet, 2002). Isolation by distance (IBD) analysis (Rousset, 1997) was performed using the Mantel test between the geographic distance matrix (ln km) and the $F_{ST}/(1-F_{ST})$ matrix. Significance was estimated based on 10,000 permutations in NTSys software v. 2.10s (Rohlf, 2000).

Barriers of gene flow between 10 Dalmatian pyrethrum populations were identified by applying Monmonier's maximum difference algorithm in Barrier software v. 2.2 (Manni et al., 2004) based on Cavalli-Sforza's chord distances. Input data consisted of the geographic coordinates for each of the 10 populations, the original genetic distance matrix based on Cavalli-Sforza's chord distances, and 1,000 genetic distance matrices based on Cavalli-Sforza's chord distances created using the bootstrap method. Based on the original distance matrix the first four barriers were determined and tested for significance using the bootstrap matrices (Guan et al., 2010; Griffiths et al., 2020). Barriers with bootstrap values greater than 75% in all their segments were retained (Gagnon and Angers, 2006).

Isolation by environmental distance (IBED) analysis was performed using the Mantel test to examine the relationship between the ecological distance matrix and the $F_{ST}/(1-F_{ST})$ matrix. Significance was estimated based on 10,000 permutations in NTSys software v. 2.10s (Rohlf, 2000). A partial Mantel test was used to examine the relationship between the ecological

distance matrix and the $F_{ST}/(1-F_{ST})$ matrix, accounting for the linear correlation with the geographic distance matrix (ln km). Excluding the effect of geographic distance on genetic distance, the correlation between residual ecological distance and residual genetic distance represents isolation by environmental distance (Mendez et al., 2010).

3.6.4. Biochemical and genetic distance correlation

To analyze relationship between biochemical and genetic distance Mantel test between the biochemical distance matrix and the Cavalli-Sforza chord distance matrix was performed. Significance was estimated based on 10,000 permutations in NTSys software v. 2.10s (Rohlf, 2000).

4. RESEARCH RESULTS

4.1. Results of chemical analyses

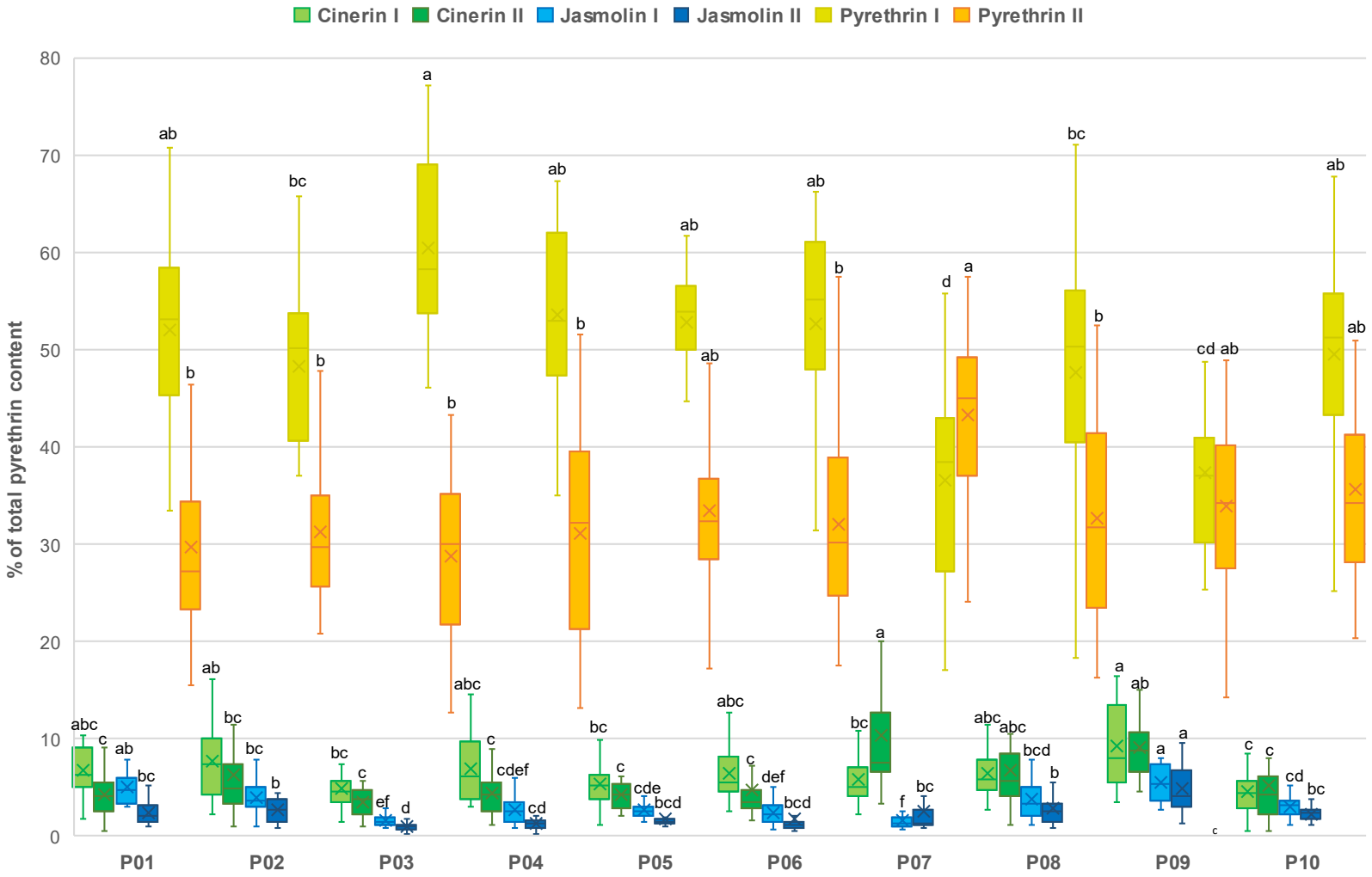
4.1.1. Pyrethrin content and composition

Identification and quantification of six pyrethrin compounds were performed by HPLC using the internal standard method. Highly significant differences ($P < 0.001$) were found between populations in all six pyrethrin compounds (Graph 1).

The lowest pyrethrin I content was observed in the individual from P06 Čiovo (12.33% of total pyrethrin content), while the highest content was observed in the individual from P03 Mali Lošinj (77.17% of total pyrethrin content). Pyrethrin II content ranged from 12.68% of total pyrethrin content in P03 Mali Lošinj to 61.12% of total pyrethrin content in P10 Konavle. In most individuals (156) pyrethrin I content was higher than pyrethrin II. The only population in which all individuals showed dominance of pyrethrin I content over pyrethrin II content was P03 Mali Lošinj. Conversely, in 75% of individuals from P07 Biokovo, pyrethrin II content was higher than pyrethrin I content.

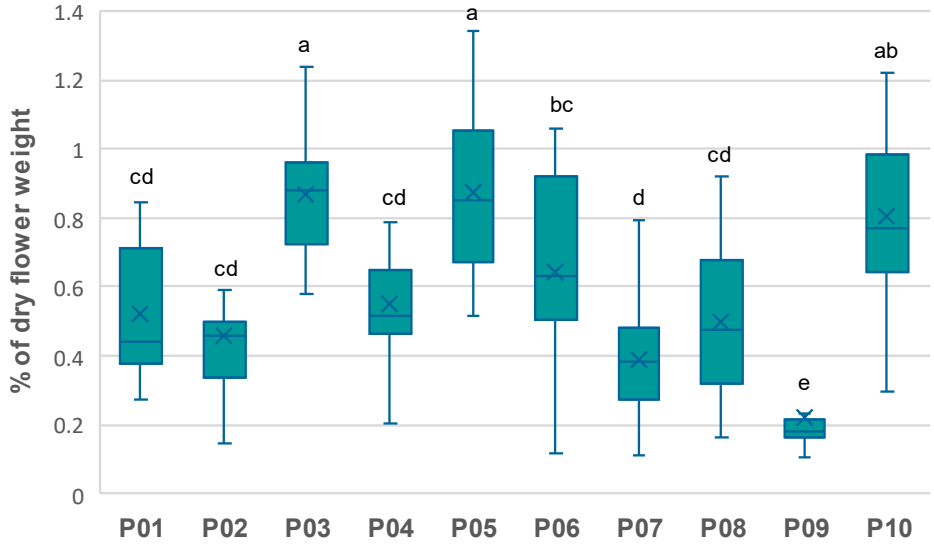
The lowest cinerin I content was observed in the individual from P10 Konavle (0.43% of total pyrethrin content), while the highest content was observed in the individual from P09 Pelješac (16.46% of total pyrethrin content). The content of cinerin II ranged from 0.43% of total pyrethrin content in P10 Konavle to 27.64% of total pyrethrin content in P07 Biokovo.

The lowest jasmolin I content was observed in the individual from P07 Biokovo (0.55% of total pyrethrin content), while the highest was observed in the individual from P01 Cres (10.94% of total pyrethrin content). The content of jasmolin II ranged from 0.22% of total pyrethrin content in P04 Ugljan to 10.57% of total pyrethrin content in P07 Biokovo.

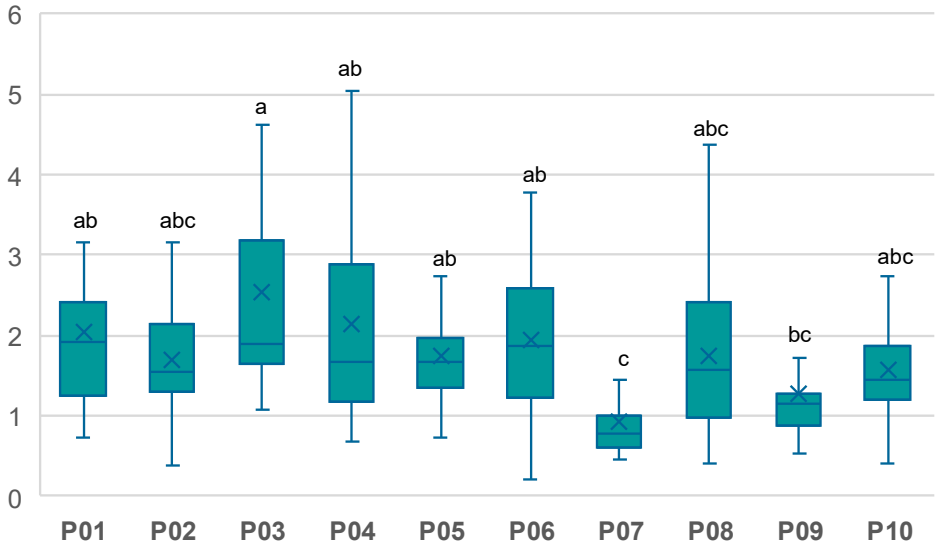


Graph 1. Content of six pyrethrin compounds in 10 natural populations of Dalmatian pyrethrum. Populations marked with the same letter are not significantly different at *P<0.05* with regards to each pyrethrin compound.

The total pyrethrin content (expressed as % of dry flower weight) ranged from 0.10% in P09 Pelješac to 1.35% in P05 Zlarin while the average total pyrethrin content across all samples was 0.58% (Graph 2). The pyrethrin I/pyrethrin II ratio ranged from 0.21 in P06 Čiovo to 5.88 in P03 Mali Lošinj (Graph 3).



Graph 2. Total pyrethrin content in 10 natural populations of Dalmatian pyrethrum. Populations marked with the same letter are not significantly different at *P<0.05*.



Graph 3. Pyrethrin I/pyrethrin II ratio in 10 natural populations of Dalmatian pyrethrum. Populations marked with the same letter are not significantly different at $P < 0.05$.

Jasmolin II showed the highest degree of variability (81.28%) and pyrethrin I the lowest (25.54%) across all samples. The population P07 Biokovo had the highest variability of pyrethrin I and the lowest variability of pyrethrin II (Table 5). In terms of total pyrethrin content, the lowest variability was found in P03 Mali Lošinj (19.93%), while P09 Pelješac had the highest (50.89%).

Table 5. Variability of natural Dalmatian pyrethrum populations based on six pyrethrin compounds and total pyrethrin content.

	Coefficient of variation						
	Cinerin I	Cinerin II	Jasmolin I	Jasmolin II	Pyrethrin I	Pyrethrin II	Total pyrethrin content
P01	38.05%	50.78%	40.67%	54.22%	20.05%	32.09%	38.34%
P02	48.70%	75.87%	45.35%	59.77%	22.96%	25.08%	41.95%
P03	38.47%	43.32%	36.10%	44.86%	16.61%	32.17%	19.93%
P04	50.55%	54.91%	54.45%	49.78%	17.74%	34.98%	25.61%
P05	43.77%	32.88%	30.93%	45.77%	13.99%	24.65%	28.46%
P06	44.09%	98.01%	51.68%	121.21%	24.16%	34.08%	44.81%
P07	49.65%	59.58%	69.99%	102.77%	29.65%	20.74%	45.91%
P08	36.33%	76.54%	53.63%	65.28%	27.15%	31.36%	44.31%
P09	47.59%	33.29%	32.93%	47.31%	21.57%	27.21%	50.89%
P10	64.22%	82.70%	41.42%	39.52%	24.69%	28.46%	30.44%
Avg	50.66%	74.44%	60.37%	81.28%	25.54%	30.53%	49.31%

Total pyrethrin content was positively correlated with pyrethrin I (r = 0.422; P < 0.001) and negatively correlated with all other pyrethrin compounds (Table 6). The highest correlation was observed between pyrethrin I and cinerin II (r = -0.818; P < 0.001).

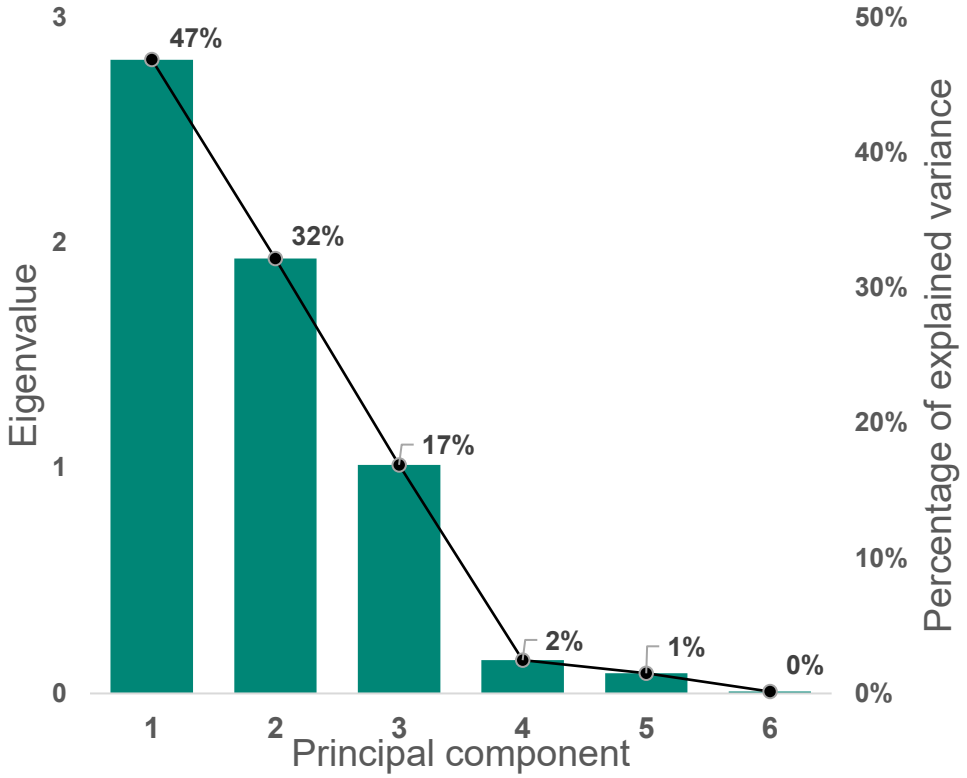
Table 6. Pearson's correlation coefficient between six pyrethrin compounds and total pyrethrin content.

Variable	Pyrethrin I	Pyrethrin II	Cinerin I	Cinerin II	Jasmolin I	Jasmolin II	Total pyrethrin content
Pyrethrin I		***	ns	***	ns	***	***
Pyrethrin II	-0.780		***	***	***	***	ns
Cinerin I	-0.024	-0.521		***	***	ns	***
Cinerin II	-0.818	0.406	0.321		ns	***	***
Jasmolin I	0.061	-0.456	0.401	-0.053		***	***
Jasmolin II	-0.592	0.237	0.093	0.451	0.460		***
Total pyrethrin content	0.422	-0.024	-0.429	-0.517	-0.369	-0.372	

[ns]non-significant; [*]significant at P < 0.05; [**]significant at P < 0.01; [***]significant at P < 0.001

4.1.2. Principal component analysis

Principal component analysis based on six pyrethrin compounds was performed on samples of *T. cinerariifolium*. The first three principal components had eigenvalues greater than 1 and explained 95.9% of the total variance (Graph 4). Pyrethrin I was significantly negatively correlated and pyrethrin II and cinerin II showed highly significant positive correlation with the first principal component. Cinerin I and jasmolin I showed strong positive correlation with the second principal component (Table 7).



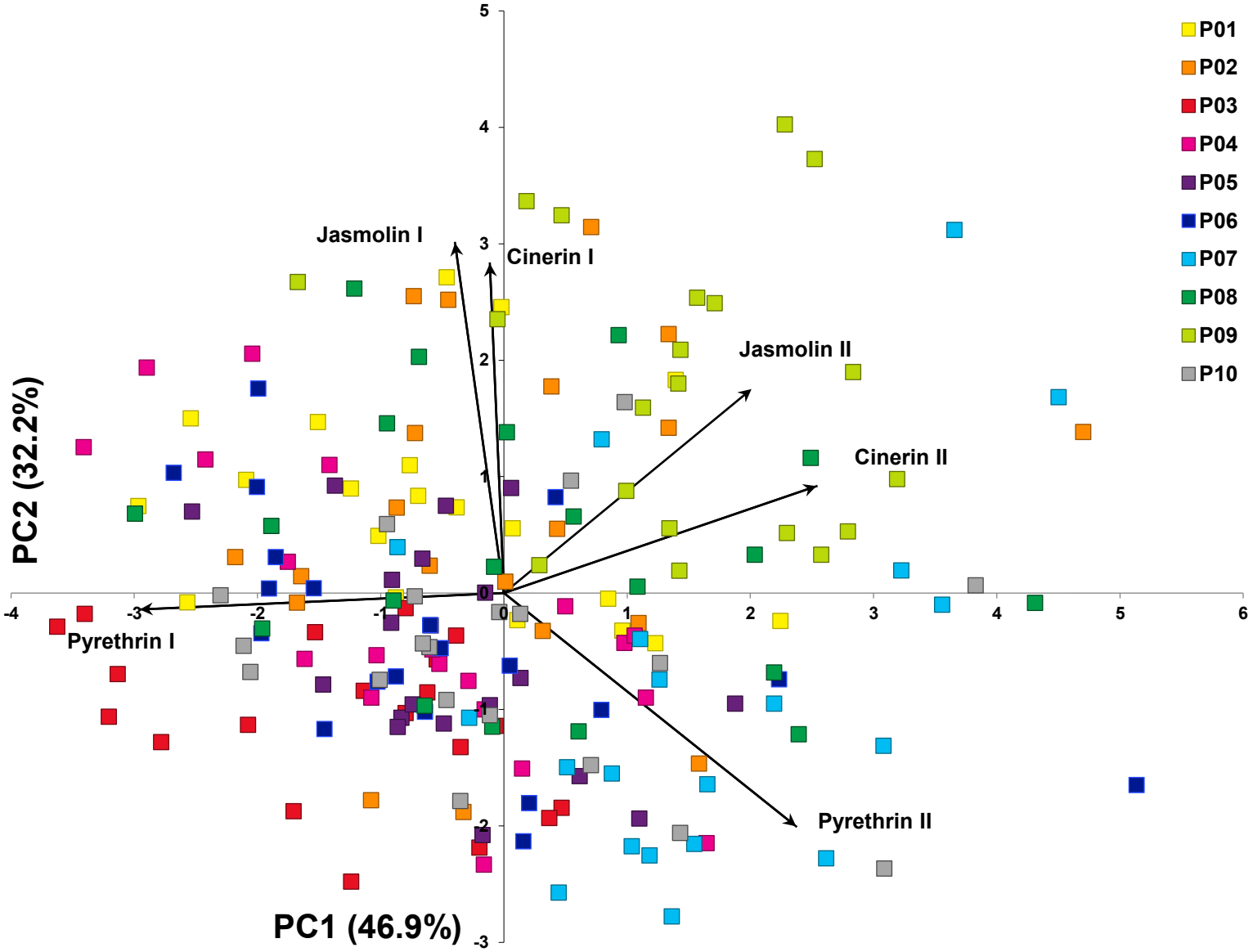
Graph 4. A scree plot of explained variance and eigenvalues for Principal components from the PCA.

The first principal component explained 46.9% of the total variance and separated individuals with high pyrethrin I content from those characterized by higher levels of cinerin II, jasmolin II and pyrethrin II. The second principal component separated individuals based on cinerin I and jasmolin I content (Graph 5).

Table 7. Pearson correlation coefficients between six pyrethrin compounds and scores of the first three Principal components

Pyrethrin compound	PC 1		PC 2		PC 3	
Cinerin I	-0.043	ns	0.800	***	-0.552	***
Cinerin II	0.817	***	0.252	***	-0.376	***
Jasmolin I	-0.105	ns	0.820	***	0.473	***
Jasmolin II	0.626	***	0.479	***	0.468	***
Pyrethrin I	-0.991	***	-0.038	ns	0.034	ns
Pyrethrin II	0.792	***	-0.561	***	0.119	ns

nsnon-significant; *significant at $P < 0.05$; **significant at $P < 0.01$; ***significant at $P < 0.001$



Graph 5. Biplot of Principal component analysis based on pyrethrin content.

4.1.1. Cluster analysis and ANOVA (chemotypes)

Cluster analysis grouped the individuals into four clusters (chemotypes) based on the values of the pseudo-F statistic and Cubic Clustering Criterion (Figure 13). The ranges of pyrethrin compounds, total pyrethrin content, and pyrethrin I/pyrethrin II ratio for each chemotype are shown in Table 8, and the geographic distribution of chemotypes is shown in Figure 14.

Table 8. Pyrethrin compounds (expressed as % of total pyrethrin), total pyrethrin content (expressed as % of dry flower weight) and pyrethrin I/pyrethrin II ratio in four chemotypes of Dalmatian pyrethrum.

		Chemotype							
		P1		P2		C1/J1		P1/P2	
n[a]		17		23		26		134	
Cinerin I	MIN	3.32		1.84		4.35		0.43	
	MAX	14.47	a*	9.76	b	16.46	c	11.74	b
	AVG	8.13		5.14		10.99		5.47	
Cinerin II	MIN	0.96		6.06		3.39		0.43	
	MAX	3.36	a	27.64	b	20.00	c	12.36	d
	AVG	2.08		12.74		8.82		4.59	
Jasmolin I	MIN	1.06		0.68		2.11		0.55	
	MAX	6.34	a	6.18	b	10.94	c	7.09	b
	AVG	3.95		2.63		6.17		2.59	
Jasmolin II	MIN	0.22		1.43		1.24		0.43	
	MAX	1.59	a	9.55	b	10.57	b	9.89	c
	AVG	0.81		4.67		4.51		1.68	
Pyrethrin I	MIN	59.66		12.33		20.91		34.78	
	MAX	77.17	a	35.74	b	57.45	c	67.82	d
	AVG	68.47		27.11		42.41		51.72	
Pyrethrin II	MIN	12.68		37.40		14.29		20.38	
	MAX	20.16	a	61.12	b	37.00	c	52.03	d
	AVG	16.56		47.71		27.10		33.95	
Total pyrethrin content	MIN	0.12		0.12		0.10		0.15	
	MAX	0.98	a	1.03	b	0.66	b	1.35	a
	AVG	0.60		0.33		0.30		0.68	
Pyrethrin I/Pyrethrin II ratio	MIN	3.16		0.21		0.62		0.68	
	MAX	5.88	a	0.83	b	4.02	c	3.33	c
	AVG	4.23		0.58		1.68		1.66	

[a]number of individuals assigned to chemotype; *values followed by the same letter are not significantly different at $P < 0.05$.

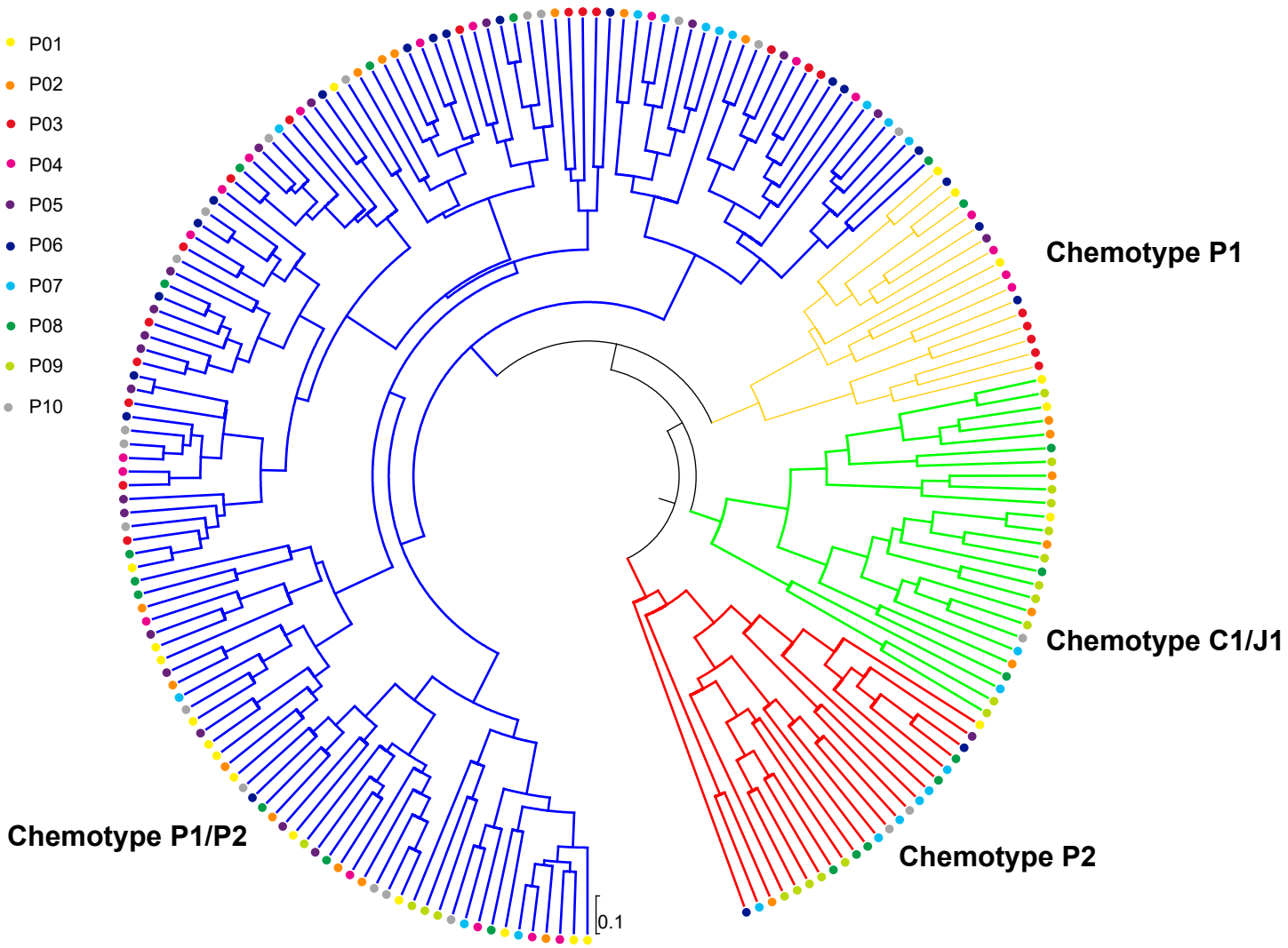


Figure 13. UPGMA dendrogram of cluster analysis of 10 Dalmatian pyrethrum populations from Croatia with four chemotypes indicated.

Highly significant differences ($P < 0.001$) were found between chemotypes based on all variables analyzed (Table 8). Chemotype P1 was characterized by high pyrethrin I content (68.47% of total pyrethrin content) and extremely low content of pyrethrin II, cinerin II and jasmolin II (Graph 6). The pyrethrin I/pyrethrin II ratio was highest in this chemotype (5.88). A total of 17 individuals from six populations were sorted into chemotype P1, with its incidence decreasing towards the south. Chemotype P1/P2 was characterized by a high content of pyrethrin I (51.72% of total pyrethrin content) and pyrethrin II (33.95% of total pyrethrin content), and the highest total pyrethrin content (0.68% of dry flower weight). It was the largest and most dominant chemotype (134 individuals) in all populations except P09 Pelješac. Chemotype P2 was characterized by the highest pyrethrin II content (47.71% of total pyrethrin content) and the lowest pyrethrin I/pyrethrin II ratio (0.58) and was more present in the southern populations. Finally, chemotype C1/J1 was characterized by the highest cinerin I (10.99% of total pyrethrin content) and jasmolin I (6.17% of total pyrethrin content) content and relatively high cinerin II and jasmolin II content. The chemotype was present in the northernmost and southernmost populations (Figure 14).

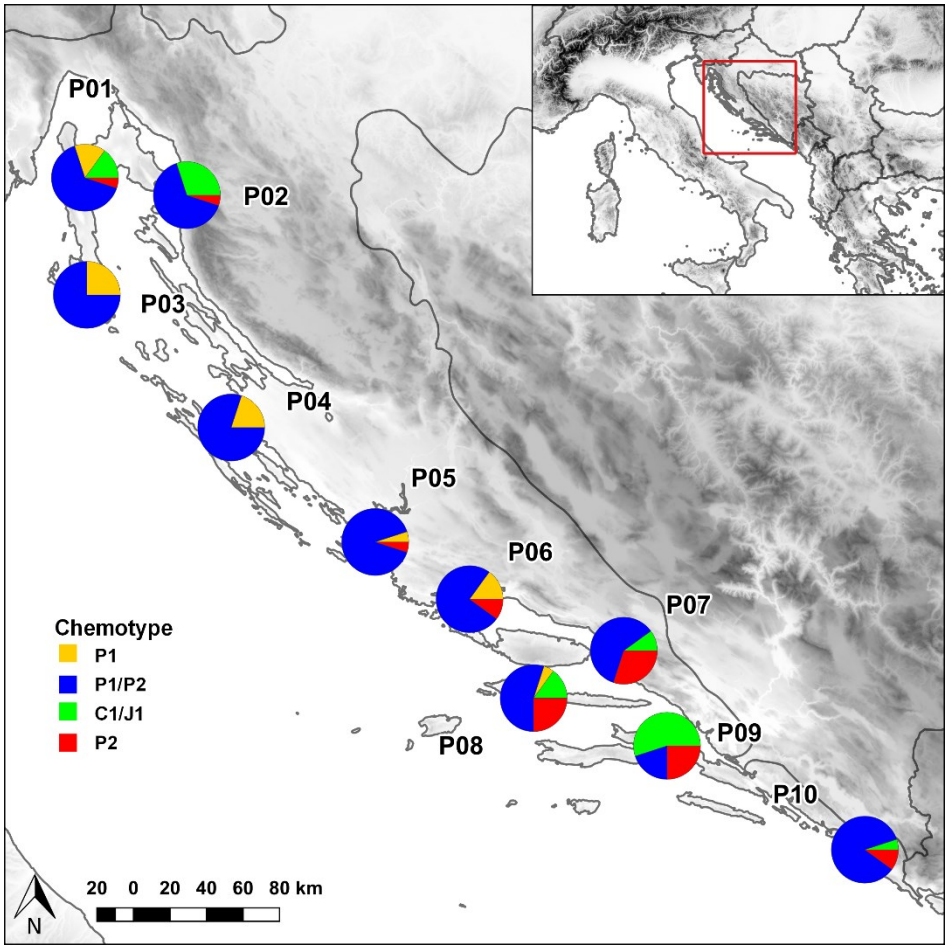
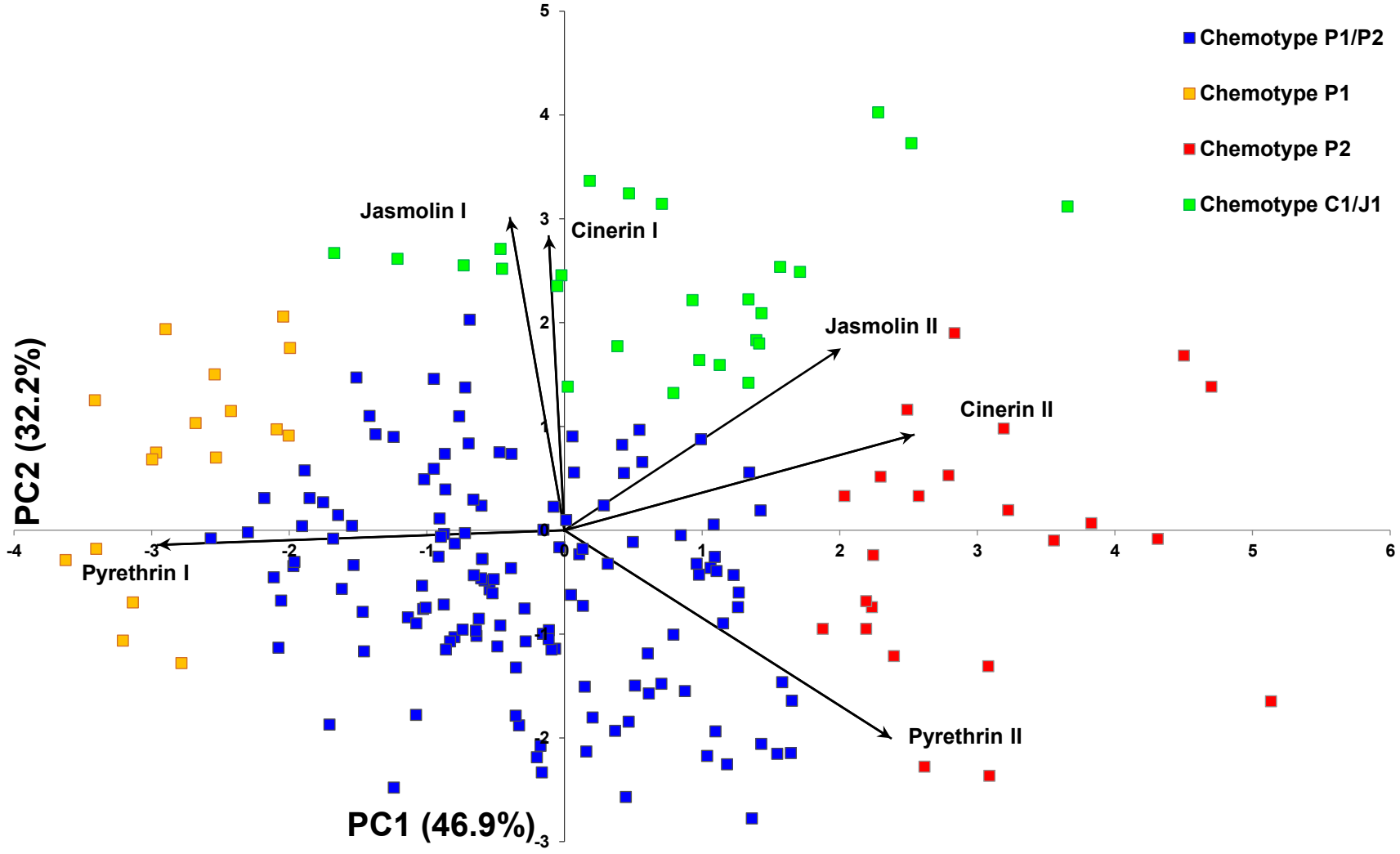


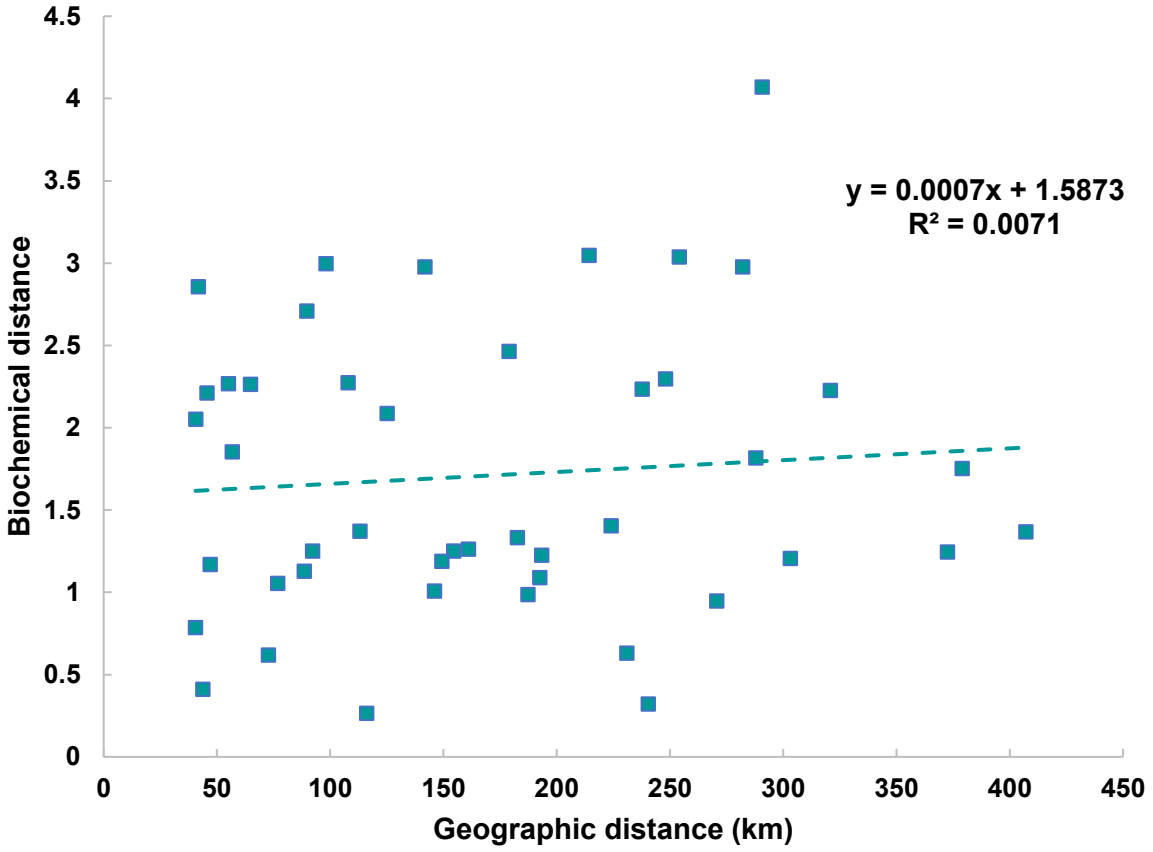
Figure 14. Geographic distribution of Dalmatian pyrethrum chemotypes.



Graph 6. Biplot of Principal component analysis based on pyrethrin content. Individuals are indicated by the assigned chemotype.

4.1.2. Spatio-ecological analyses

The average biochemical distance between Dalmatian pyrethrum populations (Euclidean distance based on the scores of the first three principal components) was 1.710. Based on biochemical composition the smallest biochemical distance was observed between populations P04 Ugljan and P06 Čiovo (0.263) and the largest between populations P03 Mali Lošinj and P09 Pelješac (4.069) as shown in Appendix 1. The correlation between the biochemical distance matrix and the geographic distance matrix (ln km) was not statistically significant after 10,000 permutations of the Mantel test (r = 0.074, P_{Mantel} = 0.693). Using linear regression of biochemical distances on geographic distances regression line was calculated, and the coefficient of determination was R^2 = 0.007 (Graph 7).

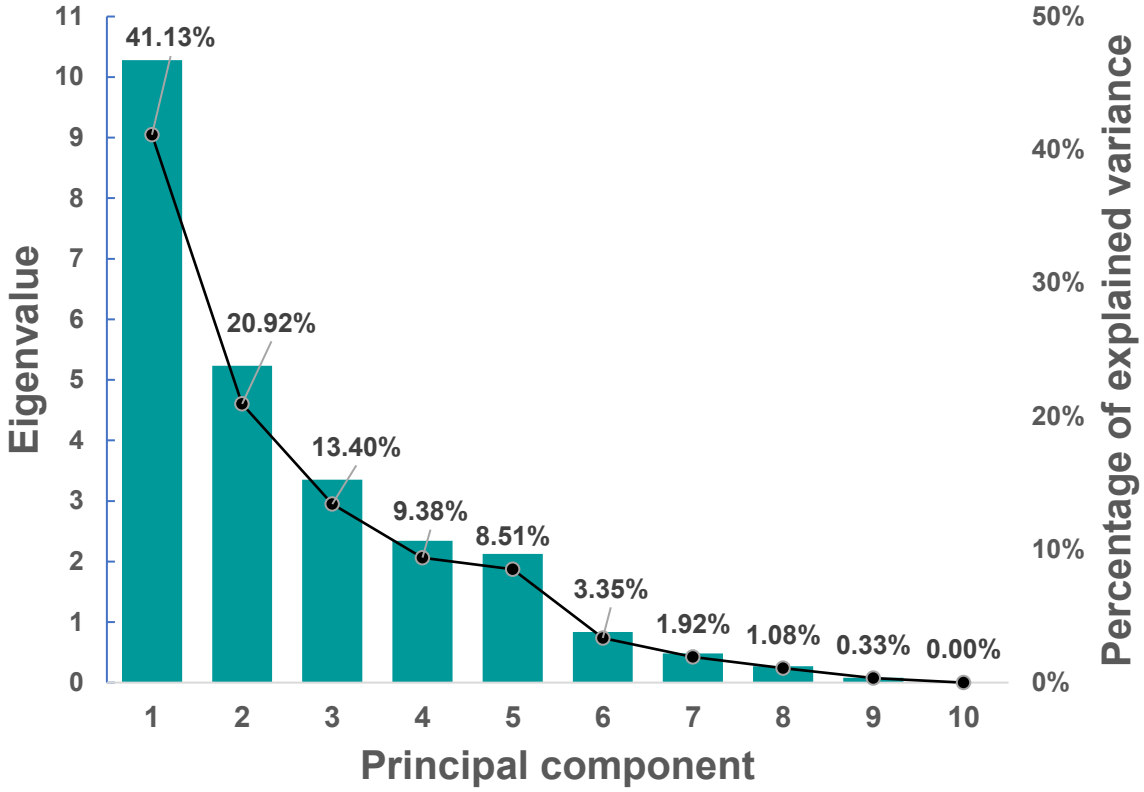


Graph 7. Regression of biochemical distance on geographic distance based on 10 Dalmatian pyrethrum populations.

To investigate the relationship between environmental factors and pyrethrin content and composition, a correlation analysis with spatio-ecological variables was performed. Among the variables related to temperature (BIO1-11), the most important was mean temperature of driest quarter (BIO9) with a highly significant ($P < 0.001$) moderate correlation with total pyrethrin content and pyrethrin I. A positive correlation was also found between altitude and pyrethrin II and cinerin II content. Parameters related to temperature range (diurnal, annual, isothermality) were highly significantly correlated with jasmolin I only.

Among the variables related to precipitation (BIO12-19), the most important was the precipitation of the warmest quarter (BIO18), in agreement with the correlation with the mean temperature of the driest quarter (BIO9) mentioned earlier, since the warmest period overlaps/coincides with the driest period (summer) in the studied area. Similarly to the variable BIO9, BIO18 was highly significantly correlated with total pyrethrin content. Among the other environmental parameters tested, significant correlation was observed between soil clay content and total pyrethrin content, pyrethrin I, pyrethrin II and cinerin II (Appendix 2).

Based on the 25 spatio-ecological variables, PCA was performed, which revealed that the first five PCs have eigenvalues greater than 1 and explain 93.33% of the total variance (Graph 8). First PC explained 41.13% of the total variance and had a strong positive correlation with BIO1 (Annual Mean Temperature), BIO5 (Maximum Temperature of Warmest Month), BIO8 (Mean Temperature of Wettest Quarter), BIO9 (Mean Temperature of Driest Quarter), BIO10 (Mean Temperature of Warmest Quarter), BIO11 (Mean Temperature of Coldest Quarter) and strong negative correlation with ALT (Altitude) and COAST (Distance to shoreline). Second PC explained 20.92% of the total variance and had a strong positive correlation with BIO12 (Annual Precipitation), BIO13 (Precipitation of Wettest Month), BIO16 (Precipitation of Wettest Quarter) and BIO17 (Precipitation of Driest Quarter) as shown in Table 9.



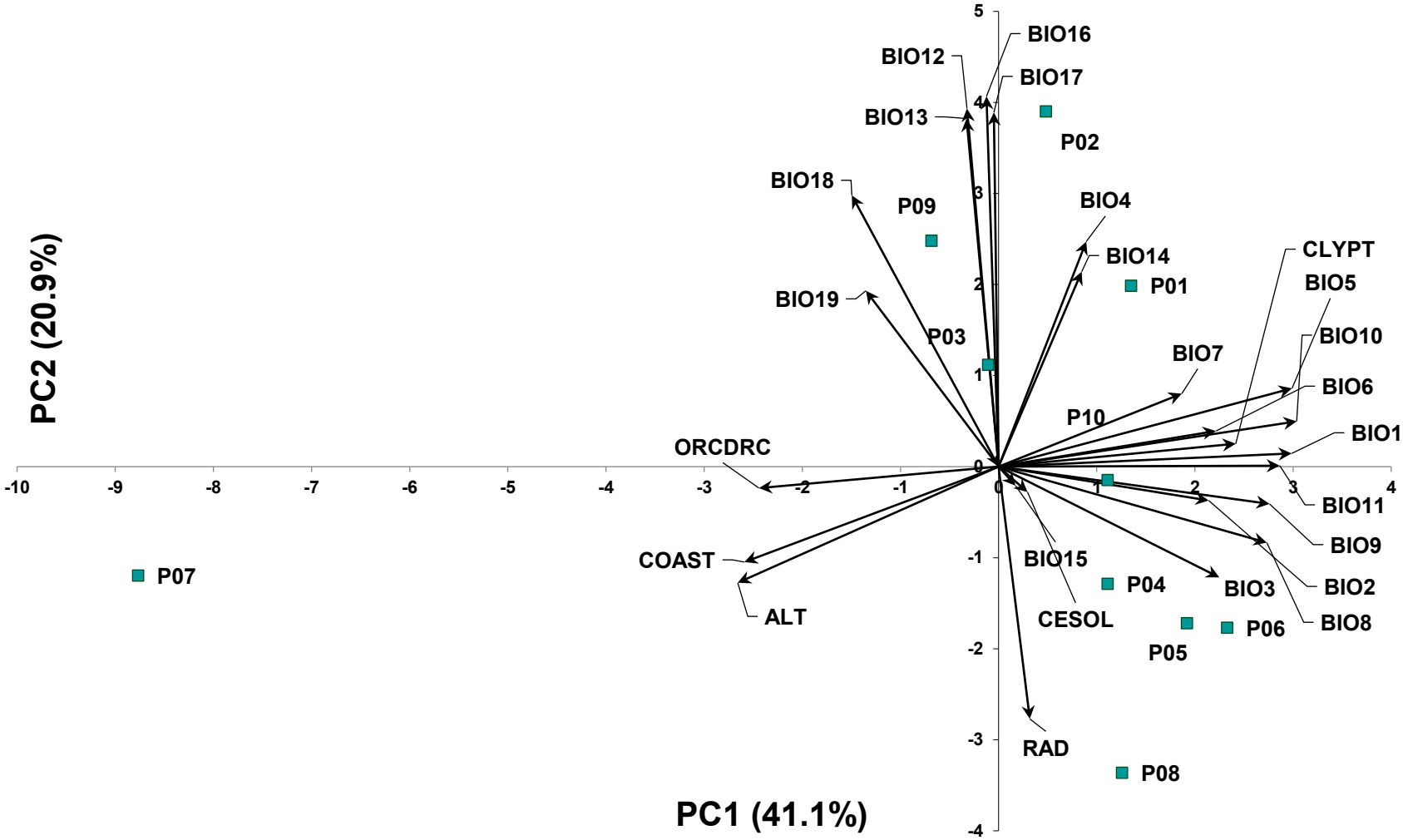
Graph 8. A scree plot of explained variance and eigenvalues of Principal components from the PCA.

PCA enabled the separation of populations in a multivariate spatio-ecological space (Graph 9). The first PC separated populations with greater daily and seasonal temperature variation, higher soil organic carbon content, altitude, and distance from the shoreline from those with higher temperatures and higher clay content. The second PC separated populations based on amount of precipitation.

Table 9. Pearson correlation coefficients between 25 spatio-ecological variables and scores of the first three Principal components.

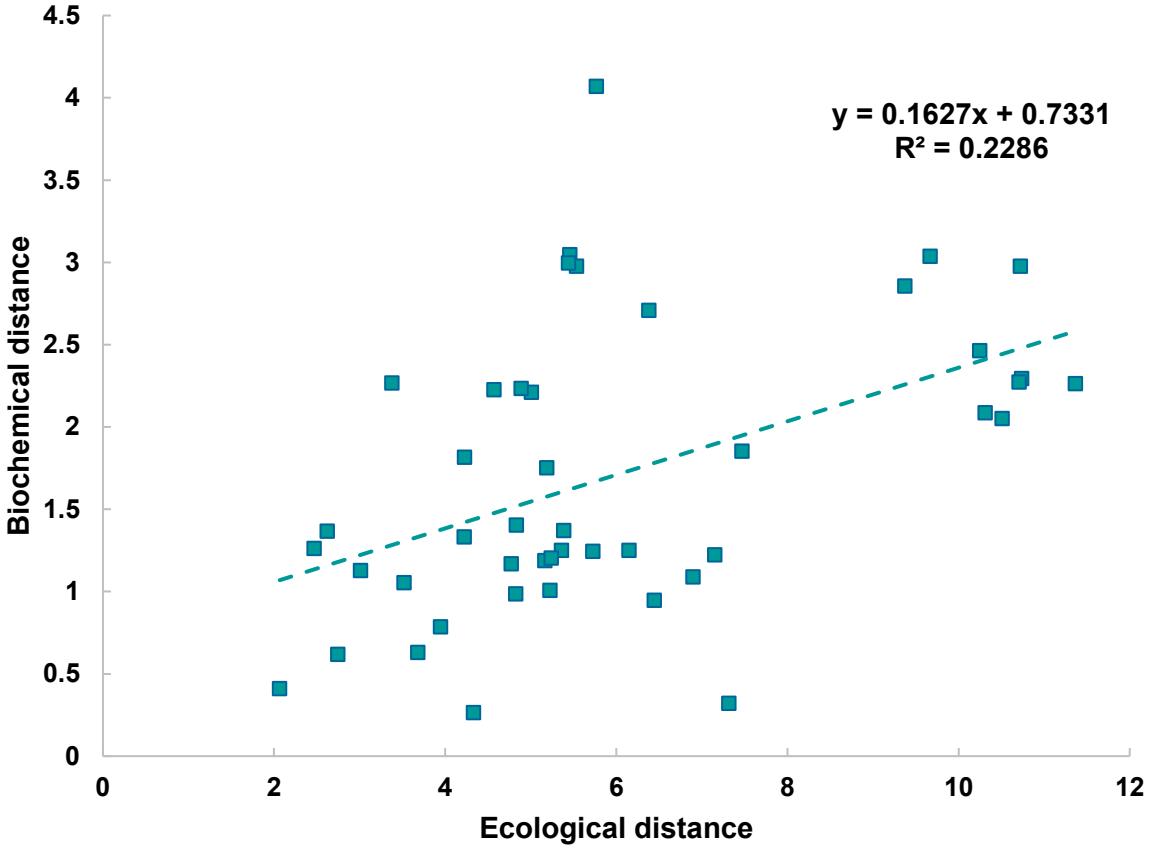
Variable	PC 1		PC 2		PC 3	
ALT	-0.852	**	-0.292	ns	0.232	ns
COAST	-0.830	**	-0.240	ns	0.370	ns
BIO1	0.957	***	0.033	ns	-0.189	ns
BIO2	0.686	*	-0.085	ns	0.698	*
BIO3	0.721	**	-0.278	ns	0.578	ns
BIO4	0.286	ns	0.565	ns	0.614	ns
BIO5	0.959	***	0.197	ns	0.022	ns
BIO6	0.710	*	0.089	ns	-0.637	*
BIO7	0.599	ns	0.183	ns	0.719	*
BIO8	0.876	***	-0.191	ns	-0.041	ns
BIO9	0.885	***	-0.094	ns	-0.235	ns
BIO10	0.974	***	0.114	ns	-0.101	ns
BIO11	0.919	***	0.002	ns	-0.236	ns
BIO12	-0.103	ns	0.900	***	0.190	ns
BIO13	-0.105	ns	0.874	***	0.250	ns
BIO14	0.271	ns	0.490	ns	-0.508	ns
BIO15	0.055	ns	-0.048	ns	0.327	ns
BIO16	-0.040	ns	0.930	***	0.308	ns
BIO17	-0.015	ns	0.890	***	-0.184	ns
BIO18	-0.480	ns	0.682	*	-0.189	ns
BIO19	-0.433	ns	0.442	ns	0.048	ns
CESOL	0.094	ns	-0.064	ns	0.128	ns
CLYPT	0.775	**	0.058	ns	-0.046	ns
ORCDRC	-0.783	**	-0.054	ns	-0.345	ns
RAD	0.102	ns	-0.634	*	0.331	ns

[ns]non-significant; *significant at $P < 0.05$; **significant at $P < 0.01$; ***significant at $P < 0.001$



Graph 9. Biplot of Principal component analysis based on 25 spatio-ecological variables.

The correlation between the biochemical distance matrix and the ecological distance matrix was not statistically significant after 10,000 permutations of the Mantel test (r = 0.478, P_{Mantel} = 0.964). Using linear regression of biochemical distances on ecological distances regression line was calculated, and the coefficient of determination was E^2 = 0.229 (Graph 10). The ecological distance matrix is shown in Appendix 3.



Graph 10. Regression of biochemical distance on ecological distance based on 10 Dalmatian pyrethrum populations.

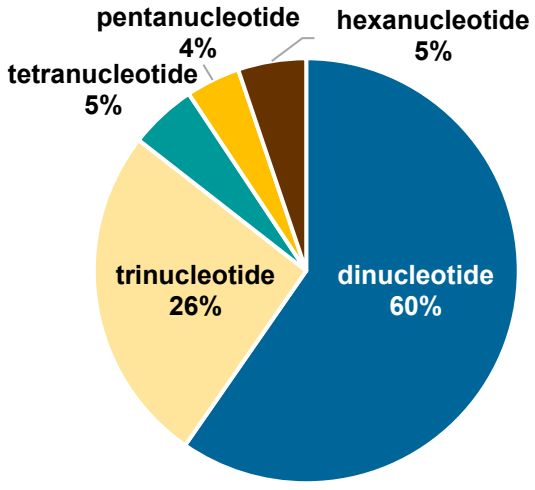
4.2. Results of microsatellite markers development

A total of 796,130,142 high-quality reads with a length of 151 bp (120 Gbp in total) were obtained by using High-throughput sequencing of *T. cinerariifolium* with a G-C content of 35.89%. The first approach (*de novo* assembly on a portion of the data) yielded 923,207 contigs. The total length of the assembled contigs was 409.5 Mbp, with a length of the longest contig of 82,765 bp and N50 assembly value of 451 bp. The assembly had a mean G-C content of 34.34%. The second approach (overlapping pair-end reads were assembled) yielded 5,986,468 sequences with a total length of 1,311 Mbp and a mean G-C content of 34.41% (Table 10).

Table 10. Summary of *de novo* assembly and SSR mining

Category	**First Approach**	**Second Approach**	**Combined results**
Total number of contigs examined	923,207	5,986,468	6,909,675
Total size of examined contigs (bp)	409,503,869	1,311,396,942	1,720,900,811
Total number of identified SSRs	11,652	23,904	35,556
Number of SSR containing contigs	9,973	21,407	31,380
Number of contigs containing more than one SSR	1,267	2,005	3,272

The obtained contigs (923,207) and sequences (5,986,468) were screened for microsatellites with different repeat motifs with the MISA tool, excluding mono-nucleotide repeats. A total of 35,556 microsatellite loci were identified in 31,380 SSR-containing contigs. The majority of SSR-containing contigs (89.6%) contained only one microsatellite locus. Dinucleotide repeats were most common, followed by trinucleotide motifs (Graph 11). The most common dinucleotide motifs were AT/AT, accounting for 29.6%,



Graph 11. Prevalence of microsatellite repeats types in contigs of *T. cinerariifolium*.

followed by AG/CT (15.1%) and AC/GT (15%). Among trinucleotides, AAT/ATT (10.4%) was the most common motif, followed by AAC/GTT (4.5%) and AGT/ATC (3.1%).

Filtering the microsatellite loci according to the selection criteria resulted in 1,006 sequences that were checked against the draft genome of *T. cinerariifolium*. This further narrowed the selection to 56 sequences that were used for primer design. After initial testing of the primers on five samples from different populations, 17 microsatellites that showed good amplification and polymorphism were selected for further testing on 20 samples from population P08 Hvar.

The analysis performed in MicroChecker revealed no evidence of scoring errors due to stuttering or large allele dropout. The presence of null alleles was detected at four loci (TcUniZg007, TcUniZg009, TcUniZg020 and TcUniZg037). A total of 94 alleles were detected ranging from 3 (TcUniZg032) to 13 (TcUniZg006) alleles per locus. Values of expected heterozygosity ranged from 0.197 (TcUniZg020) to 0.864 (TcUniZg008) with an average value of 0.545. Observed heterozygosity ranged from 0.083 (TcUniZg020) to 0.7083 (TcUniZg008) with an average value of 0.483 (Table 11).

The probability of identity varied between loci from 0.043 (TcUniZg008) to 0.668 (TcUniZg020) with combined non-exclusion probability of identity for all loci of 1.94×10^{-11}. The polymorphic information content varied from 0.178 (TcUniZg020) to 0.824 (TcUniZg008) with an average value of 0.492. Of the 17 markers tested, 8 were classified as moderately polymorphic (*PIC* > 0.44), while two microsatellite markers were classified as highly polymorphic (*PIC* > 0.70) (Table 11).

Table 11. Characterization of 17 developed microsatellite markers from a natural population (P08) of *T. cinerariifolium*.

Locus name	GenBank acc. no.	Primer sequence (5′ to 3′)	Repeat Motif	Size (bp)	N_a	H_O	H_E	F_{IS}	Sign	P_{null}	*PIC*	*PI*
TcUniZg004	MW498263	F: TGATCTTTTAAATTTGGAACTGAA R: GAAAGCTTCCTCTACCTCCTTG	$(GT)_{10}$	248-256	4	0.167	0.235	0.290	ns	-	0.219	0.605
TcUniZg005	MW498264	F: CCAGATCATTTACTTAAAATGGAACA R: TACAACACTGGTGGCGTCAT	$(AC)_8$	217-223	4	0.304	0.449	0.322	ns	-	0.384	0.370
TcUniZg006	MW498265	F: CGACGGTTGGTGTGTGTATC R: CCATACGTGTCTCTTTCTCTTTGA	$(GA)_{10}$	224-280	13	0.542	0.633	0.145	ns	-	0.605	0.158
TcUniZg007	MW498266	F: GCTTCACATGGTTCGTCTCTG R: GCTTCACATGGTTCGTCTCTG	$(CA)_8$	191-203	5	0.333	0.505	0.339	ns	0.091	0.432	0.318
TcUniZg008	MW498267	F: TGCGATGATGATGATTGAGAG R: ATGGCAGAACATTCAACACAA	$(GT)_{11}$	116-146	11	0.708	0.864	0.180	ns	-	0.824	0.043
TcUniZg009	MW498268	F: TCTCCTTCTTCCTCCTGCAA R: GGATGTTTGTTGTGTTCATTGG	$(CA)_{11}$	106-118	6	0.583	0.774	0.246	ns	0.111	0.716	0.098
TcUniZg010	MW498269	F: CATACCTCCGCCCTTGATTA R: CCAAGACCCACTTTTTGGTG	$(TG)_8$	180-194	6	0.667	0.757	0.120	ns	-	0.697	0.110
TcUniZg012	MW498270	F: TCATCATCAACAAAATATCCAAGAA R: CCACCGACCACCTCATAATC	$(CA)_{10}$	244-254	5	0.391	0.542	0.277	ns	-	0.485	0.266
TcUniZg013	MW498271	F: ACATAACGTCGGAGGCATCA R: TGAGTTGGGTGCGTTACAAA	$(TA)_8$	216-222	4	0.375	0.323	-0.160	ns	-	0.288	0.495
TcUniZg014	MW498272	F: AGCATAGACTGACTGTTCCTTCA R: CCATATTCATCACAGCCTACGA	$(TG)_{12}$	216-230	6	0.542	0.531	-0.021	ns	-	0.488	0.263
TcUniZg017	MW498273	F: AAGGCTGCGCTTCTTAACAG R: TAGCCATGCCTGGGTACTTC	$(TA)_{10}$	258-274	7	0.542	0.704	0.230	ns	-	0.639	0.146
TcUniZg019	MW498274	F: AATGTGTGACTAATGGTCCTCAGA R: TGTTACTTAATTATAACATGCGGCCTA	$(TA)_8$	116-124	5	0.583	0.720	0.190	ns	-	0.654	0.137
TcUniZg020	MW498275	F: ACCACCAATACAAATACACCTTC R: GCAGAGGCTCGAGCTAGGAC	$(CA)_7$	113-117	3	0.083	0.197	0.576	ns	0.111	0.178	0.668
TcUniZg023	MW498276	F: CACAAATCCTTCACCTGTCAAA R: GCCAGTGGCAGAAGAGAAGT	$(AC)_9$	240-250	3	0.458	0.433	-0.059	ns	-	0.350	0.405
TcUniZg032	MW498277	F: GAAATCAAGTGCGGATACGA R: TTTCCATATTGTGTTTTGGGTTC	$(CAT)_8$	106-115	3	0.500	0.592	0.155	ns	-	0.506	0.250
TcUniZg037	MW498278	F: GGACGGGATTACAGAAGGTG R: TCGACCTCATTATGCTGCTG	$(CAA)_7$	249-258	4	0.125	0.299	0.582	*	0.146	0.266	0.528
TcUniZg038	MW498279	F: GGAGCCAAATACTAGCCTTCAA R: CGTTAGTCATCCGTGAGCAA	$(TTG)_6$	151-163	5	0.542	0.702	0.228	ns	-	0.634	0.150

N_a – number of alleles; H_O – observed heterozygosity; H_E – expected heterozygosity; F_{IS} – inbreeding coefficient; Sign - significant deviations from Hardy-Weinberg equilibrium after sequential Bonferroni corrections: ns – non-significant; *P<0.05*; P_{null} – null allele frequency; *PIC* – polymorphic information content; *PI* – probability of identity

4.3. Results of molecular analyses

4.3.1. Within-population diversity

Out of 17 microsatellite markers developed, twelve microsatellite loci used based on their performance on test samples in the molecular analyses of the Dalmatian pyrethrum natural populations yielded a total of 121 alleles, ranging from 4 (TcUniZg005) to 25 (TcUniZg006) with an average of 10.083 alleles per population. Polymorphism information content (*PIC*) ranged from 0.377 to 0.842 with an average of 0.588 (Table 12).

Out of 120 tests, null alleles were suggested in nine population-locus combinations (7.5%). The frequencies of null alleles ranged from 0.092 (microsatellite locus TcUniZg006 in population P05 Zlarin) to 0.184 (microsatellite locus TcUniZg009 in population P04 Ugljan).

Table 12. Informativeness of 12 microsatellite markers used in molecular analyses of 10 Dalmatian pyrethrum populations.

Locus	Repeat Motif	Size Range (bp)	N_a	*PIC*
TcUniZg005	(AC)8	200-206	4	0.479
TcUniZg006	(GA)10	204-266	25	0.634
TcUniZg008	(GT)11	94-126	15	0.842
TcUniZg009	(CA)11	87-99	7	0.726
TcUniZg012	(CA)10	224-256	13	0.591
TcUniZg013	(TA)8	138-246	10	0.448
TcUniZg014	(TG)12	200-214	8	0.539
TcUniZg017	(TA)10	238-264	12	0.612
TcUniZg019	(TA)8	97-105	5	0.684
TcUniZg023	(AC)9	223-235	5	0.377
TcUniZg032	(CAT)8	91-121	8	0.471
TcUniZg038	(TTG)6	129-237	9	0.648
Minimum			4	0.377
Maximum			25	0.842
Average			10.083	0.588
Total			121	

N_a – number of alleles; *PIC* – polymorphic information content

Parameters describing within-population diversity of 10 Dalmatian pyrethrum populations are presented in Table 13. Allelic richness (N_{ar}) varied from 2.86 (P07 Biokovo) to 5.02 (P05 Zlarin). A total of 36 private alleles were observed across 10 populations. The highest number of private alleles was observed in population P10 Konavle (7). The values of observed

heterozygosity (H_O) varied from 0.46 (P06 Čiovo) to 0.61 (P10 Konavle). The inbreeding coefficient (F_{IS}) showed no significant deviation from the Hardy-Weinberg equilibrium in seven populations ($P > 0.05$). Significant deficiency of heterozygotes was observed in populations P01 Cres, P05 Zlarin, and P06 Čiovo ($P < 0.05$).

Wilcoxon signed rank test assuming a two-phased model implemented in BOTTLENECK identified significant bottleneck events only in population P07 Biokovo.

Table 13. Molecular diversity of 10 Dalmatian pyrethrum populations based on 12 microsatellite markers.

Pop ID	Locality	n	N_a	N_{ar}	N_{pr}	H_O	H_E	F_{IS}		$P_{Bottleneck}$
P01	Cres	20	5.000	4.647	5	0.508	0.557	0.087	**	0.810
P02	Senj	20	4.750	4.477	4	0.576	0.581	0.009	ns	0.575
P03	Mali Lošinj	18	4.417	4.277	2	0.568	0.617	0.080	ns	0.235
P04	Ugljan	16	3.833	3.770	3	0.524	0.516	-0.016	ns	0.867
P05	Zlarin	20	5.333	5.015	3	0.565	0.632	0.106	**	0.741
P06	Čiovo	20	3.500	3.367	5	0.463	0.486	0.048	*	0.455
P07	Biokovo	20	2.917	2.864	1	0.466	0.475	0.018	ns	**0.001**
P08	Hvar	20	4.500	4.238	3	0.558	0.560	0.003	ns	0.810
P09	Pelješac	20	4.583	4.337	3	0.592	0.581	-0.018	ns	0.311
P10	Konavle	20	4.917	4.537	7	0.608	0.571	-0.066	ns	0.575

n – number of individuals; *Na* – average number of alleles; *Nar* – Allelic richness; *Npr* – number of private alleles; H_O – observed heterozygosity; H_E – expected heterozygosity; F_{IS} – inbreeding coefficient (^{ns}non-significant value; *significant at $P < 0.05$; **significant at $P < 0.01$; ***significant at $P < 0.001$); $P_{Bottleneck}$ – probability results of Wilcoxon signed-ranks test used to assess population bottleneck (P values lower than 0.05 are indicated in bold typeface)

4.3.2. Genetic differentiation and structure

Average Cavalli-Sforza chord distance between analyzed populations was 0.088. Cavalli-Sforza chord distance varied from 0.049 between population P02 Senj and population P09 Pelješac to 0.173 between populations P07 Biokovo and P03 Mali Lošinj. Population distance matrix based on Cavalli-Sforza chord distances is shown in Appendix 4.

Based on the genetic distance matrix between 10 populations of Dalmatian pyrethrum a Neighbor-joining tree was constructed. The pattern of grouping showed the existence of two clades supported by a bootstrap value of 79% (Figure 15). The first clade includes populations P01 Cres, P03 Mali Lošinj, P04 Ugljan, P05 Zlarin, P08 Hvar and P10 Konavle. In the second clade the separation of P07 Biokovo and P09 Pelješac supported by the bootstrap value of 68%, from populations P02 Senj and P06 Čiovo was observed.

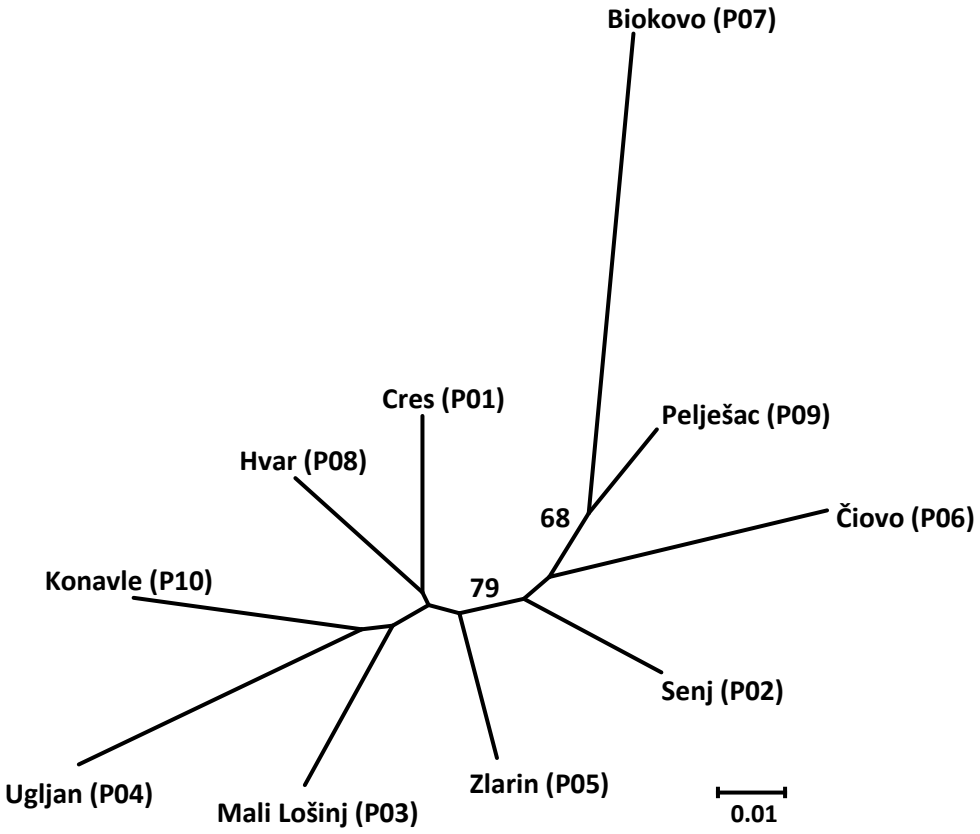
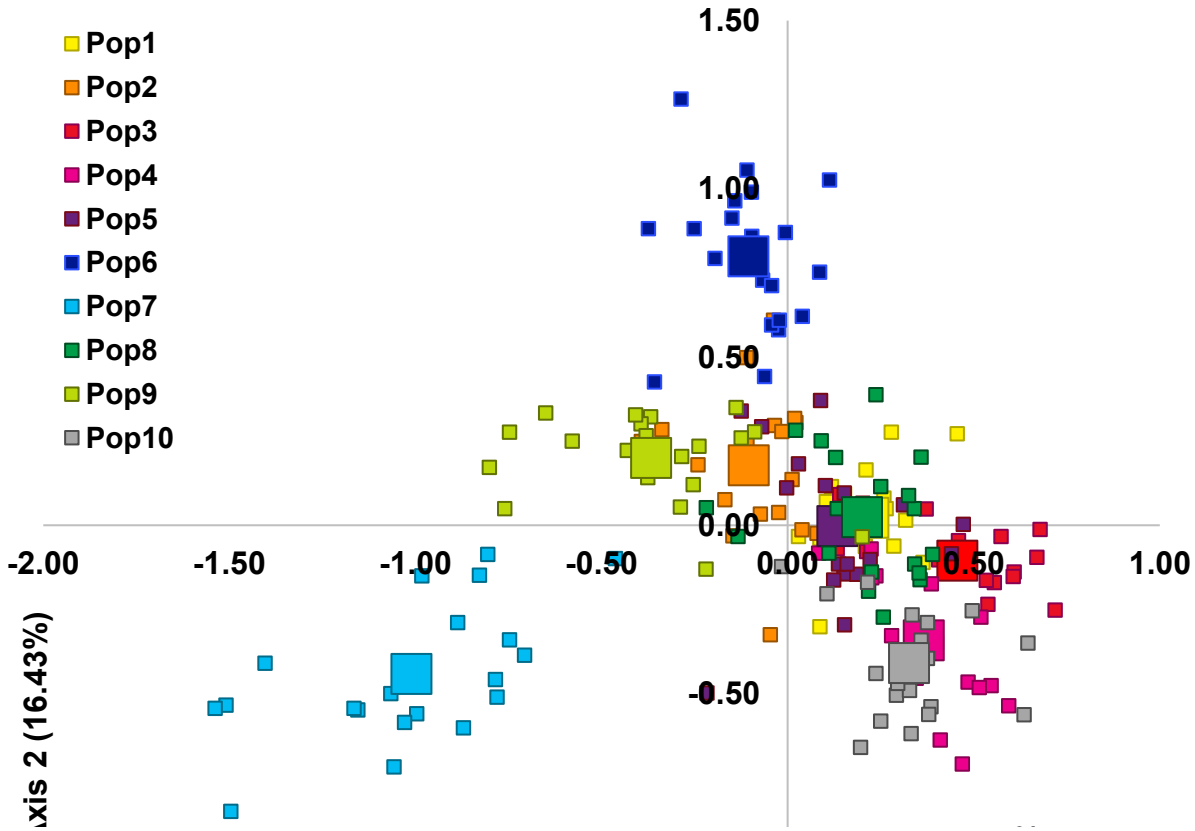


Figure 15. Unrooted Neighbor-joining tree of 10 Dalmatian pyrethrum populations. Bootstrap values greater than 50% are indicated on the branches.

The distance based on the proportion of shared alleles varied from 0.042 between two individuals from population P06 Čiovo to 0.917 in two cases (between two individuals from populations P06 Čiovo and P10 Konavle, and between two individuals from populations P03 Mali Lošinj and P07 Biokovo). The average distance based on the proportion of shared alleles between 194 individuals was 0.557.

No large clades supported by bootstrap value higher than 50% can be observed on the Neighbor-joining tree based on pairwise distances between individuals. Separation of all individuals from P07 Biokovo from other populations can be observed, but again this separation is not supported by a sufficiently high bootstrap value (Appendix 5).

The results of the factorial correspondence analysis are shown in Graph 12. The first two FCA axes accounted for a cumulative 39.80% of the total inertia. On both axes, a clear differentiation of P07 Biokovo from the other populations can be seen, with the population occupying a distinct area of multivariate space. Furthermore, differentiation of populations P02 Senj, P06 Čiovo and P09 Pelješac from other populations is evident along both axes with clear definition between them, while little genetic differentiation can be observed between populations P01 Cres, P05 Zlarin and P08 Hvar.

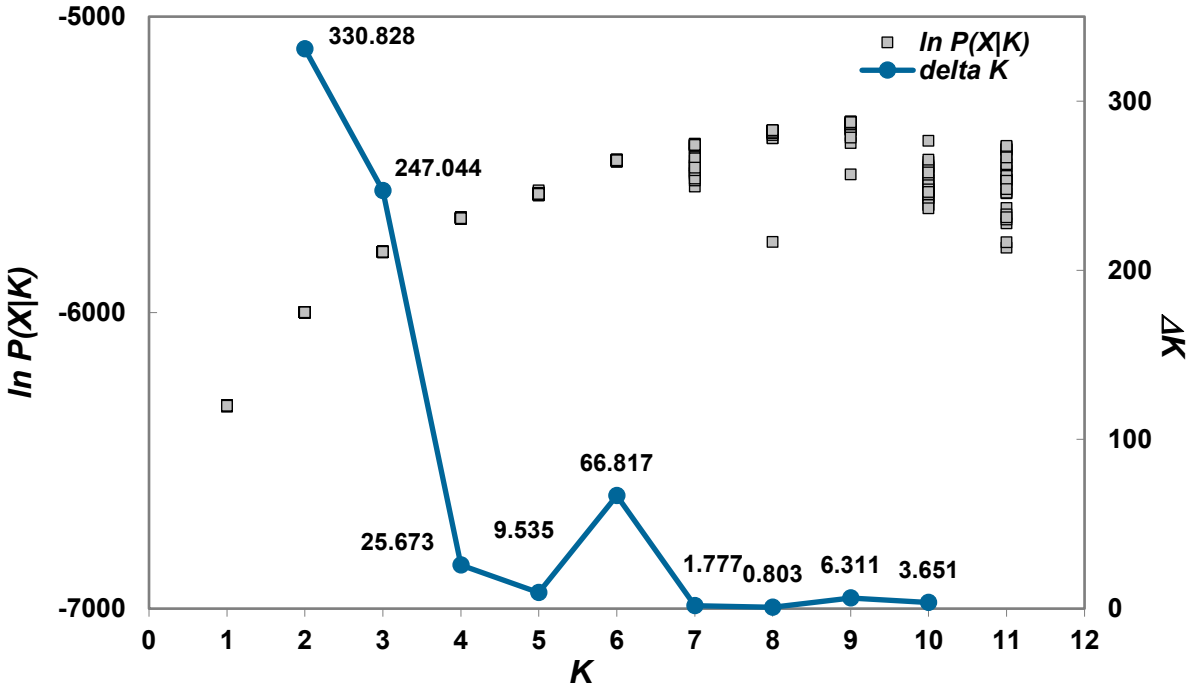


Graph 12. FCA of 200 Dalmatian pyrethrum individuals from 10 natural populations. Population barycentres are represented with larger squares.

The analysis of molecular variance (AMOVA) showed that most of the genetic diversity was due to differences between individuals within populations (87.03%) while the rest (12.97%) can be attributed to differences between populations.

Average value of genetic differentiation (F_{ST}) between all pairs of population was 0.129 (Appendix 6). The highest F_{ST} was determined between populations P04 Ugljan and P07 Biokovo (0.270) while the lowest F_{ST} was determined between populations P02 Senj and P09 Pelješac (0.037).

After performing a Bayesian model-based clustering method, the highest ΔK value was observed for K = 2 (330.83), followed by the ΔK value for K = 3 (247.04). The average estimates of the likelihood of the data, *ln P(X|K)* continued to increase with higher K, with low standard deviation at K =2 and 3 (Graph 13). Based on this information, the proportions of ancestry of all individuals in each cluster were calculated at K = 2 and 3.



Graph 13. Average estimates of the likelihood of the data [*ln P(X|K)*] and ΔK values for different numbers of ancestral populations (K = 1 to 11) based on Bayesian model-based clustering.

At K = 2, all populations were assigned to one of two ancestral populations, except for P02 Senj and P05 Zlarin, which were of mixed origin (Figure 16a). At the intrapopulation level, all individuals of population P04 Ugljan were assigned to cluster A and all individuals of P07

Biokovo were assigned to cluster B. Other populations contained varying numbers of individuals of mixed origin, with the highest number of individuals of mixed origin found in populations P02 Senj, P05 Zlarin, and P08 Hvar.

At *K* = 3, populations P05 Zlarin and P08 Hvar were of mixed origin, while other populations were assigned to one of the three ancestral populations, in particular P07 Biokovo was the only population assigned to cluster C (Figure 16b). At the intrapopulation level, all individuals from population P04 Ugljan were assigned to cluster A, and all individuals from population P07 Biokovo were assigned to cluster C. For *K* = 3, the proportion of individuals classified as of mixed origin was much lower than for *K* = 2, with population P01 Cres achieving the highest value.

The proportions of membership for both *K* = 2 and 3 at the intrapopulation level are shown in Graph 14.

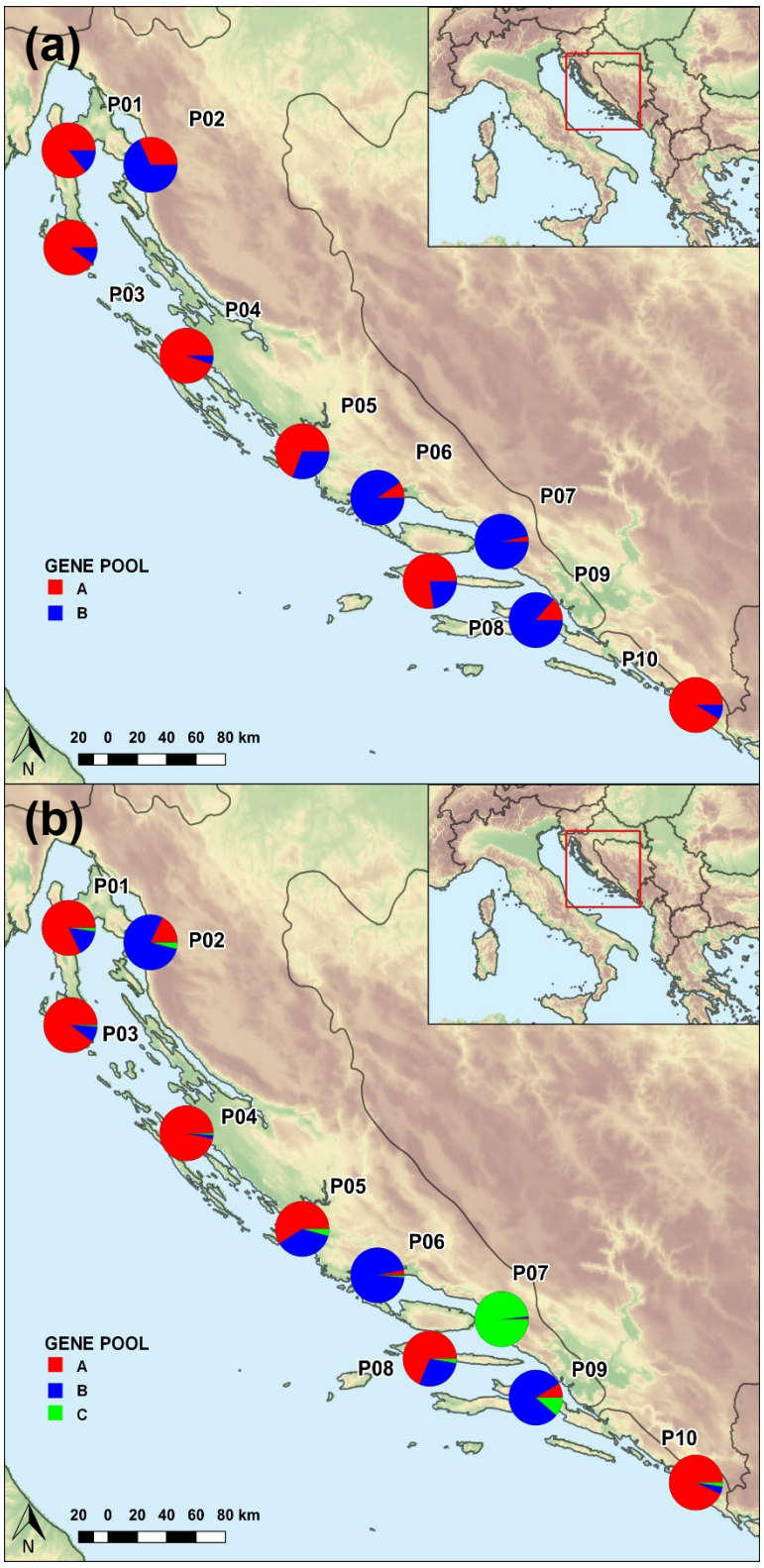
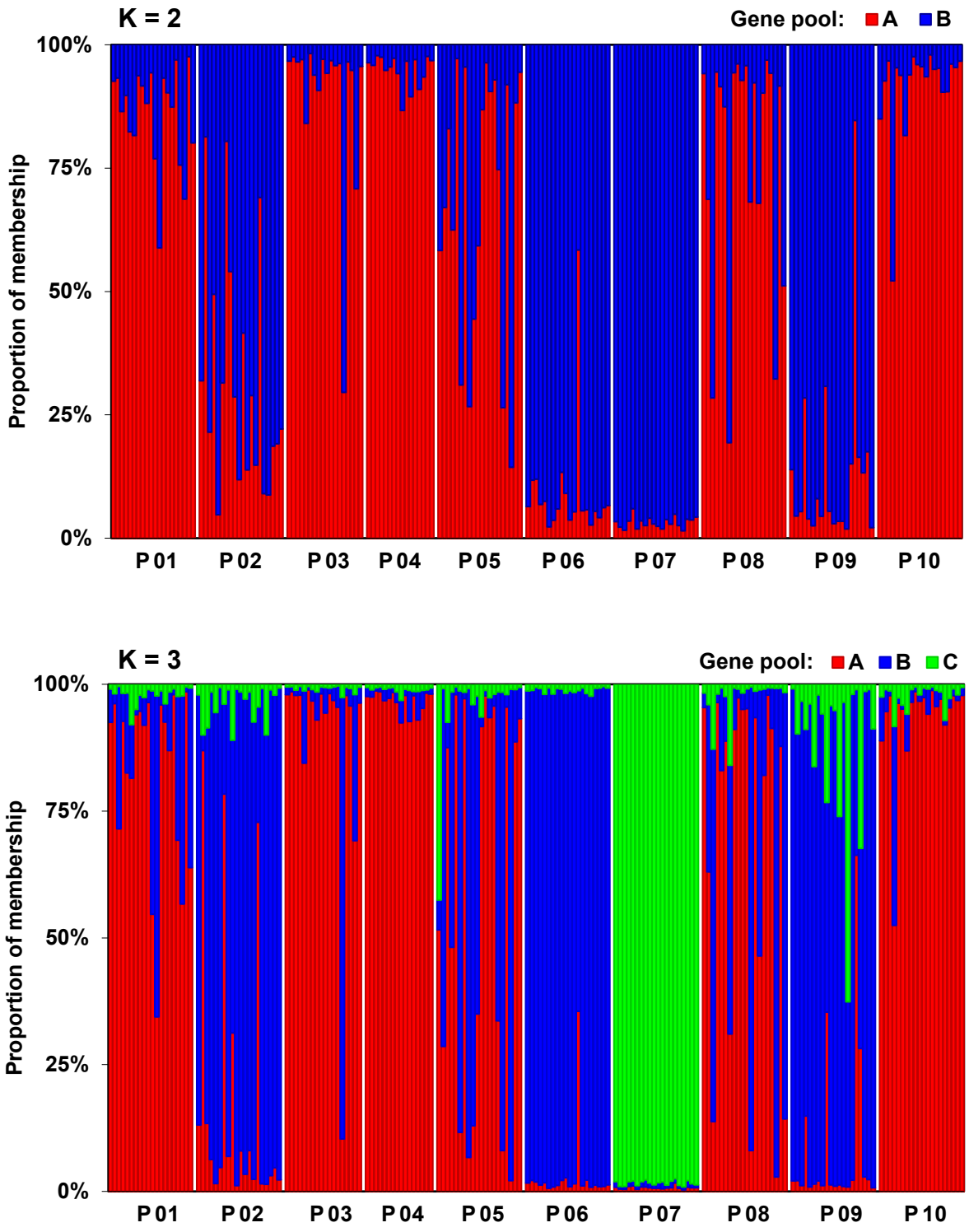


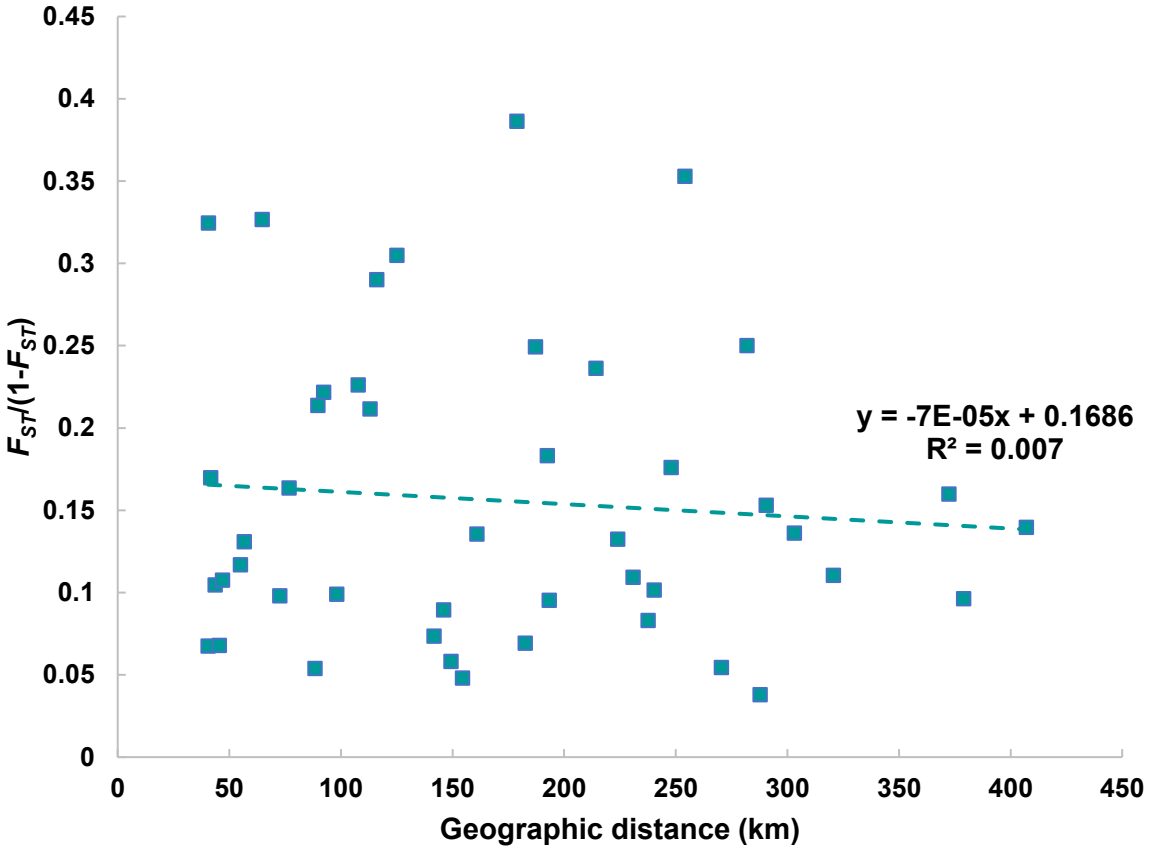
Figure 16. Bayesian analysis of *T. cinerariifolium* population structure assuming *K* = 2 (a), and *K* = 3 (b).



Graph 14. Genetic structure of 10 Dalmatian pyrethrum populations assuming *K* = 2 and 3. Each individual is represented by a vertical line divided into colors corresponding to distinct cluster.

4.3.3. Spatial genetics

Correlation between $F_{ST}/(1- F_{ST})$ matrix and distance matrix (*ln* km) was r = -0.047 and was not significant (P_{Mantel} = 0.375) after 10,000 permutations of the Mantel test. Determination coefficient was R^2 = 0.007 indicating that only 0.7% of genetic differentiation between analyzed populations can be explained by geographic distance (Graph 15).



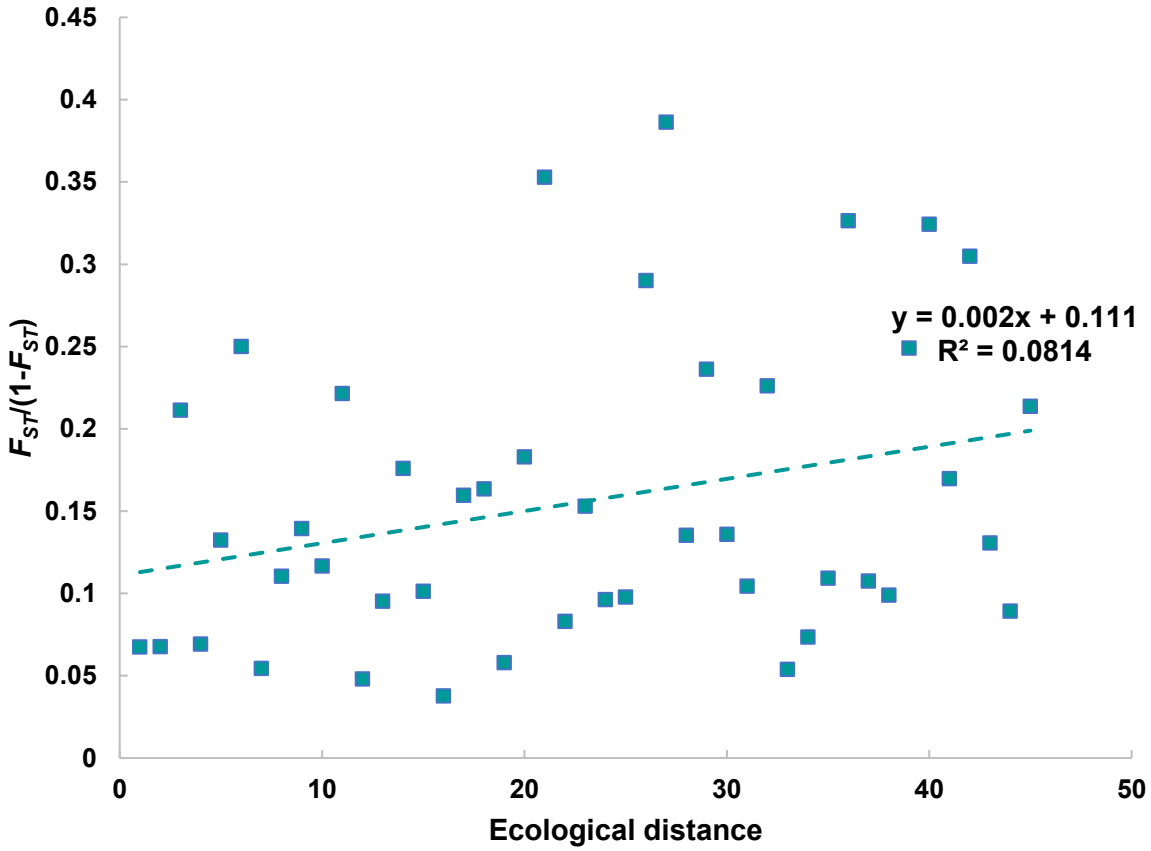
Graph 15. Regression of genetic distance on geographic distance based on 10 Dalmatian pyrethrum populations.

Using the Monmonier's maximum difference algorithm four barriers were determined based on Cavalli-Sforza's chord distances between populations and tested using 1000 bootstraps. Each of the barriers consisted of one to three segments obtained via Vornoi tessellation. Barrier a was determined between population P07 Biokovo and populations P06 Čiovo, P08 Hvar and P09 Pelješac with bootstrap values higher than 75% in all segments (Figure 17). Barrier b separated population P10 Konavle from population P09 Pelješac also

with high bootstrap value (88.6%). Barrier c separated northern populations P01 Cres, P02 Senj and P03 Mali Lošinj from the populations from the middle and southern Adriatic. Barrier c was only supported by bootstrap values in the segment separating mainland population P02 Senj from the more southern populations (96.4%), while the segment separating island populations (P01 Cres and P03 Mali Lošinj) from the more southern populations was not supported by bootstrap (69.6%). Barrier d determined between population P08 Hvar and populations P04 Ugljan, P05 Zlarin and P06 Čiovo was not supported by bootstrap (45.2%).

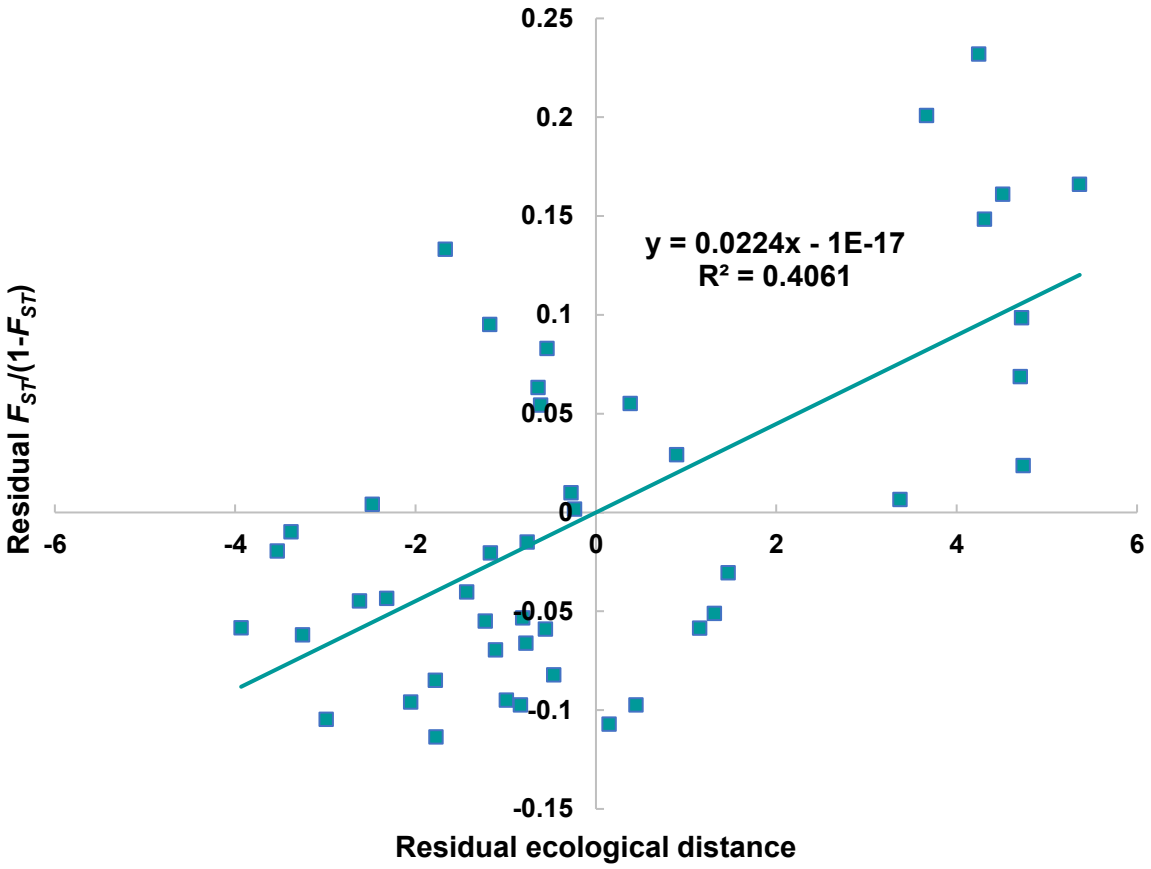
Figure 17. Gene flow barriers between 10 Dalmatian pyrethrum populations.

Correlation between genetic distance matrix [$F_{ST}/(1-F_{ST})$] and ecological distance matrix was r = 0.6366 and was not significant (P_{Mantel} = 0.992) after 10,000 permutations of the Mantel test. Determination coefficient was R^2 = 0.081 indicating that only 8.14% of genetic differentiation between analyzed populations can be explained by ecological distance (Graph 16).



Graph 16. Regression of genetic distance on ecological distance based on 10 Dalmatian pyrethrum populations.

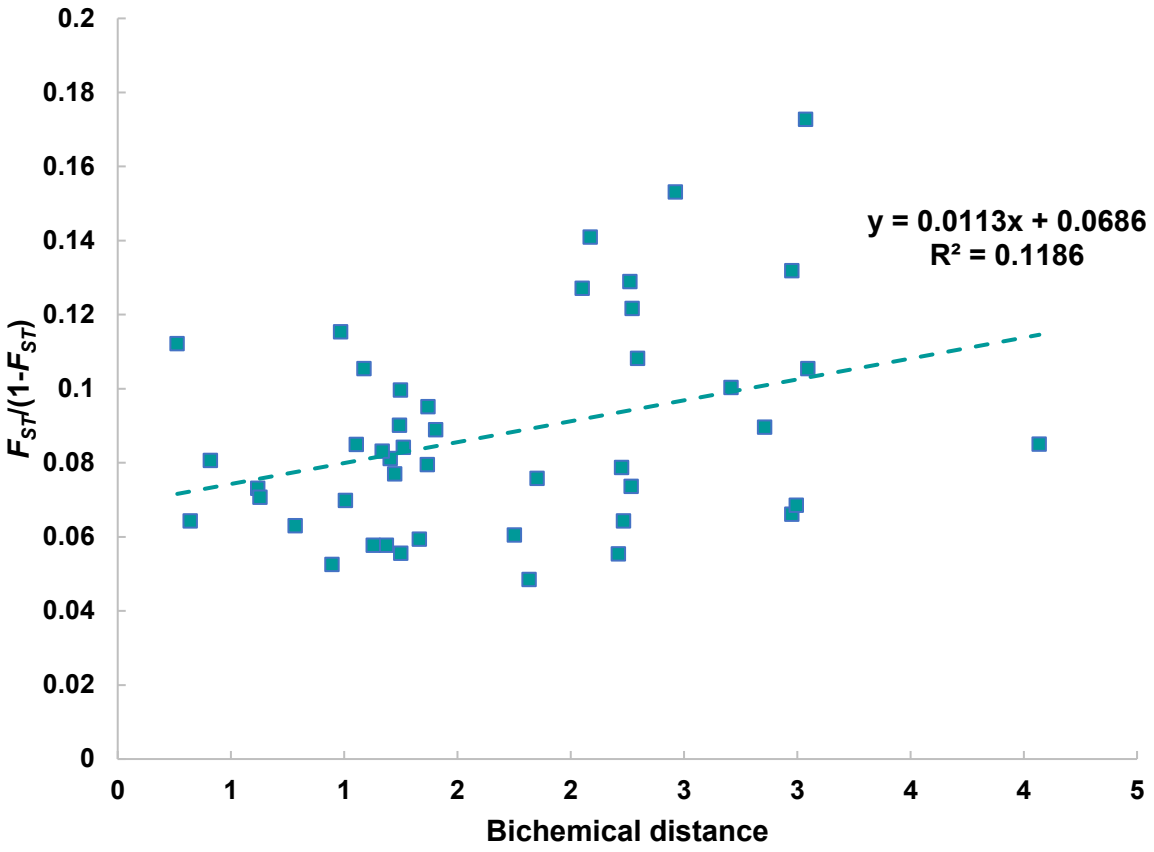
Partial Mantel test was used to examine the relationship between ecological distance matrix and genetic distance matrix [$F_{ST}/(1- F_{ST})$] with geographic distance excluded. The correlation between residual ecological distance and residual $F_{ST}/(1- F_{ST})$ values was high (r = 0.637) but not significant (P_{Mantel} = 0.992). The determination coefficient R^2 = 0.406 indicated that 40.61% of the genetic differentiation between populations can be explained by isolation by environmental distance (Graph 17).



Graph 17. Isolation by environmental distance: Regression of residual genetic distance [residual F_{ST}/(1- F_{ST}) values] on residual ecological distance excluding geographic distance between 10 Dalmatian pyrethrum populations.

4.4. Results of biochemical and genetic distance comparison

Correlation between genetic distance matrix (Cavalli-Sforza's chord distances) and biochemical distance matrix was $r = 0.344$ and was not significant ($P_{Mantel} = 0.907$) after 10,000 permutations of the Mantel test. Determination coefficient was $R^2 = 0.119$ (Graph 18).



Graph 18. Regression of biochemical distance on genetic distance (Cavalli-Sforza's chord distance) between 10 Dalmatian pyrethrum populations.

5. DISCUSSION

5.1. Biochemical diversity

Detailed analysis of the content and composition of pyrethrin in the natural populations of Dalmatian pyrethrum showed high biochemical diversity with significant differences between populations. The highest pyrethrin I content was found in population P03 Mali Lošinj, reaching up to 77.17% of the total pyrethrin content, much higher than reported in a previous study on the chemical diversity of Dalmatian pyrethrum for any population (Grdiša et al., 2013). Similarly, the lowest pyrethrin I content found in P06 Čiovo (12.33% of the total pyrethrin content) was much lower than any reported in the same study.

The pyrethrin II content ranged from 12.68% of the total pyrethrin content (P03 Mali Lošinj) to 61.12% of total pyrethrin content (P10 KOnavle). The higher pyrethrin II than pyrethrin I content in most individuals from P07 Biokovo is in contrast to a previous study where pyrethrin I content was dominant in all three populations from the same area (P07 Mt Biokovo) (Grdiša et al., 2013). This illustrates the advantage of sampling a larger number of individuals from populations, as opposed to using representative population samples, in revealing a more precise relationship between individual pyrethrin compounds in populations (Petersen et al., 2005).

The range of total pyrethrin content was also broader (0.10-1.35% of dry flower weight) than that reported in a previous study (0.36-1.30% of dry flower weight) (Grdiša et al., 2013). Although different extraction methods were used, in both studied populations Mali Lošinj and Zlarin islands had the highest in total pyrethrin content, while populations Biokovo and Pelješac had the lowest total pyrethrin content. These results are also consistent with previous reports on pyrethrin content in natural populations: 1.2% (Ambrožič Dolinšek et al., 2007), 1.1-1.3% (Ban et al., 2010), and 1-1.2% (Babić et al., 2012). Compared to commercial cultivars resulting from decades of selection and breeding for increased pyrethrin content, with pyrethrin content reaching up to 2.50% of dry flower weight (Morris et al., 2006) or 3.0% and more (Hitmi et al., 2000), it is clear that Croatia needs to make significant efforts to revitalize its pyrethrum industry and become competitive on the world market.

The highest PI/PII ratio (5.88) was found in the individual from P03 Mali Lošinj, and the lowest (0.21) in the individual from P06 Čiovo. The average PI/PII ratio of the population higher than 2 was observed in P03 Mali Lošinj (2.54), P04 Ugljan (2.14) and P01 Cres (2.05), while the lowest value was 0.92 observed for the population P07 Biokovo. Low PI/PII ratios were also

observed for other southern populations (P08 Hvar, P09 Pelješac, P10 Konavle). For comparison, in the previous study the PI/PII ratio ranged from 0.64 to 3.33 (Grdiša et al., 2013). The values of PI/PII ratio observed in some of the Croatian populations are notably higher than those previously reported for natural populations: 1.0 (Casida, 1973), 0.90–1.30 (Maciver, 1995) 1.30 (Marongiu et al., 2009) and 1.45 (Ambrožič Dolinšek et al., 2007), but more similar to the value of some breeding lines, 0.47 to 3.50 (Bhat, 1995).

The highest degree of variability (81.28%) across all populations was shown for jasmolin II content, while pyrethrin I showed the lowest degree of variability (25.54%). The population from P07 Biokovo had the highest variability of pyrethrin I and the lowest variability of pyrethrin II compared to the other populations. In terms of total pyrethrin content, the lowest variability was found in P03 Mali Lošinj (19.93%), and the highest in P09 Pelješac (50.89%).

Cluster analysis grouped individuals into four different chemotypes (P1, P2, C1/J1, P1/P2). When comparing with the results of a previous study on chemical diversity of Dalmatian pyrethrum (Grdiša et al., 2013), some agreement between the defined chemotypes can be observed, despite the difference in the level at which the cluster analysis was performed (representative population samples vs. population individuals). Chemotype P1 from this study matches well with chemotype A from the aforementioned study, not only by commonality of the main characteristics, but also geographically (A - Osor, Cres and Senj; P1 - Cres, Lošinj, Ugljan Zlarin, Čiovo, Hvar). Similarly, chemotype P1/P2 could be considered analogous to chemotype B (B - Mali Lošinj, Zlarin, Konavle; PI/PII: Lošinj, Ugljan, Zlarin and Konavle). It is worth mentioning that this study is conducted at the intrapopulation level, which leads to a higher resolution of the data and opens the possibility of an individual approach in selecting the best pyrethrin profiles, since all collected samples are stored *ex situ*. It should also be noted that the two studies have used different extraction methods (MSPD vs. UAE). While MSPD tends to extract more cinerin and jasmolin compounds, UAE seems to be optimized for higher total pyrethrin content. A preliminary comparison between the extraction efficiencies of MSPD and UAE was performed (Grdiša et al., 2020), but the results were inconclusive and a more detailed comparison of the two methods for pyrethrin extraction is required.

The chemotypes are also expected to differ in their insecticidal potential. Based on insecticidal potential the best chemotype is likely to be P1, followed by P1/P2, as both are characterized by high levels of total pyrethrin content, pyrethrin I content and PI/PII. The greatest biochemical potential is observed in the Mali Lošinj population and the lowest in Biokovo population. However, this population is located at the border of the ecological range

of the species and is adapted to harsh mountain conditions and should be considered in breeding programs for the development of lines resistant to continental climate.

In order to explain the biochemical diversity of the studied populations, a correlation between the biochemical distance matrix and the geographical distance matrix (*ln* km) was performed, which showed no significant correlation between the matrices.

Correlations of individual pyrethrin compounds and total pyrethrin content with 25 spatio-ecological variables were also calculated and showed a relationship between certain environmental factors and pyrethrin content. In this study, the most optimal pyrethrin composition was observed on the islands of Ugljan and Zlarin, where the climate is characterized by low annual precipitation and high annual temperature. In fact, temperature and the amount and seasonal distribution of rainfall are the main factors affecting pyrethrin content, while flower yield is also influenced by soil structure and fertility (Muturi et al., 1969). Optimum pyrethrum growth can be achieved at an average daily temperature of 12-15 °C. Average temperatures above 21 °C inhibit flowering entirely (Wandahwa et al., 1996) and temperatures below 17 °C for a period of six weeks are required to initiate flowering. Pyrethrin accumulation is also susceptible to heat stress during flowering period (Suraweera et al., 2020). According to trials conducted in Tasmania, temperatures above 30 °C are detrimental to pyrethrin accumulation during flowering (Greenhill, 2007). The influence of rain on flower yield is probably twofold: directly by providing soil moisture for good growth and indirectly by reducing the influence of daytime temperature (Muturi et al., 1969). Pyrethrum grows well in areas where annual rainfall is 1000 to 1400 mm, while amounts above 1400 mm increase the incidence of root rot and bud diseases (Parlevliet, 1970). It is important that annual rainfall is well distributed (monthly rainfall of ≈ 100 mm) but with sufficient dry periods to allow weed control. In this study, a significant negative correlation was observed between both total pyrethrin content and pyrethrin I content and altitude. This is in some contrast to previous studies which found that pyrethrin content increased with altitude, on average by 0.15% per 360 m (Kroll, 1964; Parlevliet, 1970). These studies were conducted in high altitude areas near the equator, where more uniform annual climatic conditions result in a longer pyrethrum flowering period (eight to nine months) and higher pyrethrum yields compared to temperate regions. Clay content was highly significantly correlated with pyrethrin I and total pyrethrin content. Based on previous studies, pyrethrum tolerates different soil types with different pH responses (Wandahwa et al., 1996), but the best results were observed on fertile, deep and well drained soils (Muturi et al., 1969). Probably the most important property of soils in which pyrethrum grows is water retentive capacity (Muturi et al., 1969).

Principal component analysis based on environmental variables clearly separated populations by geographic location. The first PC separated P07 Biokovo from the rest of the southern populations based on altitude and temperature-related variables, indicating a relationship between altered biochemical profile and the extreme environmental conditions to which the population is exposed. The second PC separated the northern populations, characterized by higher rainfall, from central and southern Adriatic populations.

Correlation analysis between the biochemical distance matrix and the ecological distance matrix showed that 22.9% of the biochemical variation could be explained by ecological differences but was not statistically significant. Similar results were obtained in studies on *Helichrysum italicum* (Roth) G. Don (Ninčević, 2020).

5.2. Development of microsatellite markers

Molecular markers are a valuable tool in diversity studies and plant breeding programs. To date, the only marker system that has been used to assess the genetic diversity of *T. cinerariifolium* natural populations is amplified fragment length polymorphism (AFLP) (Grdiša et al., 2014). This study revealed the existence of different gene pools and assessed genetic diversity between geographically distinct populations, but taxon-specific marker systems for the species such as microsatellite markers (SSRs), have not yet been developed and applied in the analysis of genetic diversity of Dalmatian pyrethrum. Compared to AFLP markers, SSR markers have properties such as multi-allelic variation, reproducibility, random distribution across the genome and codominant inheritance that make them valuable in genetic diversity research (Zalapa et al., 2012).

Despite being the source of the most widely used natural insecticide pyrethrin (Jeran et al., 2020), very few genomic data on the species are publicly available. Only 381 nucleotide records are available in NCBI (accessed on 14th March 2021), which is extremely low compared to other Mediterranean species that have economic value, such as basil (25,216), olive (470,493) or grapevine (1,186,456), suggesting that genetic research on Dalmatian pyrethrum is seriously behind.

Next generation sequencing was performed using two approaches. The first approach, in which a portion of the data was assembled de novo, resulted in 923,207 contigs. The total length of the resulting contigs was 409.5 Mbp, representing 4.3% of the estimated haploid genome size. The second approach, which merged overlapping pair end reads, yielded 5,986,468 sequences with a total length of 1,311 Mbp, representing 13.7% of the estimated

genome size (Mlinarec et al., 2019). The mean G-C content of *T. cinerariifolium* sequences obtained in this study was 34.4%, similar to the G-C content found in the genome of *H. italicum* (Baruca Arbeiter et al., 2021).

Of the 35,556 microsatellites discovered, dinucleotide repeats were the most common (60%), followed by trinucleotide repeats (26%). Tetra-, penta-, and hexanucleotide repeats together accounted for 14% of SSRs in the *T. cinerariifolium* genome. A similar distribution pattern of di- and trinucleotide repeats in this species has been reported in other species of the family Asteraceae, such as *H. italicum* (Baruca Arbeiter et al., 2021), *Helianthus annuus* L. (Badouin et al., 2017), and *Conyza canadensis* (L.) Cronquist (Peng et al., 2014).

The high frequency of A-T rich microsatellite repeats in the genome of Dalmatian pyrethrum is consistent with other dicot species such as *H. italicum* (Baruca Arbeiter et al., 2021), *Cucumis sativus* L. (Cavagnaro et al., 2010) and *Nicotiana* spp. (Wang et al., 2018).

Among the 17 developed microsatellite markers tested on 20 individuals from one population, observed heterozygosity varied from 0.083 (TcUniZg020) to 0.708 (TcUniZg008) with an average of 0.438, while expected heterozygosity varied from 0.197 (TcUniZg020) to 0.864 (TcUniZg008) with an average of 0.545. High values of observed heterozygosity are expected in *T. cinerariifolium*, a typical outcrossing perennial species with extensive gene flow (EL-Bakatoushi and Ahmed, 2018; Baruca Arbeiter et al., 2021). The polymorphic information content (*PIC*) varied from 0.178 (TcUniZg020) to 0.824 (TcUniZg008) with an average of 0.492. A low *PIC* was observed at the microsatellite loci TcUniZg004, TcUniZg013, TcUniZg020 and TcUniZg037 ($PIC < 0.290$), where only a small number of alleles were present ($N_a < 5$), suggesting that these loci are not suitable for use in genetic diversity analysis of the species.

Microsatellite loci abundance increases with genome size, but as species genome size increases, PCR amplification efficiency often decreases due to the dilution effect. The proportion of available target DNA decreases in a template DNA volume, and the amount of non-specific primer binding increases (Garner, 2002). This poses a major challenge in designing SSRs for species (such as *T. cinerariifolium*) with large genomes by classical methods. Next generation sequencing allows for rapid and cost-effective development of microsatellite markers in non-model species, although it should be noted that this method can still be costly and time-consuming in species with large genomes compared to other species (Schoebel et al., 2013). These 17 loci represent an initial set of microsatellite markers for use in population studies of Dalmatian pyrethrum.

5.3. Genetic diversity

Microsatellite markers are widely used in the genetic diversity analysis (Rešetnik et al., 2016), genome fingerprinting (Zietkiewicz et al., 1994), and germplasm conservation (Guzmán et al., 2020). Until recently, the process of their development using classical methods has proven to be time-consuming and expensive (Li et al., 2018), but with the advent of NGS, the cost of microsatellite marker development is greatly reduced (Abdelkrim et al., 2009). This technique has already been applied in the development of Expressed Sequence Tag SSRs (EST-SSRs) for *Chrysanthemum nankingense* Hand.-Mazz., a species from the genus to which Dalmatian pyrethrum once belonged (Wang et al., 2013).

The genetic diversity of 194 individuals from 10 populations of Dalmatian pyrethrum was analyzed using 12 microsatellite loci. A total of 121 alleles were detected in the studied populations with an average of 10.08 alleles per locus, indicating high genetic diversity. The values of observed heterozygosity were moderate on average (H_O = 0.543), which is expected in an outcrossing species (Ninčević, 2020). High values of expected heterozygosity (H_E = 0.557) indicate high genetic diversity of the analyzed populations. The highest number of private alleles was found in P10 Konavle, the southernmost population (N_{pr} = 7), while the lowest was found in P07 Biokovo (N_{pr} = 1). In P07, the Wilcoxon sign-rank test also showed recent bottleneck events, which are a result of decrease in population size.

The genetic distance between population pairs (Cavalli-Sforza chord distance) varied from 0.049 between P02 Senj and P09 Pelješac to 0.173 between P07 Biokovo and P03 Mali Lošinj. The Neighbor-Joining tree based on these distances shows the separation of two major clades (supported by a bootstrap value of 79%). The larger clade can be characterized as an island clade consisting of populations P01 Cres, P03 Mali Lošinj, P04 Ugljan, P05 Zlarin, P08 Hvar, with the addition of P10 Konavle, the southernmost mainland population in the sample. The second clade can be characterized as a mainland clade consisting of P02 Senj, P07 Biokovo and P09 Pelješac, with the addition of an island population - P06 Čiovo. Population P07 Biokovo also showed clear separation from the other populations (supported by a bootstrap value of 68%). This is somewhat in contrast to a previous study on Dalmatian pyrethrum, which showed a clear separation of populations into northern and southern clade based on AFLP markers (Grdiša et al., 2014). The pattern of geographic separation of populations into northern and southern is well documented in other Mediterranean species with similar range such as Dalmatian pyrethrum based on AFLP markers, with the split occurring mainly in the Kvarner Bay region (Jug-Dujaković et al., 2020; Ninčević, 2020).

The FCA results showed the same pattern of splitting as the NJ tree. Populations from the 'mainland' clade of the NJ tree clearly separated from populations from the 'island' clade of the NJ tree along the first axis of the FCA biplot. The Biokovo population separated most clearly from all other populations in a separate quadrant of the biplot, while the other populations from the clade also showed good differentiation from each other. Populations from the 'island' clade showed less genetic differentiation on the biplot, especially populations P01 Cres, P05 Zlarin and P08 Hvar.

AMOVA results showed much greater genetic variation within Dalmatian pyrethrum populations (87.03%), which is characteristic of outcrossing plant species (Nybom and Bartish, 2000). Similar results were obtained in the previous study on the genetic diversity of Dalmatian pyrethrum (Grdiša et al., 2014) and in studies of other Mediterranean plant species such as *Salvia officinalis* L. (Jug-Dujaković et al., 2020) and *H. italicum* (Ninčević, 2020). Differentiation between populations of Dalmatian pyrethrum was low (F_{ST} = 0.129), suggesting gene flow between populations (Wright, 1949). Compared to previous study of Dalmatian pyrethrum, the populations are more differentiated, which may be a result of the lower population number and their geographic distribution within this study.

In the Bayesian population structure analysis, the average likelihood estimates [*ln P(X|K)*] were calculated for each of the 30 runs for each *K* (*K* = 1 to 11). As K increased, the likelihood estimates also increased. The highest *ΔK* was observed for *K* = 2 (330.83). The second highest *ΔK* was for *K* = 3 (247.04). At higher *K* values, the *ΔK* values decreased (0.80 – 66.82) and the variance of [*ln P(X|K)*] increased, thus probability of more than three gene pools was considered less likely. The proportions of ancestry of all individuals in each gene pool were calculated for *K* = 2 to 3. The results were consistent with those obtained using distance-based methods. At *K* = 2, populations in which gene pool A was predominant were mostly island populations, with the addition of the Konavle population, while gene pool B was predominant in mainland populations and Čiovo. Populations from Senj, Zlarin and Hvar were the most admixed populations in the sample. At *K* = 3, gene pool C was predominant only in the Biokovo population, which is consistent with other results indicating isolation of the population from the rest of the sampled populations. Gene pool C was also previously determined using AFLP markers, with three populations from Biokovo (Ravna Vlaška, Lađena and Kotiški Stanovi) showing a large proportion of ancestry from this gene pool (Grdiša et al., 2014).

To explain the genetic differentiation of the studied populations, an IBD analysis was performed between the $F_{ST}/(1- F_{ST})$ matrix and the distance matrix (*ln* km), which showed no significant correlation between the matrices and that only 0.7% of the genetic differentiation

between samples can be explained by IBD. One of the major causes of IBD are pollen and seed dispersal limitations (Ghazoul, 2005). Our results suggest substantial gene flow between the majority of populations and differ somewhat from a previous genetic study in which IBD explained a larger proportion of genetic differentiation (5.6%) (Grdiša et al., 2014). These results are also supported by the number of barriers identified using Monmonier's maximum difference algorithm. Only four barriers were present based on the sampled populations, and only two of them were supported by high bootstrap values in all sections, separating populations P07 Biokovo and P10 Konavle from other populations and from each other.

Genetic differentiation was better explained by IBED. Since ecological variables are often correlated with geographical distance, the partial Mantel test was used to examine the relationship between the ecological distance matrix and the genetic distance matrix [$F_{ST}/(1-F_{ST})$] excluding geographical distance, which explained 40.61% of genetic differentiation, much higher than in the previous study (12.3%) (Grdiša et al., 2014). Although a high correlation was found between the two matrices (0.637), the correlation itself was not significant. This could be explained by the presence of null alleles and the rather low variability of the microsatellite loci used, rather than the small number of populations in the study (Landguth et al., 2012; Séré et al., 2017).

When compared, the results of molecular analyses and spatial genetics suggest different causes for the current pattern of genetic diversity in Croatian populations of Dalmatian pyrethrum. Previous study on the genetic diversity of Dalmatian pyrethrum (Grdiša et al., 2014), as well as studies on some other species with Mediterranean distribution (Surina et al., 2011; Lakušić et al., 2013; Jug-Dujaković et al., 2020) suggest that the existence of microrefugia in Kvarner Bay area caused the current patterns of genetic diversity. The results of this study suggest that the observed genetic diversity of Dalmatian pyrethrum is the result of several factors. Human influence should be particularly considered, as there are records of a long history of cultivation and overexploitation of the species, as well as fragmentation and loss of habitat in recent decades due to urbanization of the coastal region. Unfortunately, no detailed records, which could explain the current genetic structure, exist about the source of plant material used for cultivation in different parts of the Adriatic. Even considering the role of environmental factors in shaping the genetic structure of Dalmatian pyrethrum, we can only presume the exact course of events that led to the current state of the populations. Nevertheless, the high genetic variability of the species represents a potential source of genes for resistance to various abiotic and biotic factors, as well as much needed data for efficient management and protection of this endemic species (Schaal et al., 1998). It would be

interesting to compare the genetic diversity of natural Dalmatian pyrethrum populations with that of cultivated populations in countries from different regions such as Australia, Ecuador and Rwanda, where Dalmatian pyrethrum breeding programs are still very active.

5.4. Correlation between biochemistry and genetics

The correlation between genetic and biochemical distances was moderate but not significant. Compared to a previous study on chemical and genetic diversity of Dalmatian pyrethrum (Grdiša, 2011), a higher level of correlation was observed in this study (r = 0.344 vs. r = 0.069), but both results were not statistically significant. Investigating the relationship between biochemistry and genetics of economically important species is an important step in determining appropriate cultivation and breeding steps to improve the yield of target crops (Echeverrigaray et al., 2001; Li et al., 2013). Once a good connection between the two is established, association mapping can be used to identify quantitative trait loci (QTL) linking observable traits to genotypes (Fusari et al., 2012; Guan et al., 2019).

6. CONCLUSIONS

1. The total pyrethrin content varied from 0.10% to 1.35% of the dry flower weight in sampled individuals. On average, the highest total pyrethrin content was observed in the populations from Mali Lošinj and Zlarin (0.87% of dry flower weight) and the lowest in populations from Pelješac (0.22%) and Biokovo (0.37%). The average total pyrethrin content over all 200 samples was 0.58%.
2. A trend of decreasing pyrethrin I content as well as PI/PII ratio (an important indicator of pyrethrum extract quality) towards the south was observed. This might be related to the long tradition of pyrethrum cultivation and selection for higher pyrethrin yield in the northern parts of the Adriatic (mainly on the islands of Krk, Cres and Lošinj). On the contrary, pyrethrin II (and cinerin II) content increased with lower latitude.
3. The population from Biokovo mountain showed a unique chemical profile when compared to other analyzed populations, with pyrethrin II as the dominant component (43.18% of the total pyrethrin content), the lowest average PI/PII (0.92), with 75% of individuals having a PI/PII ratio lower than 1.
4. Cluster analysis assigned individuals to four clusters, which can be considered as different chemotypes. Chemotype P1 (followed by P1/P2) proved to be the most promising for future plant breeding programs, as it is characterized by the highest total pyrethrin content, pyrethrin I content and PI/PII ratio, which are important measures of insecticidal activity.
5. The populations with the highest biochemical potential are those from islands of Mali Lošinj and Zlarin. These populations should be used as a source of potentially desirable genotypes in terms of pyrethrin quantity and quality.
6. Correlations of biochemical distance with geographical and ecological distances were not statistically significant.
7. A total of 17 microsatellite markers were successfully developed for Dalmatian pyrethrum (*T. cinerariifolium*), 14 of them contained a dinucleotide motif, while three of them contained a trinucleotide motif. Based on their performance in the test samples, 12 loci were selected and successfully used in genetic diversity analysis of natural populations of Dalmatian pyrethrum.
8. AMOVA showed that most of the genetic diversity in the studied populations was due to differences between individuals within populations (87.03%), while the rest (12.97%) was due to differences between populations.

9. Both the FCA and the NJ tree, based on Cavalli-Sforza chord distances, separated the populations into two clades (bootstrap value of 79%), the ‘mainland’ clade consisting of the populations from Senj, Biokovo and Pelješac with the addition of the island population of Čiovo, and the ’island’ clade consisting of the populations Cres, Mali Lošinj, Ugljan, Zlarin and Hvar with the addition of the mainland population Konavle.
10. The existence of two gene pools (A and B) was determined. Gene pool A was dominant in the island populations (with addition of Konavle), while gene pool B was dominant in the mainland populations (with inclusion of Čiovo). At $K = 3$, gene pool C was introduced and was dominant only in the population from Biokovo.
11. A low correlation between genetic and geographical distances was found, explaining 0.7% of genetic differentiation. The correlation between genetic and ecological distances excluding geographical distance was much higher, explaining 40.2% of genetic differentiation.
12. The correlation between genetic and biochemical distances was moderate but not significant.

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8. AUTHOR'S BIOGRAPHY

Filip Varga was born on January 27th, 1989 in Čakovec. He attended the Medical High School in Varaždin (Medical Laboratory Technician), where he graduated in 2008. In the same year, he enrolled in undergraduate studies of biology at the University of Zagreb, Faculty of Science and graduated in 2011 with a thesis titled 'Ferns of Palaeophytic'. He continued his education at the graduate studies of experimental biology (module Botany) at the University of Zagreb, Faculty of Science. In 2014 he graduated with the thesis 'Digitizing, geocoding and analysis of Ivo Trinajstić's herbarium collection in Croatian Natural History Museum'.

In 2014 he worked at the University of Zagreb, Faculty of Pharmacy and Biochemistry on design of complete infrastructure and preventive care of the herbarium collection Fran Kušan. From 2014-2015 he worked as an external collaborator for digitization and geocoding of botanical information at the State Institute for Nature Protection. Since July 2015 he has been working as an assistant at the Faculty of Agriculture, Department of Seed Science and Technology, teaching the practical part of the courses 'Molecular Biodiversity and Evolution' and 'Applied Analysis of Spatial Data Using R'. In 2016, he enrolled in the postgraduate doctoral studies of Agricultural Sciences.

He actively participated in the implementation of several national and international projects: 'Molecular and Phytochemical Characterization by RAMAN Spectroscopy of Economically Important Indigenous Medicinal Plants' (2016-2017), 'Stvaranje tetraploidnog dalmatinskog buhača (*Tanacetum cinerariifolium*) s udvostručenim brojem kromosoma u svrhu povećanja sadržaja prirodnog insekticida piretrina' (2016-2017), and 'Genetic background of Dalmatian pyrethrum (*Tanacetum cinerariifolium* /Trevir./ Sch. Bip.) insecticidal potential' (2017-2021). He actively participates in the implementation of the following projects: 'The Centre of Excellence for Biodiversity and Molecular Plant Breeding' (2018-2023), 'Genome-wide microsatellite identification and the complete chloroplast genome assembly of *Tanacetum cinerariifolium*, *Salvia officinalis* and *S. fruticosa'* (2020-2021), 'CEKOM 3LJ' (2020-2023) and 'Twinning Open Data Operational' (2020.2023). He is a member of the Center of Excellence for Biodiversity and Molecular Plant Breeding (Crop-BioDiv), WP08- Dalmatian Pyrethrum / Sage and a member of the Working Group for Medicinal and Aromatic Plants of the National Program for Conservation and Sustainable Use of Plant Genetic Resources for Food and Agriculture in the Republic of Croatia.

Filip Varga completed his scientific training in applied GIS analysis at Utrecht University, Utrecht, Netherlands; ecological niche modeling at Transmitting Science, Capellades (Barcelona), Spain; open data at the University of Zagreb, Faculty of Organization and Informatics, Varaždin, Croatia; and next-generation sequencing at Exaltum Ltd. in Zagreb, Croatia.

He is a member of AMAPSEEC (Association for Medicinal and Aromatic Plants of Southeast European Countries) and HBoD (Croatian Botanical Society). His research interests include genetic and biochemical diversity and conservation of plant genetic resources, applied spatial analysis in botany and agriculture, ethnobotany, open data in science.

He co-authored and published five (5) a1 scientific papers, two (2) a2 scientific papers and participated as author or co-author in 26 international and national scientific conferences.

Scientific publications:
a1)

1. Jeran N., Grdiša M., Varga F., Šatović Z., Liber Z., Dabić D., Biošić M. (2020). Pyrethrin from Dalmatian pyrethrum (*Tanacetum cinerariifolium*/Trevir./Sch. Bip.): biosynthesis, biological activity, methods of extraction and determination. Phytochem Rev. doi:10.1007/s11101-020-09724-2
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a2)

1. Grdiša M., Varga F., Ninčević T., Ptiček B., Dabić D., Biošić M. (2020). The extraction efficiency of maceration, UAE and MSPD in the extraction of pyrethrins from Dalmatian pyrethrum. Agric Conspec Sci 85 (3): 257–267
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9. APPENDICES

Appendix 1. Biochemical distance matrix between populations of Dalmatian pyrethrum. The distance is Euclidean distance based on the scores of the first three principal components.

Population	P01	P02	P03	P04	P05	P06	P07	P08	P09	P10
P01	0.000									
P02	0.784	0.000								
P03	2.210	2.267	0.000							
P04	1.370	1.249	1.053	0.000						
P05	1.332	1.250	1.187	0.618	0.000					
P06	1.404	1.223	1.088	0.263	0.410	0.000				
P07	2.975	2.295	3.037	2.462	2.272	2.262	0.000			
P08	0.946	0.320	2.234	1.261	1.128	1.169	2.051	0.000		
P09	2.225	1.816	4.069	3.047	2.976	2.996	2.856	1.851	0.000	
P10	1.367	1.244	1.752	1.204	0.629	0.985	2.086	1.006	2.708	0.000

Appendix 2. Correlations of six pyrethrin compounds and total pyrethrin content with 25 ecological variables.

Ecological variable	Cinerin I		Cinerin II		Jasmolin I		Jasmolin II		Pyrethrin I		Pyrethrin II		Total pyrethrin content	
BIO1	0.013	ns	-0.345	***	0.218	**	-0.071	ns	0.352	***	-0.320	***	0.272	***
BIO2	0.093	ns	-0.169	*	0.290	***	0.087	ns	0.076	ns	-0.122	ns	0.062	ns
BIO3	0.071	ns	-0.173	*	0.290	***	0.070	ns	0.080	ns	-0.115	ns	0.069	ns
BIO4	0.078	ns	-0.091	ns	0.225	**	0.087	ns	0.010	ns	-0.056	ns	0.013	ns
BIO5	0.070	ns	-0.335	***	0.290	***	-0.019	ns	0.322	***	-0.328	***	0.217	**
BIO6	-0.019	ns	-0.305	***	0.115	ns	-0.126	ns	0.366	***	-0.314	***	0.254	***
BIO7	0.121	ns	-0.148	*	0.288	***	0.109	ns	0.064	ns	-0.128	ns	0.035	ns
BIO8	-0.042	ns	-0.331	***	0.148	*	-0.148	*	0.310	***	-0.229	**	0.266	***
BIO9	-0.123	ns	-0.433	***	0.015	ns	-0.285	***	0.456	***	-0.289	***	0.435	***
BIO10	0.020	ns	-0.354	***	0.239	***	-0.064	ns	0.351	***	-0.322	***	0.272	***
BIO11	0.017	ns	-0.326	***	0.212	**	-0.060	ns	0.340	***	-0.315	***	0.253	***
BIO12	0.234	***	0.135	ns	0.399	***	0.342	***	-0.187	**	-0.040	ns	-0.320	***
BIO13	0.121	ns	0.082	ns	0.367	***	0.259	***	-0.134	ns	-0.026	ns	-0.215	**
BIO14	-0.078	ns	-0.227	**	-0.093	ns	-0.201	**	0.261	***	-0.145	*	0.295	***
BIO15	-0.255	***	-0.191	**	-0.168	*	-0.236	***	0.193	**	0.000	ns	0.366	***
BIO16	0.185	**	0.073	ns	0.353	***	0.276	***	-0.127	ns	-0.052	ns	-0.225	**
BIO17	0.287	***	0.086	ns	0.351	***	0.239	***	-0.092	ns	-0.125	ns	-0.366	***
BIO18	0.235	***	0.266	***	0.186	**	0.289	***	-0.245	***	0.024	ns	-0.422	***
BIO19	-0.056	ns	0.132	ns	0.046	ns	0.091	ns	-0.181	*	0.160	*	-0.078	ns
RAD	-0.036	ns	0.100	ns	-0.055	ns	0.115	ns	-0.144	*	0.136	ns	0.059	ns
CESOL	-0.146	*	-0.005	ns	0.141	*	0.115	ns	-0.037	ns	0.046	ns	0.163	*
CLYPPT	-0.064	ns	-0.319	***	0.269	***	-0.052	ns	0.304	***	-0.261	***	0.263	***
ORCDRC	-0.145	*	0.238	***	-0.336	***	-0.050	ns	-0.168	*	0.225	**	-0.025	ns
ALT	-0.060	ns	0.387	***	-0.167	*	0.156	*	-0.405	***	0.356	***	-0.252	***
COAST	0.039	ns	0.418	***	-0.072	ns	0.232	**	-0.449	***	0.335	***	-0.383	***

[ns]non-significant; [*]significant at $P < 0.05$; [**]significant at $P < 0.01$; [***]significant at $P < 0.001$

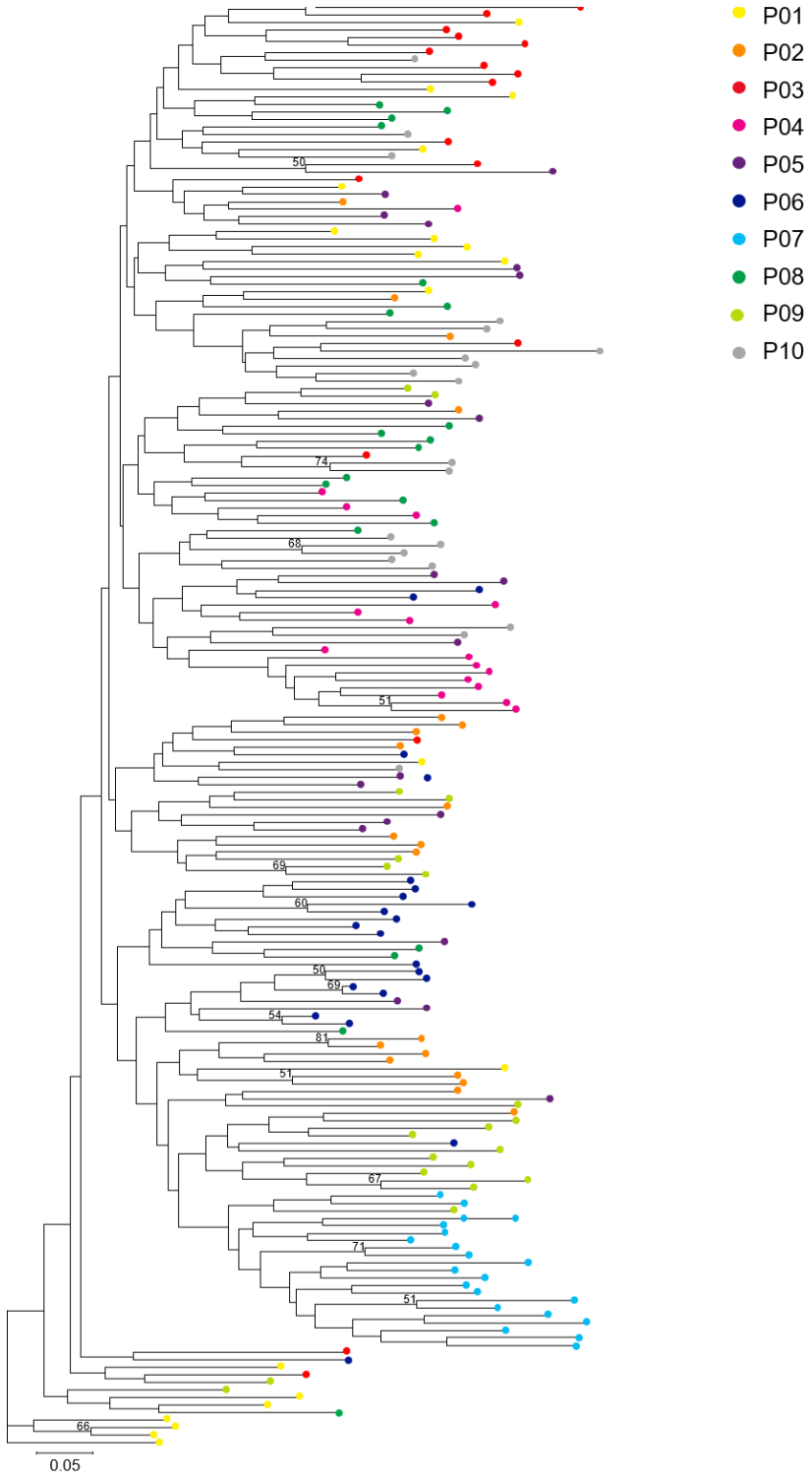
Appendix 3. Ecological distance matrix between populations of Dalmatian pyrethrum. The distance is Euclidean distance based on the scores of the first four principal components.

Population	P01	P02	P03	P04	P05	P06	P07	P08	P09	P10
P01	0.000									
P02	3.947	0.000								
P03	5.007	3.379	0.000							
P04	5.388	5.360	3.521	0.000						
P05	4.224	6.148	5.168	2.747	0.000					
P06	4.833	7.153	6.899	4.333	2.067	0.000				
P07	10.725	10.739	9.669	10.247	10.710	11.366	0.000			
P08	6.446	7.317	4.890	2.468	3.011	4.775	10.512	0.000		
P09	4.570	4.228	5.767	5.459	5.535	5.442	9.373	7.468	0.000	
P10	2.622	5.728	5.190	5.241	3.683	4.827	10.312	5.226	6.383	0.000

Appendix 4. Genetic distance matrix (Cavalli-Sforza chord distance) between populations of Dalmatian pyrethrum.

Population	P01	P02	P03	P04	P05	P06	P07	P08	P09	P10
P01	0.0000									
P02	0.0630	0.0000								
P03	0.0554	0.0736	0.0000							
P04	0.0951	0.0996	0.0849	0.0000						
P05	0.0594	0.0556	0.0577	0.0731	0.0000					
P06	0.0889	0.0770	0.1054	0.1122	0.0806	0.0000				
P07	0.1319	0.1082	0.1727	0.1531	0.1217	0.1289	0.0000			
P08	0.0525	0.0643	0.0643	0.0842	0.0577	0.0831	0.1271	0.0000		
P09	0.0787	0.0485	0.0850	0.1054	0.0661	0.0685	0.0896	0.0758	0.0000	
P10	0.0795	0.0901	0.0605	0.0811	0.0707	0.1154	0.1409	0.0698	0.1003	0.0000

Appendix 5. Neighbor-joining tree based on the proportion of shared alleles between all individuals. Bootstrap values larger than 50% are denoted on the branches.



Appendix 6. F_{ST} matrix between populations of Dalmatian pyrethrum. All values are significant at $P < 0.001$ after 10,000 permutations.

Population	P01	P02	P03	P04	P05	P06	P07	P08	P09	P10
P01	0.000									
P02	0.064	0.000								
P03	0.064	0.103	0.000							
P04	0.175	0.180	0.143	0.000						
P05	0.068	0.047	0.056	0.091	0.000					
P06	0.119	0.089	0.156	0.227	0.098	0.000				
P07	0.195	0.146	0.256	0.270	0.178	0.239	0.000			
P08	0.053	0.092	0.076	0.120	0.053	0.098	0.237	0.000		
P09	0.100	0.037	0.133	0.191	0.070	0.090	0.139	0.116	0.000	
P10	0.123	0.136	0.087	0.119	0.100	0.199	0.229	0.081	0.175	0.000