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Research Article

Genetic diversity of seven-spotted ladybug populations in Iran using cytochrome oxidase gene analysis

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Abstact

Lady beetles, particularly the seven-spotted lady beetle, play an essential role as biological control agents against aphids and other pests in agricultural ecosystems. Studying their molecular biology can provide insights into their adaptation, behavior, and potential for pest control. Recent progress in genomic technologies has led to a better understanding of the molecular mechanisms involved in these processes. In this study, we examined the genetic structure of Iranian populations of this species by sequencing the cytochrome oxidase gene. Analysis of thirty-three genetic sequences (positions 1-576) showed a haplotype diversity of 0.945, with 25 distinct haplotypes identified. The nucleotide diversity was relatively low ($\pi = 0.00658$), revealing a discrepancy between haplotype diversity and nucleotide diversity. This pattern suggests the presence of a genetically diverse population with conserved genomic regions, likely resulting from recent population expansion or selective pressures. Our findings provide important information about population dynamics and offer valuable insights for conservation strategies and ecological planning.

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Introduction

The seven-spotted lady beetle *Coccinella septempunctata* Linnaeus, 1758 is originally from Europe and Asia, but it is now found throughout the Middle East, India and North America (USA and Canada) (Babu & Azam 1987; Omkar & Shefali Srivastava 2002). This ladybug species exhibits distinctive physical characteristics. Adults measure between 5-8 mm in length and feature vibrant red elytra adorned with seven black spots. The head, pronotum, and legs display dark coloring (Sayed 2016; Roy & Brown 2018). The Seven-spot ladybird is a predatory insect that feeds on various aphid species throughout its life cycle. During winter, it overwinters as an adult in protected areas such as foliage, dead plant material, and leaf litter. The widespread success of this species can be attributed to its ecological adaptability, which is based on both genetic and phenotypic variations (Honek & Martinková 2005; Honek *et al*. 2007; Cantrell 2011; Hodek & Michaud 2013).

Biological control methods, employing organisms such as nematodes, arthropods, and fungi to suppress insect pests, have been a cornerstone of crop protection for over a century. This eco-friendly approach not only offers significant ecological advantages but also provides substantial economic benefits for both farmers and the agricultural industry. For successful use of biological control agents, all key factors must be carefully considered. This includes monitoring potential natural enemies like parasitoids and predators that may attack the biocontrol agents themselves. Environmental conditions, host specificity, and compatibility with other pest management methods should also be evaluated (Kadono-Okuda *et al*. 1995; Gurr & Wratten 1999; Gutierrez *et al*. 1999; Gurr *et al*. 2000; Krafsur *et al*. 2005; Sethuraman *et al*. 2020).

Biological control methods, though effective, come with challenges and risks. Introducing foreign organisms into ecosystems can have unintended consequences. Recent advances in population genomics have significantly expanded our understanding of biological control. This emerging technology has opened up new avenues for researchers to study this method more comprehensively. Population genetics has proven valuable in understanding evolutionary changes in pest populations and control agents. By analyzing genetic data over time, scientists gain insights into how these organisms adapt and evolve (Clausen 1978; Kajita *et al*. 2006; Sethuraman *et al*. 2020). Moreover, population genetics provides crucial information about both short-term and long-term effects of releasing biological control organisms into the wild. This knowledge is essential for predicting how these organisms will behave and interact with their surroundings over extended periods. Genomic data analysis also allows researchers to monitor key factors that are critical for the success of pest management programs. By examining genetic variations within control agent populations, scientists can identify traits that contribute most effectively to pest suppression and those that may pose risks to non-target species. These advances in population genomics are revolutionizing our approach to biological control, enabling us to make more informed decisions about which organisms to use, how to deploy them effectively, and how to mitigate potential risks. With comparing genetic sequences across multiple populations, scientists aims to elucidate patterns of genetic diversity and potential barriers to gene flow that may influence the species' distribution trends (Sethuraman *et al*. 2020; Webster *et al*. 2023, Heuertz *et al*. 2023).

This shift towards population genomics reveals insights into long-term changes in pest populations and control organisms. This new perspective enhances our ability to develop and maintain efficient biological pest control methods (Gassmann *et al.* 2009; Guillemaud *et al*. 2011; Rius *et al*. 2015). Mitochondrial DNA (mtDNA) serves as an effective tool for tracing genetic relationships within species. Among mtDNA genes, cytochrome oxidase I (COI) stands out as one of the most widely used in systematic and population studies (Javonillo *et al.* 2010; Castro *et al.* 2014; Abdolahadi *et al.* 2022). This approach goes beyond immediate effects, considering factors such as shifts in population size, natural *vs*. artificial selection, genetic mixing, and co-evolutionary processes. The

use of *COI* gene analysis extends beyond short-term impacts, examining long-term evolutionary dynamics and demographic changes across generations. By studying this gene, researchers can gain insights into complex biological phenomena that shape the genetic makeup of populations over time (Chan *et al*. 2021). Understanding these evolutionary dynamics is crucial for developing effective biological control strategies (Kolbe *et al*. 2004; Dlugosch & Parker 2008; Phillips *et al*. 2008; Lombaert *et al*. 2014).

Materials and Methods

Sample collection

In this study, samples related to 33 populations were collected from eleven provinces of Iran, encompassing Hamadan, Tehran, Kordestan, East Azerbaijan, West Azerbaijan, Kermanshah, Mazandaran, Khuzestan, Hormozgan, Yazd and Ardabil (Table 1). These provinces were chosen due to their varying climates to explore how environmental factors and geographical distance influence genetic variation within populations. All samples were carefully collected from alfalfa plants (*Medicago sativa* L.), and all specimens were female ladybugs. They were stored inside alcohol jars 85% ethanol). Until molecular analysis, these samples were kept at -20°C.

DNA extraction

Adult insect DNA extraction was conducted using the TOP General Genomic DNA Purification Kit from Topaz Gene Company (Topaz Gene Kavosh Company, Karaj, Alborz Province, Iran) While carefully following the manufacturer's protocol, certain adjustments were made to optimize the process. Post-extraction, DNA yield and purity were assessed through agarose gel electrophoresis.

The mitochondrial cox1 gene was amplified using the universal primers cox1F 5’-ATTCAACCAATCATAAAGATATTGG-3’ and cox1RTAAACTTCTGGATGTCCAAAAAATCA-3’ (Hebert et al. 2004).

PCR reaction was performed in a final volume of 30 µL containing 100 ng of DNA, 0.2 U of Taq DNA polymerase, 1.5 mM $MgCl_2$, 200 nM dNTPs, and 0.3 µM of each primer. The PCR cycling parameters for the *CO1* gene amplification consisted of 35 consecutive cycles, with each cycle incorporating three distinct phases: denaturation at 94°C for 30 seconds, annealing at 50°C for 1 minute, and extension at 72°C for 1 minute.The resulting PCR products were visualized using a 1% agarose gel treated with ethidium bromide under UV (Sayed 2016).

PCR products were purified using GEL and PCR purification. kit (Yekta Tajhiz Azma), and subsequently sequenced by Bi-directional Sanger Sequence analysis at Macrogen company. We edited the raw sequences using BioEdit version 7.0.9.0 software, developed (Hall 1999). Molecular analyses were conducted using MEGA version 7.0.26 (Kumar *et al*. 2016) for sequence alignment, genetic distance calculations, and phylogenetic tree reconstruction (Figure 1). To confirm species identity, the sequences were compared using BLAST with the NCBI GenBank database. Genetic diversity metrics including nucleotide substitutions, mutation rates, and interpopulation genetic distances were quantified using the Kimura 2-parameter model. Neutrality tests, haplotype distribution, and nucleotide diversity indices were computed with DNAsp v5 (Librado & Rozas 2009). Phylogenetic relationships were inferred through maximum likelihood analysis (1,000 bootstrap replicates) in MEGA 7.0.26 (Kumar *et al*. 2016). Population structure was assessed via hierarchical analysis of molecular variance (AMOVA), FST statistics, and neutrality tests implemented in Arlequin v3.5.2.2 (Excoffier *et al*. 2005). Finally, haplotype networks were reconstructed using PopART v1.7.2 (Leit & Bryant 2015), enabling robust inference of evolutionary trajectories and population connectivity.

Table 1. Sampling localities and geographical origin of *Coccinella septempunctata* populations analysed in this research.

Province Name	Collection Location	Code	Longitude	Latitude	Height
Ardabil (Pars Abad)	Ebrahim Abad	1	747063	4390142	50
	Majid Abad	2	752694	4391617	45
	Iran Abad	3	752248	4385195	60
East Azerbaijan	Sarab	4	718615	4199958	1670
	Tabriz	5	599048	4217401	1310
	Ajabshir	6	578385	4145687	1287
West Azerbaijan	Oromiyeh	7	506168	4176939	1300
	Salmas	8	478958	4221533	1379
	Khoye	9	485087	4273263	1274
Tehran	Shar Rey	10	543734	3934460	1030
	Kahrizak	11	529477	3929715	1000
	Eslam Shahr	12	518457	3926461	1032
Yazd	Cham	13	240096	3520162	1419
	Ashkezar	14	236936	3544993	1174
	Fahraj	15	270711	3516106	1277
Khuzestan (Ahvaz)	Jasanieh	16	282555	3478935	18
	Hamidieh	17	252292	3483227	20
	Kut Abdollah	18	279158	3456741	22
Hormozgan (Jask)	Divel	19	626054	2847749	20
	Shahrak	20	609686	2845085	20
	Divel	21	625803	2847094	20
Mazandaran (Myandorood)	Tabagh deh	22	697100	4072357	2
	Tabagh deh	23	695724	4070752	3
	Tabagh deh	24	697348	4070642	2
Hamedan	Malayer	25	267929	3802523	1640
	Bahar	26	266689	3865497	1720
	Razan	27	320102	3919403	1856
Kordestan	Saghez	28	617426	4013554	1748
	Dehgolan	29	713600	3909009	1833
	Kamyaran	30	673881	3849356	1391
Kermanshah	Kangavar	31	767915	3817493	1486
	Harsin	32	735464	3794827	1527
	Mahidasht	33	667950	3794056	1362

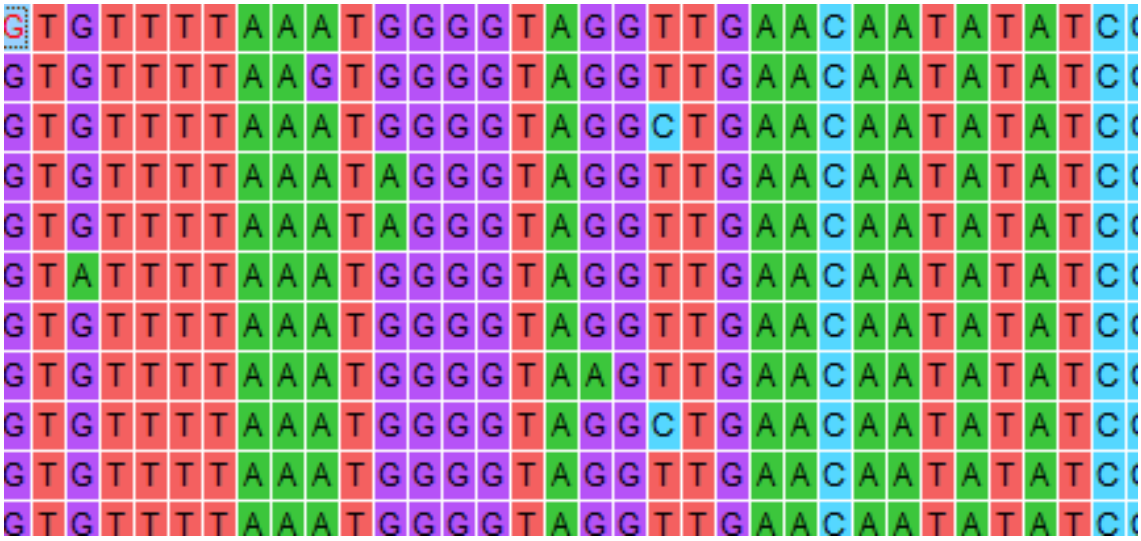

Figure 1. Multiple sequence alignment of mitochondrial *COI* gene fragments from *Coccinella septempunctata* populations using MEGA software.

Results and Discussion

In this research thirty three DNA sequences were analyzed from various populations focusing on a portion of the *COI* gene. This gene provides useful information for reconstructing evolutionary relationships between insect species (Deagle *et al*. 2014; Porter & Hajibabaei 2018; Singh *et al.* 2022 Magoga *et al.* 2022). While it could be argued that the genetic makeup at functional gene locations (which potentially contribute to selected traits) would provide the most accurate forecast of population performance (Mathur *et al.* 2023). Furthermore, it's likely that loci and alleles influencing performance are context-dependent across different populations and species (Dunbar *et al.* 2007; Rodríguez *et al.* 2014; Graves & Weinreich 2017). So in this research, 33 DNA sequences were analyzed from various populations focusing on a portion of the *COI* gene. These sequences were compared against existing records in Genbank and the World Barcoding Center. The results showed high levels of similarity (95-100%) between the analyzed sequences and those stored in databases. Our study is the first attempt to identify this biological control agent molecularly in Iran. The aim of the present study is to create a molecular database of the mitochondrial *COI* gene of this predator species, which can be of great value to the field of entomology. One of the results of this research is the addition of 33 new sequences with accession numbers PQ605655, PQ605744-PQ605751 and PQ606373-PQ606396 to GenBank.

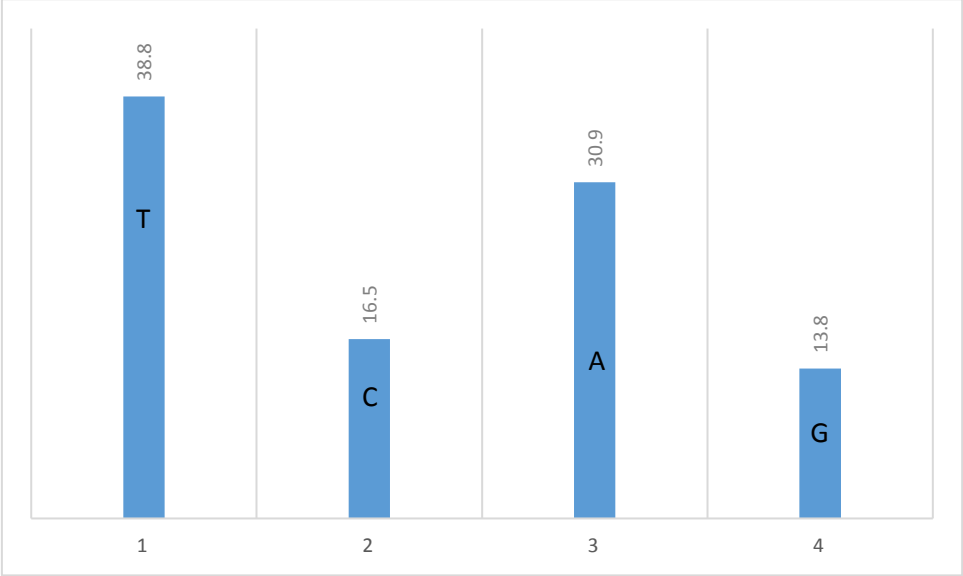

Figure 2. The average nucleotide composition of the 576 bp segment of cocinella septempunctata in 33 studied populations. T = Thymine, C = Cytosine, A = Adenine, G = Guanine.

The genetic analysis of the seven-spotted ladybug populations revealed a relatively conserved genome with limited genetic diversity. The dataset consisted of 33 sequences, all of which were used in the analysis, covering a region of 576 sites (positions 1-576). Notably, this region had no alignment gaps or missing data, indicating high-quality sequence information. The nucleotide composition of 576 base pairs was analyzed across 33 studied populations. The results showed that Thymine made up 38.8% of the bases, Cytosine accounted for 16.5%, Adenine comprised 30.9%, and Guanine represented 13.8% (Figure 2). The genetic diversity was found to be low, with only 32 variable (polymorphic) sites out of 576 total sites, representing a mere 5.56% of the analyzed region.

The observation of 25 haplotypes across 33 population could be due to several factors, including small population sizes, geographical isolation, or genetic bottlenecks. The uneven distribution of haplotypes suggests that some populations may be experiencing genetic drift, leading to fixation of

certain haplotypes while others go extinct. The genetic diversity analysis revealed notable regional differences in haplotype distribution among the studied populations. Haplotype number ten was the most widespread, appearing in eight different populations (codes 10, 11, 25, 27, 28, 29, 31, 32). This suggests that haplotype ten may be particularly well-adapted to certain environmental conditions prevalent in these regions (Table 2).

Table 2. Distribution of haplotypes of different populations of in *Coccinella septempunctata* from different provinces. Information about population codes is listed in Table 1.

H	Code of cities with haplotype
H1	1
H2	2
H۳	3
H4	4
H5	5
H6	6
H7	7
H8	8
H9	9
H10	10,11,25,27,28,29,31,32
H11	12
H12	13
H13	14
H14	15
H15	16
H16	17
H17	18
H18	19
H19	20
H20	21
H21	22
H22	23
H23	24
H24	26
H25	30,33

Notably, the highest levels of nucleotide diversity were observed in Mazandaran province, while the lowest levels were detected in Tehran province (Table 3). This geographic pattern in genetic diversity suggests important ecological and environmental factors influencing the evolution and adaptation of this species across its range. The different ecological conditions between Mazandaran province and Tehran province are likely to be effective for genetic diversity, so that in Mazandaran, there are potentially more diverse habitats, which support genetic diversity.

The maximum likelihood phylogenetic analysis of Iranian *C. septempunctata* populations, using (*Hippodamia variegata* Goeze, 1777) as an outgroup, revealed 25 distinct haplotypes organized into well-defined monophyletic clades (Figure 3). This finding demonstrates substantial genetic diversity within Iranian populations, with each haplotype representing a unique genetic variant. The well-defined monophyletic clades indicate clear evolutionary relationships between different populations, suggesting recent evolutionary divergence events. The presence of 25 distinct haplotypes suggests possible geographic isolation or adaptation to different environments within Iran. This genetic diversity is particularly significant given the ecological importance of *C. septempunctata* as a predator of agricultural pests Understanding these genetic relationships could inform both conservation efforts and biological pest control strategies, potentially enhancing the effectiveness of these beneficial insects in agricultural settings.

Table 3. Genetic diversity indices of the mitochondrial gene of *COI* for *Coccinella septempunctata* in each province. N = Number of populations, π = Nucleotide diversity, Hd = Haplotype diversity

Province	N	π	Hd
E. Azerbaijan	3	0.00343	1.00000
W. Azerbaijan	3	0.00231	1.00000
Tehran	3	0.00116	0.66667
Mazandaran	3	0.00448	1.00000
Khuzestan	3	0.00347	1.00000
Kermanshah	3	0.00232	0.66667
Hormozgan	3	0.00348	1.00000
Yazd	3	0.00232	1.00000
Kordestan	3	0.00231	0.66667
Hamedan	3	0.00231	0.66667
Ardabil	3	0.00231	1.00000
Total	33	0.00658	0.94508

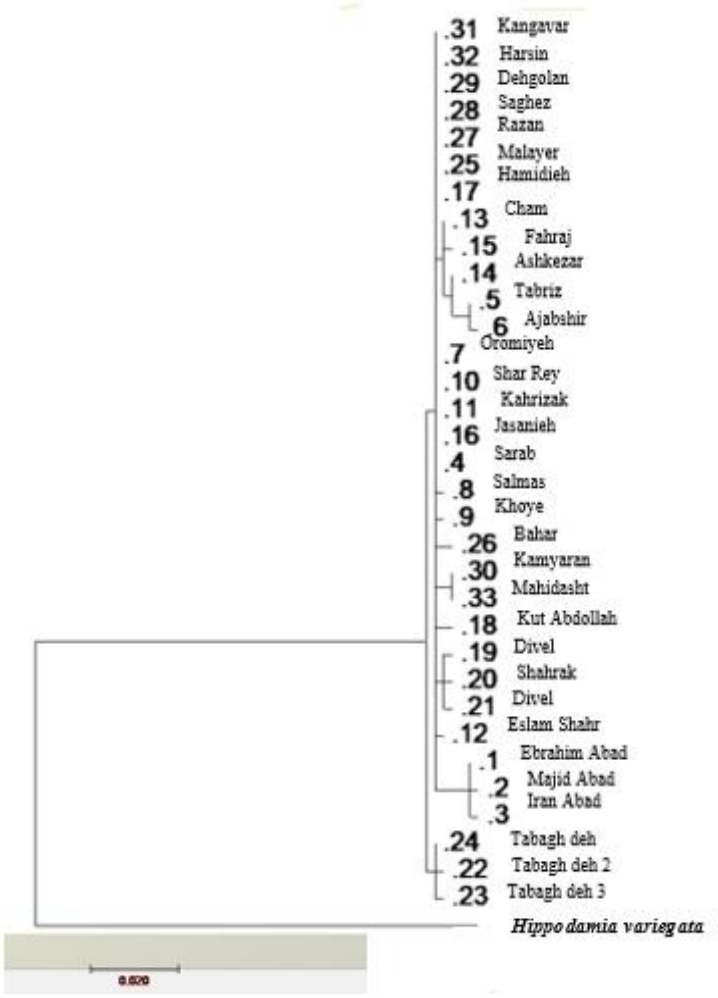

Figure 3. Maximum likelihood phylogenetic tree of 33 populations of *Coccinella septempunctata* based on the 576 bp segment COI (cytochrome c oxidase subunit I) gene. Sample codes correspond to provinces as follows: 1–3 = Ardabil, 4–6 = East Azerbaijan, 7–9 = West Azerbaijan, 10–12 = Tehran, 13–15 = Yazd, 16–18 = Khuzestan, 19–21 = Hormozgan, 22–24 = Mazandaran, 25–27 = Hamedan, 28–30 = Kordestan, 31–33 = Kermanshah. *Hippodamia variegata* was used as the outgroup to root the tree. The tree shows that samples from different provinces are interspersed, indicating a lack of strong phylogeographic structure among populations.

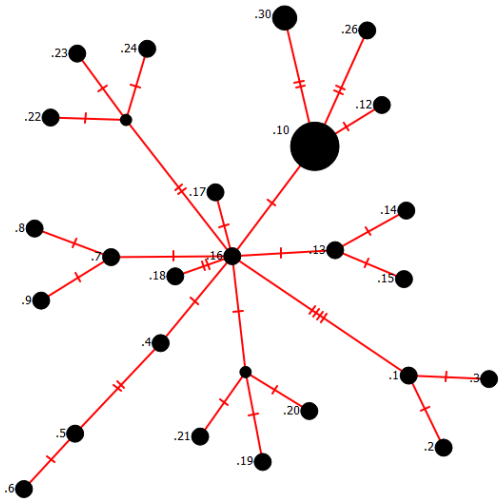

Figure 4. Haplotype network using popART software obtained.

Table 4. In Iran, pairwise comparisons of *Coccinella septempunctata*. Lower italic values represent genetic distances, while upper italic values denote index deviation. Results reveal low to moderate differentiation (0.001–0.014).

	Ardabil	AzarebaijanW	AzarebaijanE	Tehran	Yazd	Khuzestan	Hormozgan	Mazandaran	Hamedan	Kordestan	Kermanshah
Ardabil		0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
West Azarebaijan	0.013		0.003	0.003	0.003	0.002	0.003	0.003	0.003	0.003	0.003
East Azarebaijan	0.011	0.008		0.003	0.003	0.002	0.003	0.003	0.003	0.003	0.003
Tehran	0.011	0.007	0.005		0.003	0.002	0.003	0.003	0.001	0.001	0.001
Yazd	0.011	0.006	0.006	0.005		0.002	0.003	0.003	0.003	0.003	0.003
Khuzestan	0.010	0.006	0.005	0.004	0.005		0.002	0.003	0.002	0.002	0.002
Hormozgan	0.012	0.008	0.006	0.006	0.006	0.005		0.003	0.003	0.003	0.003
Mazandaran	0.013	0.010	0.008	0.008	0.008	0.007	0.009		0.003	0.003	0.003
Hamedan	0.011	0.008	0.006	0.002	0.006	0.005	0.006	0.008		0.001	0.001
Kordestan	0.011	0.008	0.006	0.002	0.006	0.005	0.006	0.008	0.002		0.001
Kermanshah	0.011	0.008	0.006	0.002	0.006	0.005	0.006	0.014	0.002	0.002	

The genetic distance analysis of C. septempunctata across Iranian regions (Table 4) reveals low to moderate differentiation (0.001–0.014), with the largest between Kermanshah–Mazandaran (0.014). Western populations (Hamedan, Kordestan, Kermanshah) showed minimal genetic distances from Tehran, suggesting high gene flow that may be due to human activities such as agricultural trade or natural dispersal mechanisms. Ardabil populations displayed uniform genetic distances (0.011-0.013) and low index deviations (0.004), reflecting relative isolation and limited genetic diversity, likely due to geographical or ecological barriers.

The results of this study reveal variation within the populations studied across different provinces in Iran (Figure 5). The data show genetic distance values within populations from eleven provinces, with three populations sampled per province. Lower genetic distances indicate greater genetic similarity within a population. Tehran has the lowest genetic diversity, suggesting high genetic similarity among its individuals. In contrast, Mazandaran exhibits the highest genetic diversity, indicating more genetic variability within its population. Higher genetic diversity within a province may reflect better habitat quality, larger population sizes, or reduced effects of genetic drift. Conversely, lower diversity could indicate smaller populations, habitat fragmentation, or stronger genetic drift. Comparing within-population diversity to genetic distances between populations can reveal patterns of gene flow and population structure. Further analyses using multiple loci or genetic markers would provide a

more comprehensive understanding of genetic diversity at different scales.

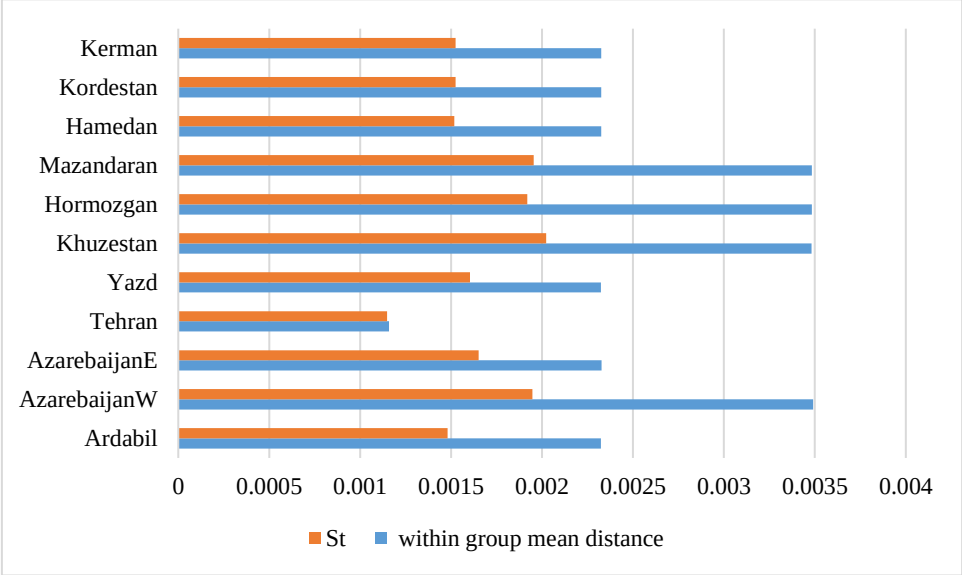

Figure 5. Within-group mean distance (range: 0-0.004) for *Coccinella septempunctata* from selected Iranian provinces. Lower values indicate higher internal homogeneity.

COI gene sequencing offers powerful species identification capabilities, but creating comprehensive databases for major insect families remains a significant challenge. To overcome this, researchers should analyze multiple samples of the same species using efficient DNA extraction, amplification, and sequencing techniques targeting various genetic markers. Developing effective methods and integrated identification protocols would greatly benefit entomological studies.

There are concerns about potential threats to specialized aphid-eating ladybugs, though this remains unproven. Several factors suggest these species might face challenges: their specialization likely makes them less adaptable genetically compared to generalist species. This aligns with observations of genetic variation in some generalist species. Specialist ladybugs rely on a narrower range of prey or habitats. If aphid populations decline or habitats degrade, specialists have fewer options for survival. Even if habitat tracking is possible, loss and fragmentation of suitable habitats poses greater challenges for specialists. They have fewer nearby habitats to disperse to, as they are generally more sedentary than generalists. Aphid-mediated competition from generalist species could further suppress specialist populations. If these scenarios prove partially correct, specialized ladybug species may face significant challenges in the future. Their vulnerability stems from their narrow ecological niches and limited adaptability, which could make them less resilient to environmental changes or disruptions in their food chains (Völkl 1995; Majerus 2002; Murrell & Barton 2017; Sloggett 2021). Therefore, it seems necessary to investigate and monitor the genetic diversity of these species. In this study, the Mantel test failed to detect a significant association between genetic and geographical distances. Specifically, the analysis did not find evidence supporting a relationship between the genetic diversity of populations and their physical separation ($r=0.08347$, $p= 0.197$) (Nei, 1973).

The result of the test Neutrality (Tajima's D = -1.85352) was obtained. The genetic analysis of seven-spotted ladybug populations reveals a complex interplay between genetic diversity, environmental factors, and ecological pressures.

The limited genetic diversity observed in these populations suggests that they may have experienced recent bottlenecks or strong selective pressures. However, regional differences in nucleotide diversity indicate that ecological factors play an important role in shaping genetic adaptation across different regions.

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مقاله پژوهشی

تنوع‌ژنتیکی جمعیت‌های کفشدوزک هفت‌نقطه‌ای در ایران با استفاده از ژن سیتوکروم اکسیداز

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چکیده:

کفشدوزک‌ها، به ویژه کفشدوزک هفت‌نقطه‌ای، نقش حیاتی در اکوسیستم‌های کشاورزی به عنوان عوامل کنترل بیولوژیک شته و آفات دیگر ایفا می‌کنند. درک زیست‌شناسی مولکولی آن‌ها می‌تواند بینشی درباره سازگاری، رفتار و پتانسیل کنترل آفات ارائه دهد. پیشرفت‌های اخیر در فن‌آوری‌های ژنومی، درک عمیق‌تری از مکانیزم‌های مولکولی زیربنایی این فرآیندها را فراهم کرده است. این مطالعه با هدف بررسی ساختار ژنتیکی جمعیت‌های این گونه در ایران، از طریق توالی‌یابی ژن سیتوکروم اکسیداز انجام شد. نتایج تحلیل سی‌وسه توالی ژنتیکی (نواحی ۵۷۶–۱)، مقدار تنوع ژنتیکی در سطح هاپلوتیپ‌ها را برابر با ($H = 0.945$) نشان داد. بیست‌وپنج هاپلوتیپ متمایز شناسایی گردید. در عین حال، میزان تنوع نوکلئوتیدی ($\pi = 0.00658$) نسبتاً پایین به دست آمد که نشان‌دهنده اختلاف بین تنوع در سطح هاپلوتیپ و تنوع در سطح نوکلئوتید است. این تناقض نشان‌دهنده یک ذخیره ژنتیکی متنوع با نواحی حفاظت‌شده ژنومی است که احتمالاً به‌دلیل گسترش جمعیتی اخیر یا تأثیر فشارهای انتخابی به وجود آمده است. نتایج این بررسی، اطلاعات ارزشمندی در رابطه با دینامیک جمعیتی، راهبردهای حفاظت و برنامه‌ریزی مطالعات زیست‌محیطی فراهم می‌کند.

کلمات کلیدی: تنوع نوکلئوتیدی، ژنتیک جمعیت، مدیریت اکولوژیک، هاپلوتیپ