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Original Article

An integrative approach to resolving the taxonomic status of *Praon exsoletum* (Nees) (Hymenoptera, Braconidae) biotypes: molecular and ecological perspectives

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ABSTRACT. The spotted alfalfa aphid parasitoid *Praon exsoletum* (Nees) (Hymenoptera: Braconidae, Aphidiinae) exhibits considerable intraspecific morphological variability, and its taxonomic status, whether it comprises distinct subspecies or geographic biotypes, has long been debated. A previous morphometric study failed to separate geographic populations, prompting the present molecular and ecological investigation. Here, we analyzed the mitochondrial *COI* barcode region from four biotypes: two from Iran (east and west) and two external (Spain and USA, the latter historically referred to as *Praon palitans* (Muesebeck), with *Praon volucre* (Haliday). Phylogenetic analyses using Maximum Parsimony, Minimum Evolution, Maximum Likelihood, and Bayesian Inference consistently placed the Iranian biotypes in a separate subclade from the European and American ones, though node support was generally weak across all methods (bootstrap values 33–78%; posterior probabilities 52–58%). Within Iran, sequences were identical, and overall genetic distances were very low (K2P: 0.0% within Iran, 0.3–0.5% between Iran and external biotypes). Canonical Correspondence Analysis (CCA) of 15 Iranian field sites, incorporating three parasitoid species, *P. exsoletum*, *Trioxys complanatus* Quilis, and *Aphidius smithi* Sharma & Subba Rao, and environmental variables (altitude, climate type, geographical direction), revealed no significant species–environment relationships. Despite the lack of statistical significance, *T. complanatus* tended to associate with hot, dry, south-eastern sites, while *P. exsoletum* was broadly distributed. The discordance between genetic structure and morphological uniformity suggests that Iranian populations of *P. exsoletum* may represent an evolutionarily distinct lineage in the early stages of divergence, but the evidence is insufficient to support formal subspecific recognition. This study highlights the need for nuclear markers and finer-scale ecological data to fully resolve the status of this important biological control agent.

KEYWORDS: Biological control, CCA, *COI*, DNA barcoding, Cryptic diversity, Iran

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INTRODUCTION

The spotted alfalfa aphid (SAA), *Therioaphis trifolii* (Monell) (Hemiptera: Aphididae), is a globally distributed and economically significant pest of alfalfa (*Medicago sativa* L.) (Jovičić et al. 2017). Native to the Mediterranean region and southwest Asia, this monophagous aphid has invaded many temperate and subtropical regions, causing severe yield losses through direct sap-feeding and the transmission of plant viruses (Valenciano et al. 2024). In the absence of effective natural enemies, populations of *T. trifolii* can reach damaging levels rapidly, necessitating chemical interventions that often disrupt integrated pest management (IPM) programs (Aeschlimann 1981). Classical biological control has therefore been a cornerstone of SAA management, relying heavily on the introduction and conservation of specialist hymenopteran parasitoids (Schlinger & Hall 1960, 1961; Hughes et al. 1987).

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Among the most effective natural enemies of *T. trifolii* are solitary endoparasitoids of the subfamily Aphidiinae (Hymenoptera: Braconidae). In particular, *Praon exsoletum* (Nees) (tribe Praini) and *Trioxys complanatus* Quilis (tribe Aphidiini) have been widely deployed and have established successfully in many parts of the world (Starý 1976). *Praon exsoletum* is a monophagous and thermophilic species that is considered one of the principal parasitoids of SAA. It is characterized by a unique external pupation behavior; unlike most other aphidiines, the mature larva spins a cocoon beneath the mummified aphid (Kavallieratos et al. 2005). The species was originally restricted to the Palearctic region, but was intentionally introduced into North America in the 1950s for SAA control, where it has since become established and widely distributed (van den Bosch et al. 1959; Johnson 1987). Despite its ecological and economic importance, the taxonomic status of *P. exsoletum* has remained contentious. Early systematic works recognized a high degree of intraspecific variability in morphological traits, host range, and geographic distribution. Mackauer (1959) formally divided the species into two subspecies: *P. exsoletum exsoletum*, distributed in Europe (excluding the Mediterranean basin), and *P. exsoletum palitans*, with a circum-Mediterranean distribution. However, subsequent authors questioned the validity of this split, arguing that the morphological characters used were variable and overlapped extensively between populations (Starý 1976, 1979; Johnson 1987). Starý (1979) synonymized the two subspecies, a view later adopted by several comprehensive taxonomic treatments (Kavallieratos et al. 2005; Tomanović et al. 2006). Nevertheless, the concept of distinct geographical “biotypes” or “ecotypes” persisted in the applied literature, with some studies still referring to *P. palitans* as a separate entity for biological control purposes (Vail et al. 2001; Gutierrez & Ponti 2013). This ambiguity is not merely academic; accurate identification of natural enemies is fundamental to the success of biological control programs, because cryptic diversity can mask differences in host preference, climatic adaptation, and efficacy that directly affect establishment and impact (Rosen 1986; Derocles et al. 2012).

An earlier work (Haddadian et al. 2016) directly addressed the taxonomic uncertainty through a comprehensive morphometric analysis of 13 geographic biotypes of *P. exsoletum* from Iran, central and western Europe, as well as the USA. Using both traditional multivariate morphometrics of 18 somatic characters and geometric morphometrics of forewing shape, we found significant phenotypic variation among biotypes, particularly in antennal structure, stigma dimensions, and wing venation. However, discriminant function analysis yielded only 45.23% correct assignment, and principal component analyses of wing shape revealed extensive overlap among all geographic groups. Crucially, the biotypes from the USA (originally introduced as *P. palitans*) and Europe did not form clusters distinct from the Iranian populations, and there was no morphometric support for the delineation of subspecies. The study concluded that morphological characters alone are insufficient to resolve the status of the putative subspecies of *P. exsoletum*, highlighting the need for alternative and more sensitive approaches.

The advent of molecular systematics, particularly DNA barcoding using the mitochondrial cytochrome c oxidase subunit I (*COI*) gene, has revolutionized the identification of aphid parasitoids and the detection of cryptic species. In Aphidiinae, *COI* sequences have proven highly effective for species delimitation, revealing previously hidden diversity in several genera, including *Praon* Haliday, 1833, *Aphidius* Nees, 1819, *Lysiphlebus* Förster, 1863, and *Ephedrus* Haliday, 1833 (Derocles et al. 2012; Mitrovski-Bogdanović et al. 2013, 2014; Petrović et al. 2013; Tomanović et al. 2014). A key advantage of *COI* is its relatively high rate of molecular evolution, which often allows discrimination at the species and even population level, whereas morphological divergence may lag behind genetic differentiation (Hebert et al. 2003). In the *Praon abjectum* group, for example, the combined use of *COI* sequences and geometric morphometrics led to the discovery and description of two new cryptic species, demonstrating that morphological stasis can mask significant genetic divergence (Mitrovski-Bogdanović et al. 2013). Similarly, within the *Praon dorsale-yomenae* complex, molecular markers uncovered multiple distinct taxa that were later supported by subtle morphometric differences (Mitrovski-Bogdanović et al. 2014). These studies exemplify modern integrative taxonomy, in which multiple and independent lines of evidence, morphology, DNA sequences, and ecology, are combined to rigorously test species boundaries (Dayrat 2005; Padial et al. 2010). In this context, the application of molecular methods to the *P. exsoletum* complex is a logical and necessary next step. If subspecies or cryptic species exist, genetic markers are more likely than morphology to reveal such structure, especially if divergence is

recent or constrained by stabilizing selection on morphological traits. Notably, several studies on aphid parasitoids have documented cases where *COI* variation was strongly partitioned geographically or by host affiliation, even when morphological differences were minimal or absent (Kos et al. 2011; Tomanović et al. 2013; Stanković et al. 2015). Conversely, there are also instances where substantial morphological variation is not accompanied by corresponding genetic divergence, suggesting phenotypic plasticity or local adaptation without speciation (Sandrock et al. 2011). Thus, for a cosmopolitan parasitoid like *P. exsoletum*, which occupies a wide range of climatic and ecological conditions, it is imperative to examine whether molecular data support the existence of divergent lineages that may correspond to subspecies, ecotypes, or host-associated races.

In addition to genetic markers, ecological niche differentiation can provide crucial evidence for evolutionary divergence. If geographically separated populations have adapted to distinct environmental conditions, they may exhibit differential distributions along climatic gradients and show significant associations with specific habitat variables. Canonical Correspondence Analysis (CCA) is a powerful multivariate ordination technique that links species occurrence data with environmental predictors, allowing the detection of non-random patterns in community composition and species-environment relationships (ter Braak & Šmilauer 2002; Lepš & Šmilauer 2003). While CCA has been widely used in studies of insect ecology and distribution (e.g., Freier et al. 2004; Tanabe et al. 2008; Lozan et al. 2011), its application to aphid parasitoids has been limited. A notable exception is the work of Rakhshani et al. (2008), who used CCA to examine the influence of altitude, host aphid species, and geographical location on the distribution of wheat aphid parasitoids in Iran, but found no significant associations, suggesting that other factors (e.g., microclimate, farming practices) might be more influential. For *P. exsoletum* and *T. complanatus*, which together dominate the parasitoid guild on alfalfa in Iran, preliminary ecological observations indicate different seasonal activities and possibly different thermal preferences (Starý 1976), but a formal ecological analysis of their biotypes has never been conducted.

The present study is therefore designed as a direct follow-up to our earlier morphometric anslysis (Haddadian et al. 2016), employing molecular and ecological approaches to test the hypothesis that the geographic biotypes of *P. exsoletum* represent genetically and ecologically distinct entities. Specifically, we first analyze DNA sequences of the barcode region of *COI* from representative biotypes, including both internal (eastern and western Iran) and external populations (Spain and the USA, the latter corresponding to the nominal *P. palitans*). We then reconstruct phylogenetic relationships using multiple analytical methods (Maximum Parsimony, Maximum Likelihood, Neighbor-Joining, and Bayesian Inference) and compute pairwise genetic distances (Kimura 2-parameter) to quantify the degree of divergence. In parallel, we perform a CCA using environmental variables (altitude, climate type, and geographical direction) to explore whether the composition and distribution of *P. exsoletum* biotypes (together with the co-occurring parasitoids *T. complanatus* and *Aphidius smithi* Sharma & Subba Rao) are structured by ecological factors. By integrating molecular and ecological evidence with the previously published morphological results, we aim to provide a comprehensive assessment of the status of the *P. exsoletum* complex and to contribute to a sound taxonomic foundation for the biological control of the spotted alfalfa aphid.

MATERIAL AND METHODS

Study sites and specimen collection. Parasitoid specimens of *P. exsoletum*, *T. complanatus*, and *Aphidius smithi* were obtained from 14 alfalfa fields across Iran, as well as from Spain, the Czech Republic, and the United States between 2011 and 2016 (Table 1). Alfalfa leaves bearing mummified aphids were collected and stored in ventilated plastic containers at room temperature until adult parasitoid emergence. Emerged wasps were preserved in 96% ethanol and kept at −20 ˚C until DNA extraction. Host aphids were slide-mounted and identified as *Therioaphis trifolii maculata* using the key of Rezwani (2001). Parasitoids were identified using the taxonomic keys of Kavallieratos et al. (2005) and Tomanović et al. (2006). *Trioxys complanatus* and *A. smithi* were included in the ecological analysis because they co-occur with *P. exsoletum* in alfalfa fields across Iran and together represent the dominant parasitoid guild associated with *T. trifolii* in the study region (Rakhshani et al. 2010; Nazari et al. 2012).

Their inclusion allowed a more comprehensive assessment of how environmental variables shape the distribution of the entire parasitoid community attacking the spotted alfalfa aphid. It should be noted that *P. exsoletum* (tribe Praini) is phylogenetically distinct from *T. complanatus* and *A. smithi* (both in tribe Aphidiini). This phylogenetic divergence may partly account for the observed differences in their ecological preferences. All voucher specimens are deposited in the Insect Collection of the Department of Plant Protection, Razi University, Kermanshah, Iran.

For the molecular analyses, four *P. exsoletum* biotypes were selected: two Iranian biotypes, Songhor (Kermanshah province, designated as the "western" biotype) and Nikshahr (Sistan & Baluchestan province, "eastern" biotype), and two biotypes from Spain and the USA (the latter historically referred to as *P. palitans*). Two species from the tribe Praini, *Praon volucre* (voucher from Canada) and *Areopraon silvestre* (voucher from France, were included as outgroups (Table 1).

Molecular Analysis. Total genomic DNA was extracted from individual male wasps using a high-yield DNA purification kit (CinneGen Inc., Iran) according to the manufacturer's instructions, with minor modifications. A 658-bp fragment of the mitochondrial cytochrome c oxidase subunit I (*COI*) gene was amplified using the universal primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al. 1994). PCR reactions were performed in a total volume of 25 µL containing 1 µL of each primer (10 pmol/µL), 12.5 µL of 2× Master Mix (Ampliqon, Denmark), 1 µL of template DNA, and 9.5 µL of nuclease-free water. The thermal cycling conditions were: initial denaturation at 95 ˚C for 5 min; 30 cycles of 94 ˚C for 60 s, 54 ˚C for 61 s, and 72 ˚C for 50 s; and a final extension at 72 ˚C for 7 min. PCR products were electrophoresed on a 1% agarose gel, purified, and bidirectionally sequenced by Bioneer Corporation (South Korea) using the forward primer. Forward and reverse sequences were edited and assembled in FinchTV v.1.4.0. The resulting consensus sequences were aligned using ClustalW integrated in MEGA 5.2 (Tamura et al. 2013), which was the current and widely used version at the time the analyses were conducted. The alignment contained no indels or stop codons, ruling out the presence of nuclear mitochondrial pseudogenes. All newly generated sequences were submitted to GenBank.

Phylogenetic trees were reconstructed using four methods. Maximum Parsimony (MP) analysis was performed with a heuristic search using the tree-bisection-reconnection (TBR) branch-swapping algorithm. Minimum Evolution (ME) analysis was conducted using the Tajima–Nei distance. Maximum Likelihood (ML) analysis employed the HKY85 substitution model with gamma-distributed rate variation (HKY+G), identified as the best-fit model in MEGA. Bayesian Inference (BI) was carried out in MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001) with two independent runs of four Markov chains for 10 million generations, sampling every 1000 generations; the first 25% of trees were discarded as burn-in. Node support for MP, ME, and ML was assessed with 10 000 bootstrap pseudoreplicates; for BI, posterior probabilities (PP) are shown. Pairwise genetic distances were calculated using the Kimura two-parameter (K2P) model.

Database mining. To evaluate the availability of additional *Praon exsoletum*, *COI* sequences and to place our biotypes within a broader taxonomic framework, we performed a BLAST search of the NCBI GenBank nucleotide database (accessed on 11 June 2026) using the four newly generated *COI* sequences as queries. All significantly similar sequences (*E-value = 0.0; identity ≥ 95%*) belonging to the tribe Praini were retrieved, and representative accessions were compiled for comparison (Table 5).

Ecological analysis. For the ecological analysis, 15 sampling sites were selected, spanning the geographical and climatic ranges of the three parasitoid species (Table 2). Three environmental variables were recorded for each site: (**i**) altitude (m a.s.l., quantitative); (**ii**) climate type (qualitative, with seven categories: hot, temperate, temperate mountainous, semi-arid and warm, hot and dry, temperate and warm, and hot and semi-humid); and (**iii**) geographical direction (qualitative, four categories: north-east, south-east, west, south-west). Long-term climatic data (30-year averages of mean annual temperature and annual precipitation) for each site were obtained from the Iran Meteorological Organization. Based on these data, and following the climatic regionalization of Iran proposed by Dinpashoh et al. (2004), sites were assigned to one of the seven qualitative climate categories. The species composition at each site was expressed as the number of emerged female individuals.

Table 1. Sampling locations and specimen data for *Praon exsoletum* biotypes used in molecular analysis (Host aphid: *Therioaphis trifolii*; Host plant: *Medicago sativa*).

Biotype code	Country	Location	Geographic coordinates	No. of specimens
IR-W (West)	Iran	Songhor (Kermanshah)	34°77'N, 47°59'E	1 (male)
IR-E (East)	Iran	Nikshahr (Sistan & Baluchestan)	26°15'N, 60°10'E	1 (male)
SP	Spain	Madrid	40°19'N, 03°51'W	1 (male)
US	USA	California	37°48'N, 122°16'W	1 (male)

* Data for two outgroup taxa, *Praon volucre* (Canada, GenBank: KR882784.1) and *Areopraon silvestre* (France, GenBank: JX507446.1), were retrieved from NCBI GenBank.

Table 2. Ecological dataset for 15 sampling sites, including parasitoid species (♀♀) composition and environmental variables.

Region	Coordinates	Altitude (m)	Climate type	Location	*P. exsoletum*	*T. complanatus*	*A. smithi*
Shush	32°19'N, 48°24'E	112	Hot	South-West	8	15	0
Dezful	32°20'N, 48°30'E	143	Hot & Semi-humid	South-West	0	6	0
Gilan-e-Gharb	34°13'N, 45°55'E	800	Temperate	West	6	0	5
Kangavar	34°40'N, 48°00'E	1460	Semi-arid & Warm	West	14	37	6
Bisotun	34°23'N, 47°26'E	1300	Temperate	West	15	0	16
Songhor	34°47'N, 47°59'E	1700	Temperate Mountainous	West	13	0	5
Firozan	34°52'N, 48°32'E	1507	Temperate Mountainous	West	26	19	0
Bam	29°07'N, 58°14'E	1050	Hot & Dry	South-East	41	21	0
Jiroft	28°40'N, 57°44'E	650	Hot & Dry	South-East	9	7	30
Nikshahr	26°15'N, 60°10'E	450	Hot	South-East	25	25	0
Sarbaz	27°20'N, 61°09'E	915	Hot & Dry	South-East	23	12	0
Zabol	31°00'N, 61°32'E	480	Hot & Dry	East	0	18	0
Bejestan	34°30'N, 58°05'E	1250	Hot & Dry	North-East	0	6	13
Sabzevar	36°15'N, 57°40'E	977	Temperate & Warm	North-East	0	23	0

The relationship between parasitoid species composition and the measured environmental variables was examined using Canonical Correspondence Analysis (CCA) in CANOCO for Windows 4.5 (ter Braak & Šmilauer, 2002). Qualitative environmental variables were coded as dummy variables. The statistical significance of the overall ordination was tested using a Monte Carlo permutation test with 999 unrestricted permutations ($a = 0.05$). Ordination diagrams were produced with CanoDraw for Windows.

RESULTS

Molecular analysis. A 658-bp fragment of the mitochondrial *COI* gene was successfully amplified and sequenced for the four *P. exsoletum* biotypes (Table 1). No insertions, deletions, or stop codons were detected in the aligned sequences, confirming that all amplicons represent functional mitochondrial copies. BLAST searches against the NCBI nucleotide database returned the highest similarity (98–99%) to several *Praon* sp. BOLD-2016 vouchers (e.g., KR800053.1, KR873543.1) and to Aphidiinae sp. BOLD:AAE7186 sequences, confirming the correct identification of the specimens. Phylogenetic trees reconstructed using Maximum Parsimony (MP), Minimum Evolution (ME), Maximum Likelihood (ML), and Bayesian Inference (BI) recovered identical topologies, consistently separating the four *P. exsoletum* biotypes from the outgroup taxa. Within the *P. exsoletum* clade, the two internal Iranian biotypes (Songhor, eastern Iran, and Nikshahr, western Iran) formed a well-defined subclade, whereas the two external biotypes (Spain and the USA) grouped in a second subclade. The complete set of phylogenetic

reconstructions is presented in Figure 1, with each tree panel labeled according to the inference method. Although the topology was identical across all four methods, node support was generally low and varied among the analyses. In the ME and ML trees, the internal biotypes clustered with bootstrap values of 78% and 74%, respectively, while the external biotypes were supported by 33% (MP) to 58–74% (ME/ML). In the Bayesian analysis (BI), the internal subclade received a posterior probability (PP) of 52%, and the external subclade a PP of 58%. The two internal biotypes (Songhor and Nikshahr) showed identical *COI* sequences, resulting in a pairwise K2P genetic distance of 0.0%. The genetic distance between the external biotypes (Spain vs. USA) was 0.5%. Distances between the internal and external groups ranged from 0.3% (Iran vs. USA) to 0.5% (Iran vs. Spain) (Table 3). The mean interspecific distance between *P. exsoletum* samples and the outgroup taxa was 2.0% (*A. silvestre*) and 1.8% (*P. volucre*), respectively, which is an order of magnitude higher than the intraspecific divergences observed within *P. exsoletum*. The 2.0% K2P distance between *P. exsoletum* and *A. silvestre* is consistent with the high sequence identities (95–98%) observed between various genera within the tribe Praini (Table 5). An updated BLAST search of the NCBI GenBank database (accessed on 11 June 2026) using the four *P. exsoletum*, *COI* sequences as queries retrieved only three nucleotide records specifically identified as *P. exsoletum*, confirming that this species is poorly represented in public databases. To provide an evolutionary context for the specimens, a list of the highest-scoring matches from the tribe Praini was compiled. These sequences consistently showed highly significant similarity (*E-value = 0.0*) and high identity (95–99%) to the biotypes analyzed in this study, confirming their correct assignment to the tribe (Table 5). The closest relatives identified include *Praon* spp., *P. volucre*, *P. abjectum*, *P. dorsale*, *P. unicum*, and *A. silvestre*.

Ecological analysis. The parasitoid community at these sites was composed of three species: *P. exsoletum*, *T. complanatus*, and *A. smithi* (Table 2). *P. exsoletum* was recorded at 11 sites, mostly in western and southeastern Iran, with the highest abundance at Bam (41 individuals). *T. complanatus* occurred at 10 sites, being particularly abundant at Kangavar (37 individuals) and Nikshahr (25 individuals). *A. smithi* was present at 6 sites, mainly in the central and western regions. The Canonical Correspondence Analysis (CCA) ordination diagram for the three species and 15 sites, constrained by altitude, climate type, and geographical direction, is shown in Figure 2. The first two canonical axes explained 34.7% and 21.2% of the species variance, respectively, and 58.0% and 80.6% of the species–environment relationship. However, the Monte Carlo permutation test revealed that the overall ordination was not statistically significant ($F = 0.796$, $p = 0.640$ for all canonical axes; Table 4). The eigenvalue of the first axis (0.260) was also non-significant ($F = 4.549$, $p = 0.340$).

Despite the lack of overall significance, the weighted averages of environmental variables indicated certain trends. Among the qualitative variables, the hot and dry climate category (*weighted average = 0.558*) and the south-east geographical direction (0.521) received the highest weights. Altitude, the only quantitative variable, had a weighted average of 0.759. Species positions in the ordination space showed a tendency for *T. complanatus* to be associated with hot and dry, south-eastern sites, while *P. exsoletum* appeared more centrally distributed, and *A. smithi* was located towards the cooler, temperate-mountainous regions (Fig. 2). However, these associations were not statistically validated (Monte Carlo test: $F = 0.796$, $p = 0.640$ for all canonical axes).

Table 3. Pairwise Kimura two-parameter (K2P) genetic distances (%) among *Praon exsoletum* biotypes and outgroup taxa.

	1	2	3	4	5	6
1. *P. exsoletum* (Nikshahr, Iran)	–					
2. *P. exsoletum* (Songhor, Iran)	0.000	–				
3. *P. exsoletum* (Spain)	0.005	0.005	–			
4. *P. exsoletum* (USA)	0.003	0.003	0.005	–		
5. *Areopraon silvestre* (France)	0.020	0.020	0.022	0.020	–	
6. *Praon volucre* (Canada)	0.018	0.018	0.020	0.018	0.012	–

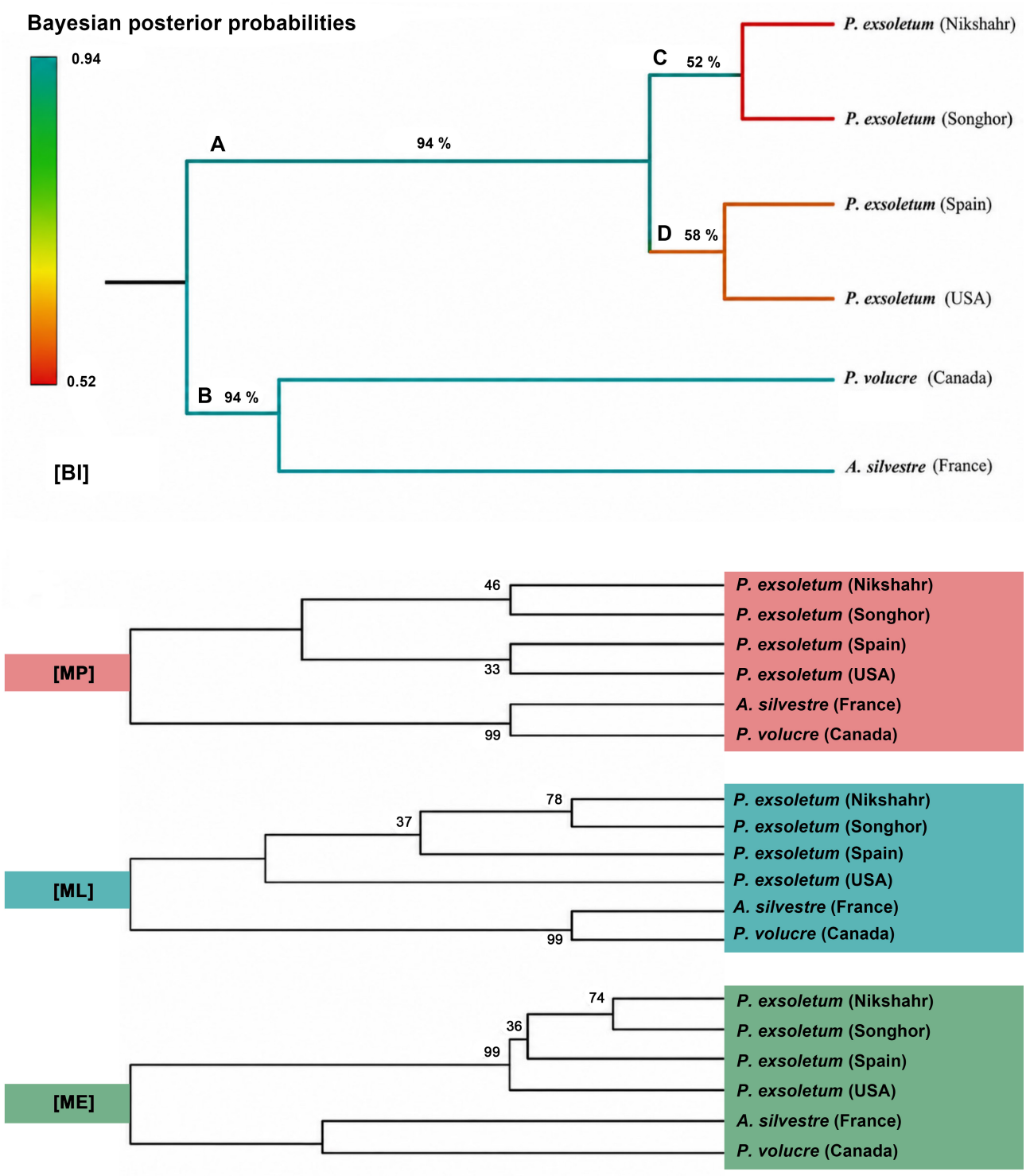

Figure 1. Phylogenetic trees reconstructed from *COI* sequences of *Praon exsoletum* biotypes using four different inference methods. The Maximum Parsimony (MP), Minimum Evolution (ME), and Maximum Likelihood (ML) trees show bootstrap support values (≥50%) above branches, while the Bayesian Inference (BI) phylogram displays posterior probabilities (≥50%). *Areopraon silvestre* and *Praon volucre* were used as outgroups.

Table 4. Summary of Monte Carlo permutation test results for the CCA.

Test	*F*-ratio	*p*-value
First canonical axis	4.549	0.340
All canonical axes	0.796	0.640

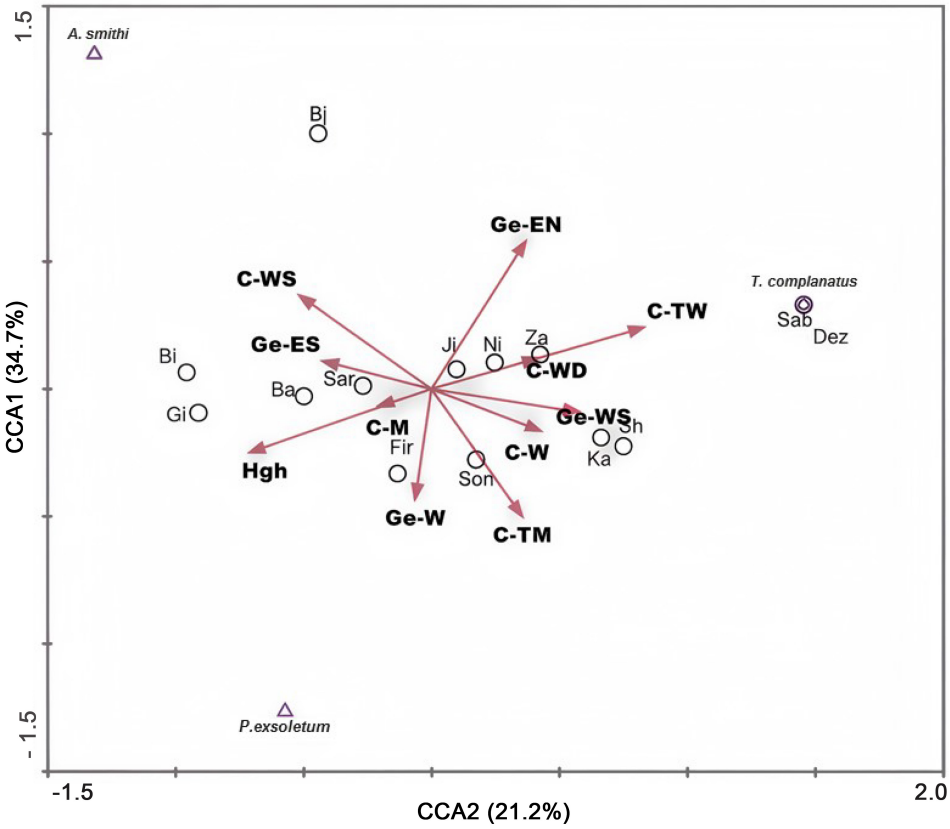

Figure 2. Canonical Correspondence Analysis (CCA) ordination diagram based on the abundance of three parasitoid species at 15 sampling sites. Species are shown as triangles: *Praon exsoletum*, *Trioxys complanatus*, and *Aphidius smithi*. Sampling sites are shown as open circles with the following abbreviations: Bi: Bisotun, Gi: Gilan-e Gharb, Ba: Bam, Sar: Sarbaz, Fir: Firozan, Son: Songhor, Ji: Jiroft, Ni: Nikshahr, Za: Zabol, Ka: Kangavar, Dez: Dezful, Bj: Bejestan, Sh: Shush, Sab: Sabzevar. Environmental variables are represented as arrows (quantitative: Hgh: Altitude) and centroids (qualitative). Geographical directions: Ge-W: West, Ge-WS: Southwest, Ge-ES: Southeast, Ge-EN: Northeast. Climate types: C-W: Hot, C-TM: Temperate mountainous, C-WD: Hot and dry, C-WS: Hot and semi-humid, C-TW: Temperate and warm. CCA1 and CCA2 explained 34.7% and 21.2% of the species variance, respectively.

Table 5. Representative *COI* sequences of the tribe Praini identified through BLAST searches of the NCBI GenBank database using *Praon exsoletum* sequences as queries.

Species	Accession Number	BOLD Process ID	Query Cover (%)	E value	Identity (%)
Praon sp. BOLD-2016	KR800053.1	BIOUG02720-A01	94	0.0	99
Praon sp. BOLD-2016	KR873543.1	BIOUG00862-D03	94	0.0	99
Areopraon silvestre	JX507446.1	–	96	0.0	98
Areopraon silvestre	KT753299.1	–	91	0.0	98
Praon volucre	KR882784.1	BIOUG05725-E01	94	0.0	98
Praon volucre	JX507440.1	–	96	0.0	96
Praon unicum	KC211023.1	–	94	0.0	97
Praon unicum	EU574904.1	–	94	0.0	96
Praon dorsale	JX507439.1	–	96	0.0	96
Praon occidentale	EU574903.1	BIOUG01913-A02	94	0.0	96
Praon abjectum	JX507438.1	–	96	0.0	95
Praon brevistigma	KU374143.1		94	0.0	96
Praon bicolor	KP983849.1		88	0.0	97
Praon humulaphidis	EU574905.1		94	0.0	96

DISCUSSION

The present study was designed as a direct extension of our earlier morphometric investigation of *P. exsoletum* biotypes (Haddadian et al. 2016). In that work, neither traditional morphometrics nor geometric morphometrics of the forewing could separate the putative subspecies, with extensive overlap observed across all geographic groups. This outcome motivated the application of molecular and ecological tools in the current study. When the results of the three methodological approaches are now considered together, a clear discordance emerges: while morphology failed to detect any subdivision, the *COI* sequences consistently placed the internal Iranian biotypes in a subclade distinct from the European and American biotypes, a pattern recovered by all four phylogenetic methods employed (MP, ME, ML, BI). Ecological data, in turn, showed no significant species–environment correlations at the scale examined.

This discordance between morphological stasis and shallow molecular divergence aligns well with patterns increasingly documented in the Aphidiinae, highlighting the efficiency of integrative molecular approaches. As noted earlier, in the *P. abjectum* group and the *P. dorsale-yomenae* complex, *COI* barcoding successfully revealed cryptic lineages and distinct taxa that were barely separable by morphological characters (Mitrovski-Bogdanović et al. 2013, 2014). Comparable patterns were also reported in cereal aphid parasitoids of the genus *Aphidius*, where mitochondrial markers discriminated among species that overlapped extensively in forewing shape and stigma dimensions (Kos et al. 2011; Tomanović et al. 2013). The pattern we observed in *P. exsoletum*, genetic divergence without clear morphological separation, therefore fits squarely within this growing body of evidence, suggesting that mitochondrial DNA markers often possess higher resolving power than morphological traits when evolutionary divergence is recent (Hebert et al. 2003). This raises fundamental questions about the evolutionary status of geographic populations within *P. exsoletum* and reinforces the value of integrative taxonomy for resolving species complexes of biological control importance. It should be noted that the taxon sampling for the molecular analysis was limited to four biotypes of *P. exsoletum*, which reflects the poor representation of this species in public sequence databases. An updated BLAST search of GenBank confirmed that only three nucleotide records of *P. exsoletum COI* are currently available worldwide. This limitation has been recognized, and the results should be interpreted with appropriate caution. Consequently, the present molecular findings should be regarded as preliminary, pending the availability of additional *P. exsoletum* sequences from a broader geographic range. Future studies, ideally with access to the original raw data, should re-evaluate these findings using updated phylogenetic software, expanded molecular datasets, and broader geographic sampling to further strengthen evolutionary inference. Although the observed separation between Iranian and Western biotypes cannot be considered definitive, it provides a preliminary indication of a geographic genetic structure within this species. Nevertheless, the retrieved sequences from the tribe Praini, including *P. volucre*, *P. abjectum*, *P. dorsale*, *P. unicum*, and *A. silvestre* (Table 4), consistently showed high similarity ($E = 0.0$; *identity* $\geq 95\%$) to the biotypes analyzed in this study, supporting their correct taxonomic assignment. Future studies incorporating nuclear markers and broader geographic sampling would be valuable to further validate the patterns observed here.

This result is particularly striking given that the biotype from the USA has historically been referred to as *P. palitans* and was introduced into North America from Mediterranean source populations for the biological control of the spotted alfalfa aphid (van den Bosch et al. 1959; Schlinger & Hall 1960). Our molecular data suggest that this introduced population retains genetic affinities with European rather than Iranian populations, and that the Iranian biotypes may represent a distinct evolutionary lineage. Such morphological stasis accompanying shallow but geographically structured genetic divergence has been reported in several other aphid parasitoid complexes. In the *P. abjectum* group, Mitrovski-Bogdanović et al. (2013) found that *COI* sequences revealed three distinct taxa that were barely separable by morphology; the subtle wing shape differences eventually detected were concentrated in the stigma and radial vein, similar to the landmarks identified as variable in our *P. exsoletum* biotypes. Likewise, in the *P. dorsale-yomenae* complex, molecular markers uncovered multiple distinct species that were later supported by fine-scale morphometric differences, leading to the description of new taxa (Mitrovski-Bogdanović et al. 2014). The cereal aphid parasitoids *Aphidius*

uzbekistanicus, *A. rhopalosiphi*, and *A. avenaphis* also exemplify this pattern, with *COI* revealing cryptic lineages that were not consistently identifiable using morphological keys (Kos et al. 2011; Tomanović et al. 2013). These examples suggest that in aphid parasitoids, mitochondrial DNA may register incipient lineage sorting well before morphological differentiation becomes apparent or taxonomically useful.

Several non-mutually exclusive explanations can account for this pattern. First, the *COI* gene is a neutral or nearly neutral marker whose rate of sequence evolution may outpace the rate of morphological change, particularly if morphological traits are under stabilizing selection imposed by shared ecological pressures (Bickford et al. 2007). In *P. exsoletum*, the conserved phenotype across geographically distant populations may reflect strong functional constraints associated with host exploitation; all studied biotypes are strictly monophagous on *T. trifolii*, a host that presents a relatively uniform resource across its range. Second, the low mitochondrial divergence observed (0.0% within Iran, 0.3–0.5% between Iran and external samples) indicates that the split between internal and external lineages is evolutionarily recent, likely dating to the Pleistocene or Holocene, and morphological divergence may simply require longer periods of reproductive isolation to accumulate. Third, the possibility of male-biased gene flow cannot be discounted; if dispersal is primarily male-mediated, mitochondrial DNA (which is maternally inherited) would show greater geographic structure than nuclear markers or morphology. This scenario has been proposed for other parasitoid wasps (e.g., Sandrock et al. 2011) and warrants testing in *P. exsoletum* using nuclear loci such as *ITS2* or microsatellites. It is also important to consider the alternative explanation: that the observed mitochondrial divergence reflects persistent ancestral polymorphism (incomplete lineage sorting) rather than ongoing evolutionary separation. The weak bootstrap support (33–78%) and low posterior probabilities (52–58%) in our phylogenetic analyses are consistent with this interpretation. Under incomplete lineage sorting, the mitochondrial gene tree does not accurately reflect the species tree, particularly when divergence is recent and effective population sizes are large. Distinguishing between incipient speciation and ancestral polymorphism would require sampling of multiple unlinked nuclear markers and applying coalescent-based species delimitation methods (Fujita et al. 2012). Our CCA did not detect statistically significant relationships between the measured environmental variables and parasitoid species composition at the 15 study sites. This finding mirrors the results of Rakhshani et al. (2008), who examined wheat aphid parasitoids in Iran and found no significant correlations with altitude, geographic location, or host aphid species. The lack of significance in both studies may reflect several factors. First, the spatial scale of sampling (hundreds of kilometers) may be too coarse to capture the microclimatic gradients that actually influence parasitoid establishment and activity. Second, the environmental variables we selected, altitude, broad climate categories, and geographic direction, are indirect measures that may not adequately proxy for the proximal factors affecting parasitoids, such as temperature regimes during critical life stages, host aphid phenology, or agronomic practices (tillage, pesticide use, irrigation) (Langer & Hance 2004). Third, the inclusion of the generalist parasitoid *A. smithi*, which attacks multiple aphid species beyond *T. trifolii*, may have introduced ecological noise into the species matrix, obscuring patterns specific to the specialist parasitoids.

Despite the lack of statistical significance, the weighted averages from the CCA suggest certain trends that merit attention. The hot and dry climate category and south-east geographical direction received the highest weighted averages, and *T. complanatus* showed a tendency to associate with these conditions in the ordination space. This observation is consistent with the biological literature: Stary (1976) noted that *T. complanatus* is adapted to warm conditions and prefers lowlands with hot, humid climates, while *P. exsoletum* has a broader thermal tolerance and is active primarily in spring and autumn. In our study, *T. complanatus* was the dominant parasitoid at sites such as Nikshahr and Bam (southeastern Iran), characterized by hot and dry summers, whereas *P. exsoletum* was more widely distributed, including in cooler temperate-mountainous regions like Firozan and Songhor. These distributional tendencies may reflect niche differentiation between the two competing parasitoid species, a hypothesis originally proposed by Stary (1976) and supported anecdotally by field observations in Iran (Rakhshani et al. 2010; Nazari et al. 2012). More targeted sampling along explicit elevational and climatic transects, combined with microclimatic data loggers, would be required to test this hypothesis formally.

A notable finding of our molecular analysis is the complete absence of *COI* divergence between the two Iranian biotypes from Songhor (western Iran) and Nikshahr (southeastern Iran), despite a geographic separation of approximately 1500 km. This genetic homogeneity across a large geographic distance stands in contrast to the substantial morphological variation observed among Iranian biotypes in our earlier study (Haddadian et al. 2016). Several explanations can be proposed. First, this pattern may indicate high levels of ongoing gene flow among Iranian populations of *P. exsoletum*, possibly facilitated by the movement of infested alfalfa hay or by wind-assisted dispersal of adult parasitoids. Alfalfa is cultivated intensively across Iran, and the continuous distribution of this host plant may facilitate extensive gene flow among parasitoid populations, preventing genetic differentiation across the sampled geographic range. Second, the morphological variation documented among Iranian biotypes may be predominantly environmentally induced (phenotypic plasticity) rather than genetically based. In aphid parasitoids, host size and quality are known to influence parasitoid body size, wing dimensions, and even antennal structure (Žikić et al. 2010; Alejandra Parreno et al. 2016). If aphid populations feeding on alfalfa of varying quality (due to differences in soil fertility, irrigation, or plant genotype) produce parasitoids of differing sizes, this could account for the observed morphological variation in the absence of genetic differentiation. Finally, the possibility that the *COI* barcode region is too conserved to detect population-level structure within Iran must be considered. The mitochondrial *COI* gene, while highly effective for species-level discrimination, may lack the resolution to detect intraspecific phylogeographic structure in recently expanded populations (Hebert et al. 2003; Derocles et al. 2012). Our study exemplifies both the strengths and the challenges of integrative taxonomy in aphid parasitoids. The combination of morphometrics, DNA barcoding, and ecological analysis provided a more nuanced understanding of the *P. exsoletum* complex than any single method could have achieved alone. However, the discordance among datasets, morphology unresolved, genetics weakly structured, ecology non-significant, also illustrates that integration does not always lead to immediate taxonomic resolution. As noted by Padial et al. (2010) and Schlick-Steiner et al. (2010), integrative taxonomy is a process of iterative hypothesis testing, not a single analytical step. In our case, the available evidence suggests that the Iranian populations of *P. exsoletum* may represent a distinct evolutionary lineage that is in the early stages of divergence, but the data are insufficient to justify formal taxonomic recognition at this time. The taxonomic status of *P. exsoletum* populations has direct practical implications for biological control programs targeting the spotted alfalfa aphid. If the Iranian biotypes represent a distinct ecotype or incipient subspecies with unique climatic adaptations or host-exploitation traits, they may offer complementary attributes to the European and American populations already widely used in classical biological control. For instance, Iranian populations from hot and dry southeastern regions (e.g., Nikshahr, Bam) may possess heat tolerance traits that could enhance parasitoid establishment in similarly arid alfalfa-growing regions of the world, such as parts of Australia, the Middle East, and North Africa, where SAA remains a significant pest (Gutierrez & Ponti, 2013). Conversely, if the genetic differences are neutral with respect to fitness, the existing introduced populations may be adequate for most control programs.

A related consideration is the risk associated with the introduction of novel genotypes. The USA biotype analyzed in this study was originally introduced from Mediterranean Europe as *P. palitans* and has been established in North America for over six decades (van den Bosch et al. 1959). The low genetic distance (0.3%) between this population and the Iranian biotypes suggests that any genetic incompatibility is unlikely. However, the introduction of Iranian genotypes could, in theory, disrupt locally adapted gene complexes or introduce undesirable traits (e.g., hyperparasitoid susceptibility). A precautionary approach, involving host-specificity testing and life-table comparisons under quarantine conditions, would be advisable before any deliberate introduction of new biotypes (van Lenteren et al. 2006).

AUTHOR'S CONTRIBUTION

The authors confirm their contribution to the paper as follows: M. Haddadian: field sampling, laboratory work, data collection, data analysis; A. Zamani: supervising, methodology, drafting the manuscript, revising and editing; A. Marefat: supervision of molecular analyses, interpretation of molecular data; E. Rakhshani: taxonomic identification, revising and editing the manuscript. The authors read and approved the final version of the manuscript.

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AVAILABILITY OF DATA AND MATERIAL

The specimens listed in this study are deposited in the collections of the department of Plant Protection, University of Zabol, and Department of Plant Protection, Razi University, Kermanshah, and are available from the curator upon request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study only included arthropod material, and all required ethical guidelines for the treatment and use of animals were strictly adhered to in accordance with international, national, and institutional regulations. No human participants were involved in any studies conducted by the authors for this article.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this paper.

GENERATIVE AI STATEMENT

The authors declare no generative AI tools were used in the preparation of this manuscript, nither in data analysis.

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رویکرد تلفیقی در تبیین وضعیت تاکسونومیک بیوتیپ‌های *Praon exsoletum* (Nees) (Hymenoptera, Braconidae): چشم‌انداز روش‌های مولکولی و بوم‌شناختی

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چکیـده: زنبور *Praon exsoletum* (Nees) (Hymenoptera: Braconidae, Aphidiinae)، پارازیتویید شته خال‌دار یونجه، تنوع قابل‌توجهی در ویژگی‌های ریخت‌شناختی درون‌گونه‌ای داشته و وضعیت تاکسونومیک آن، مبنی بر اینکه آیا شامل زیرگونه‌های متمایز یا بیوتیپ‌های جغرافیایی است، از دیرباز مورد بحث بوده است. پیش از این، یک مطالعه مورفومتریک، نتوانست جمعیت‌های جغرافیایی این گونه را از یکدیگر تفکیک کند؛ ازاین‌رو، در پژوهش حاضر، بررسی‌های مولکولی و بوم‌شناختی انجام شد. در این مطالعه، ناحیه بارکد ژن میتوکندریایی *COI* از چهار بیوتیپ شامل دو بیوتیپ از ایران (شرق و غرب) و دو بیوتیپ خارجی (اسپانیا و ایالات متحده آمریکا؛ مورد اخیر که در گذشته با نام *Praon palitans* (Muesebeck) شناخته می‌شد، همراه با *Praon volucre* (Haliday)) مورد تجزیه و تحلیل قرار گرفت. تحلیل‌های فیلوژنتیک با استفاده از روش‌های Maximum Parsimony، Minimum Evolution، Maximum Likelihood و Bayesian Inference به‌طور یکنواخت نشان دادند که بیوتیپ‌های ایرانی در یک زیرشاخهٔ مجزا از بیوتیپ‌های اروپایی و آمریکایی قرار می‌گیرند، هرچند میزان حمایت از گره‌ها در تمامی روش‌ها عموماً ضعیف بود (مقادیر بوت‌استرپ: ۳۳ تا ۷۸ درصد؛ احتمالات پسین: ۵۲ تا ۵۸ درصد). در داخل ایران، تمامی توالی‌ها یکسان بودند و فاصله‌های ژنتیکی نیز بسیار اندک به دست آمد (K2P برابر صفر، درون ایران و ۰/۳ تا ۰/۵ درصد بین ایران و بیوتیپ‌های خارجی). همچنین، Canonical Correspondence Analysis (CCA) بر روی ۱۵ محل نمونه‌برداری در ایران، با در نظر گرفتن سه گونه پارازیتویید شامل *P. exsoletum*، *Trioxys complanatus* Quilis و *Aphidius smithi* Sharma & Subba Rao و متغیرهای محیطی (ارتفاع، نوع اقلیم و جهت جغرافیایی)، هیچ رابطه معنی‌داری میان گونه‌ها و متغیرهای محیطی نشان نداد. با وجود عدم اکتساب نتایج معنی‌دار آماری، *T. complanatus* گرایش به حضور در مناطق گرم، خشک و جنوب‌شرقی داشت، در حالی که *P. exsoletum* پراکنش گسترده‌ای را در سراسر منطقه مطالعه نشان داد. عدم انطباق میان ساختار ژنتیکی و یکنواختی ریخت‌شناختی نشان می‌دهد که جمعیت‌های ایرانی *P. exsoletum* ممکن است یک دودمان تکاملی متمایز را در مراحل آغازین واگرایی تشکیل دهند، اما شواهد موجود برای توجیه شناسایی رسمی آن‌ها در سطح زیرگونه کافی نیست. این مطالعه بر ضرورت استفاده از نشانگرهای هسته‌ای و داده‌های بوم‌شناختی با مقیاس مکانی دقیق‌تر برای روشن شدن کامل وضعیت این عامل مهم کنترل زیستی تأکید می‌کند.

واژگـان کلیدی: مهار زیستی، CCA، *COI*، بارکدینگ DNA، تنوع پنهان، ایران