https://doi.org/10.15407/dopovidi2025.04.027
UDC 632.937.1

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Molecular-biological diagnostic as strategy for quality control and genetic purity of beneficial insects *Trichogramma* spp.

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The study focuses on the development of molecular diagnostic methods for verifying the genetic purity of Trichogramma spp. entomophagous insect cultures used in biological pest control. Analysis of the internal transcribed spacer ITS2 of ribosomal DNA revealed interspecific differences and identified mixed populations. Polymerase chain reaction, sequencing, and nucleotide sequence comparison ensured accurate determination of genetic consistency. The results demonstrated impurities in Trichogramma pintoi and T. evanescens samples, whereas T. brassicae and T. ahea confirmed purity. To maintain the effectiveness of biological control, regular monitoring of genetic purity is recommended every three months after two generations. Implementing these methods reduces chemical pesticide reliance, promoting ecosystem stability and organic product quality.

Keywords: *genetic purity, molecular diagnostics, Trichogramma spp., biological control, ITS2, sequencing, polymerase chain reaction.*

Introduction. In recent years, biotechnological processes for the production of biological plant protection products have become increasingly important. The populations of Europe, America, and a number of othercountries are giving preference to environmentally friendly plant products, despite the fact that the latter are more expensive in retail chains in many countries of the

Citation: Melnychuk M.D., Spyrydonov V.G., Yu W., Dall'Agata M., Boychinov B. Molecular-biological diagnostic as strategy for quality control and genetic purity of beneficial insects *Trichogramma* spp. *Dopov. Nac. akad. nauk Ukr.* 2025. No. 4. P. 27—32. https://doi.org/10.15407/dopovidi2025.04.027

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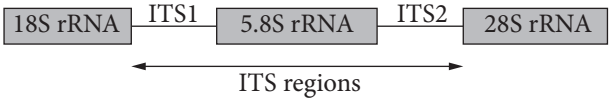


Fig. 1. The ITS region structure in four different species of commercially used insects

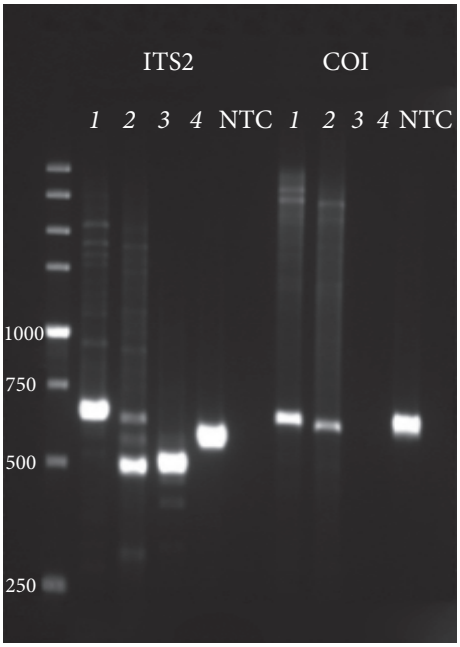


Fig. 2. Agarose gel electrophoresis of PCR-amplified ITS2 region from *Trichogramma* spp. DNA bands were visualized on a 1.0 % agarose gel stained with SYBR Green I: *1* — *T. pintoi*; *2* — *T. evanescens*; *3* — *T. brassicae*; *4* — *T. ahea*; NTC — Non template control ►

world. Thus, the Research Institute of Organic Agriculture of Switzerland (FiBL) showed that Switzerland is the leader among the countries of the world with the highest level of consumption of organic products per capital, followed by Denmark, Luxembourg, Austria and Sweden. At the same time, Germany is the leader in the organic food market with an financial indicator of 15,9 billion euro, followed by France (12,7 billion), Italy (3,9 billion) and Switzerland (3,7 billion). The world map of organic products looks quite predictable – Europe leads (with an "organic" budget of 54,5 billion euros), followed by North America (53,9 billion) and Asia (13,7 billion) (http://organic.world.net.statistics.org/) [1].

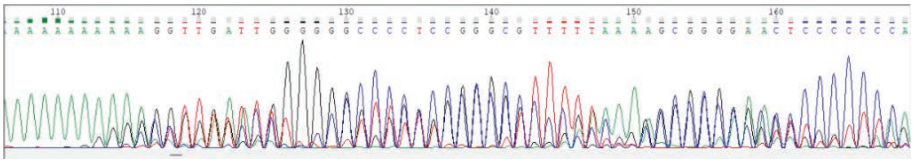
In Ukraine, for example, back in 2021, the government approved a National Economic Strategy program until 2030, which outlined plans to transfer 3 % of Ukraine's total agricultural land (about 1,3 million hectares) to organic production [2].

Egg parasitoids of the genus Trichogramma (Hymenoptera: Trichogrammatidae) are extensively used against agricultural pests of both annual and perennial crops [3].

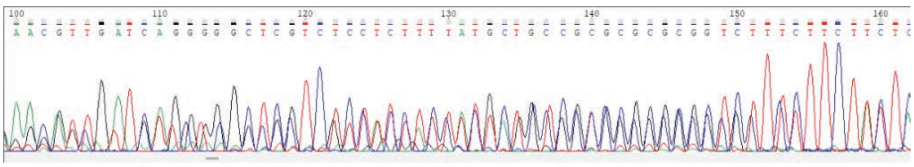
Pure cultures ensure that beneficial insects used in biological control programs are consistent in their behavior and effectiveness. Maintaining pure cultures preserves the genetic integrity of insect entomophagous lines. Hybridization or contamination with other species or strains can dilute the desired features such as high fertility, specific host preferences, and environmental tolerance. In 2007, investigations of the genetic structure of the internal noncoding transcribed spacer 2 (ITS2) of ribosomal DNA of *Trichogramma pintoi* Voeg were conducted. The performed PCR-analysis with the following nucleotide sequence determination of ITS2 region allowed us to reveal essential interspecific distinctions. The data obtained can be used to identify the species of *T. pintoi* [4].

Table 1. **Primer sequences for ITS2 region amplification**

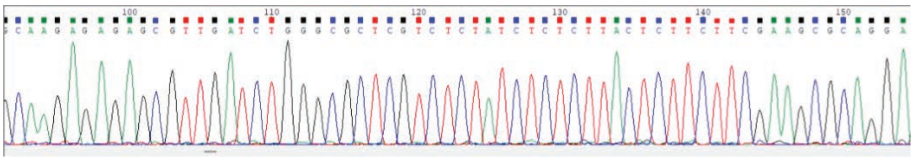
Name	Sequence (5' → 3')
Trich ITS2-F	TGTGAACTGCAGGACACATG
Trich ITS2-R	GTCTTGCCTGCTCTGAG



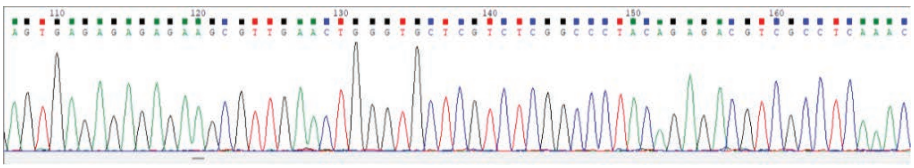
Sample 1. (*T. pintoi*, not confirmed)



Sample 2. (*T. evanesces*, not confirmed)



Sample 3. (*T. brassicae*, confirmed)



Sample 4. (*T. ahea*, confirmed)

Fig. 3. Sequencing chromatograms of PCR-amplified ITS2 products from *Trichogramma* spp. Samples 1 (*T. pintoi*) and 2 (*T. evanescens*) exhibit mixed sequence peaks, indicating contamination or hybrid populations. Samples 3 (*T. brassicae*) and 4 (*T. ahea*) show pure chromatograms of single-species, confirming genetic purity

On the basis of special practical importance, we are considering the possibility of creating effective test-systems and subsequent molecular biological control of various *Trichogramma* lines, which provide both purity and genetic consistency through molecular diagnostic methods. In particular, we use amplification and sequence analysis of the area of internal transcribed sequence 2 (ITS2) (Fig. 1) stocks, belonging to the genus *Trichogramma*: *T. pintoi*, *T. evanescens*, from Ukraine, and *T. brassicae* and *T. ahea* from Bulgaria were examined.

Methods. *DNA Extraction.* DNA was extracted from four *Trichogramma* spp. samples (provided by customers), using the GEB multisource DNA extraction kit in accordance with the manufacturer's protocol. Briefly, frozen insect samples (approximately 100 µg) were homogenized in 200 µL of lysis buffer supplemented with Proteinase K (20 mg/mL) and incubated at 60 °C. Nucleic acids were bound to a silica spin column by centrifugation at maximum speed for 1 minute. After binding, the columns were washed twice with 75 % ethanol-containing wash buffer, air-dried, and eluted in TE buffer (10 mM Tris-HCl, 1,0 mM EDTA, pH 8,0). The purified DNA was immediately used for downstream PCR amplification.

Table 2. **PCR cycling conditions**

Step	Temperature, °C	Time	Repeats
1	95	5 min	1
2	95	30 sec	30
3	55	30 sec	30
4	72	1 min	30

PCR Amplification. Amplification of the internal transcribed spacer 2 (ITS2) region was performed using GEB PCR Master Mix composed of 50 mM *Tris*-HCl (pH 8,8), 50 mM KCl, 0,25 mM dNTPs, 3 mM $MgCl_2$, and 2,5 U Taq DNA polymerase per reaction. Each 25 μL reaction contained 5 pmol of forward (Trich ITS2-F) and reverse (Trich ITS2-R) primers [4] (Table 1) and 100 ng of extracted DNA.

PCR conditions are given in Table 2.

PCR products were separated by electrophoresis on a 1,0 % agarose gel at 100 mA for 20 min and visualized with SYBR Green I DNA stain using a gel documentation system.

Sequencing and Sequence Analysis. Amplicons were purified and subjected to Sanger sequencing using the BigDye Terminator Kit ("Applied Biosystems", USA). Sequencing was performed on a Genetic Analyzer System 3500 ("Applied Biosystems"). The obtained sequences were analyzed using the BLAST tool (Basic Local Alignment Search Tool) to compare them with reference sequences and confirm species affiliation based on the similarity of ITS2 regions.

Results. PCR amplification successfully generated products from all four *Trichogramma* samples. The agarose gel electrophoresis confirmed the presence of distinct bands corresponding to the expected ITS2 fragment size across all samples (Fig. 2).

Sequencing results further revealed that samples 1 and 2 from the Ukrainian stock contained mixed populations, indicating contamination or hybridization between species. Meanwhile, samples 3 and 4 from the Bulgarian insect stock corresponded to pure cultures, with sequences matching reference species with high fidelity (Fig. 3).

Discussion. Molecular diagnostic methods for verifying the genetic purity of *Trichogramma* spp. entomophagous insect cultures used in biological pest control have become a widely used approach [4—6]. In particular, the combination of morphological trait analysis and molecular techniques (such as analysis of the mitochondrial cytochrome oxidase I (*COI*) gene) enabled the identification of eight *Trichogramma* species from sixteen geographic populations [5]. Additionally, Ivezić et al., 2021 [6] developed primers based on the ITS2 region of the rRNA gene for a multiplex PCR assay aimed to identify two common *Trichogramma* species: *T. brassicae* Bezdenko, 1968 and *T. evanescens* Westwood, 1833, associated with *Ostrinia nubilalis* (Hübner, 1796) (Lepidoptera: Crambidae) in Serbia.

Our study highlights the usefulness of molecular methods, particularly amplification and sequencing of the ITS2 region, for the genetic validation of beneficial insect cultures in biological control programs. While electrophoresis confirmed the amplification success, sequencing provided the definitive method for species identification and the detection of genetic impurities.

The identification of mixed species within some of the tested samples emphasizes the critical need for routine genetic monitoring in the mass production of *Trichogramma spp*. Contamination with closely related species could result in reduced biocontrol efficiency due to variability in host preference, environmental tolerance, and reproductive traits.

The presence of pure lines in two samples (*T. brassicae* and *T. ahea*) confirms that stringent colony management methods allow genetic stability to be maintained. These pure lines are essential to ensure predictable field performance, regulatory compliance, and customer trust.

Given the findings we recommend implementing molecular quality control measures at regular intervals (every three months or after two breeding generations) to identify and eliminate genetic inconsistencies. In addition, promoting the use of the "genetically controlled" label on commercial biological control *Trichogramma* agents could serve as a powerful marketing

tool, signaling reliability and superior quality to users increasingly focused on sustainable agricultural practices.

Conclusion. Maintaining pure cultures of entomophagous insect lines is fundamental to the success of biological control programs. This ensures consistency, preserves genetic integrity, prevents disease, supports research and development, ensures regulatory compliance, and underpins the commercial production of high-quality biological control agents. All these factors collectively contribute to sustainable management of pest populations and the reduction of chemical pesticide use, promoting healthier ecosystems and agricultural practices.

Our research has revealed that some insect cultures of the entomophagous species *Trichogramma spp*. are not entirely pure. They consist of a mixture of different species. As a result, we have identified an opportunity to test *Trichogramma* cultures for genetic consistency. To maintain quality control we recommend retesting the cultures for genetic purity confirmation every three months following two generations. These results can also serve as a valuable marketing tool, assuring customers high-quality products labeled as "Genetically controlled insect".

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Received 21.05.2025

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МОЛЕКУЛЯРНО-БІОЛОГІЧНА ДІАГНОСТИКА ЯК СТРАТЕГІЯ КОНТРОЛЮ ЯКОСТІ І ГЕНЕТИЧНОЇ ЧИСТОТИ КОРИСНИХ КОМАХ *TRICHOGRAMMA* spp.

Дослідження спрямовано на розроблення методів молекулярної діагностики для підтвердження генетичної чистоти культур ентомофагів роду *Trichogramma*, які використовуються у біологічному контролі та біозахисті рослин від комах-шкідників. За результатами аналізу внутрішнього транскрибованого спейсера ITS2 рибосомної ДНК виявлено міжвидові відмінності та ідентифіковано змішані популяції трихограми. Порівняння нуклеотидних послідовностей секвенованих фрагментів ITS2 уможливило точність визначення генетичної однорідності культур ентомофагів. Встановлено наявність домішок у зразках *Trichogramma pintoi* та *T. evanescens*, тоді як у *T. brassicae* та *T. ahea* підтверджено однорідність та генетичну чистоту. Для підтримки ефективності біологічного контролю рекомендовано регулярний моніторинг генетичної чистоти кожні три місяці після двох генерацій. Впровадження і валідація таких методів в Україні сприятиме ефективності біозахисту і забезпечить зменшення використання пестицидів заради збалансованості функціонування екосистем та підвищення якості й безпечності органічної рослинної продукції.

Ключові слова: *генетична чистота, молекулярна діагностика, Trichogramma spp., біологічний контроль, ITS2, секвенування, полімеразна ланцюгова реакція.*