DOI: 10.2478/frp-2019-0021
Available online: www.lesne-prace-badawcze.pl

Leśne Prace Badawcze / *Forest Research Papers*
Wrzesień / September 2019, Vol. 80 (3): 227–232

REVIEW ARTICLE

e-ISSN 2082-8926

DNA barcoding: A practical tool for the taxonomy and species identification of entomofauna

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Abstract. DNA barcoding is an innovative system designed to provide rapid, accurate, and automatable species identification by using short, standardized gene regions as internal species codes. The mitochondrial cytochrome C oxidase I (COI) gene was proposed by Paul Hebert as an official marker for animals, because of its small intraspecific but large interspecific variation. Since the launch of the project Barcode of Life, this simple technique has caught the interest of taxonomists, ecologists and plant-quarantine officers charged with the control of pests and invasive species.

The great diversity of insects and their importance have made this group a major target for DNA barcoding. In most cases, the identification of insect species by traditional methods based on morphological features requires specialist knowledge and is labor-intensive. DNA barcoding aims at meeting the challenge of monitoring and documenting the biodiversity of insects. The utility of DNA barcoding for identifying small insects, cryptic taxa or rare species, as well as many species of forest entomofauna that are impossible to discriminate morphologically throughout all of their life stages, is a subject discussed in this review. Due to its usefulness, also in Poland in the Forestry Research Institute, a method for identifying selected species of saproxylic beetles based on the sequence of the COI region was developed. In the future, this method will be used to assess the state of biodiversity and the naturalness of forest ecosystems. Therefore, this and other future implications of this promising new technique are also discussed here.

Keywords: DNA barcodes, entomofauna, species identification, taxonomy

1. Introduction

Insects are the most diverse group of animals, with over a million species described and another million pending description or simply undiscovered (Grimaldi, Engel 2005). In acquiring useful knowledge about any group of organisms, the basic requirement is the ability to describe, classify and then identify individual taxa of this group. The identification of numerous insect species by traditional methods based on morphological features requires specialist knowledge and is time consuming. Moreover, this diagnosis is often based on the morphological differences of adult insect forms, which severely restricts the identification of specimens in juvenile phases (Balakrishnan 2005). In many cases, due to the lack of diagnostic features or the large variability of these features, the DNA barcoding method provides the possibility of identifying organisms quickly and precisely (Hebert et al. 2003, 2004). The use of DNA sequences to obtain the information on the taxonomic similarities of an unknown specimen consists of sequencing a short fragment of the mitochondrial sequence encoding the subunit of the cytochrome oxidase I gene (cox1 CO1) and comparing this with the reference library of the barcodes of known species. This method is effective, especially in identifying small, cryptic insects (Burns et al. 2008; Huemer et al. 2014) or rare species (Hebert et al. 2004; Sinclair, Gresens 2008; Sweeney et al. 2011; Anderson et al. 2013; Jackson et al. 2014) and attempting to link different stages of insect development (Carew et al. 2005; Ekrem et al. 2007, 2010; Zhou et al. 2007, 2009; Stur, Ekrem 2011; Webb et al. 2012).

Received: 12.06.2019 r., accepted after revision: 9.09.2019 r.

2. The taxonomic identification of insects

The development of molecular phylogenetics has revolutionised taxonomy. Numerous research studies show how the analysis of the mitochondrial cytochrome C oxidase I (COI) locus can challenge existing taxonomic boundaries. For the stag beetle family, DNA barcoding confirmed the differences between several *Lucanus* species and *Lucanus cervus* L. subspecies (Cox et al. 2013). Similarly, COI analysis enabled the species of two groups of beetles from the Hydrophilidae and Scarabaeidae families to be distinguished (Monaghan et al. 2005). Based on mtDNA analyses, Jordal and Kambestad (2014) performed a taxonomic revision of two species of bark beetles, *Pityophthorus micrographus* L. and *Pityophthorus pityographus* Ratz. DNA barcodes have also been used to identify tropical butterflies of the genus *Perichares* (Lepidoptera, Hesperiidae). Since their first description in 1775, this group often included specimens that only superficially matched the described species. After mtDNA analysis, it turned out that some species are incorrectly assigned, and many of them belong to a complex of cryptic species (Burns et al. 2008). The correct assignment of specimens to a species, especially in the case of cryptic species, has important implications for taxonomic, evolutionary and biodiversity studies, and their presence amongst pests also has economic consequences.

DNA barcode analysis also allows ambiguities to be removed in determining species, resulting from the morphological differences between representatives of different castes in the case of social insects, such as ants (Smith et al. 2008), or differences in the morphology of male and female species with high sexual dimorphism (Janzen 2005). mtDNA analysis is an important tool in nature inventorying and biodiversity protection, facilitating both the identification of specimens living today as well as the description of taxa from museum collections, and, most importantly, it allows these samples to be linked to reconstruct the history of species. The inventory project of the geometer moth family (Geometridae) in Costa Rica demonstrates the possibility of using DNA barcodes to describe new species (Chacon et al. 2012). Caterpillars of five butterfly species – *Opsiphanes quiteria* Stoll, *Opsiphanes tamarindi* Felder, *Opsiphanes bogotanus* Dist., *Opsiphanes invirae* Hübner and *Opsiphanes fabricii* Boisduval – are very often encountered. A sixth species in the genus *Opsiphanes*, whose adult males are very similar to male specimens of the common *O. fabricii,* were identified as *Opsiphanes cassina fabricii* (DeVries 1987; Bristow 1991). An analysis of the DNA barcode of these species showed that *O. cassina fabricii* is a new species for Costa Rica, which, due to its morphological similarity to the common species, was overlooked until now.

The effectiveness of the DNA sequencing method in a selected, standard region of the genome to distinguish species (Hebert et al. 2003, 2004) was initiated by international research programmes that resulted in the barcode libraries available in the international GenBank databases (Clark et al. 2016) and the BOLDSystems (Barcode of Life Data System) (Ratnasingham, Hebert 2007). In the first years of operation, 430,000 insect barcodes were collected, representing approximately 50,000 species (30% of all known species) (Silva-Brandão et al. 2009; International Barcode of Life 2010b). Webb (2012) provided the largest set of DNA barcode data for water insects. He collected 4,165 sequences from the mitochondrial region of the COI subunit gene, representing 354 species of mayflies (Ephemeroptera) from Canada, Mexico and the United States. This allowed previously misidentified species to be corrected and extended knowledge about the levels of variation of the COI locus, both within and between species. Thanks to a project using DNA barcoding to prepare genetic and morphological keys to identify species of the Tanytarsini tribe of flies on Spitsbergen and Bear Island (Stur, Ekrem 2011), DNA barcode libraries have been developed for all Arctic species. Tanytarsini is a large and diverse group of lake flies belonging to the *Chironomidae* family. The developed keys allow the identification of the Tanytarsini species' larvae, pupae and finally metamorphosed specimens on Svalbard.

3. Protection of endangered species

In the context of protecting endangered species, it is worth mentioning that the DNA barcode method has the additional advantage of allowing the use of only small anatomical fragments instead of whole individuals (Svensson et al. 2009). This eliminates the need to obtain whole specimens, which is particularly important in the case of small populations. The flagship taxon of significance for the protection of invertebrates in Europe is the genus *Osmoderma* (hermit beetles), comprising a complex of four species, including *Osmoderma barnabita* Motsch. – strictly associated with old, hollow trees, which was intensively studied by Landvik et al. (2017). In the study cited above, the authors focused on explaining the phylogeographic structure of the east European population of *O. barnabita*, based on specimens from Latvia and Finland, as well as on previously published sequences and museum specimens. A sequence analysis of the mitochondrial COI gene has identified 26 closely related haplotypes whose diversity decreases from south to north. The observed distribution of *O. barnabita* also pointed to the recent expansion of this species in eastern Europe. This knowledge has a huge impact on the protection and control of the species, which in Poland and in all other countries is legally protected. The problem

of identifying rare and endangered species also applies to the stag beetle family (Lucanidae). More than 90 species of the genus *Lucanus* have been described, but in many cases, the validity of isolating taxa is questionable. Sexual dimorphism and variability in body size as well as the lack of diagnostic phenotypic features in the larvae further complicate the systematics of *Lucanidae*. Their classification is changing and is under discussion. Recent studies (Cox et al. 2013) describe the seven species of *Lucanus* in the western Palearctic*: Lucanus cervus* L.*, Lucanus ibericus* Mötsch., *Lucanus orientalis* Kraatz, *Lucanus tetraodon* Thungerg, *Lucanus (Pseudolucanus) barbarossa* Fab., *Lucanus* (P.) *busignyi* Planet and *Lucanus* (P.) *macrophyllus* Kraatz; some of them are considered endangered. It is difficult to set specific conservation priorities without proper identification; therefore, *Lucanus* was used to analyse the 3′-end of the COI gene for identifying species and subspecies (Cox et al. 2013). The sequenced COI fragment distinguished several studied *Lucanus* species and alleged *L. cervus* subspecies.

4. Control of forest tree pests

In an era of increased trade in wood and wood products, the risk of accidentally introducing alien insect species is increasing. Quick and correct identification is crucial to detecting invasive alien species that may exhibit greater pathogenicity in areas beyond their range. Sequencing the mtDNA region of the COI gene region enabled a DNA barcode library of European beetles to be established, which includes *Ips typographus* L.*, Plagionotus detritus* L.*, Rhagium inquisitor* L.*, Rhagium mordax* De Geer*, Saperda scalaris* L.*, Xylotrechus rusticus* L., *Peltis grossa* L. and *Protaetia* sp. Similar projects were implemented in Finland (Pentinsaari et al. 2014) and Germany (Hendrich et al. 2015), presenting DNA barcodes for 4,330 species from northern and central Europe. Similarly, the first DNA reference library was developed for about 100 *Agrilus* species from the Northern Hemisphere based on three mitochondrial markers: cox1-5′ (DNA barcode fragment), cox1-3′ and rrnL. All data, including sample images and geographic data (dx.doi.org/10.5883/DS-AGRILUS1), are available in barcode database format, which is thus far the largest collection of data for the identification of species from this genus. All of the more than 3,000 species of the genus *Agrilus* feed on parts of vegetation, causing serious damage to trees. Their quick identification is the first step in undertaking further protective measures. Molecular research can, therefore, be an effective tool for controlling pests on a global scale. About 151 species of beetles, pests found in Europe and North America, have been identified using DNA barcodes, including *Ips acuminatus* Gyll. and *I. typographus* L. (Jordal, Kamberstad 2014). Therefore, DNA barcode analysis can serve not only as a new taxonomic tool but also as a standard system for quick identification and monitoring.

The genus *Xylotrechus* (Coleoptera: Cerambycidae), represented in Greece by four species, damaged olive tree plantations in 2014. The pests *Xylotrechus stebbingi* Gahan and *X. rusticus* L. were identified based on morphological features in conjunction with the analysis of the DNA barcode (Levidara et al. 2018).

Preventing the introduction of invasive alien species into forest ecosystems is a high priority goal for countries with extensive forest resources. Studying DNA barcodes within the family Erebidae (Lepidoptera) revealed intraspecies differences between *Lymantria dispar* L., *Lymantria mathura* Moore and *Lymantria sinica* Moore (Stewart et al. 2016). To help identify these insects, a TaqMan® test kit was developed, which can identify all three *L. dispar* subspecies and five additional *Lymantria* species that threaten North American forests. The test suite is a ‘molecular key’ (analogous to a taxonomic key) and includes several parallel single and multiplex qPCR reactions. Each reaction uses a combination of primers and probes designed to distinguish taxa, enabling their rapid and accurate identification. Analysing molecular data can quickly identify an unknown sample, including the juvenile stages of insects. Successive studies confirm the high potential of COI barcodes to identify the larvae of the flat bark and longhorn beetle families: *Cucujus cinnaberinus* Scop.*, R mordax* De Geer and *R. inquisitor* L. (Ziganshina et al. 2018). Using DNA barcodes in research also provides the possibility to identify rare species and forest pests at various stages of development. Wu et al. (2017) applied an integrated approach not previously used, but which allows the results to be cross-verified by simultaneous rearing larvae to the adult stage. The DNA barcodes generated in these studies supplement the data in the event of inadvertently transported pests such as *Saperda* sp. and *Xylotrechus* sp.

5. Controversy about the usefulness of DNA barcodes

The DNA barcode has many advantages that allow a large number of samples to be quickly identified even by non-experts. However, there is some controversy regarding the use of DNA barcodes in the classification system of organisms, both at the stage of specimen identification and in the discovery of new species (Meier 2008). The assignment of taxonomic names to unknown specimens is performed using DNA reference libraries and depends on the number of representatives of each species contained in the database. This process may be affected by errors when the samples contained in the library are incorrectly described or do not reflect the overall genetic diversity of the species. Therefore, the most

important factor determining the accuracy of species identification is the wealth of available barcode libraries (Ekrem et al. 2007). In fact, most identification errors are due to a lack of reference data (Virgilio et al. 2010). The identification of new species using DNA barcodes requires high accuracy and ceases to be effective when there is high genetic diversity within a given species (Davis, Nixon 1992; DeSalle et al. 2005). The identification results may be adversely affected by heteroplasmy (Song et al. 2008), that is, the coexistence of several mitochondrial haplotypes in one individual, reported for many insects (Gellissen, Michaelis 1987; Bensasson et al. 2000; Brower 2006; Rubinoff et al. 2006; Magnacca, Brown 2010a, b). It is estimated that a quarter of the taxa of the described animals are not monophyletic (Funk, Omland 2003), which may also be the source of errors in the analyses. Different species may also appear polyphiletic or paraphyletic because of incomplete sorting of mitochondrial DNA lines or introgression. Such situations are quite common (e.g. Kaila, Ståhls 2006; Burns et al. 2010; Žurovcová et al. 2010). The selection of appropriate statistical methods and interpretation of the results of genetic variation between specimens are also problematic. The development of algorithms for identification based on the DNA barcode is still a challenge in the field of bioinformatics.

Despite these issues, Collins and Cruickshank (2013) were optimistic about the future of DNA barcoding because the advantages of the method outweigh its disadvantages. And identification itself based on DNA barcodes can be performed in two stages: initial identification using the COI barcode and detailed identification using additional molecular and morphological data for a specific group of insects.

6. Summary

The importance of biodiversity research in forests is increasing, as exemplified by the implementation of large -scale inventories of extensive forest complexes in selected forest districts and the Białowieża Forest. This is due to the need to know the number of species associated with forest ecosystems, which is crucial, amongst others, for the designation of valuable natural areas, sustainable forest management, determining its impact on biodiversity and understanding the causes of range changes, including the expansion or disappearance of certain species. Thus far, an overwhelming number of inventory studies have been conducted using traditional methods, that is, based on the taxonomic identification of morphological features, less often anatomical. Such research often encountered difficulties with identification, the lack of specialists, the ability to include larger forest areas in the studies and the high costs of implementation. Molecular analysis techniques can help here. Sometimes it is the only method that can provide a clear result, for example, to distinguish between cryptic (twin) species or forms difficult to identify, such as larvae. Molecular techniques are also indispensable in the case of "difficult samples," such as fragments of insect bodies remaining in humus, based on which the parent species may not be able to be determined in the traditional manner. The DNA barcoding technique is now a common practice in supporting the identification and classification of living organisms (Hebert et al. 2003), because this code is the same at every life cycle stage of a specific organism for both sexes. Barcode sequences sent to databases are easily available and subsequent analyses can be repeated by anyone. However, there are many taxonomical gaps in the databases. Many sequences or even entire genomes are available for intensively studied groups and model organisms, but the vast majority of species have no sequence data and are waiting to be described (Sanderson et al. 2003).

Conflict of interest

The authors declare no potential conflicts.

Acknowledgements and source of funding

The research was financed from the forest fund of the State Forests National Forest Holding – DGLP 500-449.

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Authors' contribution

I. Sz-B. – concept, literature review, manuscript preparation; K.S. – literature review, manuscript preparation, proofreading.

Translated by: Barbara Przybylska