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PCR-RFLP method to distinguish *Frankliniella occidentalis*, *Frankliniella intonsa*, *Frankliniella pallida* and *Frankliniella tenuicornis*

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Abstract: Thrips from the genus *Frankliniella* (Thysanoptera, Thripidae) are phytophagous on crops and wild plants. Some of them cause slight economic damage, however, others including *F. occidentalis* and *F. intonsa* are responsible for considerable losses in crop production. Moreover, they constitute a double threat for host plants by not only feeding on them but also vectoring viruses, some of which are on the quarantined list of the European Plant Protection Organization. The rapid detection and differentiation between more and less harmful *Frankliniella* species is, therefore, important in order to combat the pests at the time of their appearance. In this study, we have undertaken to develop a method of detecting *F. occidentalis, F. intonsa, F. pallida,* and *F. tenuicornis.* The protocol is based on PCR amplification of ITS1 rDNA fragments of these insects using universal primers pair giving products of slightly distinct length for studied insects. Restriction enzymes digestion which is easy to interpret, allows for visible differentiation of all these *Frankliniella* species. The method was shown to be species-specific and sensitive. Even single specimens in either the larvae or adult stage could be distinguished.

Key words: *Thrips*, molecular diagnostics, pest control, detection, *Frankliniella occidentalis, Frankliniella pallida, Frankliniella tenuicornis, Frankliniella intonsa*

Introduction

Thrips are one of the most important pests occurring on vegetables and ornamental plants in greenhouses, and on vegetables in fields in Poland. About 6,000 species of thrips are known throughout the world, with about 600 in Europe, of which about 220 occur in Poland (Kucharczyk 2007). About 230 species are listed in the genus *Frankliniella*, with up to 130 further names placed into synonymy (Nakahara 1997). This high rate of synonymy is due to unrecognized variability in size and color of many species. In Poland, species included in the *Frankliniella* genus (Thysanoptera, Thripidae) are represented mainly by *Frankliniella occidentalis* (Pergande), *Frankliniella intonsa* (Trybom), *Frankliniella pallida* (Uzel), *Frankliniella tenuicornis* (Uzel), and rarely by *Frankliniella nigriventris* (Uzel) and *Frankliniella tristis* (Priesner) (Sierka and Gocyła 2004). All of these are phytophagous on crops and wild plants and are responsible for direct damages (feeding and oviposition wounds). Moreover, *F. occidentalis* and *F. intonsa* are important vectors of tospoviruses such as tomato spot wilt virus (TSWV), tomato chlorotic spot virus (TCSV), groundnut ringspot virus (GRSV) or impatiens necrotic spot virus (INSV) (Wang *et al*. 2010).

Frankliniella occidentalis (western flower thrips) is on the EPPO A2 List of pests recommended for regulation as quarantine pests (EPPO). This pest is highly polyphagous with a wide host range. It spreads from horticultural and ornamental crops (such as pepper, beans, carnations, roses and gerbera) to strawberry plants and trees (table grapes and peach). With regard to their impact on agricultural systems, western flower thrips can be considered as the most invasive pest introduced into Europe and responsible for the greatest damage in crops. For example it can cause up to 70% yield losses of greenhouse cucumber (Marullo 2002). *Frankliniella intonsa* (flower thrips), which in some seasons can cause significant damage, is found in Poland (Pobożniak 2011). It also has a wide host plant range, spreading from field crops to greenhouse and wild plants and can colonize Rosaceae (apple, pear, cherry, plum) and herbaceous plants (grasses). *Frankliniella pallida* can not be considered as a pest, since it lives mainly on wild plants such as *Trifolium, Ranunculus, Cichorium, Leucanthemum, Brassica nigra* (Marullo 2003). *Frankliniella tenuicornis* is host-specific on wild and cultivated Gramineae. Its injuries produce grain malformations or complete sterilization. Morphological identification of thrips

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species is not easy and possible only for professional taxonomists.

Proper identification of species is essential to successfully control the number of pests. The earlier a pest is identified the better the chance to take appropriate steps to limit its spreading. It is also important to distinguish between non-pest thrips species, pests causing moderate losses and species causing huge damages in crops in order to take immediate measures to fight the latter. Due to the similarity between *Frankliniella* species, especially in the larval stadium and similar host range, morphological identification is difficult, especially for non-taxonomists.

For this reason molecular biology can be very useful for rapid, sensitive and reliable identification. Molecular diagnostic protocols for identification of economically important pest species exist for many insects, including thrips (Kraus 1998; Moritz 2000; Obrepalska-Steplowska *et al*. 2008; Huang *et al*. 2009; Pumariño *et al*. 2011; Varadínová *et al*. 2015). Also several protocols were developed for quarantine thrips species (e.g. Przybylska *et al*. 2015). In this study PCR-RFLP (Polymerase Chain Reaction − Restriction Fragment Length Polymorphism) was used which allows for the amplification of a conserved region of DNA sequence using PCR followed by the digestion of the amplified fragment with appropriate restriction enzymes which enables the detection of genetic variation between identified species. There are two other protocols for distinguishing thrips species using PCR-RFLP assay (Brunner *et al*. 2002; Mainali *et al*. 2008) but not for all four species analyzed in this study. Other protocols for thrips detection are based on real-time PCR (Huang *et al*. 2010), PCR (Zhang *et al*. 2012) or Loop-mediated Isothermal Amplification (LAMP) (Przybylska *et al*. 2015).

Most molecular diagnostic protocols are based on mtDNA or rDNA regions. There are some protocols for thrips species based on *mtCOI* (Brunner *et al*. 2002; Zhang *et al*. 2012) but there are some reports about several intraspecific variants of this region in the case of thrips (Rebijith *et al*. 2012) which might result in an ambiguity of detection with this marker. We chose the ITS1 rDNA region because sequences analysis done in our previous study (Przybylska *et al*. 2015) demonstrated its interspecific variations between *Frankliniella* and *Thrips* species and the stability within the studied species. Therefore we found appropriate fragments which made it possible to design universal primers and the amplification of DNA from all four analyzed species. Moreover, the ITS1 region differs in length for each studied species. The method described in this study makes it possible to detect four *Frankliniella* species and differentiate between them without DNA isolation.

Materials and Methods

Insect samples

Material for the study consisted of populations of different thrips species considered as positive controls (*F. occidentalis, F. intonsa, F. pallida, F. tenuicornis*) and negative controls. There remaining species are listed in table 1. For molecular analysis both larvae and adult specimens were taken.

Genomic DNA extraction from thrips samples

Thrips samples were collected and suspended in 70% ethylene alcohol. DNA extraction was performed from 1–3 thrips specimens using DNeasy Blood & Tissue Kit (Qiagen) and obtained in the final volume of 100 µl and concentration 5–10 ng · µl^{-1}.

Design of four *Frankliniella* species specific primers

Sequences of 18S-ITS1-5,8S-ITS2-28S rDNA region from a number of thrips species (*F. occidentalis, F. intonsa, F. pallida, F. tenuicornis, Thrips palmi, T. tabaci, T. major, T. trehernei, T. sambuci, T. simplex, T. roepkei*), obtained during our previous study (Przybylska *et al*. 2015) and *F. schultzei* sequence downloaded from NCBI databases (National Center for Biotechnology Information) were aligned using BioEdit software (Hall 1999) and regions conserved only for *Frankliniella* species were chosen for designing forward and reverse primers amplifying products of different lengths. Next, this *Frankliniella* species sequences alignment was used to indicate restriction enzymes sites which would allow for differentiation between *F. occidentalis*, *F. intonsa*, *F. pallida* and *F. tenuicornis*.

PCR assay specific for *Frankliniella* species

Degenerate primers pair designed for this study (FrUNIF: GATRCGACTGTCAGAGWAC and FrUNIR: GATACCGACACTTCATCTG), giving products of distinct lengths for each tested species, were tested in PCR reaction with DNA isolated from populations of *F. occidentalis*, *F. intonsa*, *F. pallida,* and *F. tenuicornis.* To test method reproducibility, ten *F. occidentalis* populations and seven *F. intonsa* populations were tested. Moreover, the combination of DNA isolated from *F. occidentalis* and *F. intonsa* mixed in equimolar ratio in one reaction tube was included. To exclude cross-reactions, DNA isolated from many other *Thrips* species (Table 1) was also tested as well as no template control to test for the reagents contamination. All PCR reactions were performed in the mixture containing 1 µl DNA template, 1 µM of each primer, 5 µl of DreamTaq Master Mix (Thermo Scientific) and sterile, distilled water up to 10 µl. The amplification conditions were as follows: denaturation for 3 min at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 57°C, 30 s at 72°C; the final extension for 5 min at 72°C (Mastercycler Personal, Fermentas). After reaction, 3 µl of the final mixture were run on 1% agarose gel and visualized with Midori Green stain (Nippon) in the gel documentation system (Infinity, Vilber) and the remaining volume of the PCR product was taken for restriction enzyme digestion in RFLP analyses.

Restriction Fragment Length Polymorphism (RFLP) assay

RFLP assay was performed using 7 µl of PCR product as a template. To each reaction 1 µl of FastDigest Buffer (Thermo Scientific), and 5 U of FastDigest enzymes *Tru1I*

Table 1. *Thrips* and *Frankliniella* populations used in this study

Species	Origin	Host plant	GenBank accession number
Frankliniella occidentalis 1	Poland	*Cucumis sativus*	KM886242
Frankliniella occidentalis 2	Poland	*Dendranthema indica*	–
Frankliniella occidentalis 3	Poland	*Cucumis sativus*	–
Frankliniella occidentalis 4	Poland	*Cucumis sativus*	–
Frankliniella occidentalis 5	Poland	*Cucumis sativus*	–
Frankliniella occidentalis 6	Poland	*Solanum lycopersicum*	–
Frankliniella occidentalis 7	Poland	*Cucumis sativus*	–
Frankliniella occidentalis 8	Poland	*Eupatorium* sp.	–
Frankliniella occidentalis 9	Poland	*Cucumis sativus*	–
Frankliniella occidentalis 10	Poland	*Solanum melongena*	–
Frankliniella intonsa 1	Poland	*Nemesia strumosa*	KM886243
Frankliniella intonsa 2	Poland	*Pelargonium hirsutum*	–
Frankliniella intonsa 3	Poland	*Lysimachia vulgaris*	–
Frankliniella intonsa 4	Poland	*Rosa* sp.	–
Frankliniella intonsa 5	Poland	*Trifolium* sp.	–
Frankliniella intonsa 6	Poland	*Gentiana pneumonanthe*	–
Frankliniella intonsa 7	Poland	*Gentiana asclepiadea*	–
Frankliniella pallida	Poland	*Sedum acre*	KM886244
Frankliniella tenuicornis	Poland	*Zea mays*	KM886245
Thrips major	Poland	*Sambucus nigra*	KM877309
Thrips menyanthidis	Poland	*Menyanthes trifoliata*	–
Thrips nigropilosus	Poland	*Ocimum basilicum*	–
Thrips palmi	Japan	*Cucumis sativus*	KM877305
Thrips origani	Poland	*Origanum vulgare*	–
Thrips physapus	Poland	*Ceantaurea jacea*	–
Thrips roepkei	Poland	*Solanum dulcamara*	KM877310
Thrips simplex	Poland	*Gladiolus* sp.	KM877312
Thrips sambuci	Poland	*Sambucus nigra*	KM877311
Thrips tabaci	Poland	*Inula salicifolia*	KM877307
Thrips trehernei	Poland	*Tragopogon pratensis*	KM877313

and *HinfI* and sterile distilled water up to 10 µl were added. Reaction was carried out for 30 min in 37°C, and then the PCR-RFLP products were separated in 2.5% agarose gel and visualized with Midori Green stain (Nippon) UV lamp integrated in the gel documentation system (Infinity, Vilber)

PCR-RFLP detection without DNA isolation

PCR-RFLP assay was tested for *F. occidentalis* and *F. intonsa* specimens using one larva and without DNA extraction. To assess repeatability, five larvae from each species were taken and each one was crushed in a separate tube in 20 µl of sterile distilled H_2O. The obtained suspensions (2 µl) were taken as templates for PCR-RFLP analysis. Reactions were done under the previously described conditions.

Results

Design of *Frankliniella* species specific primers and restriction enzymes sites

In the first part of the study PCR primers were designed which enabled the detection of four analyzed *Frankliniella* species. Obtained sequences of primers were analyzed by last searches to exclude potential cross-reactions with non-target species. The BLAST analysis showed that the designed primers hybridize only to *Frankliniella* sequences. In the obtained alignment consisting of *Frankliniella* species, the species-conservative regions were indicated as targets for primers hybridization sites. Simultaneously, fragments localized between regions of primers hybridization and showing interspecies variability were found to serve as targets for species-specific restriction enzymes digestion, so they were suitable for four *Frankliniella* species differentiation (Fig. 1).

PCR assay specific for *Frankliniella* species

Under the described conditions, the PCR reaction gave single products with the following lengths: 326 bp for *F. occidentalis*, 237 bp for *F. intonsa*, 280 bp for *F. tenuicornis* and 250 bp for *F. pallida*. No cross-reactions were observed with thrips species taken as negative control: *T. major*, *T. menyanthidis*, *T. nigropilosus*, *T. origani*, *T. palmi*, *T. physapus*, *T. roepkei*, *T. sambuci*, *T. simplex*, *T. tabaci* and *T. trehernei* as well as no product was obtained for reaction mixture without insects' DNA template (Fig. 2). The reaction was repetitive for ten analyzed *F. occidentalis* populations and seven *F. intonsa* populations. Moreover, the combination of *F. occidentalis* and *F. intonsa* in one tube gave two bands with sizes specific for those species (Fig. 3A).

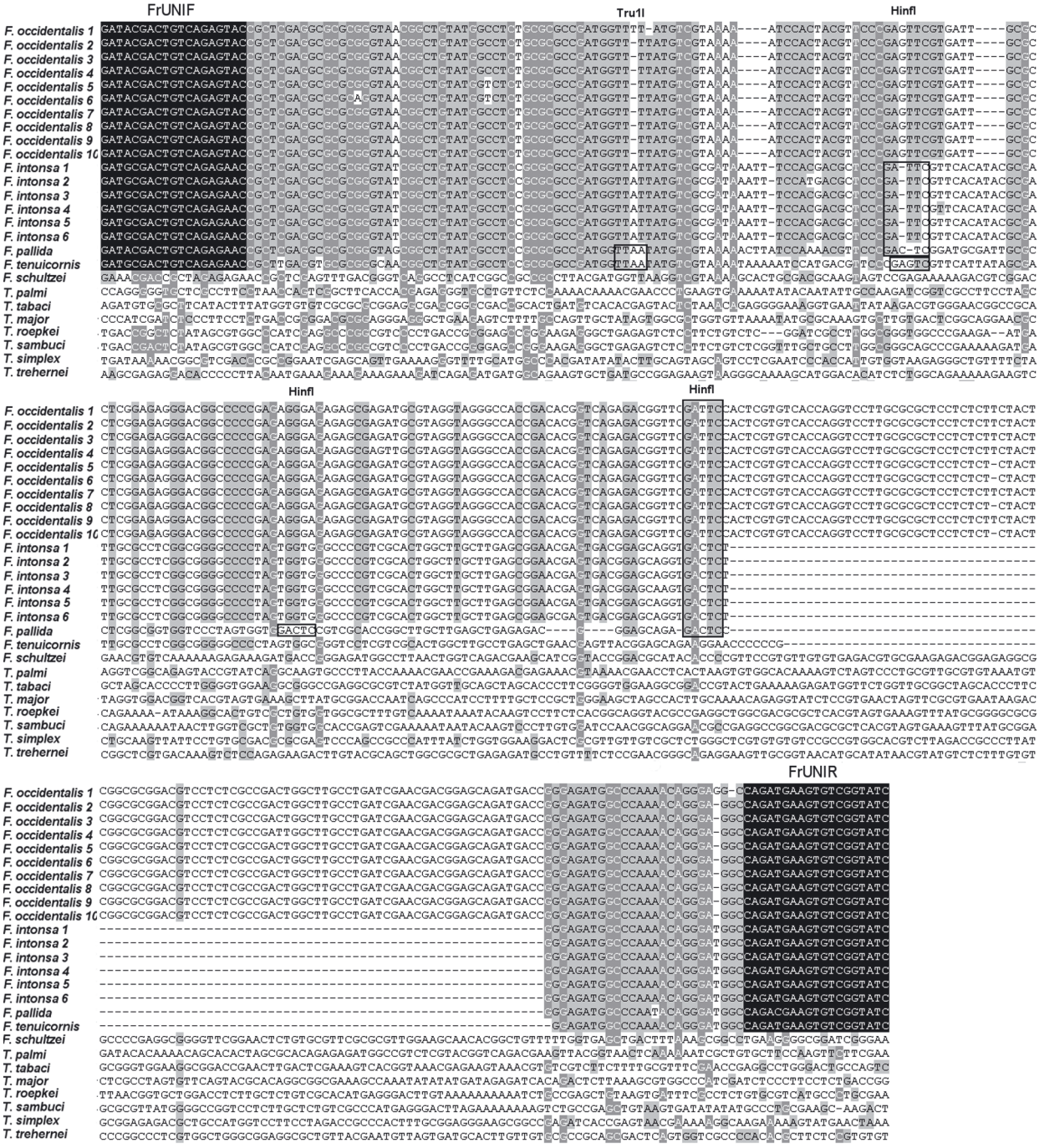

Fig. 1. Multiple sequence alignment of the rDNA region of analyzed thrips species with FrUNIF/R primers sequences (black) and restriction enzymes digestion sites (in frames). Visualization in GenDoc software (Nicholas *et al.* 1997)

PCR-RFLP assay

The PCR protocol allows for detection of four aforementioned *Frankliniella* species. To differentiate between them two restriction enzymes were selected (*TruI, HinfI*) and applied in the extended version of PCR-RFLP. The patterns of the RFLP analysis were characteristic for each species (Fig. 3B) and their lengths were as follows: 190 bp and 147 bp for *F. occidentalis*; 102 bp, 95 bp and 50 bp for *F. intonsa*; 68 bp, 50 bp, 44 bp, 42 bp and 35 bp for *F. pallida* and 151 bp, 68 bp and 36 bp for *F. tenuicornis* according to bioinformatics tools (NEBcutter V2.0) used for prediction of products fragments sizes after restriction enzymes digestion of *Frankliniella* DNA sequences used in this study. After gel electrophoresis some bands of similar sizes migrated together and some smaller ones were less visible on agarose gel but RFLP patterns differed significantly between analyzed species.

PCR-RFLP sensitivity and detection without DNA isolation

The sensitivity of the PCR-RFLP method was determined for larva of *F. occidentalis* and *F. intonsa.* Single larva samples (without DNA isolation) were subjected to PCR-RFLP under the conditions described above. The method proved to be sufficiently sensitive to detect even one larva crushed in water without DNA extraction. Results were positive for all five replicates of *F. occidentalis* larvae as well as for *F. intonsa* larvae (Fig. 4). This also confirms the repeatability of the method.

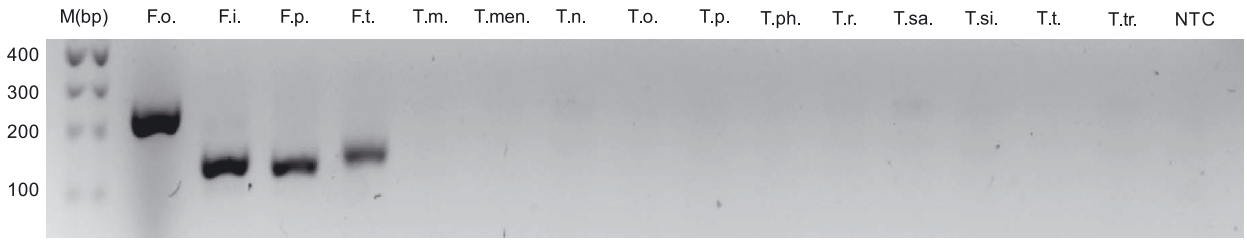

Fig. 2. The electrophoretic separation of PCR products obtained with FrUNIF/FrUNIR primers in agarose gel. Obtained products sizes were as follows: *F. occidentalis* – 326 bp, *F. intonsa* – 237 bp, *F. tenuicornis* – 280 bp, *F. pallida* – 250 bp; F.o. – *F. occidentalis*, F.i. – *F. intonsa*, F.p. – *F. pallida*, F.t. – *F. tenuicornis*, T.m. – *Thrips major*, T.men. – *T. menyanthidis*, T.n. – *T. nigropilosus*, T.o. – *T. origani*, T.p. – *T. palmi*, T.ph. – *T. physapus*, T.r. – *T. roepkei*, T.sa. – *T. sambuci*, T.si. – *T. simplex*, T.t. – *T. tabaci*, T.tr. – *T. trehernei*, NTC – no template control, M – marker of molecular weight (Nova 100 bp, Novazym)

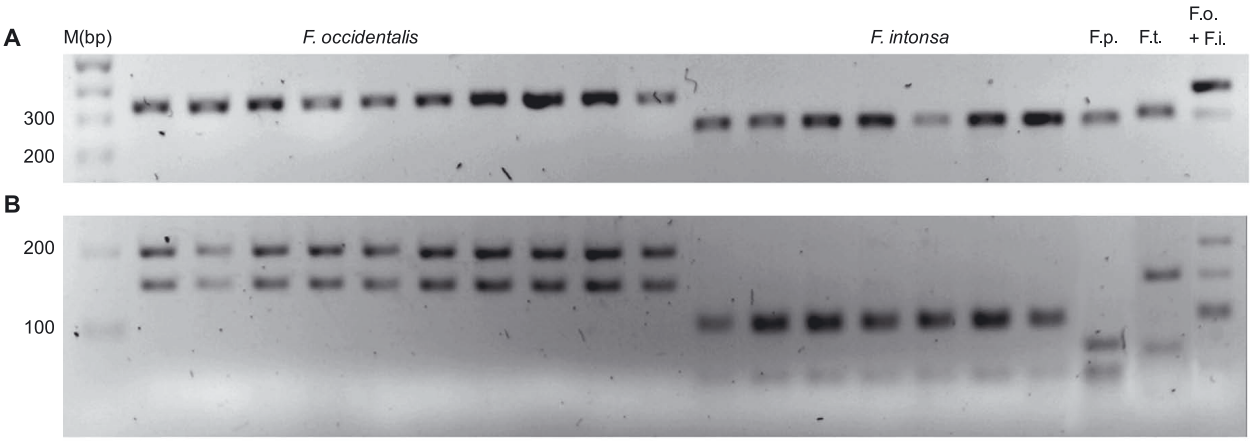

Fig. 3. Electrophoretic separation and visualization of: A – PCR reaction results with FrUNIF/FrUNIR primers with 10 populations of *F. occidentalis*, seven populations of *F. intonsa*, one population of *F. pallida* and one population of *F. tenuicornis*; B – PCR-RFLP products obtained using *TruI* and *HinfI* restriction enzymes for DNA obtained from all analyzed *Frankliniella* populations; F.p. – *F. pallida*, F.t. – *F. tenuicornis*, F.o. – *F. occidentalis*, F.i. – *F. intonsa*. PCR-RFLP products sizes are as follows: 190 bp and 147 bp for *F. occidentalis*; 102 bp, 95 bp and 50 bp for *F. intonsa*; 68 bp, 50 bp, 44 bp, 42 bp and 35 bp for *F. pallida* and 151 bp, 68 bp and 36 bp for *F. tenuicornis* according to bioinformatics tool used for prediction of products fragments sizes after restriction enzymes digestion (NEBcutter V2.0). After gel electrophoresis some bands of similar size migrated together during electrophoresis; M – marker of molecular weight (Nova 100 bp Novazym)

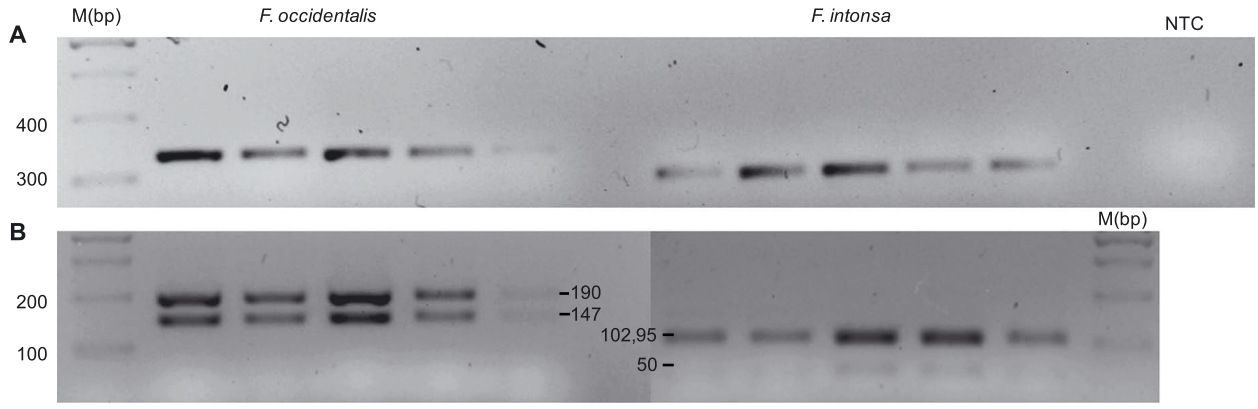

Fig. 4. The separation of PCR products during electrophoresis in agarose gels: A – PCR products obtained with FrUNIF/FrUNIR primers on one larva without DNA extraction step; B – RFLP reaction results for one larva without DNA extraction step; NTC – no template control, M – marker of molecular weight (Nova 100 bp Novazym)

Discussion

Thrips species in the genus *Frankliniella* are polyphagous pests that feed on a variety of ornamental and vegetable hosts. *F. occidentalis* and *F. intonsa* are both efficient vectors of viruses and are major pests feeding on a number of crop species. Since most *Frankliniella* species are highly polyphagous and are commonly found on plants as larvae which usually have not developed the morphological characteristics of adults, identification at a species level requires growth to adulthood (Huang *et al.* 2010). Classical morphological studies regarding insects belonging to the *Frankliniella* genus species show deficiencies in identifying both interspecific and natural strains of the same species. These are characterized by considerable phenotypic variation relating to morphological traits, such as size and position of the ocellar setae. But it is not always easy, because *F. intonsa* is very similar to *F. occidentalis.* These less stable traits should therefore be coupled to other distinctive features to make the identification more reliable. Accordingly, molecular biology techniques are a valuable addition to traditional phenotypic methods of pest thrips recognition (Moritz *et al*. 2004).

There are some PCR-RFLP protocols for *F. occidentalis* and *F. intonsa* (Mainali *et al*. 2008), *Hercinotrips femoralis*, *F. occidentalis*, *T. palmi*, *T. tabaci* and *Heliothrips haemorrhoidalis* (Brunner *et al*. 2002) as well as for *F. occidentalis*, *F. intonsa*, *T. hawaiiensis*, *T. coloratus*, *T. flavus*, *T. palmi*, *T. tabaci* and *T. setosus* (Toda and Komazaki 2002). However, to date there is no method which can differentiate four of the species analyzed in this study. Furthermore, there is no molecular diagnostics protocol for *F. pallida* and *F. tenuicornis*. To fill this gap we have undertaken to develop a protocol which would make it possible to both detect and differentiate the four *Frankliniella* species.

The method described by Mainali *et al*. (2008) to distinguish *F. occidentalis* and *F. intonsa* species may result, as stated by the authors, in false positives for *F. intonsa* when one sample contains both species. This deficiency has been eliminated in our protocol which allows for the differentiation of *F. intonsa* and *F. occidentalis* even in the preliminary PCR reaction, before RFLP, when both species are mixed in a single sample (Fig. 3A). As a product of this reaction two single bands with sizes specific for each species (*F. occidentalis* – 326 bp, *F. intonsa* – 237 bp) are observed. Since *F. occidentalis* and *F. intonsa* are found sympatrically in fields and greenhouses (Ullah and Lim 2015), proper identification of this species is particularly relevant.

Protocol is useful for identification all *Frankliniella* species occurring frequently not only in Poland but also in other countries. *Frankliniella nigriventris* and *F. tristis* which were not tested during this study have been reported in Poland only a few times so far and their significance is marginal (Sierka and Gocyła 2004). Moreover, their rDNA sequences in public databases are lacking. In our preliminary study we first aligned all known *Frankliniella* sequences from GenBank including one additional *Frankliniella* species not tested in our study, which until now has not been reported in Poland – *F. schultzei*. *Frankliniella schultzei* is a polyphagous pest feeding on various ornamental and vegetable hosts in different parts of the world including Europe. This pest can cause both direct and indirect damages to crop. It feeds on pollen and floral tissue, leading to flower abortion. Indirect damage is due to virus transmission (Kakkar *et al*. 2010). The sequence analysis done showed that the PCR primers described in this study are not able to bind to rDNA of *F. schultzei* and thus to amplify DNA of this species (Fig. 1).

The PCR-RFLP method described in this study is fast, simple and relatively cheap because it allows for the identification of one larva of *F. occidentalis* and *F. intonsa* even without DNA extraction and it does not require any additional equipment except for a thermal cycler and electrophoresis set.

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