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A NEW METHOD FOR SPECIES IDENTIFICATION OF POULTRY BASED ON 12S-rRNA FRAGMENT POLYMORPHISM

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Abstract

European Union regulations require producers of food and animal feeds to provide reliable information on the qualitative composition of products. Development of methods to determine differences between declared and actual components has become a major research challenge in recent years. The present study attempts to develop a method for rapid determination of poultry species based on analysis of the mitochondrial DNA 12S-rRNA region using PCR/RFLP. Two alternative methods for identification of four poultry species are presented, each using one pair of primers and two restriction enzymes. Using the first pair of primers, mtDNA fragments of 102 and 103 bp were amplified for chickens (*Gallus gallus*) and turkeys (*Meleagris gallopavo*), respectively, and those of 110 and 112bp for ducks (*Anas platyrhynchos*) and geese (*Anser anser*), respectively. *BslI* enzyme was used to distinguish between chickens and turkeys, and *Tsp509I* to distinguish between geese and ducks. The second pair of primers amplified a mtDNA fragment of about 222 bp. The use of the *MnlI* enzyme enabled the chicken and turkey components to be identified, and in the case of ducks and geese *Sau3AI* was additionally used to distinguish between these two species based on the presence of restriction site in the duck PCR product. The application of the method in laboratory practice could help to put into practice the European Union requirements concerning the qualitative analysis of food products and animal feeds.

Key words: 12S-rRNA, PCR-RFLP, poultry, species identification.

Analysis of mtDNA is a useful tool in identifying the species of breeding and wild animals. This is possible due to the presence of many mtDNA copies in every cell and greater resistance of mtDNA to degradation compared to nuclear DNA, thus ensuring good reaction sensitivity. These characteristics make it possible to study genetic material that is difficult to analyse because of its trace amounts, degradation caused by atmospheric agents, or thermal treatment used during manufacture of feed mixtures or food products. Poultry is a group of animals that attracts the interest of food and animal feed producers. In light of current regulations, producers are obliged to provide reliable information on the qualitative composition of products. It is realized, however, that without regular monitoring, the substitution of more expensive poultry

species with cheaper materials with inferior properties will continue to be a problem. In response to this market demand, it seems appropriate to develop a method for rapid identification of poultry species.

Material and methods

DNA was isolated from chicken, goose, duck and turkey blood samples using the Promega Wizard kit, according to the manufacturer's protocol.

The following primers were used:

F1: 5'-ttaagccacacccccacgggta-3' R1: 5'-ctggcacaagatttaccaaccctg-3

F2: 5'ctgggattagataccccactatgc-3 R2: 5'tcattagaggtgggctggcgac-3

These sequences, designed using Primer3 software (Rozen and Skaletsky, 1998), restrict a mitochondrial DNA (mtDNA) 12S-rRNA fragment in poultry, i.e. in *Gallus gallus* (NC_001323), *Meleagris gallopavo* (NC_010195), *Anas platyrhynchos* (NC_009684) and *Anser anser* (NC_011196). For PCR products determined in silico, homologous sequences were determined using BLAST (Altschul et al., 1997) to find if they amplify mammalian DNA, thus making their specificity more reliable.

The optimum reaction mixture was: 1 × Buffer; dNTPmix – 0.8 mM; AmpliTaq Gold polymerase – 1.25 U; $MgCl_2$ – 2.4 mM, each primer – 0.5 µM, DNA – 1 µl. Total volume of the reaction mixture was 25 µl.

The amplification was performed using a thermal program of 95°C for 3 min, 30 × (94°C for 1 min, 60°C for 1 min, 72°C for 1 min) 72°C for 30 min.

For the PCR products obtained, restriction enzymes that identify the species of the analysed material were determined using Nebcutter software (Vincze et al., 2003).

Results

The results were analysed by electrophoretic separation with a 2.5% agarose gel. The size of DNA fragments was determined as the absolute number of base pairs (bp) by comparison with known size marker (25 bp DNA).

Figure 1 presents the result of PCR/RFLP analysis obtained using the first pair of primers. They amplify a 12S-rRNA fragment of 102 bp for hens, 103 bp for turkeys, 112 bp for geese, and 110 bp for ducks. To distinguish between hens and turkeys we used the *BslI* enzyme that has a restriction site at 83 bp for chicken PCR product only. The *Tsp509I* enzyme determined for geese cut the PCR products into fragments of 30 and 82 bp, which enables it to be distinguished from a duck product.

The second pair of primers (Figure 2) amplified a fragment of 222 to 224 bp depending on the species identified. Such small differences prevent distinguishing between species and only provide information on the presence or absence of components originating from any of the species investigated. Only the use of *MnlI* and *Sau3AI* enzymes enabled further identification. After digestion of PCR products with *MnlI* enzyme, restriction patterns with bp size of 101/69/54 and 121/61/40 were obtained

for chickens and turkeys, respectively. For ducks and geese, *Sau3AI* was additionally used because the *MnlI* enzyme produced an almost identical restriction pattern for these species. *Sau3AI* enzyme had a restriction site in duck PCR product, which enabled it to be distinguished from the goose PCR product.

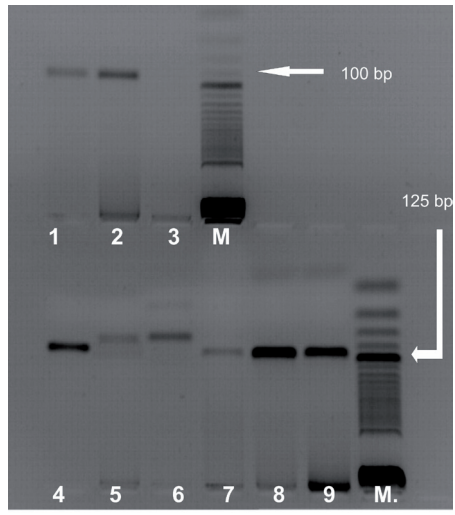

Figure 1. PCR amplification of 102–112 bp products and their restriction profile. Lanes 1, 2 , 8 and 9 contain a PCR product, in which the matrix was DNA isolated from *Gallus gallus*, *Meleagris gallopavo*, *Anas platyrhynchos* and *Anser anser*, respectively. In lanes 4–5, restriction profile *Bsl I* for *Meleagris gallopavo* and *Gallus gallus*, and *Tsp509I* for *Anser anser* and *Anas platyrhynchos* (6,7). In lane 3, negative control of PCR. M – size marker (25 bp)

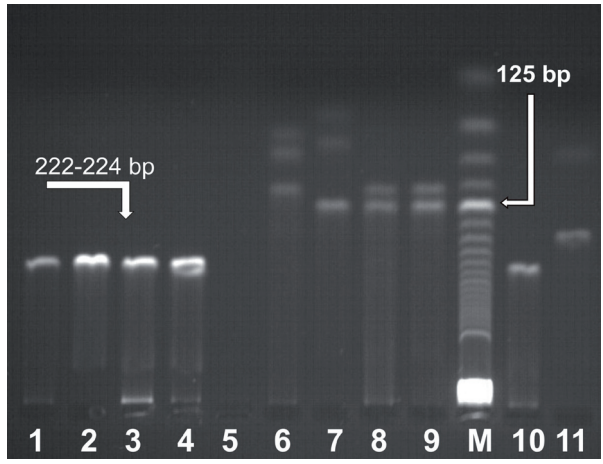

Figure 2. PCR amplification of 222–224 bp products and their restriction profile. Lanes 1–4 contain a PCR product, in which the matrix was DNA isolated from *Gallus gallus*, *Meleagris gallopavo*, *Anas platyrhynchos* and *Anser anser*, respectively. In lane 5, negative control of PCR. M – size marker (25 bp).
In lanes 6–11, restriction profile *Mnl I* for *Gallus gallus*, *Meleagris gallopavo*, *Anas platyrhynchos*, *Anser anser*, and *Sau3AI* for *Anas platyrhynchos*, *Anser anser*. M – size marker (25 bp)

Discussion

European Union regulations require producers of food and animal feeds to provide reliable information on the qualitative composition of products (Zipfel and Zipfel, 2000). In the present study, we wanted to check declared components against actual components. The results describe two alternative methods for identification of four poultry species, each using one pair of primers and two restriction enzymes. We used a sequence of the 12S-rRNA conservative region (Rodrigez et al., 2003). Sequence analysis of this mtDNA fragment is often used for species identification of animal components in food products and animal feeds (Ha et al., 2006). It enables distinguishing between different numbers of poultry species. The first study describes methods for distinguishing between just two species (Rodríguez et al., 2001), but a later study makes it possible to distinguish between four species (Rodríguez et al., 2003). These analyses are performed in multiplex reactions or when using restriction enzymes. A common forward primer flanking sequences for all species and separate reverse primers, homologous with one of the analysed sequences, are often used in multiplex reactions (Rodríguez et al., 2003). This method yields PCR products of different length specific for a given species, which provides a basis for their identification as a result of electrophoretic analysis. This method, like that presented in our study, makes it possible to identify four poultry species, but may be inadequate for material derived from several species because of the common forward primer, whose amount may be difficult to assess in this case. The method discussed in our study does not have these limitations and enables the analysis regardless of the amount of individual components.

An important step in poultry identification was a study by Martín et al. (2007). These authors designed a multiplex reaction amplifying poultry DNA, but small differences in PCR product lengths between individual species may make this analysis difficult. In contrast, PCR fragment sizes obtained using our method present no problems during the identification because there are sufficient differences between them.

Another PCR/RFLP method makes it possible to distinguish between material derived from hen, duck and turkey using as many as four restriction enzymes (Girish et al., 2007). In contrast, the method presented in our study seems to introduce favourable changes, which enable the analysis to be performed using two restriction enzymes.

Sequencing of the amplified fragment is another method that can be used for species identification. Although it guarantees 100% success, this method shows limitations during routine studies because of the cost and duration of full analysis.

In conclusion, the PCR/RFLP analysis of the mitochondrial DNA 12S-rRNA fragment polymorphism provides valuable information on species identification and is a useful tool in qualitative analyses. After being checked with raw components other than blood and processed components, the method presented in this paper could be applied for routine control of animal products.

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Nowa metoda identyfikacji gatunkowej drobiu na podstawie polimorfizmu fragmentu 12S-rRNA

STRESZCZENIE

Przepisy unijne wymagają od producentów żywności oraz karmy dla zwierząt rzetelnej informacji na temat składu jakościowego produktu. Opracowanie sposobów sprawdzenia składników deklarowanych z rzeczywistymi stało się ważnym wyzwaniem badawczym w ostatnich latach. W przedstawionej pracy podjęto próbę opracowania metody pozwalającej w szybki sposób określić gatunek drobiu na podstawie analizy regionu 12S-rRNA mitochondrialnego DNA przy zastosowaniu metody PCR/RFLP. Prezentowane wyniki opisują dwa alternatywne sposoby identyfikacji czterech gatunków drobiu, każdy przy zastosowaniu jednej pary starterów oraz dwóch enzymów restrykcyjnych. Przy pomocy pierwszej pary starterów powielono fragment mtDNA o wielkości 102 i 103 pz odpowiednio dla kur (*Gallus gallus*) i indyków (*Meleagris gallopavo*) oraz 110 i 112 pz dla kaczek (*Anas platyrhynchos*) i gęsi (*Anser anser*). W celu rozróżnienia kur od indyków zastosowano enzym *BslI*, natomiast gęsi od kaczek *Tsp509I*. Z kolei druga para starterów umożliwiła powielenie fragmentu mtDNA o wielkości około 222 pz. Zastosowanie enzymu MnlI umożliwiło identyfikacje komponentu kurzego i indyczego, natomiast w przypadku kaczek

i gęsi dodatkowo użyto *Sau3AI*, który rozróżniał te dwa gatunki na podstawie obecności miejsca restrykcyjnego w kaczym produkcie PCR. Wdrożenie do praktyki laboratoryjnej opracowanej metody przyczyni się do wprowadzenia w życie wymogów unijnych dotyczących analizy jakościowej produktów żywności oraz karmy dla zwierząt.