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PCR identification of five species from the *Anopheles maculipennis* complex (*Diptera: Culicidae*) in North-Eastern Romania

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Abstract

The members of the *Anopheles maculipennis* complex have been incriminated for the transmission of the malaria in Europe, which was endemic until the middle of the century. The global warming and the intensification of the intercontinental travel constitute a risk of the re-emergence of the malaria in Europe, given the presence of the Anopheles vectors. The study has attempted the identification by using the PCR (Polymerase Chain Reaction) of the members of the *Anopheles maculipennis* complex from the North-eastern area of Romania from the city of Iaşi. In total there have been identified by using the PCR amplifying the ITS2 sequence of the ribosomal DNA, 217 specimens belonging to the complex of *A. maculipennis* among which: 58 *A. atroparvus*, 18 *A. melanoon*, 2 *A. labranchiae*, 52 *A. maculipennis* and 87 *A. messeae.* The ITS2 sequences of the ribosomal DNA have been compared to those of the species belonging to the *A. maculipennis* available in GenBank. The Species *A. labranchiae* is reported for the first time in Romania, being identified in the larval stage IV. The adaptation of a new species to the climatic conditions present in the North-eastern Romania, confirms the phenomenon of global warming and also the intensification of the travelling. As a result of the analysis of the *A. labranchiae* sequence, this one corresponds to the extent of 96% to the species from Italy, registered in GenBank, given the fact that a high number of the inhabitants of the municipality of Iaşi are working in this country.

Keywords

Anopheles maculipennis complex; Malaria; Europe

Introduction

The malaria was endemic in Europe until the middle of the century, in Romania the Campaign of Eradication of the malaria ending in 1965, in 1967 being declared by the World Health Organization, a country free of malaria (Nicolescu and Purcărea-Ciulacu 2012).

However, the global warming, the intensification of the travel between the continents and the demographic modifications constitute a risk of reintroducing the malaria in Europe. The malaria is rarely diagnosed in Europe, yet it represents an emergency in the medical world. The important cases of malaria imported by the immigrants in Europe coming from the endemic areas of malaria, has increased in the latest years from 14% up to 83% (Odolini *et al*. 2012; Calleri *et al*. 2011). The infections with malaria from among the immigrants may increase the risk of transmission in other areas where there exist the adequate climatic vectors and conditions (Shkurti *et al.* 2013). There have been cases of transmission of the malaria also in the airports where the mosquitoes carriers of the malaria parasites have been brought with the planes from the endemic areas of malaria (Cobo 2014).

In Europe and the Middle East the transmission of the malaria is low or absent, but there have been registered species from the genus of *Anopheles*, considered vectors of the malaria: *A.atroparvus*, *A.labranchiae*, *A.messeae*, *A.sacharovi*, *A.sergentii*, *A.superpictus* (Sinka *et al*. 2010; Nicolescu *et al.* 2004; Sedaghat *et al.* 2003).

The transmission of the malaria by the mosquitoes from the genus of *Anopheles* depends very much of the geographi-

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cal region, this capacity being acquired genetically; thus, there are vectors of local importance and secondary vectors whose role remains unclear (Mouchet *et al.* 2004).

The climatic changes with the increase of the temperature even with 0,5°C may cause an increase of the population of mosquitoes with 30–100% (Patz and Olson 2006). The factors which limit the geographical spread of the species of mosquitoes are the temperature and the humidity.

The existence of a complex of *maculipennis* species has been reported for the first time by Falleroni in 1926, describing the morphological differences from the chorion of these species. From the *Anopheles maculipennis* complex belong nine Palearctic species: in the central Europe, the most common are the *A. messeae*, *A. maculipennis sp.* and *A. Atroparvus*, *A. labranchiae* and *A. sacharovi* are indigenous along the Black Sea and the Mediterranean Sea. *A. beklemishevi*, is distributed mainly in Scandinavia, the Eastern and Western Europe.

A. sicaulti and *A. martinius* are non-European species (Kronefeld *et al*. 2012**;** Proft *et al*. 1999). The species with the widest spreading in the Europe and the Middle East is the *A.messeae* (Sinka *et al.* 2012).

All the members of this complex are considered vectors of the malaria from Europe. Taking into account the medical importance of the *maculipennis* complex, the differences regarding the vectorial capacity and the distribution of the species, which are considered to be effected at a worldwide level by the global warming, it becomes necessary the use of secure methods of identification of the species (Shkurti *et al.* 2013**)**. The vectorial capacity is an index of the capacity of transmission of the malaria vector, being influenced by this index of density of the vector (influenced by the climatic conditions: the temperature and humidity in the geographical conditions: the seasonal variations as well as the zoophylical preference or the anthropophilic ones. (Djadid *et al*. 2007; Toole 2009). Without the presence of the preferential host, the feeding behaviour can modify (Pages *et al.* 2007; Nitzulescu and Gherman 1990). The *A. atroparvus*, *A.labranchiae* şi *A.sacharovi* have been incriminated as the main vectors of the malaria in the region of Europe (Vicente *et al.* 2011).

Anopheles labranchiae, considered one of the most important vectors of the malaria in Europe, has a limited distribution in the Southern and South-eastern Europe. This fact has been reported in the South-eastern area of Spain, Corsica, the coastal areas from Italy, Sardinia, Sicily (Romi *et al.* 2001; Bietolini *et al.* 2006; Romi *et al.* 2010). In the Northern Africa the species is found in Morocco, Algeria and Tunisia (Zahar 1990). The *Anopheles labranchiae* has registered an increase in Sardinia during the last 35 years. In Sardinia have been found larvae in almost all the habitats, with the exception of those very shady (Sinka *et al.* 2010). *A.labranchiae* females easily attack the man, (Di Luca *et al.* 2009) having been reported the presence of the human blood at 86%–90,7% from the fed females. *A.labranchiae* has been involved in the transmission of the malaria in Portugal, Spain, France and Italy (Alten *et al.* 2007) a proved malaria vector along the coastal plains (up to 200 – 330 meters above the sea level) from the centre and South of the continental Italy, Sardinia and Sicily (Romi *et al.* 2012).

Materials and Methods

The Iaşi County is located in the North-eastern area of Romania and the central-eastern of Moldavia, between the parallels 46°50′ and 47°36′ northern latitude and between the meridians 26°33′ and 28°07′ eastern longitude. Captures of mosquitoes have been made in three areas of the city of Iaşi Iaşi: the Cotu Morii pond, the Ciurbeşti natural lake and the Nicolina River (Galata district).

The Ciurbeşti Lake

Is a lake of artificial plateau barrage from the Central Moldavian Plateau, formed on the territory of the Ciurbeşti village from the Miroslava commune from the outskirts of the municipality of Iaşi. It is located on the Ciurea Hill (192 meters altitude).

The Nicolina River

Is a river from the east of Romania which springs from the Bârnova commune (the County of Iaşi), near the Rotunda hill at an altitude of over 350 m and it flows into the Bahlui River on the territory of the municipality of Iaşi. The monthly average temperature of the water of the river closely follows the monthly average temperature of the air, thus a maximum of 18°C is registered in the month of July and a minimum of 0.1°C in the month of February. The riverbed is dirty and has abundant vegetation, the water being polluted by the industrial units which are on the route, as well as by the household waste.

The Cotu Morii Pond

Is a pond situated at 20 km from Iaşi, on the Iaşi-Sculeni road, near human dwellings. The water is relatively clean, with abundant vegetation.

The traps have been installed at the nightfall, at 20.00 and until 6.00 in the morning in the points considered as the most propitious for the activity of the mosquitoes. With the view of the identification of the genera and species of the mosquitoes, these are being kept in 80% ethyl alcohol. The action of collecting the samples has been made beginning from the month of May, until the month of October, the traps being located at the beginning and the end of the month for a period of two years 2010 and 2012. The traps of the mosquitoes used are made of a source of UV light source, fan, net and a recipient with saponified water for the collection of the mosquitoes.

The mosquitoes preserved in alcohol have been identified based on the keys of identification described by Becker *et al.* in 2003. The Culicidae from the *Anopheles* genus classified in the *Anopheles maculipennis* complex have been used for the extraction of the DNA and the identification by means of molecular biology techniques, being identical twin species from the morphological point of view.

In total, 1830 mosquitoes have been examined, classified in the *Culex*, *Aedes*, *Ochlerotatus* and *Anopheles* genera. In the *Anopheles maculipennis* complex 217 mosquitoes have been classified based on the morphological characteristics, the species being afterwards identified using the PCR. Adult mosquitoes and larvae of stage III and IV have been used for the identification. The ITS2 region of the DNA has been entirely amplified by the PCR, using the primers utilised by Jana Proft in 1999 (Table I). The sequence of the forward primer is complementary to the conserved region 5.8S r DNA (5'-TGTGAACTGCAGGACACATG-3') and the reverse primer makes annealed with the conserved region 28S DNAr (5'-ATGCTTAAATTTAGGGGGTA-3 ').

The amplification and sequencing of the ITS2 section is useful for detecting the intraspecific differences.

For the extraction of the DNA, the mosquitoes have been completely utilised. The extraction of the DNA has been made using two kits: – Thermo Scientific Phire Animal Tissue Direct PCR Kit and ZR Tissue & Insect DNA MiniPrep. Two PCR mixes have been utilised**:** – a mix recommended by the Thermo Scientific Phire Animal Tissue Direct PCR kit and a mix adapted according to the model of Jana Proft (1999) (Table II). 2 PCR programmes have been used, one recommended by the Scientific Phire Animal Tissue Direct PCR Kit and one adapted according to the model of Jana Proft. Both methods have given very good results.

According to the literature studies, the DNA sequences of the mosquitoes from the *A.maculipennis* complex had the following measures of: -*Anopheles maculipennis sp*: 410 bp -*Anopheles labranchiae*: 374 bp -*Anopheles messae*: 305 bp -*Anopheles melanoon*: 224 bp -*Anopheles atroparvus*: 117 bp.

The migration in the electrophoresis gel has been made using 4 µl of PCR product, 2 µl of bromophenol in gel of agarose of 2% in which 20 µl of Syber Safe DNA has been added. The migration has been realised at 80V for a period of 1h and 30 min, afterwards being read at the UV light.

Results

By using PCR the ITS2 sequence has been amplified, flanked by the 5.8S and 28S regions. The length of the sequence has varied from 117 bp *A. atroparvus* at 410 bp *A. maculipennis sp*. By means of PCR, five species belonging to the *A. maculipennis* complex: *A. atroparvus*, *A.messeae*, *A.melanoon*, *A.maculipennis sp.* and *A. labranchiae.* (Fig. 1)

The sequentiation of the sample of *A. labranchiae* from Iaşi-Romania (Fig. 2) has been verified in GenBank by using NCBI Blast: Nucleotide sequence and it corresponds in a proportion of 96% to the species of *A. labranchiae* from Italy (Cagliari), code-AY253840.1, Italy (Foggia) code-AY253841.1 and Iran code-AY842516.1. Comparing the sequences of the species of *A. labranchiae*, which comes from Italy, the Cagliari region (code AY253840.1) with those by us for the *A. labranchiae* Iaşi-Romania, we have the partial 5.8S sequence from 1–93 bp TGT→TAT and the partial ITS2 sequence from 94 –365 bp TTG→TAC, which coincide in a proportion of 96% with this species.

Table I. The universal characters of the primers (Jana Proft in 1999)

Species (primer code)	Primer nucleotide sequence (5' - 3')	m(°C)	Length of specific PCR product (bp)
Complex (universal) (5.8 –UN)	TGTGAACTGCAGGACACATG	7	±
An. maculipennis s.s. (AMA)	TATTTGAGGCCCATGGGCTA	6	410
An. atroparvus (AAT)	CGTTTGGCTTGGGTTATGA	4	117
An. messeae (AMS)	GACGCCTCACGATGACCTT	8	305
An. melanoon (AML)	TGCAAGTTGAAACCTGGGGC	9	224
An. labranchiae (ALA)	GTATCTCTGCTGCTATGGTC	6	374
An. sacharovi (ASA)	CAAGAGATGGATGTTTTACG	3	180

Table II. The PCR Programme adapted (according to the model of Jana Proft)

Step	Temperature	Time	Number of cycles
Initial denaturation	95°C	1,5 min	1
Denaturation	95°C	1 min	
Annealing	53°C	30 sec	35
Extension	72°C	1 min	
Final extension	72°C	5 min	1

The sequentiation of the sample of *A. maculipennis sp*. from Iaşi-Romania, corresponds in a proportion of 99% with the *A. maculipennis sp.* species from Turkey (Anatolia), code-JN112927.1, Turkey (Anatolia) code-JN112928.1 and Iran (Ardebil province) code-FJ210877.1. Comparing the sequences for *A. maculipennis sp.* species from Iran, the Ardebil province code-FJ210877.1 with those obtained by us for the *A. maculipennis* Iaşi-Romania, we have the partial ITS2 section from 42 -404 bp GGA→AGT, which coincides in a proportion of 99% with the species from Iran.

Anopheles messeae reported in Iaşi-Romania corresponds in a proportion of 99% with the species of *A. messeae* from Italy, code-AY365011.1, Russia (the Krasnodar region) code-FN646207.1 and Russia (the Kalmikiya region)_code-AM409767.1. It has been obtained for *A. messeae* Iaşi-Romania, the partial 5.8S sequence rRNA from 1–93 bp TGT→TAT, and the partial ITS2 region from 94–290 TTG→TGA which coincides in a proportion of 99% with the species from Russia.

The sequentiation of the sample *A. melanoon* reported in Iaşi-Romania corresponds in a proportion of 100% with the species of *A. melanoon* from Italy, code- AY365009.1, Yugoslavia (the Kotor region) code- AY238410.1 and Yugoslavia (the Montenegro region) code- AY238411.1. We have obtained for the *A. melanoon* Romania (Iaşi), the partial sequence of 5.8S rRNA from 1–94 bp TGT→TAT, and the partial ITS2 region from 95–216 TTG→ GCA which coincides in a proportion of 100% with the compared species (Fig. 3).

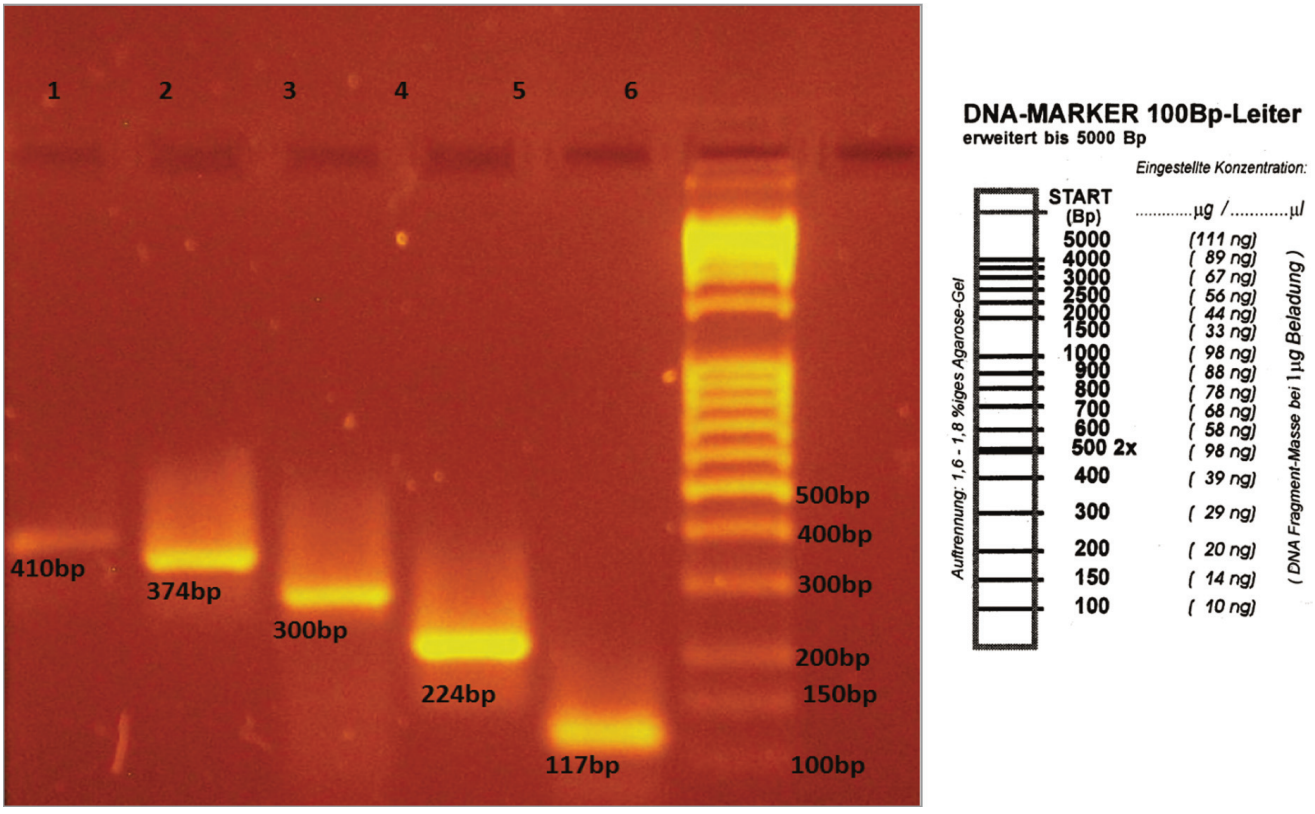

Fig.1. The PCR product specific for the five species from the *Anopheles maculipennis* complex:*1.Anopheles maculipennis s.s; 2.Anopheles labranchiae; 3.Anopheles messae; 4.Anopheles melanoon ; 5.Anopheles atroparvus;* 6.Marker 100 bp

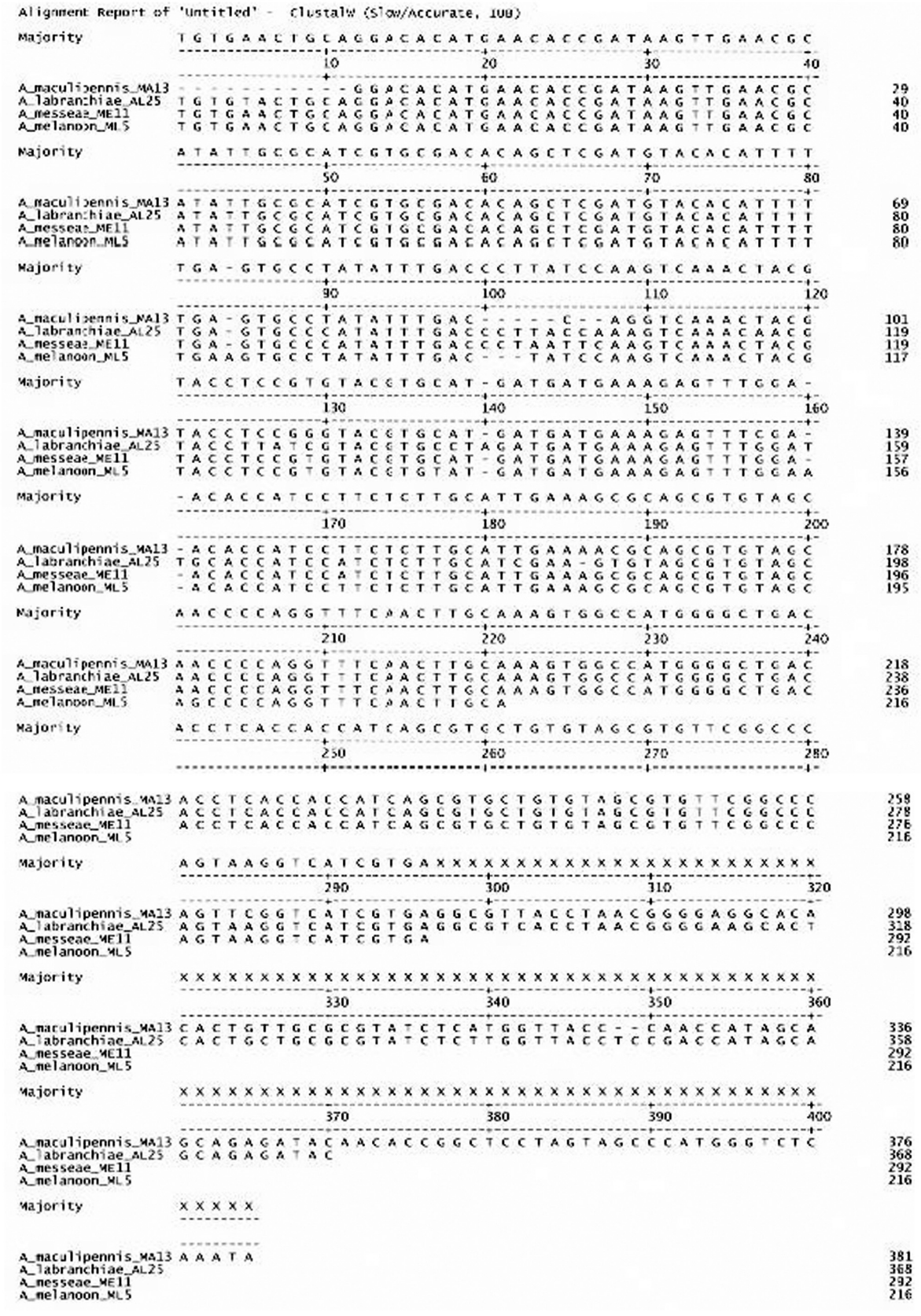

Fig. 2. The alignment of the nucleotides of the ITS2 sequence at four species from the *An. Maculipennis* complex

Discussion

In total, by means of PCR 217 specimens belonging to the *A. maculipennis* complex have been identified from which: 58 *A. atroparvus*, 18 *A. melanoon*, 2 *A. labranchiae*, 52 *A. maculipennis* and 87 *A. messeae.*

A. labranchiae is reported for the first time in Romania, the extraction of the DNA and that of the PCR being made from larvae of mosquitoes of stage IV. The two larvae of stage IV have been captured at the natural pond Ciurbeşti. As a result of the captured larval stage we can conclude the possibility of adaptation of the species of *A. labranchiae* to the climate of the city of Iaşi. The resemblance in a proportion of 96% to the species of *A. labranchiae* from Italy presents the possibility of importing the species from this country, taking into account the fact that a large part of the inhabitants of the municipality of Iaşi and the peripheral communes work in Italy (during the summer coming back in the country and thus being possible the transportation with themselves also mosquitoes from this species). The *A. labranchiae*, *A. messeae*, *A. melanoon*, *A. atroparvus* and *A. maculipennis* have been incriminated for the transmission of the malaria in Europe, the last 4 being responsible for the transmission of the malaria in Romania during the period of 1948–1963.

The specimens belonging to the *A. maculipennis sp* species, have been 12 larvae of stage III and IV and the rest adults, being captured on the Nicolina river (the Galata district), during the period of May – October 2010 and 2012. The *A. maculipennis* female is zoophile feeding itself mainly from the cattle, but also from pigs and chickens and occasionally it can also be also anthropophilic.

From the *A. messeae* species, 35 specimens have been larvae of stage III and IV and the rest adults. The specimens have been captured at the Ciorbeşti pond, Cotu Morii and on the Nicolina river (the Galata district), during the period May – October 2010 and 2012.

For the *A. melanoon*, all the identified specimens have all been in the stage of adult. The captures have been made at the Ciurbeşti pond in the months of June – July in 2012. In Romania, the species has been reported for the first time in Constanța in 2004 by Nicolescu *et al.* And a single specimen has been captured.

The *A. atroparvus* species has been identified in the captures made at the Ciurbeşti pond, all the specimens captured being adults.

All the five species identified in the city of Iaşi, are considered vectors of the malaria in Europe. The *A. labranchiae*, can contribute to the greatest extent at the re-emergence of the malaria in Romania.

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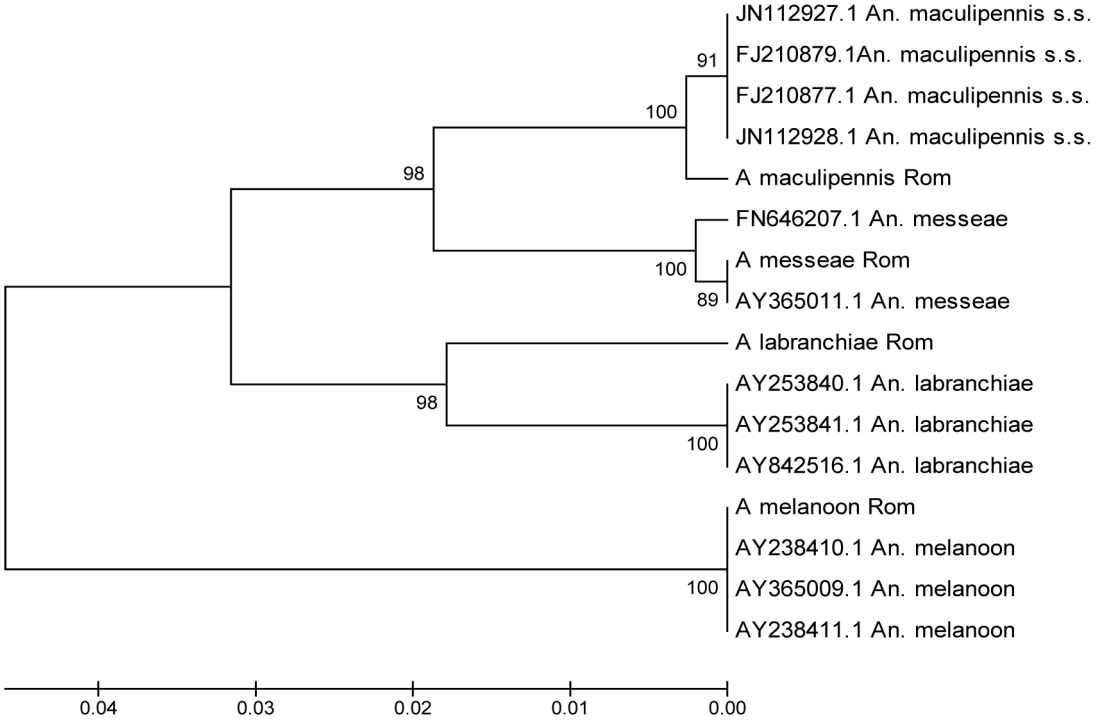

Fig. 3. The phylogenetic tree of the species identified in the city of Iaşi shows the detachment of the *A.labranchiae* and *A.maculipennis* species from Iaşi, Romania, with the presence of the intraspecific differences. In order to correct the sequences and the formation of the phylogenetic tree Geneious 6.0.6 programme has been used

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