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ABSTRACT

One of the effects of global warming is the adaptation of new vectors to the climatic conditions considered as not corresponding to the development, and also the risk of the introduction and/or re-emergence of a disease. Malaria is among the diseases whose risk of re-emergence is related to the effects of global warming. Malaria is the disease with the widest distribution on the globe. Every year, thousands of sick people or carriers of malaria travel to malaria-free countries, reintroducing the risk of the re-emergence of this disease. There is a great necessity of mapping the global distribution of malaria, due to its continuous change, and to the fact that the vectors implicated in its transmission are adapting on more and more diverse areas, being transported along with the intensification of the intercontinental voyages.

Consequently, in the present research we intended to identify the species of culicids belonging to the complex *Anopheles maculipennis*, and to establish their vector role in the transmission of malaria.

The dissertation is structured on two parts, corresponding from the point of view of the length of the subject to the rules required by the doctoral dissertation format.

The first part presents the bibliographic study of the subject chosen, with documentation based on the current literature from our country and abroad, offering the most recent data on the subject proposed on a length of 48 pages, with 7 pictures and 5 tables.

Chapter I presents aspects of the bio-ecology of culicids from Iaşi City area – as a case study, with the presentation of climatic factors influencing the development of mosquitoes: solar radiation, relief characteristics, multiannual thermal characteristics, humidity of the air, and precipitations.

Chapter II of the bibliographical research part is a morphological description based on the keys of determination of the mosquitoes belonging to the complex *Anopheles maculipennis*, in different stages of development, and the comparison of the present stage of knowledge of the complexes of species of the genus *Anopheles* transmitting malaria nationally and internationally.

Chapter III presents aspects regarding the epidemiology of malaria, due to the known pathogenicity of the mosquitoes that can produce this disease with the widest distribution on the globe. The distribution of malaria in Africa, Asia, America and Europa is described, ending the chapter with data concerning the distribution of malaria in Romania.

Chapter IV presents the biological cycle of malaria parasites, of the group of species of the genus *Plasmodium*, describing the sporogonic cycle, the schizogonic (erythrocytic) cycle, and vector or transfusion transmission of malaria.

Chapter V describes some techniques of molecular cloning, involving the assembling of some recombined DNA molecules, and their replication in the organism of the host. It describes the cloning process and its stages, from the DNA extraction to the amplification of the gene of interest, treatment with restriction enzymes of the purified DNA in order to obtain ends that can be linked with the ones of the cloning vector, and introduction of the recombined molecules in the host cell, with verification of the recombined molecules.

The second part contains our personal research, and it contains 128 pages, with 75 pictures and 28 tables, being structured on five chapters, as follows:

Chapter VI contains the analysis of the climatic data (temperature, humidity and precipitations between 1961 and 2013), with the purpose to demonstrate their favourable evolution over the life cycle of culicids, at present and up to 2030. For this purpose, we implemented a new mathematical model, the ET30 model, based on the construction of a function of interpolation of Lagrange polynomial type, which offers the possibility of a prognosis with over 95% probability. The use of this type of mathematical algorithm allows finding a

function *F,* by the interpolation of the experimental measurement, to estimate as well as possible the average temperature of each month of the year 2030, according to the thermal tendency registered in the last five decades. The program sequences of the ET30 model were calculated using FORTRAN programming language, and the data processing was realised with the help of OringiPro application.

Consequently, comparing with the period of malaria eradication in Romania, the 60's, a raise with 1.1°C has been registered up to present in Iași, and the level of the whole country raised by 0.72°C, showing the probability of adaptation of new species of culicids to our clime. According to a study realised by Jonathan A. Patz and Sarah H. Olson in 2006, in the mountains, where malaria is absent or seasonal, a warming by 0.5°C would lead to a multiplication of the populations of culicids with 30-100%, and it would also favour the development of the parasite inside the vector.

The average humidity in Iași has a decrease with 8.0% as compared to the period of malaria eradication, registering an average annual relative humidity of 64%, favourable to the development of culicids, an excessive humidity slowing down their development. Two maps were made with the current situation in Romania, and there was an extrapolation of the data to 2030, showing a raise with 0.7-0.8°C, results which coincide with those provided globally for 2030 by the most famous research institutes in the world (0.8-1.7°C), demonstrating the validity of the mathematical model we implemented, ET30.

Chapter VII contains studies referring to the collection and identification of the species of culicids belonging to the complex *Anopheles maculipennis* by techniques of molecular biology, with captures in three areas of Iași City: Ciurbești Lake, Cotu Morii pond, and Nicolina River (Galata district). The traps were installed in the evening, from 8 p.m., and were left until morning, at 6 a.m., in the areas considered the most favourable for the activity of the mosquitoes. In order to identify the genera and the species of mosquitoes, they are preserved in 80% ethylic alcohol.

The collection of the samples started in May, and lasted until October, the traps being placed at the beginning and at the end of the month, in 2010 and 2012. The mosquitoes preserved in alcohol were identified on the bases of the identification keys described by Norbert Becker in

2003. The culicids of the genus *Anopheles* classified in the complex *Anopheles maculipennis* were used for DNA extraction and identification by molecular biology techniques, being identical twin species from a morphological point of view.

A total of 1830 mosquitoes were examined and classified in the genera *Culex, Aedes, Ochlerotatus* and *Anopheles*. 243 mosquitoes were classified in the complex *Anopheles maculipennis* on the basis of morphological characteristics (identification keys given by Norbert Becker), the species being identified afterwards by techniques of molecular biology.

A total of 217 specimens belonging to the complex *Anopheles maculipennis* were identified by PCR, of which: 58 specimens of *Anopheles atroparvus,* 18 specimens of *Anopheles melanoon*, 2 specimens of *Anopheles labranchiae,* 52 specimens of *Anopheles maculipennis,* and 87 specimens of *Anopheles messeae.* They were used for the identification of the adult mosquitoes and stage III and IV larvae. The ITS2 region of the DNA was entirely amplified by PCR, using the same primers used by Jana Proft in 1999.

Anopheles labranchiae was signalled for the first time in Romania, the species being captured at Ciurbești Lake, located at the periphery of Iași City, in the commune of Miroslava. The two specimens identified were stage IV larvae, which can be conclusive in what concerns the adaptation of the species to the clime of Iași City. The resemblance in proportion of 96% with the species *Anopheles labranchiae* of Italy shows the possibility of importing the species from this country, taking into account that a great part of the inhabitants of Iași Municipality and of the surrounding localities work in Italy (they return to the country in the summer, consequently transporting with them the mosquitoes of this species). *Anopheles labranchiae* larvae develop in stagnant freshwater or salty water, in the coastal areas of Europe, preferring heat. The hibernation is as an adult female in the dark, in stables or natural cavities. The diapause can be complete or incomplete, with occasional blood meals. The females are mainly anthropophage, occasionally feeding themselves on domestic animals.

Anopheles labranchiae is an endophilic species, living in human dwellings, stables, animal sheds, and occasionally in open air, in natural shelters (tree cavities). The presence of the larvae was signalled from April to October. The flight of this species is limited to 2-5 km. *Anopheles labranchiae* has a limited distribution in the South and South-East of Europe. It was

reported in the South-East of Spain, Corsica, coastal areas of Italy, Sardinia, Sicily. In Northern Africa, the species can be found in Morocco, Alger and Tunis (A.R. Zahar 1990). *Anopheles labranchiae* registered an increase in Sardinia in the last 35 years.

The PCR samples, specific to the species of mosquitoes identified, were subjected to sequencing, and the results were compared with those registered in the GenBank; the similarities were between 96% and 100%. Consequently, the species *Anopheles labranchiae* of Romania are 96% similar with the species *Anopheles labranchiae* of Italy (Cagliari), code-AY253840.1; the sequencing of the sample of *Anopheles maculipennis sp*. of Romania (Iași) was verified in GenBank by NCBI Blast: Nucleotide sequence, and it corresponds 99% with the species *Anopheles maculipennis* of Turkey (Anatolia), code-JN112927.1; the sequencing of the sample of *Anopheles messeae* of Romania (Iași) was verified in GenBank by NCBI Blast: Nucleotide sequence, and it corresponds 99% with the species *Anopheles messeae* of Italy, code-AY365011.1; the sequencing of the sample of *Anopheles melanoon* of Romania (Iași) was verified in GenBank by NCBI Blast: Nucleotide sequence and it corresponds 100% with the species *Anopheles melanoon* of Italy, code-AY365009.1.

The global distribution of malaria depends very much on the intrinsic characteristics of the vector, of the competence of the vector (capacity of an *Anopheles* species to assure the complete development of the parasite), and its vector capacity, *Anopheles labranchiae* proving to be the most important malaria vector in Europe.

Anopheles labranchiae, Anopheles messeae, Anopheles melanoon, Anopheles atroparvus and *Anopheles maculipennis* were incriminated in the transmission of malaria in Europe, the last four being responsible for the transmission of malaria in Romania in the period 1948-1963.

Chapter VIII contains studies referring to the epidemiology of malaria in Romania in the last 37 years, with the analysis of the cases according to age, sex, occupation, and area of infestation between 2007 and 2013, in order to establish prophylactic measures.

Since 1976, when malaria was completely eradicated, and until 2012, 744 cases of malaria had been diagnosed in Romania. The highest incidence of malaria was reported in Constanta, with 229 cases, and in Bucharest, with 221 cases. Though we do not have details about the cases in Constanta concerning the occupation of the patients diagnosed with malaria,

compared to the data of 1997-2005, when 70% of the cases registered were sailors, we can state that a better training of the maritime crew, and also stricter medical investigations of those returned from malaria-endemic areas can be considered prophylactic measures.

In the period 2007-2012, 142 cases were diagnosed in Romania, all imported. *Plasmodium falciparum* was diagnosed in 52% of the cases; this is also the most dangerous species of *Plasmodium*, responsible for cerebral malaria, leading to death in a short time if the corresponding treatment is not implemented. On the globe, *Plasmodium falciparum* is responsible for 85% of the cases of malaria. Of all the species infecting the humans with malaria, *Plasmodium vivax* and *Plasmodium ovale* can develop latent hepatic stages, which can reactivate after intervals from 2 (*P. vivax*) to 4 years (*P. ovale*). The cases diagnosed in Romania between 2007 and 2012 were represented in a proportion of 12% by *P. vivax* and of only 2% by *P. ovale*.

In 57.7% of the cases diagnosed in Romania, the infestation was produced in a country from the African continent. Precise statistics concerning the real situation of malaria in Africa cannot be obtained, due to the existing problems in obtaining data from the health system, and due to the fact that many treatments and deaths are not registered. Snow *et al.* made a mapping of the world distribution of malaria in 2002, year when 515 million of clinical episodes of *P. falciparum* were registered. These global estimations are 50% higher than the ones reported by the World Health Organisation (WHO), and 200% higher for the areas outside Africa.

Africa represents 85% of the cases of malaria registered, and 90% of the deaths produced by malaria in the whole world.

Between 1997 and 2005, the cases of malaria diagnosed in Romania were represented by sailors, percentage which lowered to 0.01% between 2007 and 2012, which shows an improvement in the training of the personnel, and of the maritime transportation companies.

The situation is completely different between 2007 and 2012, when most of the cases of malaria were represented by workers. According to the statistics, the world economic crisis started in December 2007, and it seems that it is the main reason of the Romanians' immigration to very remote geographical areas, which are malaria-endemic areas, which until then did not represent a financial opportunity. Most of the people registered as workers did not graduate from higher education, or they were not qualified in a certain occupation, which is also an explanation

for the lack of documentation in what concerns the risks of travels in malaria-endemic areas. A solution for educating the population concerning the importance of the implementation of a correct prophylaxis and its duration could be the centralisation of travels in the moment of the purchase of the ticket, and automatically benefiting of medical counselling.

The following category was represented by tourists who had a prophylaxis, but not constantly, and which most of the times was not an adequate one. This shows that people are not informed properly in what concerns the importance of a prophylaxis, and the seriousness of the problem of contracting a tropical disease with a very high mortality rate in the whole world.

Between 2007 and 2012, the cases of malaria diagnosed in Romania were represented by males in a proportion of 71%, closely connected with the previous chart, illustrating the occupation. The gender is therefore directly influenced by the occupation of the people who migrated to malaria-endemic areas with professional purposes, in fields implicating male labour. In what concerns the case studies, we considered 2 cases of students, and 2 cases of workers, but with higher education, and who received prophylaxis.

As prophylactic measures, the travellers should sleep under a mosquito net impregnated with insecticide. If the voyage is long, they may purchase simple and efficient kits of impregnation containing compounds for unique impregnation (effervescent tablets, bags, bottles) which are mixed with water for the impregnation of the nets. The use of the air conditioner is not a protection against the mosquito bite; it may reduce its aggressiveness, but it does not prevent the bite. The use of electrical devices, room sprays, constitutes an alternative modality of protection against the mosquitoes. It is indicated to wear long sleeved blouses and trousers, and also to use repellents on the areas exposed (face, neck), which completes the mechanical protection given by the clothes. Also, the use of repellent sprays on clothes increases the protection from a few days to a few weeks. The clothes may also be impregnated with permethrin (insecticide with the lowest toxicity for humans - an impregnation by spraying with the dose of 1g/m^2), offering protection for a period of 2 months, and resisting to 8 washings with water and soap (consequently also protecting from the discomfort of the bite of other species of mosquitoes).

According to the data analysed, the number of travels to malaria-endemic areas with tourist or professional purposes is continuously increasing, maintaining the risk of introducing the malaria parasite in the country. Also, Iaşi City is considered a cultural city, with many universities, hosting foreign students who come from malaria-endemic areas, being possible carriers of *Plasmodium.* In correlation with the presence of the vector belonging to the complex *Anopheles maculipennis* and favourable climatic factors, the periodical introduction of malaria in the country may increase the risk of its re-emergence in the country.

Chapter IX studies the molecular mechanism developed in the moment of the infection of the liver with *Plasmodium*, with creation of transgenic parasites *Plasmodium berghei* and *Plasmodium yoelii* expressing a GFP-LUC fusion protein. The objective on long term is to allow, due to a good understanding of the mechanism of penetration and of the intracellular differentiation of sporozoites, the approach of new therapeutic possibilities or vaccines to prevent the infection of the liver with *Plasmodium*. The complexity of the parasite *Plasmodium falciparum* was a challenge for the logistic of vaccines and of immunology for more than a century, representing an impediment in the creation of a vaccine for an integral protection. This complexity is due to the capacity of the parasite to totally change the cellular and molecular exterior layer in each stage of development, process controlled by a genome with over 5000 known genes; and also to change the niches of extracellular and intracellular development.

We used *Plasmodium berghei* and *Plasmodium yoelii* from rodents as parasitic models. At these parasites, all the stages of the parasitic cycle can be studied, especially after the infection of *Anopheles* mosquitoes fed on infected mice.

Compared with *Plasmodium falciparum,* the main agent of paludism in humans, the genetic modifications are relatively easily to see in *P. berghei* and *P. yoelii,* by homologous recombination. In order to study the infection of the liver with *Plasmodium,* we used consistent *in vitro* liver, infected with cellular cultures of sporozoites, and *in vivo* models, where the mice are inoculated intravenously with sporozoites. We used a new approach for the quantification of the parasites in the liver, based on the use of transgenic parasites expressing luciferase, which allowed the detection *in vivo* of the parasites by bioluminescence after the injection of an adapted substrate (Luciferases are enzymes controlling the oxidation of luciferin into oxyluciferin,

producing photon emission). The best known luciferase is that of *Photinus pyralis* (fireflies). We used bioluminescence, which allowed the quantification of the parasites in a non-invasive way, without sacrificing the animal, allowing therefore the analysis of the same animal along the time. An important aspect of the study of liver infection with *Plasmodium* is the capacity of *in vivo* quantification of the change of parasitemia in the liver.

The strategies chosen are as follows:

1. Construction of vectors of the expression in *E. coli* using commercial plasmid (pSP-luc+NF Fusion vector, ref e4471, Promega), which contains a gene resisting to ampicillin (for the selection of recombined bacteria), and a coding sequence for firefly luciferase (LUC), flanked by multiple restriction sites. In this plasmid, 5 fragments were cloned successively: GFP, the (HSP70-1) promoter, 3'UTR (DHFR), the homologous region 5'□ (Pb or Py), the homologous region 3' (Pb or Py).

2. Choosing the transgene: We used firefly luciferase, to which a GFP fluorescent protein fused. GFP was used as a selection marker; it is a protein formed of 238 residues of amino acids, expressing fluorescence when exposed to UV light. The most frequent GFP is obtained from *Aequorea Victoria* jellyfish. Luciferases are enzymes controlling the oxidation of luciferin into oxyluciferin, producing photon emission. The best known luciferase is that of *Photinus pyralis* (fireflies).

3. Choosing the promoter: We used the HSP70 gene promoter, because it allowed a strong and constitutive expression (of all the stages) of the transgene. This promoter should allow the detection of bioluminescence emitted by *Plasmodium* precocious hepatic forms.

4. Methods of integration into the genome

The GFP-LUC expression cassette was integrated in the genome of the stable homologous recombination receptor parasite. For this, we used an approach of genic replacement, allowing the irreversible integration of the transgene after double crossing-over.

In order to allow this recombination, the expression cassette of the gene was flanked by two regions homologous to the regions 5' and 3' of the P230p gene, which is not necessary to the parasite, and, therefore, it can be replaced by another gene (for example: transgene).

5. Selection of transgenic parasites

A classical approach for the selection of transgenic parasites is the introduction as a selection marker of a gene resistant to pyrimethamine.

6. Experimental infections on laboratory mice, after a previous transfection. Transfection is a very difficult technique, the success rate being usually low. The transfection may be successful, but mutations might appear, conferring resistance to the antibiotic used. The transfection might not succeed, due to the non-destruction of all the wild parasites. If the transfected parasites contain a fluorescent marker in their structure (in our case GFP-LUC), it is recommended to check the fluorescence with the fluorescence microscope (objective x40). We collected blood from the mice containing transgenic parasites, then we placed the blood in the troughs of the plate, and examined it under the microscope. The transfected blood did not present fluorescence. According to the electrophoresis and sequencing, the introduction of the 5 fragments in plasmid was successful.

The causes of the lack of fluorescence can be either due to the low level of expression of the fluorescence markers, undetectable under the microscope, and the transfection could have been successful, or a failed transfection, or an inadequate gasification of the environment where the parasites were cultivated.

Chapter X contains the final conclusions, which are followed by the bibliography.