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Use of molecular tools for the identification of males of some scale insects (Hemiptera: Coccoidea), in pheromone traps used for monitoring and comparison with females

Abstract - Species from Pseudococcidae family were studied. It was determined that the dry males of *Planococcus citri*, and *Pseudococcus comstocki*, collected by pheromone traps could be useful for the molecular analyses too. The ITS-2 sequences of males and females in case of *Pl.citri, Planococcus ficus* and *Ps. comstocki* were identical. This molecular method could differentiate the two mealybug species and this method can be useful to have idea specimens collected by pheromone traps.

Riassunto - *Uso degli strumenti molecolari per l'identificazione di maschi di cocciniglie (Hemiptera: Coccoidea), in trappole a feromoni utilizzate per monitoraraggio e comparazione con le femmine.*

Sono state prese in considerazione specie appartenenti alla famiglia degli Pseudococcini. E' stato visto che maschi essiccati di *Planococcus citri*, e *Pseudococcus comstocki*, raccolti da trappole a feromoni possono essere utilizzati anche per analisi molecolari. La sequenza ITS-2 di maschi e femmine di *P.citri, Planococcus ficus* e *P. comstocki* è identica. Questo metodo molecolare può differenziare le due specie di cocciniglie e può essere utile per avere un'idea delle specie raccolte in trappole a feromoni.

Key words: *Planococcus citri*, *Planococcus ficus*, *Pseudococcus comstocki*, male, pheromone trapping, climate change.

The climate change already supported spread of new pest species, among them a lot of scale insect species (*Pseudococcus comstocki* (Kuwana), *Pseudococcus viburni* (Signoret), *Planococcus citri* (Risso), *Planococcus ficus* (Signoret), etc, which are very important virus vectors in different crops. These species shows a substantial northward spread in different parts of Europe especially in the last forty years (Ben-Dov et al., 2009; Kozár, 1997, 1998, 2005; Kozár and Nagy Dávid, 1986; Kozár and Szentkirályi, 2005; Pellizzari, 1991; Godinho and Franco, 2001; Sentenac and Kuntzmann, 2003; Boudon-Padieu and Maixner, 2007; Sforza et al, 2005; etc).

The pheromone traps are useful and easy method to study on spread of these pests, although they collect only males. The use of pheromone traps to monitor scale insect

populations requires some basic knowledge about morphology of males, but the identification of scale insect males is a current problem because all descriptions and keys are based only on females (Kosztarab and Kozár, 1988). Kozár *et al*. (1996, 1997) prepared morphological and biological keys for males collected by pheromone traps of *Diaspidiotus (Quadraspidiotus) perniciosus* (Comstock) because it was found that this pheromone compounds are not species specific. This result was verified by molecular method by Frey and Frey (1995). From this point of view the identification problems of the males of *Pl. citri*, *Pl. ficus,* and *Ps. comstocki* taxa collected by pheromone traps could be solved by using more detailed morphological analyses combined with molecular approaches. The aim of this work was (1) to present results of the monitoring of distribution three mealybug species in different parts of Europe, (2) to study the molecular differences of scale insect males of two mealybug species collected by pheromone traps which can occur together easily in the same pheromone traps, (3) to compare the sequences of females and males in case of *Pl. citri*, *Pl. ficus,* and *Ps. comstocki*.

MATERIAL AND METHODS

Monitoring of mealybugs was conducted by Nagykovácsi type tent trap (10x10 cm), by using (pheromone ingredients for *Pl. citri;* (+)-2,2-dimethyl-3-(1-methylethenyl) cyclobutanemethanol acetate, for *Pl. ficus;* (S)-lavandulyl senecioate-(*S*)-lavandulyl isovalerate), for *Ps. comstocki; (*2,6-dimethyl-l,5-heptadien-3-ol acetate) with Soveurode /Witasek Pflanzenschutz GmbH, Austria/ glue and Biochemtech /Biochemtech Ltd. Kishinev, Moldavia/ pheromone dispensers.

The traps were used in Austria (Wien), Slovakia (Bratislava), Serbia (Belgrade), Greece (Athens, Iraklion, and Chania), Macedonia, and in Hungary in 2009 and 2010, in different times and places (Tab 1).

Tab 1 - Number of mealybug males collected by pheromone traps in 2009.*

Country, Locality	**Time period**	***Planococcus citri***	***Planococcus ficus***	***Pseudococcus comstocki***
Hungary, Budapest, Ördögárok ú.	04.28-07.31.2009	0	0	0
Hungary, Budapest, Ördögárok ú.	07.31-09.02.2009	8	2	1
Hungary, Budapest, Ördögárok ú.	09.02-10.02.2009	0	-	0
Hungary, Budapest, Ördögárok ú.	10.02-11.02.2009	3	-	2
Hungary, Budapest, M0,Csepel	05.18-03.08.2009	0	0	0
Hungary, Budapest, M0,Csepel	03.08-03.09.2009	2	0	0
Hungary, Budapest, M3, Szilas	05.11-07.30.2009	0	0	0

Hungary, Budapest, M3, Szilas	05.11-07.30.2009	0	0	0
Hungary, Budapest, M3, Ecséd	05.11-07.30.2009	0	0	0
Hungary, Budapest, M3, Ecséd	07.30-09.09.2009	1	0	0
Hungary, Budapest, M5, Kecskemét	05.14-07.28.2009	0	0	0
Hungary, Budapest, M5, Kecskemét	07.28-09.03.2009	0	0	0
Hungary, Budapest, M5, Röszke	05.14-07.28.2009	0	0	0
Hungary, Budapest, M5, Röszke	07.28-09.03.2009	0	0	0
Hungary, Budapest, M7, Budaörs	05.06-07.30.2009	0	0	0
Hungary, Budapest, M7, Budaörs	07.28-08.29.2009	1	0	0
Hungary, Budapest, M7, Velence	05.06-07.24.2009	0	0	0
Hungary, Budapest, M7, Velence	07.08-08.31.2009	2	1	1
Hungary, Budapest, M7, Töreki	05.06-07.25.2009	0	0	0
Hungary, Budapest, M7, Töreki	07.25-10.09.2009	3	1	2
Hungary, Budapest, M7, Letenye	05.06-07.25.2009	0	0	0
Hungary, Budapest, M7, Letenye	07.25-10.09.2009	0	-	0
Hungary, Nagykovácsi, around greenhouse	04.28-07.31.2009	**22**	0	0
Hungary, Nagykovácsi, around greenhouse	07.31-09.06.2009	**95**	1	2
Hungary, Nagykovácsi, in greenhouse	05.10-06.10.2009	8	0	0
Austria, Wien	04.03-07.29.2009	0	0	0
Austria, Wien	07.29-08.28.2009	3	0	0
Greece[x], Olympus Plaza	09.20-09.26.2009	0	-	0
Greece, Kifissia	09.20-09.26.2009	14	-	0
Greece, Iraklion	09.20-09.25.2009	**48**	-	**41!**
Makedonia, Vardar bridge	09.20-09.27.2009	0	-	0
Slovakia, Bratislava	07.29-08.28.2009	**102**	0	0
Switzerland, Lausane	06.16-06.28.2009	0	0	0

[*] Between 04.04-04.10, 2010 the traps in Serbia (Beograd), Greece (Gevgelija, Thessaloniki, Olympus plaza, Kifissia, Chania), and in Hungary (Budapest) between 05.01-05.31, 2010, did not collected males of these three species.

DNA extraction, amplification, cloning and sequencing

Total genomic DNA was extracted from single individuals (males and females) using REDExtract-N-Ampl™ Tissue PCR Kit (Sigma) according to manufacturer instruction. Females and males of *P. citri*, *P. ficus* and *P. comstocki* were tested (Tab 2.). After preliminary experiments CAS5p8sFc (sense) (5'-gcgaacatcgacaagtcgaacg-cacat-3') and CAS28sB1d (antisense) (5'-ttgttttcctccgcttattaatatgcttaa-3') primer pair (Kim and Lee 2008) was selected for PCR. The primers amplified the ITS 2 sequence of nuclear DNA. PCR was performed using *Taq* DNA polymerase (Fermentas) in thermo-cycler (Eppendorf Mastercycler gradient) according to the following procedure: initial denaturation at 95 °C for 4 min, followed by 40 cycles of 95 °C for 30 sec, annealing temperature 50 °C for 30 sec, extension at 72 °C for 60 sec; final extension at 72 °C for 10 min. The PCR products were purified using Gel/PCR DNA Fragments Extraction Kit (Geneaid). Purified PCR products from 3 dry individuals of *P. citri* from pheromone traps, and three females living on *Ficus benjamina* (Budapest) and 3 females of *P. comstocki* from mass culture on potato (Van), and three males from ethanol from mass culture on potato (Van). *Pl. ficus* individuals (2 females and 2 males) were from mass culture on potato (Van). Each were cloned into CloneJet (Fermentas) vector and inserted into *Escherichia coli* DH5α competent cells. All cloning steps were based upon standard molecular biology protocols (Sambrook et al. 1989). The recombinant plasmids isolated from selected colonies were sequenced using pJET1.2 forward and reverse primers, the PCR products of two *P. citri*, and *P. comstocki* were sequenced by CAS5p8sFc and CAS28sB1d primers by an automated DNA sequencer (Applied Biosystem Gene Analyzer 3100). Sequence comparisons were performed using Wisconsin Package version 10.0 Genetic Computer Group (GCG) sequence analysis software (Devereux et al. 1984).

RESULTS AND DISCUSSION

The pheromone trap catches show that only *Pl. citri* males were collected (Tab 1.) and this species was caught only in Greece (Kifissia, Iraklion) in field conditions. In other places such as in Hungary (Nagykovácsi), or Slovakia (Bratislava) the insects caught on outdoor plants probable originate from greenhouses plants. In these places, this species probably could not overwinter, or may be only in mild winter.

The males collected by *Ps. comstocki* pheromone traps in Greece (Crete, Iraklion) belongs to *Pl. citri* according to the molecular data, which was abundant in park on *Catalpa* sp., *Ficus* sp., *Morus* sp. and other plants, and their males could appear accidentally in *Ps. comstocki* traps.

On the other hand *Pl. ficus* and *Ps. comstocki* were not found in studied places and times. The one or two males caught by traps in some places probable belong to other genera and species.

Tab 2 - Origin and typing of mealybugs studied here.

Species	Origin	type	Vocher no.	GeneBank accesion no.	Sequencing
Pseudococcus comstocki	Turkey Van University laboratory culture	female	KozarF No. 9254	hm628568	801 bp
Pseudococcus comstocki	Turkey Van University laboratory culture	female	KozarF No. 9254	hm628569	801 bp
Pseudococcus comstocki	Turkey Van University laboratory culture	male, dry	KozarF No. 9254	hm628570	801 bp
Pseudococcus comstocki	Turkey Van University laboratory culture	male, in ethanol	KozarF No. 9254	hm628571	801 bp
Pseudococcus comstocki	Korea			fj430147	1357 bp
Pseudococcus comstocki	France		09000067	gu134668	709 bp
Pseudococcus comstocki	France			Gu134669	709 bp
Ps.com-PCR1	Turkey Van University laboratory culture	male, ethanol	KozarF No. 9254		739 bp
Ps.com-PCR2	Turkey Van University laboratory culture	male, dry	KozarF No. 9254		741 bp
Planococcus citri	Iraklion, Crete, *P.comstocki* feromone trap	male, dry	KozarF No. 9461	hm628572	773 bp
Pl.citri PCR1	Hungary	male, dry	Kozar F. No 9203		712 bp
Pl.citri PCR2	Hungary	male, dry	Kozar F. No 9462		714 bp
Planococcus citri	Hungary,	female	KozarF No. 9255	hm628573	773 bp
Planococcus citri	Hungary,	female	KozarF No. 9255	hm628574	773 bp
Planococcus citri	Iraklion, Crete, *P.comstocki* feromone trap	male, dry	KozarF No. 9461	hm628575	773 bp
Planococcus citri	Hungary,	female	KozarF No. 9204	hm628576	773 bp
Planococcus citri	Korea			fj430145	1332 bp
Planococcus citri	France		08089inra	gu134678	688 bp
Planococcus citri	France		08002inra	gu134675	688 bp
Planococcus minor	France		0803223	gu134676	686 bp
Planococcus ficus	France		082558inra	gu134677	692 bp
Planococcus ficus	Turkey	male	Kozar No9587/1		770 bp
Planococcus ficus	Turkey	female	Kozar No9587/2		770 bp

Characteristics of sequences

It was determined that the used DNA extraction procedure was suitable to handle adequate quantity and quality DNA from dry males from pheromone traps (6-7 months kept at laboratory condition), and males from ethanol or from living males and females.

The PCR product size (without the primers) of ITS2 sequences were 801 bp for *Ps. comstocki*, 773 bp for *Pl. citri* and 770 bp for *Pl. ficus*. The sequence variation among *Ps. comstocki* males and females were between 0.375 – 0.0 and among *Pl. citri* between 0.26 and 0.00 (See Tab 3. and 4.). The amplified ITS2 sequences were compared with the similar sequences in the Genebank. It is very interesting that *Ps. comstocki* collected and characterized in Korea (1357 bp Genebank accession no. fj430147), and France (709 bp Genebank accession no.gu134668 and gu134669) showed very high identity with species collected in Turkey (in case of gu134668 and hm628568 there is 100 %) The same tendency could be observed in the case of *Pl. citri* and *Pl. ficus*. *Pl. citri* individuals collected and characterized in France, Korea or Hungary showed 99.9 - 100 % identity. ITS2 sequences of *Pl. ficus* individuals also showed 99.9 – 100 % identity regardless of their origin, but differed from *Pl. citri* (90.4% identity). *Pl. minor* were also included in the comparison test and showed 99.7 % identity with *Pl. citri*. Considerable divergence was observed between the *Ps. comstocki* and *Pl. citri* (53.3 % – 56.1% identity), which give possibility to identify and distinguish the males of these species (Fig. 1).

The very low sequence variation of different individuals regardless their origin and state (living, dry material or conserved in ethanol) indicate that ITS-2 molecular marker is suitable method for reliable characterization and differentiation of *Pl. citri* and *Ps. comstocki* species. It was determined that males from the pheromone traps kept in 6-7 months in laboratory condition were suitable for molecular work. On basis of molecular data it was possible to separate the males of *Ps. comstocki* and *Pl. citri*. The high divergence for the ITS 2 region suggest that it should be possible to design species-specific primers and discriminate *Ps. comstocki* and *Pl. citri* males based on PCR method.

The molecular data could be a very important tool for the verification of the males collected by pheromone traps, to exclude the accidentally present specimens in the trap, and other species, for which the pheromone compound could serve as attractant. This result is especially important in quarantine and ecological studies of distribution of important pest species in new localities.

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*Tab 3 - Identity of ITS2 sequences of Ps. comstocki collected from Korea (fj430147), France (gu134668 and gu134669) and Turkey (hm628568 – hm628571). (*Ps.com-*PCR1 Kozár F. ref. no. 9254 and* Ps.com-*PCR2 Kozár F. ref. no. 9254 are PCR products of separate individuals).*

	gu134668	**gu134669**	**hm628568**	**hm628569**	**hm138570**	**hm138571**	***Ps.com*-PCR1**	***Ps.com*-PCR2**
fj430147	99.30	99.4	99.2	99.1	99.1	99	99.1	98.9
gu134668		99.9	100	99.9	99.7	99.9	99.9	99.7
gu134669			99.9	99.7	99.6	99.7	99.7	99.6
hm628568				99.8	99.8	99.9	99.9	99.7
hm628569					99.6	99.8	99.7	99.6
hm138570						99.6	100	99.6
hm138571							99.6	100
Ps.com-PCR1								99.6

Tab 4 - Identity of ITS2 sequences of Pl. citri *collected from Korea (fj430145), France (gu134675 and gu134678) and Hungary (hm628572-hm628575),* Pl. ficus *and* Ps. comstocki *(HM628569), (*Pl.citri-*PCR1 Kozár F. ref. no. 9203 and* Pl.citri-*PCR2 Kozár F. ref. no. 9462 are PCR products of separate individuals).*

	gu 134675	**gu 134678**	**hm 628576**	**hm 628575**	**hm 628574**	**hm 628573**	**hm 628572**	**gu 134676**	**gu 134677**	***Pl.citri* PCR1**	***Pl.citri* PCR2**	***Pl.ficus* t579-L1/1**	***Pl.ficus* T579-L3/1**	**HM 628569**
fj430145	100	99,9	99,9	99,9	100	99,9	100	99,7	90,4	100	100	91.4	91.5	55.6
gu134675		99,9	100	100	100	100	100	100	90,4	100	100	90,4	90,6	53.3
gu134678			99,9	99,9	99,9	99,9	99,9	99,6	90,2	99,9	99,9	90.3	90.4	53.5
hm628576				100	99,9	99,7	99,9	99,7	90,4	100	100	91,4	91,5	56.0
hm628575					99,9	99,7	99,9	99,7	90,4	100	100	91,3	91,5	55.9
hm628574						99,9	100	99,7	90,4	100	100	91,5	91,6	56,0
hm628573							99,9	99,7	90,4	100	100	91,4	91,5	56,0
hm628572								99,7	90,4	100	100	91,5	91,6	56.1
gu134676									90,4	99,7	99,7	90,4	90,5	49.9
gu134677										90,4	90,4	99,9	100	54.3
Pl.citri-PCR1											100	90,7	90,9	54.3
Pl.citri-PCR2												90,8	90,9	54.4
Pl. ficus t579-L1/1													99,9	57.1
Pl. ficus t579-L3/1														57.1

Fig. 1 - Comparison of ITS 2 sequences of Ps. comstocki *and Pl. citri males.*

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Percent Similarity: 55.906   Percent Identity: 55.906

        Match display thresholds for the alignment(s):
                    | = IDENTITY
                    : =   5
                    . =   1

 PcomstockiF.seg x PlanococcuscitriA5.seg January 12, 2011 09:56

                .         .         .         .         .
       1 TGCGGcCCTCGTG.ACCAAAGAGTCCTGGGCCACGCCTGTCTGAGGGTCG 49
         |||||||||||| | || ||||||||||||||||||||||||||||||||
       1 TGCGGCCCTCGCGAaCAAAAGAGTCCTGGGCCACGCCTGTCTGAGGGTCG 50
                .         .         .         .         .
      50 GTCATACGTGTAACACGATGGTTGCGTTCTCGCGAGTTTCGCGCTCGCTC 99
         || |||||||||||||||||||||||||||||||    | |||    |
      51 GTTATACGTGTAACACGATGGTTGCGTTCTCGCGAGCGCCTCGCAGTTTT 100
                .         .         .         .         .
     100 CGCAGCAGACCAGATCGTGCGCGCTCGTCGCGCGCGTGTGTGtgAATTCA 149
         | | ||             | ||       |  |   | | |||  || |
     101 CACCGCGAGGTTAGCTCGCCCCGTGACAGACCAGATCGAGCGTGTGTTTA 150
                .         .         .         .         .
     150 CGCACGCCGCCGAG...CGCGCGAGAGCGAGTACGTTGCGCTTTGCGGCG 196
         |||||    | |||   ||||        |   ||| || |   || |
     151 CGCACATGCCAGAGCGACGCGGTTTTCGAATCGCGTCGCTCGAAGCTGAC 200
                .         .         .         .         .
     197 ACTTCGTACGCTCGAAGCTGACGATTCGTTTGCCCGCGTGACTTGTCGCG 246
            |||| ||| |  |   |     |||  | |  |||    ||  ||
     201 GACTCGTTCGCGCTGACGCGGGCCATCGCCTCCGTGCGGTGGTTAGCGTC 250
                .         .         .         .         .
     247 TCGACGGCGAACGTTCGTCGAAATATTGCGGTTCTCGCCCGCATCGATAG 296
           | || |||||||||| ||||||    |   |    |||       ||
     251 GTG.CGTCGAACGTTCGTTGAAATACACGGCGCCGATGCCGAGAAACAAG 299
                .         .         .         .         .
     297 TTCGTTCACGAACAACAACGTGTTCGACGAAAGCGTGCCGCGTGCAGCGG 346
         |||||||||||||| |    |     |||    || |    |||     |
     300 TTCGTTCACGAACACCGTGTTCGAAAACGTTGCCGCGTATAGTGGTAGAG 349
                .         .         .         .         .
     347 ACCATATGCGAACGCGATGCGGTAGATACTCGGAGAGTGCGTTGGCCGTC 396
         | |  | |||   || ||     |||   || ||      |    ||
     350 AGCGAAGGCGGTAGCTATCGTAGAGAGCGTCCGATGAACGGGCATCCAGA 399
                .         .         .         .         .
     397 GGATGTACGGGCGTCGAAAATTCTCTCTCGGGGCGACGGCGGGTGACGCG 446
         |   |   | |    ||| ||              |    | |   ||||
     400 GAGAGGTGGTGGCGAGAAGAT.............ATACGGAGGATCGCG 435
                .         .         .         .         .
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447 CGTGTTGTACGATCGCGCGTTCC.......AACACCCCCTCGTCGCTTGA 489
    ||||||||||||||||||||  |        || | |    |||  |
436 CGTGTTGTACGATCGCGCGTCTCCGAAAAAAAAAGCTTAACGTTACCGCC 485
              .         .         .         .         .
490 GAAAAAAATTATACGACGCGAAGACGGTTTTCTCTTTACACGCCAACCGC 539
    |   ||  | ||||||  |||||||||  | ||    || | || |
486 GTCGAATCTCATACGATGCGAAGACGTCTCTCGAAATAGAGGCGATAGTT 535
              .         .         .         .         .
540 GATCGTTTCCCGATCGGACTTTGAGTATTTTTTCCGCAGTTCGCCCGCCT 589
       ||||||  | |||    |  ||          ||  || ||  ||  |
536 TGCCGTTTCTCTATCCACGTACGA..........CGTTGTACGTGCGATT 575
              .         .         .         .         .
590 GCACGACGACGTTGGCGCACGTACGCGCGCGCGTTAGCGTGTGTTCGCGT 639
    || ||    |||| |||    ||  |  ||   |       |  |||||
576 GCGCG....CGTTcGcGTCGTTATTCCGGCCGTTGTCGACGAATTCGCTC 621
              .         .         .         .         .
640 CGTCATTCCGGCCGTTGTcGACGAAATTCAATAATTCGAAGACGGCACTC 689
       | |   |  ||    ||| ||     |   |       |    || ||
622 ACGCGTCATGTACGGACGCGATGATGACGATCGAGAACGGGCACACATTC 671
              .         .         .         .         .
690 GGAAGGCGATTTCGCGACTCGCGTTACGTGCGACGCACGCGAACGTACCC 739
    ||||| ||| | || |  |    | |   |||    | ||| || |
672 GGAAGACGA.TGCGAGCGTGCGATAATTCGCGTTATAAGCGTACTT.... 716
              .         .         .         .         .
740 GACACACACGCACGCATACGATTTCACGCCGACCTCAGATCAGGTAAGAC 789
      |  ||  |    ||  |  | ||||||||||||||||||||||||||
717 ..CGTACCAGGCTTCACTCTGTATCACGCCGACCTCAGATCAGGtAAGAC 764
              .
790 TACCCGCCGAAT 801
    |||||||||
765 TACCCGCCG... 773
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