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MOLECULAR IDENTIFICATION, PHYLOGENY AND HAPLOTYPE COMPOSITION OF GRAPEVINE-ASSOCIATED SOFT SCALE INSECTS (HEMIPTERA COCCIDAE) IN SERBIA

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Milošević M., Vojvodić M., Jovičić I., Graora D., Bulajić A. - Molecular identification, phylogeny and haplotype composition of grapevine-associated soft scale insects (Hemiptera Coccidae) in Serbia.

Soft scale insects (Hemiptera: Coccomorpha: Coccidae) are important grapevine pests causing direct damage by phloem sap feeding and indirect damage by promoting sooty molds growth and virus transmission. Accurate and rapid identification is critical for effective management but remains challenging due to reliance on morphological features of briefly available young, unsclerotized females. To address these limitations, two gene regions, mitochondrial cytochrome oxidase I (COI) and large ribosomal subunit (28S), were used to characterize *Parthenolecanium corni* (Bouché), *Parthenolecanium persicae* (Fabricius) and *Pulvinaria vitis* (L.) collected from vineyards, while phylogenetic analysis provided insights into their genetic relationships within Coccidae. Haplotype analysis based on COI and 28S regions revealed no genetic variability in *P. corni* and *P. persicae*, each represented by a single haplotype. In contrast, *P. vitis* exhibited higher diversity, with three 28S haplotypes and 14 COI haplotypes. By generating the first genetic data for these species from European populations, this study strengthens the molecular identification of soft scale insects and provides a valuable foundation for their monitoring, as well as future taxonomic and ecological research.

Key Words: COI, 28S, haplotypes, *Parthenolecanium, Pulvinaria*, genetic diversity

INTRODUCTION

Soft scale insects (Hemiptera: Coccomorpha: Coccidae) represent the third largest family within superfamily Coccoidea, with 1232 described species in 178 genera (Garcia Morales *et al*., 2016). According to Kondo & Watson (2022), 153 species are considered important pests in agriculture.

Soft scales are a key component of the grapevine pest complex, including *Neopulvinaria innumerabilis* (Rathvon), *Parthenolecanium persicae* (Fabricius), *Parthenolecanium corni* (Bouché) and *Pulvinaria vitis* (L.). These polyphagous species feed on a wide range of host plants and are known to cause damage in viticulture. Among them, *P. corni* has a significant negative impact on grapevines production across Europe (Hoffmann, 2002; Ioriatti *et al*., 2008; Silva *et al*., 2016; Hommay *et al*., 2020; Milošević and Graora, 2023). *P. persicae* has caused notable damage in various grapevine producing regions (Graora *et al.*, 2012; Rakimov *et al*., 2013; Masten Milek *et al*., 2021), while *P. vitis* occasionally reaches high population densities (Kosztarab and Kozár, 1988; Pellizzari, 1997; Graora *et al*., 2012; Masten Milek *et al*., 2021).

On grapevine, soft scales cause both direct and indirect damage. By feeding on plant sap from above-ground grapevine parts, they reduce plant vigor and may lead to long-term plant decay. Infestations often result in excessive honeydew secretion, which promotes the development of sooty mold, interfering with photosynthesis and reducing fruit quality (Kosztarab and Kozár, 1988; Mibey, 1997). Additionally, soft scales are vectors of grapevine viruses (Herrbach *et al*., 2017).

Accurate identification is fundamental for effective pest management. Soft scales identification relies on microscopic morphological characteristic examination of slide-mounted adult females (Kondo and Watson, 2022). However, soft scale females are particularly challenging to examine morphologically. As they mature, their bodies stretch, becoming thick, heavily sclerotized and fragile, making them unsuitable for accurate identification. Young adult females provide the most reliable morphological characters for identification. However, they are available only for a short period, often just a few days per year for univoltine species (Hodgson, 1994). Given these limitations, developing molecular identification protocols for morphologically defined soft scale pests could offer a faster, more accurate and cost-effective identification.

Despite the increasing integration of molecular techniques in entomological research, the genetic characterization of soft scale insects remains insufficiently explored. Only a limited number of sequences for the Cytochrome Oxydase Subunit I (COI), nuclear ribosomal RNA genes (18S and 28S) and elongation factor-1α (EF-1α) are available in the GenBank online database, primarily derived from Asian, North and South American specimens (Yokogawa and Yahara, 2009; Deng *et al*., 2012; Bahder *et al*., 2013; Wang *et al*., 2015; Amou-

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roux *et al*., 2016; Choi and Lee, 2020). To date, European populations remain uncharacterized at the molecular level, despite the clear need to understand their intraspecies diversity and phylogeographic patterns. Such insights are essential for developing accurate molecular diagnostics and improving large-scale monitoring and management strategies.

Given their significance as grapevine pests, rapid molecular identification is essential for improving pest management. The aims of this research were: (i) molecular identification and characterization of soft scale insects associated with grapevine in Serbia; (ii) to improve the understanding of their genetic diversity and phylogenetic relationships; (iii) to establish relationships of Serbian populations and populations of other regions; (iv) to contribute the development of molecular identification protocols; (v) to provide molecular data for two marker genes, mitochondrial cytochrome oxidase subunit 1 (COI) and 28S fragment D gene rDNA (28S) and test the suitability of different primer sets for amplification; (vi) assess haplotype diversity and population structure to reveal potential patterns of genetic differentiation among regional populations; (vii) to support future research on monitoring, population dynamics of soft scale insects and timing of potential control strategies.

MATERIAL AND METHODS

Collection of soft scale insect specimens

Soft scale insect specimens were collected in April, 2019-2021 from grapevine in vineyards in Jagodina (N43°59′26″ E21°14′14″, Elevation: 122 m a.s.l.), Bačka Palanka (N45°11′10″ E19°27′25″, Elevation: 199 m a.s.l.) and Belgrade (44°45′27″ E20°35′03″, Elevation: 146 m a.s.l.) localities in Serbia. Sampling was conducted immediately after females were formed, with approximately 100 individuals collected per locality to ensure population representativity. Females were preserved in 70% ethanol for microscopic slides preparation and in 96% ethanol for molecular analysis.

Morphological identification

Slide mounting was done according to Kosztarab & Kozár (1988). The species identification was based on morphological characters according to the keys by Gill (1988) and Kosztarab & Kozár (1988). Specimens were examined using a Leica DMLS microscope for detailed morphological analysis. Slide-mounted voucher specimens are deposited at University of Belgrade - Faculty of Agriculture, Department for Entomology and agricultural zoology.

DNA Extraction, Amplification and Sequencing

Immediately after collection, female specimens were immersed in 96% ethanol and stored in the fridge at +4°C. Before DNA extraction, specimens were soaked in nuclease-free water overnight and then dried on sterile filter paper, following the pretreatment used for scale insects DNA extraction in Wang *et al*. (2019). Three adult female specimens per locality (9 in total) were individually used for DNA extraction following the protocol of Phillips & Simon (1995).

The COI and 28S genomic markers were amplified using primers shown in Table 1. For COI gene region, two sets of primers were used, amplifying fragments of different lengths. All PCR reactions were prepared at a total reaction volume of 25 µl, containing 12.5 µl 2 X PCR Master mix (Fermentas, Lithuania), appropriate volume of primers (Table 1), 1 µl target DNA and RNase-free water to total reaction volume.

PCR amplifications were performed according to the following conditions: initial denaturation step of 5 min at 96°C, followed by 35 cycles of 45 s at 96°C, 1 min at 45°C and 1 min on 72°C, with final extension period of 10 min at 72°C (Cocuzza *et al*., 2015). Obtained amplicons were visualized using 1% agarose gel electrophoresis stained in ethidium bromide under UV transilluminator and directly sequenced in both directions using the same primers as for amplification in an automated sequencer (ABI 3730XL Automatic Sequencer Inc, Macrogen, Inc, The Netherlands). Consensus sequences were manually edited using the ClustalW (Thompson *et al*., 1994), integrated in MEGA 11 software (Tamura *et al*., 2021) and deposited in the GenBank database (https://www.ncbi.nlm.nih.gov). All generated sequences were compared with each other by calculating nucleotides (nt) similarities, as well as with previously deposited specimens available in the GenBank, using similarity search tool BLAST.

Phylogenetic analyses

The newly generated 28S sequences from Serbian soft scale specimens were analysed with total of 47 selected Coccidae sequences belonging to 22 genera (Di Sora *et al*., 2023) retrieved from GenBank (Table 2). A phylogenetic tree was inferred using the Maximum likelihood method implemented in MEGA 11 software (Tamura *et al*., 2021). Distances in 28S region were determined using Tamura-Nei model (Tamura and Nei, 1993) and all sites with gaps were omitted. The reliability of the obtained trees was evaluated using 1,000 bootstrap replicates.

Haplotype analysis of soft scale insect sequences

Haplotype analysis was performed for *P. corni* and *P. persicae* populations based on all available 28S sequences. For *P. vitis* the analysis included all available 28S and COI sequences obtained from GenBank (accessed on May 10, 2025). In total, 28S sequence analyses included 34 sequences of *P. corni*, 5 of *P. persicae*, and 14 of *P. vitis*. For the COI gene, 16 sequences of *P. vitis* were analysed. All sequences were compared by calculating nucleotide identities using MEGA X software (Kumar *et al*., 2018). The number of haplotypes, haplotype diversity, identification of polymorphic sites and the nucleotide diversity of the regions were analysed using DnaSP version 6.0 (Rozas *et al*., 2017). Haplotype composition and frequency were examined using PopART (Popula-

Table 1 – Primers used in the present study.

Gene regions	Primer name	Directions	Sequences 5`-3`	Reference	Volume
28S	S3660	Forward	GAGAGTTMAASAGTACGTGAAAC	Whiting *et al.*, 1997	5 µl
	A335	Reverse	TCGGARGGAACCAGCTACTA	Dowton and Austin, 1998	5 µl
COI	LCO1490	Forward	GGTCAACAAATCATAAAGATATTGG	Folmer *et al.*, 1994	2.5 µl
	HCO2198	Reverse	TAAACTTCAGGGTGACCAAAAAATCA	Folmer *et al.*, 1994	2.5 µl
	C1-J-2183	Forward	CAACATTTATTTTGATTTTTTGG	Simon *et al.*, 1994	2.5 µl
	C1-N-2568	Reverse	GCWACWACRTAATAKGTATCATG	Brady *et al.*, 2000	9 µl

tion Analysis with Reticulate Trees) software (Leigh and Bryant, 2015). The mutual genealogical relationships among haplotypes were visualized using the Median Joining Network algorithm implemented in the PopART software (Bandelt *et al*., 1999).

RESULTS

Morphological identification

Three soft scale insect species were identified on grapevine in Serbia: *P. corni* from Bačka Palanka locality, *P. persicae* from Belgrade locality and *P. vitis* from Jagodina and Belgrade locality.

Molecular identification

Amplification of the 28S gene region of *P. corni, P. persicae* and *P. vitis* yielded sequences of the expected lengths of approximately 500 bp. The amplification of COI region resulted in sequences length of 400 bp and 550 bp, depending on used primers. Nine sequences were generated for *P. corni, P. persicae* and *P. vitis* and deposited into GenBank (Table 3).

BLAST analysis of 28S gene region revealed that *P. corni* sequence from this study showed the highest nucleotide (nt) identity (100%) to a sequence *P. corni* from *Aristotelia chilensis* Stuntz from Chile (KY085847) and specimen from *Prunus cerasifera* L. cv. Pissardi from China (KP189563). Furthermore, *P. persicae* sequence from this study showed nt identity (100%) with *P. persicae* from *Ulmus pumila* L. from China (KP189588). Our *P. vitis* sequence showed the highest level of nt similarity (99.75%) with *P. vitis* from *Ilex cornuta* Lindley (KP189627) and *Celtis bungeana* Blume (KP189628) from China.

Analysis of the COI gene region showed that sequences *P. corni* collected from Serbia had the highest nt similarity (98.96%) with the *Parthenolecanium pruinosum* (Cocquillet) from *V. vinifera* L. from Australia (KC784922) and with a *P. corni* from an unidentified host plant from Japan (AB439534).

COI region sequence of *P. persicae* showed the highest nt similarity (98.96%) with *P. pruinosum* from *V. vinifera* (KC784922) and *P. persicae* (92.17%) from *V. vinifera* from Australia (KC784881).

Serbian sequences of *P. vitis* (approximately 400 bp) showed the highest nt similarity (98.75%) with a sequence of specimen *Pulvinaria psidii* Maskell from Peru (AB440072). In addition, the longer *P. vitis* sequence (approximately 550 bp) was identical (100% nt similarity) with sequence *P. vitis* from undefined plant from Iran (MH684599).

Phylogenetic analyses

Phylogenetic analysis based on 28S gene region was performed using three sequences from Serbian specimens along with 47 selected sequences from Coccidae family. The resulting phylogenetic tree exhibited a topology and resolution consistent with previously identified sequences available in public databases. The Serbian sequences of *P. corni*, *P. persicae* and *P. vitis* clustered with their respective reference sequences from GenBank, supported by high bootstrap values (Fig. I). Furthermore, our phylogenetic analysis confirmed the belonging of this species to the subfamily Coccinae and as expected, confirmed the closer relationship of *P. corni* and *P. persicae*, as all sequences for both species cluster within the clade of *Parthenolecanium* with the highest bootstrap support (100%). Both Serbian sequences, *P. corni* and *P. persicae*, form two well-supported clusters with their respective species from China which was supported by bootstrap value of 100%. These results confirm the identification of all threes *P. corni*, *P. persicae* and *P. vitis* and provide robust molecular evidence for their presence in Serbian vineyards.

Haplotype structure of soft scale insect sequences

Different levels of intraspecies variability were observed among the three analysed species based on 28S region. The population of 34 *P. corni* sequences as well as population of 5 *P. persicae* sequences, including respective Serbian sequences, revealed no genetic variation, and no haplotype diversity was detected. On contrary, the 28S dataset of *P. vitis*, based on 14 available sequences, consisted of three haplotypes. Hap_1 comprised 11 sequences from China and one sequence from Serbia. Hap_2 and Hap_3 were each represented by single sequence from China (Table 4). Haplotype diversity of *P. vitis* population based on 28S was 0.275, indicating a low level of genetic variation with diversity variance of 0.02202 and nucleotide diversity 0.203 (0 to 2 nt difference), indicating a very low level of nucleotide variability. The haplotype network of 28S locus (Fig. II, 1) suggests possible ancestral role

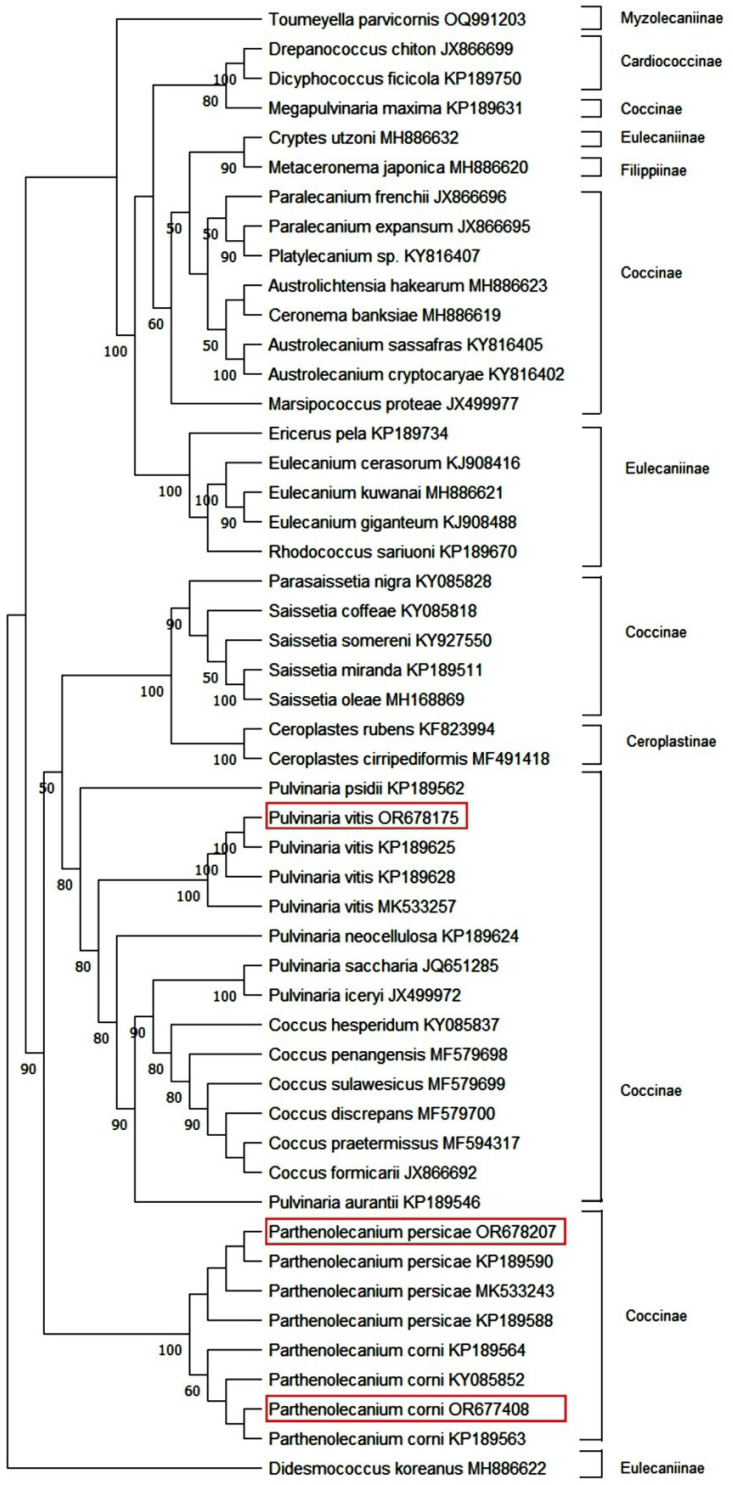

Fig. I – Maximum likelihood phylogenetic tree inferred from 28S fragment D gene among Coccidae family including *Parthenolecanium corni*, *Parthenolecanium persicae* and *Pulvinaria vitis* from Serbia together with 47 soft scale insect sequences originated from 22 genera. The tree was generated in MEGA 11 using Tamura-Nei model. Bootstrap analyses were performed with 1.000 replicates and bootstrap values (>50%) are shown next to relevant branches. Serbian sequences are marked with red rectangle.

Table 2 – Coccidae 28S sequences used for phylogenetic analysis in this study.

Acc. number	Taxa	Plant species	Locality	Author
KY816402	*Austrolecanium cryptocaryae*	*Cryptocarya* sp.	Australia	Lin *et al.* (2017)
KY816405	*Austrolecanium sassafras*	*Cryptocarya* sp.	Australia	Lin *et al.* (2017)
MH886623	*Austrolichtensia hakearum*	*Cryptocarya* sp.	Australia	Lin *et al.* (2017)
MH886619	*Ceronema banksiae*	*Cryptocarya* sp.	Australia	Lin *et al.* (2017)
MF491418	*Ceroplastes cirripediformis*	Unknown	Unknown	Wu - Unpublished
KF823994	*Ceroplastes rubens*	*Viburnum odoratissimum*	China	Deng *et al.* (2013)
MF579700	*Coccus discrepans*	Unknown	Papua New Guinea	Lin *et al.* (2017)
KY085837	*Coccus hesperidum*	*Citrus* × *sinensis*	Chile	Amouroux *et al.* (2016)
JX866692	*Coccus formicarii*	*Mangifera indica*	Thailand	Lin *et al.* (2013)
MF579698	*Coccus penangensis*	*Macaranga bancana*	Singapore	Lin *et al.* (2013)
MF594317	*Coccus praetermissus*	*Rhizophora mucronata*	Thailand	Lin *et al.* (2017)
MF579699	*Coccus sulawesicus*	*Dicotyledonous shrub*	Indonesia	Lin *et al.* (2017)
MH886632	*Cryptes utzoni*	*Acacia aneura*	Australia	Lin *et al.* (2018)
KP189750	*Dicyphococcus ficicola*	*Ficus microcarpa*	China	Wang *et al.* (2015)
MH886622	*Didesmococcus koreanus*	*Prunus persica*	China	Lin *et al.* (2018)
JX866699	*Drepanococcus chiton*	*Ficus benjamina*	Taiwan	Lin *et al.* (2013)
KP189734	*Ericerus pela*	*Ligustrum* sp.	China	Wang *et al.* (2015)
KJ908416	*Eulecanium cerasorum*	*Magnolia* × *soulangeana*	China	Deng *et al.* (2015)
KJ908488	*Eulecanium giganteum*	*Juglans regia*	China	Deng *et al.* (2015)
MH886621	*Eulecanium kuwanai*	*Sophora japonica*	China	Lin *et al.* (2018)
JX499977	*Marsipococcus proteae*	*Protea caffra*	South Africa	Sethusa *et al.* (2014)
KP189631	*Megapulvinaria maxima*	*Artocarpus heterophyllus*	China	Wang *et al.* (2015)
MH886620	*Metaceronema japonica*	*Ilex crenata*	Japan	Lin *et al.* (2018)
JX866695	*Paralecanium expansum*	*Litsea glutinosa.*	Taiwan	Lin *et al.* (2013)
JX866696	*Paralecanium frenchii*	*Banksia integrifolia*	Australia	Lin *et al.* (2013)
KY085828	*Parasaissetia nigra*	*Annona cherimola*	Chile	Amouroux *et al.* (2016)
KY085852	*Parthenolecanium corni*	Unknown	Chile	Amouroux *et al.* (2016)
KP189564	*Parthenolecanium corni*	*Ulmus pumila*	China	Wang *et al.* (2015)
KP189563	*Parthenolecanium corni*	*Prunus ceraifera* cv. *Pissardii*	China	Wang *et al.* (2015)
OR677408	*Parthenolecanium corni*	*Vitis vinifera*	Serbia	This study
KP189590	*Parthenolecanium persicae*	*Ulmus pumila*	China	Wang *et al.* (2015)
MK533243	*Parthenolecanium persicae*	*Magnolia kobus*	Korea	Choi and Lee (2020)
KP189588	*Parthenolecanium persicae*	*Ulmus pumila*	China	Wang *et al.* (2015)
OR678207	*Parthenolecanium persicae*	*Vitis vinifera*	Serbia	This study
KY816407	*Platylecanium* sp.	*Syzygium* sp.	Malaysia	Lin *et al.* (2017)
KP189546	*Pulvinaria aurantii*	*Pittosporum tobira*	China	Wang *et al.* (2015)
JX499972	*Pulvinaria iceryi*	Grass	South Africa	Sethusa *et al.* (2014)
KP189624	*Pulvinaria neocellulosa*	*Ficus* sp.	China	Wang *et al.* (2015)
KP189562	*Pulvinaria psidii*	*Ixora chinensis*	China	Wang *et al.* (2015)
JQ651285	*Pulvinaria saccharia*	*Saccharum officinarum*	South Africa	Sethusa *et al.* (2014)
KP189628	*Pulvinaria vitis*	*Celtis bungeana*	China	Wang *et al.* (2015)
KP189625	*Pulvinaria vitis*	*Salix matsudana*	China	Wang *et al.* (2015)
MK533257	*Pulvinaria vitis*	*Betula platyphylla*	Korea	Choi and Lee (2020)
OR678175	*Pulvinaria vitis*	*Vitis vinifera*	Serbia	This study
KP189670	*Rhodococcus sariuoni*	*Pyrus* × *sinkiangensis*	China	Wang *et al.* (2015)
KY085818	*Saissetia coffeae*	*Olea europaea*	South Africa	Amouroux *et al.* (2016)
KP189511	*Saissetia miranda*	*Erythrina crista-galli*	China	Wang *et al.* (2015)
MH168869	*Saissetia oleae*	Lime	Australia	Phuong (2018)
KY927550	*Saissetia somereni*	*Theobroma cacao*	Ghana	Lin *et al.* (2017)
OQ991203	*Toumeyella parvicornis*	*Pinus pinea*	Italy	Di Sora *et al.* (2023)

Table 3 – Sequences generated in the present study with Accession numbers.

Acc. No.	Species	Specimen	Gene Region	Primers used	Length	Locality
OR677408	*Parthenolecanium corni*	8	28S	S3660/A335	503 bp	Bačka Palanka
OR678207	*Parthenolecanium persicae*	4	28S	S3660/A335	504 bp	Belgrade
OR678175	*Pulvinaria vitis*	2	28S	S3660/A335	517 bp	Jagodina
OR671207	*Parthenolecanium corni*	7	COI	C1-J-2183/C1-N-2568	401 bp	Bačka Palanka
OR647748	*Parthenolecanium corni*	8	COI	C1-J-2183/C1-N-2568	387 bp	Bačka Palanka
OR593386	*Parthenolecanium persicae*	4	COI	C1-J-2183/C1-N-2568	401 bp	Belgrade
OR589444	*Pulvinaria vitis*	3	COI	C1-J-2183/C1-N-2568	401 bp	Jagodina
OR591293	*Pulvinaria vitis*	9	COI	C1-J-2183/C1-N-2568	395 bp	Belgrade
PV040718	*Pulvinaria vitis*	2	COI	LCO1490/HCO2198	528bp	Jagodina

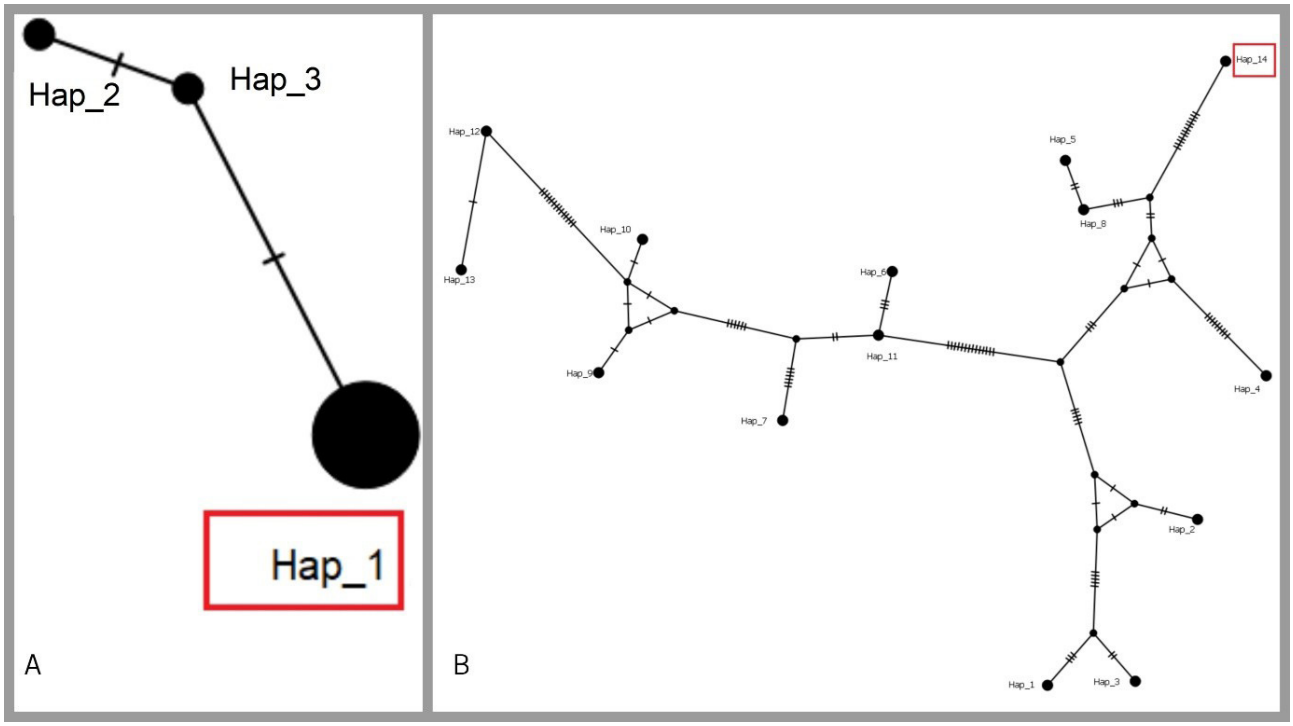

Fig. II - Median-joining networks showing the phylogenetic relationships between the haplotypes of *Pulvinaria vitis* 28S (A) and COI (B) sequences available in GenBank. Details of the sequences and haplotypes are shown in Tables 4 and 5. Haplotypes where Serbian isolates belong are labeled in red rectangles.

of Hap_1, while other haplotypes were linked to each other. Sequence from Serbia belong to major Hap_1. The relatively large genetic distance between nodes may be due to an insufficient number of sequences from other geographic regions and the apparent peripheral position of some haplotypes may change as additional sequences from other regions become available.

Analysis of the COI gene of *P. vitis* revealed 14 distinct haplotypes (Hap_1 to Hap_14), with 65 variable positions (Table 5). Most haplotypes were represented by a single sequence, except Hap_2 and Hap_3 which contained two sequences each. Serbian sequence is the single member of Hap_14. Haplotype diversity was 0.983 with variance of 0.00077, and nucleotide diversity of 0.04493 (0 to 31 nt difference). The COI haplotype network (Fig. II, 2) showed reticulated pattern with several mutationally distant lineages, indicating a relatively high level of diversity. The position of Hap_14 containing Serbian sequence at the at the periphery of the network suggests possible geographic isolation.

Table 4 – 28S sequences of *Pulvinaria vitis* used in haplotype analysis.

Haplotype	Species	Locus	Acc. No.	Continent	Country	Reference
Hap_1	*P. vitis*	28S	KP189629	Asia	China	Wang et al. (2015)
Hap_1	*P. vitis*	28S	KP189628	Asia	China	Wang et al. (2015)
Hap_1	*P. vitis*	28S	KP189627	Asia	China	Wang et al. (2015)
Hap_1	*P. vitis*	28S	MT317070	Asia	China	Li (Unpublished)
Hap_1	*P. vitis*	28S	MT317069	Asia	China	Li (Unpublished)
Hap_1	*P. vitis*	28S	MT317068	Asia	China	Li (Unpublished)
Hap_1	*P. vitis*	28S	MT317067	Asia	China	Li (Unpublished)
Hap_1	*P. vitis*	28S	MT317066	Asia	China	Li (Unpublished)
Hap_1	*P. vitis*	28S	MT317065	Asia	China	Li (Unpublished)
Hap_1	*P. vitis*	28S	MT317064	Asia	China	Li (Unpublished)
Hap_1	*P. vitis*	28S	MK533257	Asia	Korea	Choi and Lee (2020)
Hap_1	*P. vitis*	28S	OR678175	Europe	Serbia	This study
Hap_2	*P. vitis*	28S	KP189626	Asia	China	Wang et al. (2015)
Hap_3	*P. vitis*	28S	KP189625	Asia	China	Wang et al. (2015)

Table 5 – COI sequences of *Pulvinaria vitis* used in haplotype analysis.

Haplotype	Species	Locus	Acc. No.	Continent	Country	Literature
Hap_1	*P. vitis*	COI	MT241143	Asia	China	Li (Unpublished)
Hap_2	*P. vitis*	COI	MT241142	Asia	China	Li (Unpublished)
Hap_2	*P. vitis*	COI	MT241141	Asia	China	Li (Unpublished)
Hap_3	*P. vitis*	COI	MT241140	Asia	China	Li (Unpublished)
Hap_3	*P. vitis*	COI	MT241139	Asia	China	Li (Unpublished)
Hap_4	*P. vitis*	COI	MT241138	Asia	China	Li (Unpublished)
Hap_5	*P. vitis*	COI	MT241137	Asia	China	Li (Unpublished)
Hap_6	*P. vitis*	COI	MT241136	Asia	China	Li (Unpublished)
Hap_7	*P. vitis*	COI	MK543935	Asia	Korea	Choi and Lee (2020)
Hap_8	*P. vitis*	COI	MH684599	Asia	Iran	Yousefi, M. and Vahedi, A.
Hap_9	*P. vitis*	COI	KP189888	Asia	China	Wang et al. (2015)
Hap_10	*P. vitis*	COI	KP189887	Asia	China	Wang et al. (2015)
Hap_11	*P. vitis*	COI	KP189886	Asia	China	Wang et al. (2015)
Hap_12	*P. vitis*	COI	KP189885	Asia	China	Wang et al. (2015)
Hap_13	*P. vitis*	COI	KP189884	Asia	China	Wang et al. (2015)
Hap_14	*P. vitis*	COI	PV040718	Europe	Serbia	This study

DISCUSSION

During our research, three soft scale insect species were detected in commercial vineyards in Serbia based on morphological identification: *P. corni*, *P. persicae* and *P. vitis*. Molecular analysis of 28S and COI gene regions validated their identification and provided novel molecular data on European populations of soft scale insects along with insights into their evolutionary relationships within Coccidae family.

BLAST analysis of the 28S region confirmed species identity, while amplification of COI gene of Serbian specimens proved to be challenging. Universal COI primers (Folmer *et al*., 1994), which amplify longer fragments, failed to produce sequences for *P. corni* and *P. persicae*. Alternative primers targeting shorter COI fragments (Simon *et al*., 1994; Brady *et al*., 2000) were used successfully, but resulting sequences had limited taxonomic resolution due to the lack of available reference data.

Soft scale insects exhibit high intraspecies variability due to their broad geographic distribution, polyphagy and parthenogenetic reproduction. Morphological intraspecies variations have been extensively studied in *P. corni* and *P. vitis* (Saakyan-Baranova *et al*., 1971; Malumphy, 1991; Danzig, 1997; Stepaniuk and Lagowska, 2006), but molecular data remain scarce. In this study, the 28S gene region demonstrated high reliability for species identification, with sequences exhibiting 100% nt identity with the corresponding species sequences available. In contrast, COI gene showed inconsistencies in *Parthenolecanium* species. Although COI is widely used in insect identification, universal primers often fail in scale insects (Hebert *et al*., 2004; Hajibabaei *et al*., 2006; Kondo *et al*., 2008; Park *et al*., 2011; Deng *et al*., 2012), which was also observed in this study. Alternative primers (Simon *et al*., 1994; Brady *et al*., 2000), previously used for scale insects identification (Rakimov *et al*., 2013; Wang *et al*., 2015) successfully amplified the COI region in *P. corni, P. persicae* and *P. vitis* but provided limited taxonomic resolution. The 28S gene, proposed as a reliable alternative (Sethusa *et al*., 2014) demonstrated high accuracy in this study, emphasizing its potential as a genetic marker for soft scale insect identification.

The phylogenetic analysis based on 28S gene region provided further insight into evolutionary relationships within the Coccidae family reinforced expected relationships where Serbian *P. vitis* sequence clustered with *Coccus* species, indicating a close evolutionary affinity. The two *Parthenolecanium* species formed distinct clusters with *P. corni* grouped with a specimens from Chile and China, while *P. persicae* clustered with specimens from China and Korea. These results align with previous taxonomic studies on soft scale insects (Choi and Lee, 2020; Di Sora *et al*., 2023) and provide the first data on not only for Serbian but for European *P. corni, P. persicae* and *P. vitis* populations as well, establishing a base for future research in population genetics and phylogenetics.

Further molecular analysis of haplotype composition revealed different levels of intraspecies variation among the studied species. No haplotype variation was detected in *P. corni* and *P. persicae* based on the 28S region, while three well defined haplotypes were identified within the *P. vitis* population. Hap_1 was the most prevalent, comprising one sequence from Serbia and 11 from Asia (Wang *et al*., 2015; Choi and Lee, 2020). Serbian sequence was derived from *V. vinifera*, while Asian sequences were derived from specimens collected on various host plants, including *Betula platyphylla* Sukaczev, *C. bungeana*, *I. cornuta*. Hap_2 and Hap_3 were each represented by one sequence from China, associated with *Populus* sp. and *Salix matsudana* Koidzumi, respectively. These findings suggest low geographic and host-related variability within the *P. vitis* population based on the 28S gene region which is the first insight into the population structure of this important species.

The COI region provided high intraspecies resolution for *P. vitis*, revealing 14 haplotypes. The Serbian sequence formed a single haplotype (Hap_14), located at the terminal end of the haplotype network and separated by a substantial number of mutations from the other haplotypes, suggesting potential geographic isolation. Remaining haplotypes originated from Asia and were derived from a variety of host plants, including *B. platyphylla*, *C. bungeana*, *I. cornuta*, *Populus* sp., *S. matsudana*, and several unidentified plants. The COI network lacked a central dominant haplotype and showed multiple divergent lineages, consistent with the absence of a single common ancestor and similar patterns observed in other scale insect studies (Amouroux *et al*., 2017). These findings underscore the genetic complexity of *P. vitis* populations and highlight the value of COI in detecting fine-scale genetic structure, whereas 28S provides robust species-level resolution.

These findings highlight the importance of broader geographic sampling, particularly across European vineyards, to clarify regional population structure and evolutionary patterns. Incorporating additional molecular markers or alternative mitochondrial gene regions, along with an expanded dataset of European soft scale insect sequences, will enhance species-level resolution and contribute to a more robust phylogenetic framework for the Coccidae family. This study provides a valuable baseline for future research on European populations, offering initial insights into their genetic diversity and supporting the development of improved diagnostic tools. Such efforts would not only improve diagnostic tools for soft scale insects but also provide a foundation for future studies on their ecology, biogeography, and evolutionary relationships.

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REFERENCES

Amouroux P., Crochard D., Germain J.F., Correa M., Ampuero J., Groussier G., Kreiter P., Malausa T., Zaviezo T., 2017 – *Genetic diversity of armored scales (Hemiptera: Diaspididae) and soft scales (Hemiptera: Coccidae) in Chile*. – Sci. Rep., 7: 2014.

Bahder B.W., Poojari S., Alabi O.J., Naidu R.A., Walsh D.B., 2013 – Pseudococcus maritimus *(Hemiptera: Pseudococcidae) and* Parthenolecanium corni *(Hemiptera: Coccidae)* **are** *Capable of Transmitting Grapevine Leafroll-Associated Virus 3 Between Vitis x labruscana and Vitis vinifera*. – Environ. Entomol., 42(6): 1292-1298.

Bandelt H., Forster P., Röhl A., 1999 – *Median-joining networks for inferring intraspecific phylogenies.* – Mol. Biol. Evol., 16: 37-48.

Brady S.G., Gadau J., Ward P.S., 2000 – *Systematics of the ant genus* Camponotus *(Hymenoptera: Formicidae): a preliminary analysis using data from the mitochondrial gene cytochrome oxidase I.* In: Hymenoptera: evolution, biodiversity and biological control, AustinA.D. & DowtonM. Ed., CSIRO Publishing, Victoria, Australia, pp.131-139.

Choi J., Lee S., 2020 – *Molecular phylogeny of the family Coccidae (Hemiptera, Coccomorpha), with a discussion of their waxy ovisacs.* – Syst. Entomol., 45: 396-414.

Cocuzza G.E.M., Di Silvestro S., Giordano R., Rapisarda C., 2015 – *Congruence between cytochrome oxidase I (COI) and morphological data in* Anuraphis *spp. (Hemiptera, Aphididae) with a comparison between the utility of the 5'barcode and 3' COI regions*. – ZooKeys, 529: 123-144.

Danzig E.M., 1997 – *Intraspecific variation of taxonomic characters.* In: Soft Scale Insects – Their Biology, Natural Enemies and Control, Ben-DovY. & HodgsonC.J. Ed., Elsevier Amsterdam, pp.203-212.

Deng J., Yu F., Zhang T.X., Hu H.Y., Zhu C.D., Wu S.A., 2012 – *DNA barcoding of six* Ceroplastes *species (Hemiptera: Coccoidea: Coccidae) from China*. – Mol. Ecol. Resour., 12(5): 791-796.

Dowton M., Austin A.D., 1998 – *Phylogenetic relationships among the microgastroid wasps (Hymenoptera: Braconidae): Combined Analysis of 16S and 28S rDNA genes and morphological data.* – Mol. Phylogenet. Evol., 10: 354-366.

Folmer O., Black M., Hoeh W., Lutz R., Vrijenhoek R., 1994 – *DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates*. – Mol. Mar. Biol. Biotechnol., 3(5): 294-299.

Garcia Morales M., Denno B.D., Miller D.R., Miller G.L., Ben-Dov Y., Hardy N.B., 2016 – *ScaleNet: A literature-based model of scale insect biology and systematics*. Database. Retrieved February 1, 2025.

Gill R. J., 1988 – *The scale insects of California: Part 1. The soft scales (Homoptera: Coccoidea: Coccidae).* California Department of Food and Agriculture, Sacramento, CA, 118 pp.

Graora D., Sivčev L., Spasić R., Sivčev I., 2012 – *Biology and harmfulness of soft scale insects (Hemiptera: Coccidae) on the grapevine*. Proceedings of the International Symposium on Current Trends in Plant Protection, Belgrade, Serbia, pp.526-531.

Hajibabaei M., Janzen D., Burns J., Hallwachs W., Hebert P., 2006 – *DNA barcodes distinguish species of tropical Lepidoptera.* – PNAS, 103(4): 968-971.

Hebert P., Penton E., Burns J., Janzen D., Hallwach W., 2004 – *Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly* Astraptes fulgerator. – PNAS, 101(41): 14812-14817.

Herrbach E., Alliaume A., Prator C.A., Daane K.M., Cooper M.L., Almeida R.P.P., 2017 – *Vector Transmission of Grapevine Leafroll-Associated viruses.* In: Grapevine Viruses: Molecular Biology, Diagnostics and Management, MengB., MartelliG.P., GolinoD. & FuchsM., Ed., 1st ed., Springer, Cham, Switzerland, 485-503 pp.

Hodgson C., 1994 – *The scale insect family Coccidae: an identification manual to genera.* CABI, Wallingford, Oxfordshire, 639 pp.

Hoffmann C., 2002 – *Schildlaüse im Weinbau und ihre Antagonisten*. Ph.D. Thesis. University of Karlsruhe, Karlsruhe, Germany.

Hommay G., Wiss L., Reinbold C., Chadoeuf J., Herrbach E., 2020 – *Spatial Distribution Patterns of* Parthenolecanium corni *(Hemiptera, Coccidae) and of the Ampelovirus GLRaV-1 and the Vitivirus GVA in a Commercial Vineyard.* – Viruses, 12: 1447.

Ioriatti C., Lucchi A., Bagnoli B., 2008 – *Grape Areawide Pest Management in Italy.* In: Areawide Pest Management: Theory and Implementation, KoulO., CuperusG. & ElliottN. Ed., Wallingford: CABI International, pp.208-225.

Kondo T., Watson G.W., 2022 – *Chapter 2. A list of scale insect agricultural pests.* In: Encyclopedia of Scale Insect Pest, KondoT. & WatsonG.W. Ed., CABI, Wallingford, Oxfordshire, pp.8-37.

Kondo T., Watson G.W., 2022 – *Chapter 5. Collection, preservation, slide-mounting, labelling and vouchering of scale insects*. In: Encyclopedia of Scale Insect Pest, KondoT. & WatsonG.W., Ed., CABI, Wallingford, Oxfordshire, pp.548-558.

Kondo T., Gullan P., Williams D., 2008 – *Coccidology. The study of scale insects (Hemiptera: Sternorrhyncha: Coccoidea).* – Cienc. Tecnol. Agropecuaria, 9(2): 55-61.

Kosztarab M., Kozár F., 1988 – *Scale insects of Central Europe*. Akadémiai Kiadó, Budapest, 456 pp.

Kumar S., Stecher G., Li M., Knyaz C., Tamura K., 2018 – *MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms.* – Mol. Biol. Evol., 35: 1547-1549.

Leigh J. W., Bryant D., 2015 – *PopART: Full-feature software for haplotype network construction.* – Methods ecol. evol., 6: 1110-1116.

Malumphy C.P.J., 1991 – *A morphological and experimental investigation of the* Pulvinaria vitis *complex in Europe*. Ph.D. Thesis. The Natural History Museum, London.

Masten Milek T., **Šimala** M., Pintar M., 2021 – *Scale insects on vine on vine and their control in the atmosphere of new trends and reduction of pesticide* use. – Glasilo biljne zaštite, 21(3): 403-407.

Mibey R., 1997 – *Sooty moulds*. In: Soft Scale insects – Their biology, natural enemies and control. Ben-DovY. & HodgsonC.J., Ed., Elsevier, Amsterdam, pp.275-288.

Milošević M., Graora D., 2023 – *Within-plant distribution of* Parthenolecanium corni *(Bouché) (Hemiptera: Coccidae) on grapevine*. – Phytoparasitica, 51: 393-401.

Park D.S., Footit R., Maw E., Hebert P., 2011 – *Barcoding Bugs: DNA-Based Identification of True bugs (Insecta: Hemiptera: Heteroptera).* – PLoS ONE, 6(4): e18749.

Park D.S., Suh S.J., Hebert P.D., Oh H.W., Hong K.J. 2011 – *DNA barcodes for two scale insect families, mealybugs (Hemiptera: Pseudococcidae) and armored scales (Hemiptera: Diaspididae).* – Bull. Entomol. Res., 101: 429-434.

Pellizzari G., 1997 – *Coccid pest of important crops: Grapevine.* In: Soft Scale insects – Their biology, natural enemies and control, Ben-DovY. & HodgsonC.J., Ed., Elsevier, Amsterdam, pp.323-331.

Phillips A., Simon C., 1995 – *Simple, Efficient and Non-destructive DNA Extraction Protocol for Arthropods*. – Systematics, 88(3): 281-283.

Rakimov A., Ben-Dov Y., White V., Hoffmann A.A., 2013 – *Soft scale insects (Hemiptera: Coccoidea: Coccidae) on grapevines in Australia*. – Aust. J. Entomol., 52: 371-378.

Rozas J., Ferrer-Mata A., Sanchez-Del Barrio J. C., Guirao-Rico S., Librado P., Ramos-Onsins S. E., Sánchez-Gracia A., 2017 – *DnaSP 6: DNA Sequence Polymorphism Analysis of Large Data Sets*. – Mol. Biol. Evol., 34: 3299-3302.

Saakyan-Baranova A.A., Sugonyaev E.S., Sheldeshova G.G., 1971 – *Brown fruit scale (*Parthenolecanium corni *Bouché) and its parasites (Chalcidoidea). An essay of a complex investigation of host-parasite relations.* Nauka Publishers, Leningrad. 167 pp.

Saitou N., Nei M., 1987 – *The neighbor-joining method: A new method for reconstructing phylogenetic trees.* – Mol. Biol. Evol., 4: 406-425.

Sethusa M.T., Millar I.M., Yessoufou K., Jacobs A., van der Bank M., van der Bank H., 2014 – *DNA barcode efficacy for the identification of economically important scale insects (Hemiptera: Coccoidea) in South Africa.* – Afr Entomol., 22(2): 257-266.

Silva E.B., Maia M., Santos M., Cruz A., Botelho M., Franco J.C., Ribeiro H., Mexia A., 2016 – Parthenolecanium corni *(Bouché) (Hemiptera Coccidae) in vineyards in Portugal: Morphology, seasonal development, life cycle and reproduction*. – Redia, 99: 215-217.

Simon C., Frati F., Beckenbach A., Crespi B., Liu H., Flook P., 1994 – *Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers.* – Ann. Entomol. Soc. Am., 87(6): 651-701.

Stepaniuk K., Lagowska B., 2006 – *Number and arrangement variation of submarginal tubercles in adult females* Parthenolecanium corni *group (Hemiptera, Coccidae) and its value as a taxonomic character.* – Pol. J. Entomol., 75(2): 293-301.

Tamura K., Stecher G., Kumar S., 2021 – *MEGA11: Molecular Evolutionary Genetics Analysis Version 11*. – Mol. Biol. Evol., 38(7): 3022-3027.

Tamura K., Nei M., 1993 – *Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees*. – Mol. Biol. Evol., 10: 512-526.

Thompson J.D., Higgins D.G., Gibson T.J., 1994 – *CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice*. – Nucleic Acids Research, 22(22): 4673-4680.

Wang X.B., Deng J., Zhang J.T., Zhou Q.S., Zhang Y.Z., Wu S.A., 2015 – *DNA barcoding of common soft scales (Hemiptera: Coccoidea: Coccidae) in China*. – Bull. Entomol. Res., 105: 545-554.

Wang Y.S., Dai T.M., Tian H., Wan F.H., Zhang G.F., 2019 – Comparative analysis of eight DNA extraction methods for molecular research in mealybugs. – PLoS ONE, 14(12): e0226818.

Whiting M.F., Carpenter J.C., Wheeler Q.D., Wheeler W.C., 1997 – *The Strepsiptera problem: phylogeny of the holometabolous insect orders inferred from 18S and 28S ribosomal DNA sequences and morphology*. – Syst. Biol., 46(1): 1-68.

Yokogawa T., Yahara T., 2009 – *Mitochondrial phylogeny certified PGL (Paternal Genome Loss) is of single origin and haplodiploidy sensu stricto (arrhenotoky) did not evolve from PGL in the scale insects (Hemiptera: Coccoidea).* – Genes and Genetic Systems., 84(1): 57-66.