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Genetic Constitution of Parthenogenetic Form of *Polydrusus inustus* (Coleoptera: Curculionidae) – Hints of Hybrid Origin and Recombinations

Łukasz KAJTOCH and Dorota LACHOWSKA-CIERLIK

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Asexual reproduction among insects may arise spontaneously, as a result of infectious agents or hybridization between phylogenetically distinct organisms. Polyploidy, which may be the result of auto- or allopolyploidization (also associated with hybridization), is often connected with parthenogenesis. However, all studied parthenogens of weevils were apomictic, rare meiotic stages have been observed in a few of them what could suggests in their genomes besides the mutational changeability also recombination may occurs. We studied the level of heterozygosity in Internal Transcribed Spacer 2 sequences, and congruence of mitochondrial (cytochrome B) and nuclear genealogies among individuals of parthenogenetic form of *Polydrusus inustus*. High frequency (87.0%) of heterozygotes in ITS2 sequences may indicate a hybrid origin of the parthenogen. It is possible that this parthenogenetic form arose via a hybridization event between phylogenetically distinct lineages of bisexual *P. inustus*, known only from Georgia and Turkey, or between this species and one of the closely relatives from the subgenus *Scythodrusus*. Although result of partition-homogeneity test did not show significant incongruence of mitochondrial and nuclear genealogies, in the network was detected one loop. Most probable explanations for existence of that loop in parthenogenetic form and for origin of rare homozygotes, which were detected in ITS2 sequences, is gene conversion. It is supported by recently cytogenetic studies indicating rare meiotic stages during the oogenesis of triploids of *P. inustus*.

Key words: weevils, hybridization, apomictic, poliploidy, ITS2, mtDNA, gene conversion.

Łukasz KAJTOCH, Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Sławkowska 17, 31-016 Kraków, Poland.

E-mail: kajtoch@isez.pan.krakow.pl

Dorota LACHOWSKA-CIERLIK, Institute of Zoology, Department of Entomology, Jagiellonian University, R. Ingardena 6, 30-060 Kraków, Poland.

E-mail: dorota.lachowska-cierlik@uj.edu.pl

Asexual reproduction is known in most groups of vertebrates (AVISE *et al.* 1992) and invertebrates (BELL 1982; NORMARK 2003). It has originated independently many times and takes a variety of forms, but most often it is associated with parthenogenesis. In short, parthenogenesis involves the reproduction of females which produce only daughters without fertilization and pass on only maternal genes. Approximately 900 documented cases of parthenogenesis are known among insects (NORMARK 2003). Such parthenogenetic forms are thought to be short-lived in an evolutionary sense (dead-ends) (WHITE 1970; BELL 1982), but in some cases their genetic diversity is higher than in bisexuals forms (SAURA *et al.* 1976) and exclusively parthenogenetic taxonomic groups are known as “unisexual scandals” (MAYNARD SMITH 1978). Parthenogenetic reproduction without meiosis and recombination results in offspring (daugh-

ters) that are identical clones except for random mutations. Parthenogenesis has originated in at least four ways. Spontaneous parthenogenesis takes place when a mutation occurs in the genes connected to sex, meiosis or hormone levels (SIMON *et al.* 2002) or sometimes when females fail to mate (SEILER *et al.* 1961). Infectious agents such as *Wolbachia* may cause male mortality, cytoplasmic incompatibility or development of haploid eggs into females (STOUTHAMMER *et al.* 1993; WERREN *et al.* 1997). However, the most common factor triggering parthenogenesis is probably hybridization. It may arise as a result of hybridization between related species or different (phylogenetically distinct) forms of the same species (HONEYCUTT & WILKINSON 1989; STENBERG *et al.* 2003; STENBERG & LUNDMARK 2004). It is also possible that parthenogenetic genes are transmitted via crossbreeding of a female from a clonal

lineage of one species and a male from a bisexual species (INNES & HEBERT 1988; RISPE & PIERRE 1998). Identification of such hybrids is possible by examining genes from mitochondria (which are only maternally inherited) and nuclear genes (which are a combination of the genes of both parental species). In strictly asexual lineages, phylogenies based on maternally and paternally (or biparentally) inherited markers should correspond perfectly. Polyploidization is often associated with parthenogenesis. The origin of polyploidy in animals can be caused by autopolyploidy (failure of cell division during meiosis or after mitotic doubling, production of unreduced eggs) or allopolyploidy (hybridization between species or different forms of one species) (OTTO & WHITTON 2000). Asexual forms are often represented by individuals with different levels of ploidy (mostly di-, tri- and tetraploids). The genealogical interrelations between them are uncertain, i.e. if diploid or triploid parthenogens came into existence first. (LOKKI 1976; SUOMALAINEN *et al.* 1976; SAURA *et al.* 1993). Despite of the short life-span of parthenogenetic species (which are mainly also polyploids), they often have high levels of genetic diversity (SUOMALAINEN *et al.* 1987), heterogeneity in karyotype structure (JUDSON & NORMARK 1996) and are ecologically successful (LYNCH 1984; SUOMALAINEN *et al.* 1987). Parthenogenetic (often polyploid) forms have more or less outcompeted their sexual relatives. They often have wider or different geographic distributions. This situation has been described as “geographic parthenogenesis” (VANDEL 1928) and “geographic polyploidy” (STENBERG *et al.* 2003). Asexual forms are mostly distributed in environments in adverse conditions, e.g. at higher altitudes or latitudes, on islands, in disturbed or marginal habitats (CUELLAR 1994; LYNCH 1984; SUOMALAINEN *et al.* 1987). They are also considered as first colonizers of new areas, e.g. subsequent to glaciation events (SEILER 1961; KEARNEY 2005; LUNDMARK 2006; LUNDMARK & SAURA 2006).

Parthenogenetic species or forms have been identified in only a few genera of weevils (SUOMALAINEN *et al.* 1987; STENBERG *et al.* 2000, 2003). All clonal weevils that have been examined so far are apomictic, however, SIMON *et al.* (2003) mentioned the occurrence of automictic species (without citations). SUOMALAINEN (1969) and LOKKI *et al.* (1976) assumed that parthenogenesis was automictic at the beginning of the evolution of asexual lineages but changed afterwards to apomictic reproduction. This was later questioned by SAURA *et al.* (1993). However, all studied parthenogenetic forms or species of weevils were apomictic, rare meiotic stages have been postulated during the oogenesis of *Otiorhynchus sulcatus*

(SEILER 1947) and a few other parthenogens, among others – *Polydrusus inustus* (LACHOWSKA *et al.* 2008; LACHOWSKA *et al.* in preparation). The observation of stages resembling meiosis could confirm hypothesis that the apomictic parthenogenesis evolve through a stage of automixis. The evidence of chromosomes forming figures of rings, crosses and rods – like morphology could suggests that in parthenogenetic genome of weevils besides the mutational changeability also recombination (crossing-over and conversion) may occurs.

In Europe parthenogenetic weevils were studied mainly in *Otiorhynchus*, *Polydrusus* and *Strophosoma* (MIKULSKA 1960; SUOMALAINEN 1969; SUOMALAINEN & SAURA 1973; LOKKI *et al.* 1976; SUOMALAINEN *et al.* 1987; STENBERG *et al.* 1997, 2000; TOMIUK *et al.* 1994). Until now nothing is known of the origin of the parthenogenetic form of *Polydrusus inustus*. This weevil inhabits areas from the Ural and Caucasus Mts in the east to Poland and the eastern slopes of the Carpathian Foothills in the west (KOROTYAEV 1996). Populations of this weevil over most of its range consist of parthenogenetic females and, at least in Poland and Ukraine, they are triploid (LACHOWSKA *et al.* 2008). Bisexual populations are know only from Georgia and Turkey (KOROTYAEV & MELESHKO 1995; KOROTYAEV 1996). These weevils live mostly on Rosacea bushes in xerothermic habitats but sometimes are also found on dry wastelands and in strawberry fields.

The aim of this study was to assess a level of heterozygosity in individuals of the parthenogenetic form of *P. inustus* and check congruence between nuclear and mitochondrial sequences. Based on these data conclusions about origin of parthenogenesis and polyploidy and possibility of recombination events were made.

Material and Methods

Individuals of *P. inustus* were collected from most of their ranges in Poland and western Ukraine (Table 1). Weevils were sampled as adults during several expeditions between 2005-2008. Samples were first preserved in 99% ethanol and afterwards stored in 22°C. A total of 5 individuals per population were taken for analyses (except population Hal in which only 2 specimens were collected). DNA was extracted from whole insect bodies using the Dneasy Tissue Kit (Qiagen). Amplification of a fragment of the nuclear internal transcribed spacer 2 (ITS2), and mitochondrial cytochrome b (CytB), was performed using primer pairs ITS3 and ITS4 (WHITE *et al.* 1990) and CB-J-10933 and CB-N-10920 (SIMON *et al.* 1994), respectively.

Table 1

Localization of sample collection sites and distribution of ITS2 “genotypes” and CytB haplotypes in studied weevils. In bold are presented individuals which genotype position in network is uncertain

Symbol of population	Geographic coordinates	Individual	ITS2 genotypes	CytB haplotypes
Kra	N 50°02'32.1'' E 19°52'55.5''	1	RR	H1
		2	RR	H1
		3	RR	H1
		4	RR	H2
		5	RR	H2
Dal	N 50°19'58.6'' E 20°12'55.4''	1	RR	H2
		2	RR	H3
		3	RR	H3
		4	RR	H3
		5	RR	H3
Cho	N 50°22'30.5'' E 20°42'43.9''	1	RR	H1
		2	RR	H3
		3	RR	H3
		4	RR	H3
		5	RR	H3
Boc	N 51°20'33.1'' E 21°58'48.6''	1	RR	H1
		2	RR	H1
		3	RR	H1
		4	RR	H1
		5	RR	H6
Gro	N 50°48'01.6'' E 23°57'12.7''	1	RR	H1
		2	RR	H1
		3	RR	H1
		4	RR	H4
		5	RR	H4
Kor	N 50°24'24.9'' E 23°32'25.3''	1	GG	H5
		2	GG	H5
		3	GG	H5
		4	GG	H5
		5	GG	H5
Mie	N 52°19'21.3'' E 23°02'12.2''	1	RR	H5
		2	RR	H5
		3	RR	H5
		4	RR	H5
		5	RR	H5
Roz	N 49°43'03.4'' E 22°46'49.7''	1	RR	H1
		2	RR	H1
		3	RR	H1
		4	RR	H1
		5	RR	H1
Jar	N 49°58'34.3'' E 22°38'14.2''	1	RG	H1
		2	RG	H1
		3	RR	H1
		4	RR	H1
		5	RR	H1
Szc	N 49°56'28.6'' E 20°53'06.9''	1	GG	H1
		2	GG	H1
		3	GG	H1
		4	RR	H1
		5	RR	H1
Bis	N 49°58'06.5'' E 20°06'55.5''	1	RR	H1
		2	RR	H1
		3	RR	H1
		4	RR	H1
		5	RR	H1
Kal	N 51°59'14.4'' E 21°13'59.3''	1	RR	H1
		2	RR	H1
		3	RR	H1
		4	RR	H1
		5	RR	H1
Mod	N 52°25'59.9'' E 20°41'25.7''	1	RR	H1
		2	RR	H1
		3	RR	H1
		4	RR	H1
		5	RR	H1
Wlo	N 52°39'44.4'' E 19°04'11.6''	1	RR	H5
		2	RR	H5
		3	RR	H5
		4	RR	H5
		5	RR	H5
Gog	N 50°29'30.8'' E 18°02'59.5''	1	AA	H5
		2	RR	H5
		3	RR	H5
		4	RR	H5
		5	RR	H5
Glo	N 51°40'50.6'' E 16°04'27.4''	1	RR	H5
		2	RR	H5
		3	RR	H5
		4	RR	H5
		5	RR	H5
San	N 50°41'26.0'' E 21°48'40.5''	1	RR	H1
		2	RR	H6
		3	RR	H6
		4	RR	H6
		5	RR	H6
Zlo	N 49°39'58.3'' E 24°37'00.5''	1	GG	H1
		2	GG	H1
		3	GG	H1
		4	RR	H1
		5	RR	H1
Hal	N 48°36'19.9'' E 24°57'47.2''	1	RR	H1
		2	RR	H1

The cycling profile for the PCR for both DNA fragments was: 95°C for 4 min, 35 cycles of 95°C for 30 s, 50°C for 1 min, 72°C for 2 min and a final extension period of 72°C for 10 min. PCR fragments after purification (QIAquick Gel Extraction Kit for ITS2; Qiagen's QIAquick PCR Purification Kit for CytB) were sequenced using the BigDye Terminator v.3.1. Cycle Sequencing Kit (Applied Biosystems) and ran on an ABI 3100 Automated Capillary DNA Sequencer. All sequences are deposited in GenBank (Accession nos. FJ430155-FJ430158 and FJ442922- FJ442927). Sequences were checked and aligned using BioEdit v.7.0.5.2 (HALL 1999) and ClustalX (THOMPSON *et al.* 1997). No indels or stop codons were observed in either of the DNA fragments. In electrochromatograms of ITS2 sequences were found two peaks in two nucleotide positions which were clearly higher than any peaks of background. In these positions nucleotide were ascribed according to IUPAC ambiguity codes. Incongruence between the phylogenetic signals provided by different DNA fragments (mitochondrial and nuclear) was assessed by statistical evaluating the incongruence length difference (ILD) index (MICKEVICH & FARRIS 1981; FARRIS *et al.* 1994) using the partition homogeneity test implemented in PAUP* 4.9b10 (SWOFFORD 2002). A Statistical Network Analysis (CRANDALL & TEMPLETON 1999) was applied in order to verify if distribution of the mitochondrial haplotypes and nuclear "genotypes" are congruent in the studied weevils. Incongruence of these markers was used as proof for hybrid origin (GÓMEZ-ZURITA & VOGLER 2006; GÓMEZ-ZURITA *et al.* 2006). Based on mitochondrial haplotypes, a network was constructed using the Statistical Parsimony method (TEMPLETON *et al.* 1992) and the program TCS 1.21 (CLEMENT *et al.* 2000). ITS2 "genotypes" were added manually to network of CytB haplotypes, which were co-found in studied individuals.

Results

The 92 individuals of *P. inustus* from 19 localities yielded 4 ITS2 "genotypes" and 6 CytB haplotypes (Table 1, Fig. 1).

The 528 bp fragments of ITS2 showed only 2 polymorphic sites in positions 249 and 496. Most individuals (84.8%) were heterozygotes with "R" (G/A) in both positions, 11 individuals (11.9%) were homozygotes with guanine (G) in both positions, 1 individual (1.1%) was a homozygote with adenine (A) in both positions and 2 individuals (2.2%) were heterozygotes with "R" in position 249 and homozygotes with G in position 496. As *P. inustus* is triploid (LACHOWSKA *et al.* 2008)

these heterozygotes have double Gs and single As or double As and single Gs in particular ITS2 sequences. However, peaks of G were higher than A peaks in both positions, as evidenced by the sequencing electrochromatograms. The detection of a higher frequency of G homozygotes in both positions suggests that these heterozygotes are G/G/A. Because it is not clear which combination of nucleotides is correct, we present these "genotypes" as G/A (R). Individuals with the "genotype" RR were found across the entire studied range of the species. Other "genotypes" were limited to specific areas: individuals with "genotype" GG were identified in populations close to the Polish/Ukrainian border (Zlo, Kor) and Carpathian Foothills (Szc). In one population from the Carpathian Foothills (Jar), 2 individuals with "genotype" RG were detected. A single individual with an AA "genotype" was found in a population from Silesia (Gog).

A total of 5 polymorphic sites were detected in 435 bp sequences of CytB. The most common haplotype H1 was found in 49 individuals (53.2%) from most of the studied range of the species, except for the western and northern part of Poland. Haplotype H5 (26 individuals, 28.3%) was found in the latter localities along the Oder, lower Vistula and Bug valleys (Mie, Wlo, Gog and Glo) and in southeastern Poland (Kor, Roz). Haplotypes H4 (5 individuals, 5.4%) and H6 (9 individuals, 9.8%) were identified only in a limited area in the central part of the Vistula valley and Małopolska Upland, respectively. The remaining haplotypes were found in single populations: Kra in the upper Vistula valley (1 individual with haplotype H2), and Gro in the Bug valley (2 individuals with haplotype H3).

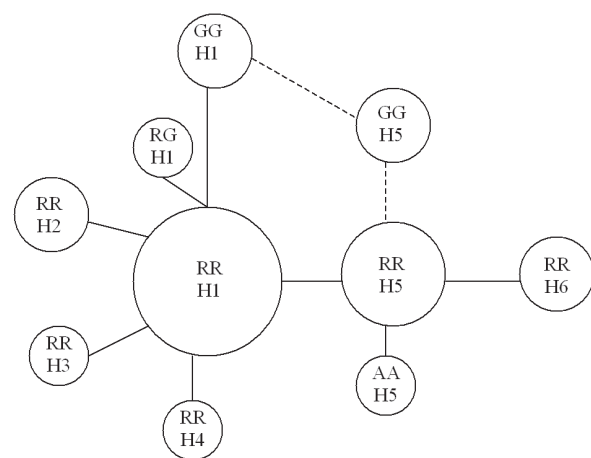


Fig. 1. Network of 6 CytB haplotypes (H1-H6) together with 4 ITS2 "genotypes" (RR, GG, RG and AA) of *P. inustus*. Sizes of circles correspond to the number of individuals with particular haplotype/genotype. Broken line represent two possible connection of GG/H5 genotype (loop).

44.5% of individuals had the most common CytB haplotype (H1) and ITS2 “genotype” RR. Only incongruence between these two DNA fragments was found in individuals which had GG “genotype” and H1 haplotype (0.5%) or H5 haplotype (0.5%).

Incongruence of mitochondrial and nuclear sequences was not detected by partition-homogeneity test which was nonsignificant for intergenomic comparison ($P=1.0$). Nonsignificant result of that test was caused by the presence of many heterozygotes RR in nuclear sequences which were not parsimony informative.

Discussion

About 45% of the individuals share the same and most common “genotype” (RR) and haplotype (H1). This most common “genotype” and haplotype are probably ancestral for the parthenogenetic form of *P. inustus*, at least for populations in central Europe, but also possibly for individuals across the range of this form.

Three sets of chromosomes were present in the studied triploid individuals of *P. inustus* (LACHOWSKA *et al.* 2008): two probably from one ancestor (double Gs in the polymorphic sites in ITS2 sequences) and one from another (single A nucleotide in polymorphic sites in ITS2 sequences). The origin of parthenogenesis and polyploidy was investigated for diploid and triploid parthenogenetic forms of *P. mollis* in Europe (STROEM 1768) (SUOMALAINEN 1969; LOKKI *et al.* 1976; TOMIUK *et al.* 1994), which revealed that parthenogenetic forms were more closely related to each other than to the bisexual form, but it was not certain which asexual form originated first. SUOMALAINEN (1969) and LOKKI *et al.* (1976) suggested that the triploid asexual form evolved from the diploid asexual, but SUOMALAINEN *et al.* (1987) and SAURA *et al.* (1993) argued for a reverse sequence of origin. The heterozygosity of both asexual forms was greater than for the bisexual. SAURA *et al.* (1993) proposed a cytological mechanism that explained the high degree of heterozygosity observed in asexual forms of this species as defective meiosis leading to diploid gametes and consequently to triploid individuals. A plate disjunction slip may at a later time lead to the origin of the diploid asexual form. However, in subsequent studies it was postulated that the high degree of heterozygosity in *P. mollis* and other asexual forms of weevils may be due to hybridization (SAURA *et al.* 1993; TOMIUK *et al.* 1994), as probably occurred during the evolution of the parthenogenetic form of *P. inustus*.

ITS2 sequences together with internal transcribed spacer 1 (ITS1) are untranslated breaks be-

tween 18S, 5.8S and 28S ribosomal DNA genes. These sequences are part of a tandemly repeated unit (family of rDNA genes) and are inherited together. A mutation which appears in one part of this unit is removed or propagated across all repeated units, therefore rDNA sequences (and among them ITS2) should mostly be homozygous. Homogenization of rDNA sequences is caused by molecular drive, gene conversion, unequal crossing-over or transposition (FOGEL & MORTIMER 1969; HOOD *et al.* 1975; SMITH 1976; DOVER 1982, 1986). This is an example of concerted evolution (BROWN *et al.* 1972; ZIMMER *et al.* 1980; NEI & ROONEY 2005). Most of the studied individuals of *P. inustus* were heterozygotes in ITS2 (87.0%), indicating that they may have descended from genetically different ancestors. Populations of *P. inustus* consist of parthenogenetic females over most of their range in eastern and central Europe (KOROTYAEV 1996). Bisexual populations of these weevils are known from Georgia and Turkey where they inhabit isolated localities in mountainous regions (KOROTYAEV 1996). They are probably ancestral to the asexual form. The most plausible scenario explaining high frequency of heterozygotes in nuclear DNA in parthenogenetic individuals of *P. inustus* is a hybridization event, which may have occurred between genetically distinct lineages of bisexual *P. inustus*. A similar scenario for the origin of parthenogenesis was described for *O. scaber* (STENBERG *et al.* 1997, 2000). Parthenogenetic forms of these weevils arose as a result of hybridization between two distinct bisexual lineages inhabiting isolated localities in the Alps and spread across most of central and northern Europe. The origin of parthenogenetic forms of *P. inustus* may be nevertheless more complicated. Along the western coast of the Caspian Sea and the northeastern coast of Black Sea, *P. inustus* coexists with the closely related species *P. pilifer* (KOROTYAEV & MELESHKO 1995; KOROTYAEV 1996). The second species also consists of a bisexual form distributed around the eastern and southern coast of the Caspian Sea and a parthenogenetic form in central Asia. In this area, a third species from the subgenus *Scythodrusus*, *P. obrieni*, (KOROTYAEV *et al.* 2004) was also recently described. It is probable that the parthenogenetic form of *P. inustus* (and possibly also *P. pilifer*) arose as a result of hybridization between different ancestral species. It is also possible that parthenogenetic genes are transmitted via crossbreeding of a female from the clonal lineage of another species and a male from *P. inustus*, as detected in many other invertebrates (INNES & HEBERT 1988; RISPE & PIERRE 1998). Indeed, such an origin of parthenogenesis is thought to be the major route to polyploidy (SIMON *et al.* 2003). In the parthenogenetic form of *P. inustus*, triploidy

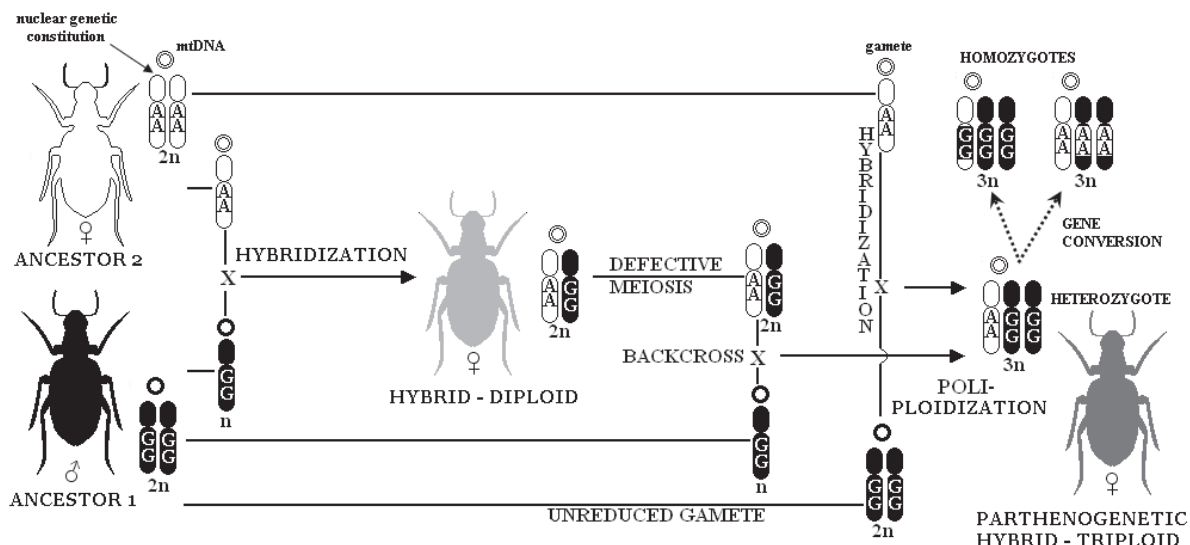


Fig. 2. Possibly scenarios of origin of parthenogenesis and ploidy in *P. inustus* via hybridization and polyploidization events (adapted from SAURA *et al.* 1993) and rare homozygotes via conversion. A and G represent nucleotides in two polymorphic sites of the ITS2 sequences. For details see text.

possibly arose as a result of cross-breeding between a diploid hybrid form with one of their ancestral taxon (phylogenetically distinct lineage or species) or cross-breeding between ancestral taxons which one had produced unreduced gamete (Fig. 2). The possible presumed deduction of the hybrid origin of the parthenogenetic form of *P. inustus* needs further investigation through a comparison of mitochondrial and nuclear sequences from all bisexual and parthenogenetic forms of *P. inustus* and its *Scythodrusus* relatives from their entire ranges.

In asexually breeding animals strict congruence between nuclear and mitochondrial genes should be observed because all parts of the genome are inherited together (as one locus; SIMON *et al.* 2003). The incongruence of mitochondrial and nuclear sequences was not detected but a few individuals with “genotypes” GG and CytB haplotypes H5 which may be connected in two ways in network (create a loop) – situation which is rather improbable in strict apomictic form. They may originate as a result of sporadic sexual breeding, convergent mutations in nuclear or mitochondrial sequences, gene conversion and crossing-over (SIMON *et al.* 2003). As males of *P. inustus* has never been detected outside Georgia and Turkey (KOROTYAEV 1996; MAZUR 1994) such a explanation is not convinced. Haplotypes H1 and H5 differ only by one mutation so it cannot be excluded that such a mutation occur twice: in lineage RR/H1 – RR/H5 and in lineage RR/H1 – GG/H1 – GG/H5. However it is also probable that GG “genotypes” originate twice in lineages: RR/H1 – RR/H5 – GG/H5 and RR/H1

– GG/H1. Identification of homozygotes in both (GG and AA) or one (RG) of the polymorphic sites in ITS2 sequences would be difficult to explain in terms of mutation. This especially applies to individuals homozygous in both nucleotide positions (249 and 496 in studied sequences), the more so because no other mutations were detected in other parts of the ITS2 sequences. Origin of homozygotes may be explained as a result of gene conversion – the replacement of one DNA fragment in one chromosome by a homologous sequence from another chromosome (i.e. loss of A allele and addition of G allele which gives GG homozygote) (Fig. 2). Conversion, which is one way of recombination, has been considered as improbable in apomictic animals, however, somatic crossing-over (endomeiosis) were offered to account for sudden bursts of variation in parthenogenetic aphids and cladoceran populations (BACCI *et al.* 1961; COGNETTI 1961). According to COGNETTI (1961) pairing of chromosomes leads to chiasma formation and gene recombination. These conclusions were nevertheless not concord with studies of SUOMALAINEN (1950) and BLACKMAN (1978) which were against possibility of endomeiotic recombination events. Recently intensive cytogenetic studies on parthenogenetic weevils (among others – *P. inustus*) reviewed rare occurrence of atypical course of apomictic oogenesis with stages similar to meiosis, in which chromosomes form bivalents and multivalent clusters (LACHOWSKA *et al.* 2008; LACHOWSKA *et al.* in preparation). These associations of chromosomes probably represents the remnants of meiosis, although it is uncertain if

these events have something to do with recombination. Detection of rare homozygous individuals among prevailing heterozygous *P. inustus* support the hypothesis that recombination (gene conversion) might occasionally happened but further research is needed to confirm it.

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