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Molecular phylogeny of European *Saga*: comparison with chromosomal data

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Abstract

The six European *Saga* species (Orthoptera Tettigoniidae) were mitochondrially analyzed for the COI gene. Results were compared with available karyological data, the two analyses producing complementary and congruent conclusions. European *Saga* species appear to be monophyletic, and probably derived from Asiatic species by a Robertsonian translocation. *Saga natoliae* Serville separated first from their common trunk, followed by *Saga hellenica* Kaltenbach and *Saga rhodiensis* Salfi. The three other species share several gene mutations and two chromosomal changes. The parthenogenetic *Saga pedo* (Pallas) from France and Balkans on the one hand and *Saga campbelli* Uvarov and *Saga rammei* Kaltenbach on the other hand represent two sister clades. Their proximity is in agreement with the possible origin of the pentaploid karyotype of *S. pedo*, by addition of haploid genomes from species close to *S. campbelli* and *S. rammei*, as proposed earlier. Molecular level of divergence indicates that the separation of French and Balkans lineages of *S. pedo* occurred between 420,000 and 650,000 years ago.

Key words: Tettigoniidae, European *Saga*, genetics, cytogenetics, phylogeny.

Introduction

In Europe, the genus *Saga* Charpentier 1825, is composed of six species, including the parthenogenetic species *Saga pedo* (Pallas 1771). These species mainly occur in Balkans, with the exception of *S. pedo* showing a much larger distribution, from Spain to Asia, and *Saga rhodiensis* Salfi 1929, which is endemic to Rhodes Island (Greece) and neighbouring continental Turkish region (figure 1). Nine other species are known in the genus, all from Minor and Central Asia. Balkans hosts a high concentration of both individuals and species whose morphological differences are not clear-cut: the criteria for defining species need refining. Up to now, the best morphological criterion seems to be the structure of the tegmina in the males (figure 2). Unfortunately, this criterion has no value in females, and thus in the parthenogenetic *S. pedo*. No genetic data are available so far, but several chromosome studies were performed, with contradictory results. The karyotype of *S. pedo* was initially thought to be tetraploid, with 68 chromosomes (Matthey, 1946). This was later confirmed by Warchalowska-Sliva *et al*. (2009), but challenged by Dutrillaux *et al*. (2009) who found 70 chromosomes and proposed a pentaploidy instead of a tetraploidy. The other species studied (table 1) are diploid, with a chromosome number ranging from 31-32 for Asiatic species to 27-28 for *Saga rammei* Kaltenbach 1965 and *Saga campbelli* Uvarov 1921 (Goldschimdt, 1946; Warchalowska-Sliva *et al*., 2007; 2009; Lemonnier-Darcemont *et al*., 2008). Notice that for these species, as for *S. pedo*, discrepancies exist between the data from Lemonnier-Darcemont *et al*. (2008) and Warchalowska-Sliva *et al*. (2009) which give different chromosome numbers for the same species. This may be due to wrong diagnoses of the species, but also to either intra-specific regional variations, presence of B (dispensable) chromosomes in some populations, or artifacts. After karyotype comparisons, a tentative reconstruction of their phylogeny was proposed, but a major difficulty remained for the position of *S. pedo*, whose polyploidy and complex karyotype was difficult to compare with those of diploid species (Lemonnier-Darcemont *et al*., 2008). The aim of this study was to develop DNA sequence comparison for the mitochondrial cytochrome oxidase subunit 1 (COI) gene. Due to the high mutation rate of mitochondrial DNA (mtDNA), it constitutes a valuable molecular tool for the identification of morphologically similar species (Avise, 1994). Moreover, its maternal inheritance is particularly suitable for *S. pedo* allowing to infer possible maternal ancestry. In this species, as in other parthenotes (Tomiuk and Loeschcke, 1992), the polyploidy may result from the juxtaposition of several haploid genomes, by exceptional fecundations by males from closely related species (Dutrillaux *et al*., 2009), which would make interpretation of nuclear DNA sequencing difficult. The good congruence between chromosomal data, summarized in table 1, and present sequencing results allows us to propose a more achieved phylogenic scheme of the European *Saga*.

Materials and methods

S. pedo is a protected species for which we obtained capture authorizations in France and Former Yugoslavian Republic of Macedonia (F.Y.R.O.M). Information about specimens analyzed in this study is given in table 2.

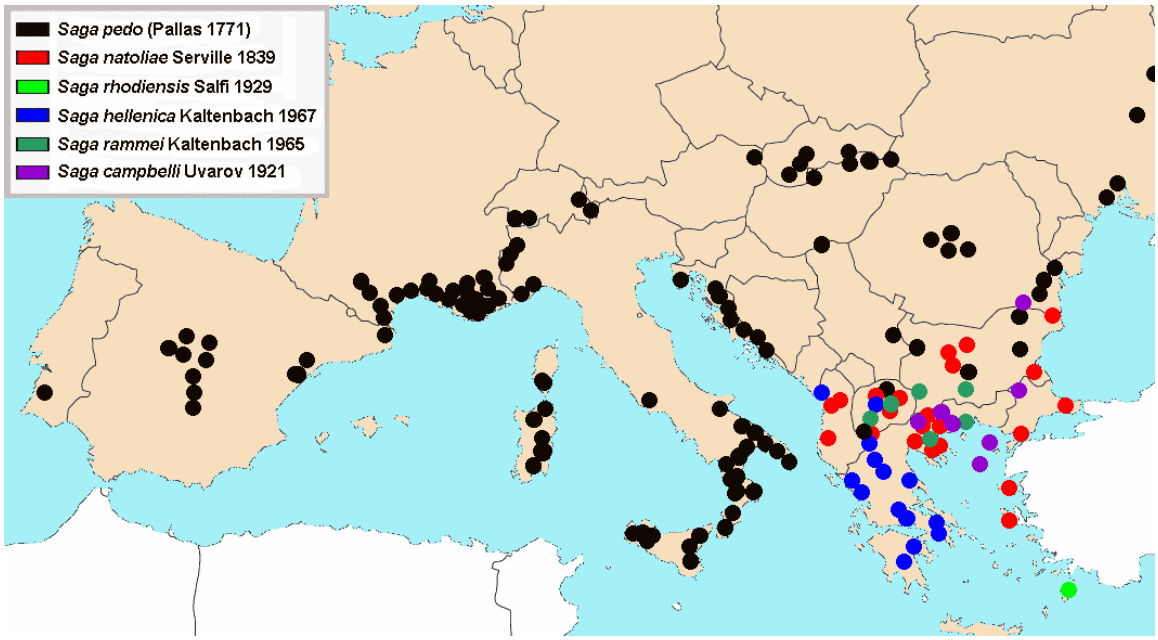

Figure 1. Distribution map of *Saga* species in Europe.

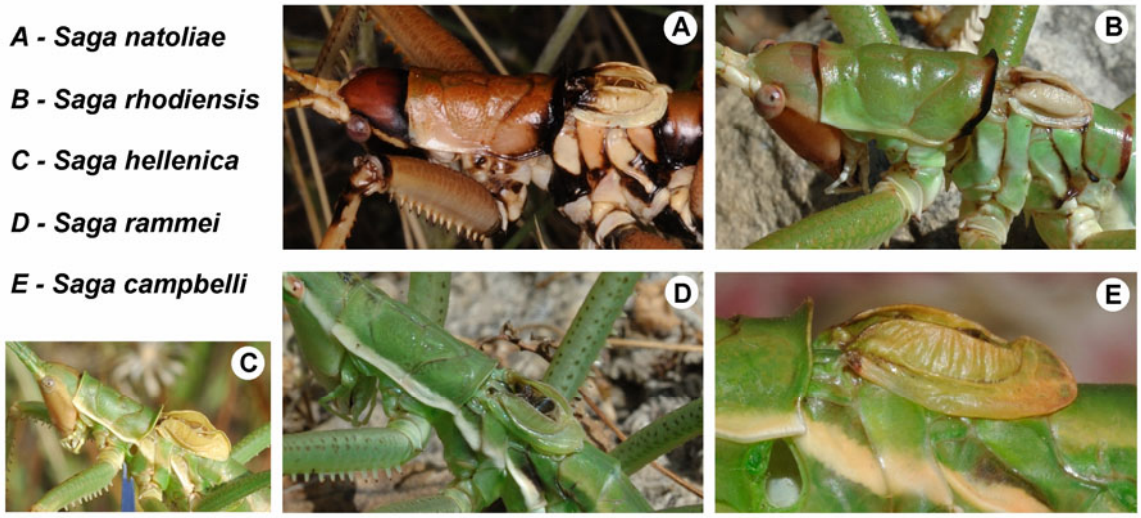

Figure 2. Tegmina morphology and partial lateral view of A) *S. natoliae* B) *S. rhodiensis* C) *S. hellenica* D) *S. rammei* E) *S. campbelli*.

Mitochondrial DNA was isolated from all specimens according to Aljanabi and Martinez (1997). A 397 bp segment of the COI gene was amplified using the primers COIF (5'- TTGGTGATGATCAAATTTATAA-3') and COIR (5'ACAAATAAAGGTGTTTGGTCTA-3'). Primers were designed according to a published DNA sequence of the species *Anabrus simplex* Haldeman 1852 (Orthoptera Tettigoniidae) (Fenn *et al*., 2007). PCR reactions (50 µL) contained 100-200 ng DNA, 1 x Taq buffer, 2 mM MgCl2, 0.2 mM of each dNTP, 50 pmoles of each primer and 1 U Taq polymerase (Invitrogen, Carlsbad, USA). The cycling conditions consisted of an initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 54 °C for 40sec and extension at 72 °C for 40 sec, with a final extension at 72 °C for 10 min. PCR products were purified using QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and on both strands sequenced by Macrogen Inc. Seoul, Korea. Sequences were deposited in GenBank (accession numbers are given in table 2). Nucleotide sequences were aligned using ClustalW (Thompson *et al*., 1997). For all sequences, base composition, nucleotide variation, polymorphic and parsimony informative sites were assessed using MEGA4 (Tamura *et al*., 2007). In order to find the model of evolution that fits the data set, we used the findmodel server (http://hcv.lanl.gov/content/hcv-db/findmodel/findmodel.html), and a phylogenetic tree, using the maximum likelihood method, was inferred using phyml online web server (Guindon *et al*., 2005). *A. simplex* sequence (accession number GU122265.1 in GenBank) was used as outgroup to root the tree.

Table 1. Summary of published data on *Saga* chromosomes.

Saga species	2N male	Nb non-acro	Ch 1 morpho	Large hetero	Ch change	References
S. ornata	31	0	A	?		Matthey, 1946
S. cappadocica	31	0	A	?		Matthey, 1948
					Rt	
S. natoliae	29	1	M	?		Warchalowska-Śliva *et al*., 2007
S. natoliae	29	1	SM	0		Lemonnier-Darcemont *et al*., 2008
					inv	
S. hellenica	29	1	M	0		Warchalowska-Śliva *et al*., 2007; Lemonnier-Darcemont *et al*., 2008
S. rhodiensis	29	1	M	0		Lemonnier-Darcemont *et al*., 2009
					Rt	
S. campbelli	27	2	M	0		Lemonnier-Darcemont *et al*., 2008; Warchalowska-Śliva *et al*., 2009
					H add	
S. rammei	23	2	M	?		Warchalowska-Śliva *et al*., 2009
S. rammei	27	2	M	1		Lemonnier-Darcemont *et al*., 2008
					Poly	
S. pedo	68	?	M?	?		Matthey, 1946
S. pedo	70	11	M or SM	1		Lemonnier-Darcemont *et al*., 2008; Dutrillaux *et al*., 2009

2N male: chromosome number in the male; Nb non-acro: number of non-acrocentric chromosomes; Ch 1 morpho: Chromosome 1 morphology, A: acrocentric, M: metacentric, SM: sub-metacentric; Large hetero: presence (1) or absence (0) of a large heterochromatic fragment in non-centromeric position; Ch change: chromosomal rearrangement separating the above and below cited species: Rt: Robertsonian translocation; inv: inversion; H add: heterochromatin addition; Poly: polyploidization.

Table 2. Collection data, number of specimens analyzed, and GenBank accession numbers for *Saga* species included in the study.

Species	Locations and origins of specimens	Number of specimens	Latitude	Longitude	GenBank accession no.
Saga pedo (Pallas 1771)					
*Saga pedo*1	Fos-sur-mer, 2008 Bouches du Rhône (FR)	1	43°28'N	04°56'E	HQ717173
*Saga pedo*2	Ohrid, 2008 (F.Y.R.O.M)	1	40°57'N	20°49'E	HQ717166
*Saga pedo*3	Breeding 2009. Origin: Ohrid, 2007 (F.Y.R.O.M)	1	40°57'N	20°49'E	HQ717172
*Saga pedo*4	Breeding 2009. Origin: Ohrid, 2007 (F.Y.R.O.M)	1	40°57'N	20°49'E	HQ717167
Saga campbelli Uvarov 1921	Breeding 2008. Origin: Messi, 2005 (GR)	3	40°56'N	25°14'E	HQ717164 HQ717163 HQ717174
Saga rammei Kaltenbach 1965	Breeding 2006. Origin: Olimbiada, 2003 (GR)	1	40°37'N	23°46'E	HQ717165
Saga rhodiensis Salfi 1929	Breeding 2009. Origin: Appolakkia, Rhodes, 2008 (GR)	1	36°03'N	27°47'E	HQ717169
Saga natoliae Serville 1839	Ierissos, 2005 (GR)	1	40°23'N	23°52'E	HQ717170
Saga hellenica Kaltenbach 1967					
*Saga hellenica*1	Pardalitsa, 2005 (GR)	1	39°30'N	20°39'E	HQ717168
*Saga hellenica*2	Kristallopigi, 2007 (GR)	1	40°39'N	21°08'E	HQ717171

Results and discussion

The sequences obtained were 397 nucleotides long and corresponded to nucleotides 1570-1967 of COI gene of *A. simplex* complete mitochondrial genome (Fenn *et al*., 2007), which is the most related species retrieved in GenBank. The presence of nuclear mitochondrial pseudogenes (numts) seems to be a common phenomenon in most eukaryotic species studied so far (Richly and Leister, 2004; Cameron *et al*., 2009). However, the absence of insertion, deletion or in-frame stop codon within the sequences studied here indicates that they correspond to functional mitochondrial COI gene fragments and are not derived from nuclear mitochondrial pseudogenes. The observed high percentage (59.3% on average) of A+T content is a common feature of animal mitochondrial genes (Brown, 1985). Among the 397 sites, 103 are variable while 64 of them are informative for parsimony. Among the 103 variable sites, 90.3% are third codon positions, while first and second codon positions are much more conserved (9.7% and 0.0% variation, respectively). This pattern is typically observed in segments under strong functional constraints. Interestingly, of the 103 variable sites, only one resulted in amino acid substitution in *Saga natoliae* Serville 1839.

The sequence divergence within the genus ranged from 0.0% (*S. pedo* 5, 6 vs. *S. pedo* 4) to 20.7% (*S. hellenica* 9 vs. *S. natoliae*). Findmodel server revealed General Time Reversible plus gamma (GTR + γ) as the model of sequence evolution that best fit our data. Based on sequence data, a Maximum Likelihood (ML) phylogenetic tree was constructed with 1000 bootstrap replicates. The main branches exhibit bootstrap values more than 50% and clearly separate the species in distinct clades (figure 3). Mega4 (Tamura *et al*., 2007) was also used to calculate the Tamura-Nei (Tamura and Nei, 1993) genetic distance to construct a neighbour-joining (NJ) phylogenetic tree (not shown). Both ML and NJ trees showed identical results.

DNA sequence and chromosome data are congruent (figure 3). The high genetic distance between *S. natoliae* and other species is associated with an inversion of chromosome 1 (table 1), which is a unique rearrangement among the studied *Saga*. The monophily of *S. rhodiensis* and *S. hellenica* fits with their similar karyotypes. Their clear separation from the clade formed by *S. pedo*, *S. rammei* and *S. campbelli* is marked by a translocation and heterochromatin addition. Finally, *S. campbelli* and *S. rammei* are monophyletic and possess similar karyotypes, variations of heterochromatin excepted. Considering old data on male karyotypes of Asiatic species (Goldschmidt, 1946; Matthey, 1946, reviewed in Warchalowska-Śliva, 1998), which reported 31,X formulae, apparently with only acrocentric chromosomes, it is likely that European *Saga* are monophyletic. Either a Robertsonian translocation or fission separates their karyotype from that of Asiatic species (table 1). Because Robertsonian translocations are much more frequent than fissions and they tend to be recurrent in a clade, the occurrence of a translocation is more likely. Consequently, Balkans would not be the cradle of the whole genus, but of the European species only, because Asiatic species would have a more primitive karyotype with only acrocentric autosomes, from which that of European species would derive by a Robertsonian evolution.

The characteristics of *S. pedo* genome deserve attention. In its complex pentaploid karyotype, most non acrocentric chromosomes are unique or in small copy number (Dutrillaux *et al*., 2009). Thus, either they were rearranged after polyploidization, or they came from fecundations by males with different karyotypes or both. Interestingly, the karyotype of the French specimens differs by one chromosome rearrangement from that of the Balkans. This may indicate their ancient separation (Dutrillaux *et al*., 2009), but the validity of this interpretation is restricted by the lack of data on the kinetic of

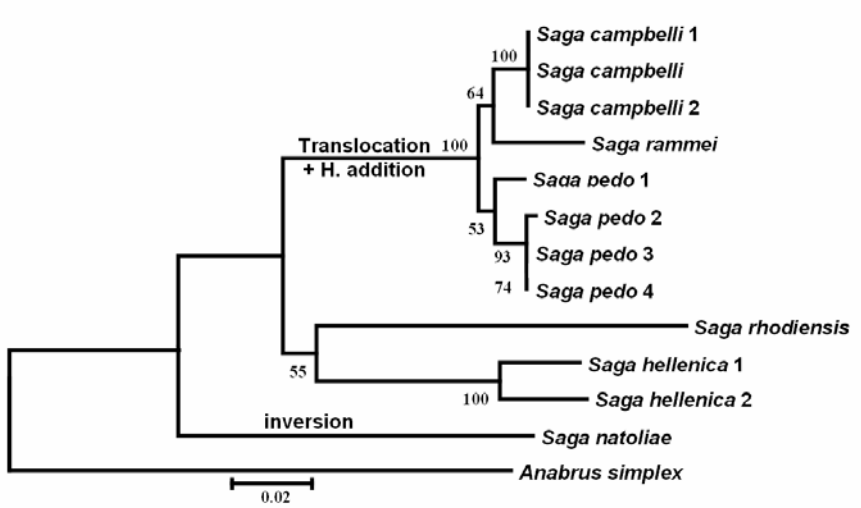

Figure 3. Phylogenetic tree based on the maximum likelihood method. Numbers above branches represent percentages of bootstrap values (1000 replicates). *A. simplex* sequence (accession number GU122265.1 in GenBank) was used as outgroup to root the tree. Bootstrap values below 50% were omitted. Chromosome changes between the different clades are indicated on the branches, except for *S. pedo*, for which all specimens have a highly derived pentaploid karyotype. H.: Heterochromatin.

chromosome rearrangements in parthenogenetic species. COI sequence data are compatible with the interpretation suggesting the existence of a common trunk, followed by a separation correlated with the geographic origin. Fortunately, the significance of their fairly low bootstrap value (53%) is strengthened by the good congruence with chromosome data. Because mitochondrial DNA is of maternal origin, thus independent on further fecundations, the differences between Balkans and French specimens is strictly related to the mutations accumulated since their separations. To estimate the age of separation, two different base substitution rates were considered: a divergence rate of 2.3% Myr, which has been obtained for mitochondrial genes in various organisms (e.g. Brower 1994, Carisio *et al*. 2004), and a divergence rate of 3.54% Myr for the *COI* gene, based on "known" dates (Papadopoulou *et al*., 2010). Considering the above molecular clocks, the separation between Balkans and French specimens might have roughly occurred 420.000 to 650.000 years ago.

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