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# Chromosomal similarities and differences among three sibling species of the *Acalles echinatus* group (Coleoptera, Curculionidae, Cryptorhynchinae)

DOROTA LACHOWSKA[1], MARIA ROŻEK[1] & MILADA HOLECOVÀ[2]

[1]*Institute of Systematics and Evolution of Animals Polish Academy of Science, Sławkowska 17, 31-016 Kraków, Poland. E-mail: dorota.lachowska-cierlik@uj.edu.pl*

[2]*Department of Zoology, Comenius University, Mlynská dolina B-1, 842-15 Bratislava, Slovakia*

## Abstract

In order to clarify the taxonomic position of three sibling species of weevils from the *Acalles echinatus* group, *A. echinatus, A. fallax* and *A. petryszaki*, cytogenetic relationships are investigated by studying the mitotic and meiotic chromosomes, including the localisation of heterochromatin by C-banding, as well as the localisation of NORs by silver impregnation. These sources of data are congruent and strongly support that the examined species are closely related. All examined species are characterised by a karyotype of the same chromosome number and sex determination system but with different morphology of chromosomes. All the analysed features, such as the centromeric index, relative length, C-bands and NORs, show that the structure of the karyotype of *A. echinatus* is more similar to that of *A. petryszaki*, whereas the karyotype of *A. fallax* is divergent. The higher chromosome number (2n = 30) in relation to the modal formula in Curculionidae (2n = 22) suggests that karyotype evolution in these species could have occurred by centric fissions of metacentric elements leading to acrocentry.

**Key words**: *Acalles*, C-bands, Coleoptera, Curculionidae, karyotype, NORs, sibling species

## Introduction

The genus *Acalles* Schoenherr, 1825 of the tribe Cryptorhynchini includes more than 300 species distributed in the Palaearctic, Nearctic, Neotropic, Australian and Oceanic regions (Alonso-Zarazaga & Lyal 1999). Up to the present, ninety taxa are known from Europe (Stüben *et al.* 2003). All Central European *Acalles* species are bisexual, apterous and largely characterised by their nocturnal activity (Smreczyński 1972, Dieckmann 1982). Due to their flightlessness and low vagility , they often live in isolated populations. The *Acalles echinatus* group (*sensu* Solari & Solari 1907) comprises 10 species, grouped together because of their triangular aedeagus but difficult to distinguish from each other (Stüben *et al.* 2003). Three of these, the close relatives - *Acalles echinatus* (Germar, 1824), *A. fallax* Boheman, 1844 (= *commutatus* Dieckmann, 1982) and *A. petryszaki* Dieckmann, 1982, live in Central Europe. The distribution ranges of *A. echinatus* and *A. fallax* are large and include Central, Western and the northern parts of Southern Europe, southern Scandinavia, the Balkan Peninsula and Eastern Europe (including the Caucasus), whereas that of *A. petryszaki* is limited to the Polish and Slovak Carpathians and Bulgarian mountains (Stara planina).The species are associated with deciduous and mixed forests and often occur together in the same locality – *A. echinatus* and *A. fallax* in the western part of Central Europe and *A. petryszaki* and *A. fallax* in the eastern part. Sympatry of *A. echinatus* and *A. petryszaki* has, however, not been recorded. These closely related species differ in the structure of their male genitalia and also in their ecology (microhabitat preference), *A. echinatus* and *A. petryszaki* being more thermophilous whereas *A. fallax* tolerates colder conditions and often occurs at higher altitudes. The first two species prefer forest of the oak–hornbeam vegetation tier, while *A. fallax* is more abundant in beech and fire–beech forests.

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Three species of *Acalles*, *A. camelus*, *A. fallax* and *A. echinatus*, and one species of the related genus *Ruteria* have so far been cytogenetically analysed. These studies were carried out on the chromosomes of meiotic stages (prophase I and metaphase I), and the results showed that the examined species have the same chromosome number (2n=30) and meioformula ($n♂=14+Xy_p$), but the structure of the karyotypes could not be described (Lachowska *et al.* 2001, 2004).

The aim of this study was to assess the cytogenetic relationships between three sibling species of the *Acalles echinatus* group in Central Europe and to compare the conclusions with the hypothesis of their relationship derived from taxonomic criteria. The karyotypes of *A. echinatus*, *A. fallax* and *A. petryszaki* are described, presenting the chromosomal morphology, C-banding pattern and silver nitrate impregnation (NORs).

## Materials and methods

Adult males were collected by sifting leaf litter and remnants of twigs in forested habitats of SW Slovakia and Poland during August–September 2005 and April–June 2007. *Acalles echinatus* was obtained in oak-hornbeam forests (*Querco-Carpinetum melicetosum uniflorae*) in Bratislava – Horský park (48°09'N, 17°05'E) and Devínska Kobyla (48°10'N, 16°59'E) near Bratislava (SW Slovakia), *A. fallax* in a beech forest (*Dentario bulbiferae – Fagetum*) near Lozorno (48°19'N, 17°03'E), Malé Karpaty Mts. (SW Slovakia), and *A. petryszaki* in thermophilous oak-hornbeam forests (*Querco-Carpinetum caricetosum pilosae*) in Czaszyn (49°26'N, 22°13'E), Beskid Niski Mts. (SE Poland), Medzilaborce – Kamenná hill (49°15'N, 21°54'E) and Udavské near Humenné (48°57'N, 21°56'E), Laborecká vrchovina hills (NE Slovakia). Voucher specimens of all species were deposited in the Institute of Systematics and Evolution of Animals PAS (Kraków, Poland).

Gonads were dissected under a stereomicroscope in several drops of hypotonic 0.9 % sodium citrate solution containing 0.005% colchicine. The gonads were transferred into a small volume of the same solution and incubated for 45–60 minutes at room temperature, after which they were fixed according to the method described by Rożek (1994), with minor modifications (Rożek & Lachowska 2001). C-banding was performed using the procedure described by Sumner (1972) as slightly modified by Lachowska *et al.* (2006). For the NOR silver staining, the method described by Howell & Black (1980) was used, again with some modifications (Lachowska *et al.* 2005). Chromosomes were classified according to Levan *et al.* (1964), and evaluation of chromosome morphology was based on ten mitotic metaphases (Table 1). Chromosome lengths were calculated as percentages of total chromosome length of the haploid set (% TCL). Spermatogonial metaphase, meiotic stages and interphase nuclei were analysed and photographed with a Nikon Eclipse 400 light microscope and CCD DS-U1 camera, using the software Lucia Image 5.0.

## Results

*Acalles echinatus*, *A. fallax* and *A. petryszaki* displayed spermatogonial metaphases I with $n♂ = 14+Xy_p$ meioformulas, and the karyotypes were asymmetric (Table 1). The karyotype of *Acalles echinatus* had three metacentric long pairs of autosomes of relative lengths of 12.65–10.62%, a fairly smaller submetacentric pair and ten acrocentric pairs with relative lengths of 5.64–3.41%. The X chromosome was a long metacentric element possesing a weaker-staining region on one of its arms, which was probably a site of NOR. This was clearly visible during the mitotic metaphase stage (Fig. 1A). The y chromosome was dot-like and of 1.80% relative length (Table 1, Fig. 1A). The diploid set of *A. fallax* included five pairs of long, metacentric autosomes of relative lengths 11.46–10.35% and arm ratios of 1.40–1.29. The remaining chromosomes were smaller, three pairs being metacentric and six pairs acrocentric with relative lengths 5.55–2.05%. The X chromosome was submetacentric and of medium size, whereas the dot-like y chromosome was the smallest element in the set (Table 1, Fig. 1B). In *A. petryszaki* the metacentric structure was detected in six pairs of

autosomes (relative length 11.10–9.15%) as well as in the X chromosome, whereas single pairs of submeta- and subtelocentrics were also recorded. The acrocentric structure was evident in six pairs of autosomes (Table 1, Fig. 1C). The analysis of meiotic plates demonstrated that the pattern of meiotic behaviour of the chromosomes was similar, the longest bivalents forming rings and the small bivalents presenting a rod-and-cross structure (Fig. 1E). Wide C-bands were found on chromosomes of *A. fallax*, which were visible also during mitotic prophases (Fig. 1F). In this species, the heterochromatin was located centromerically and terminally, whereas in *A. echinatus* the heterochromatic segments were smaller and located centromerically and intercalary (Figs. 1A). In *A. petryszaki*, centromeric heterochromatin appeared only on five autosomal pairs ($5^{th}$, $8^{th}$, $10^{th}$, $13^{th}$) (Figs. 1 C, D). The y chromosome was completely heterochromatic in *A. fallax* and *A. petryszaki* but in *A. echinatus* it also contained euchromatic parts (Figs. 1A, B, C). In *A. echinatus* and *A. petryszaki*, silver nitrate impregnation of chromosomes showed that they possess two NORs located on one autosomal pair and on their sex chromosomes. The presence of two labelled regions was detected in mitotic prophase and meiotic stages from leptotene to diakinesis, but in metaphase I only one was visible on the heterovalent $Xy_p$ (Figs. 1G, 2 A–C, E). The pachytene nucleus of spermatocytes in *A. fallax* exhibited three NORs, two on autosomal bivalents and one on the sex chromosomes (Fig. 2D).

**TABLE 1.** Relative length (% TCL) and centromeric index (AR) of particular chromosome pairs of three *Acalles* species during mitotic metaphase.

| Pair no. | *Acalles echinatus* | | *Acalles fallax* | | *Acalles petryszaki* | |
|---|---|---|---|---|---|---|
| | %TCL | AR | %TCL | AR | %TCL | AR |
| 1 | 12.65 | 1.84 | 11.46 | 1.40 | 11.10 | 1.30 |
| 2 | 12.05 | 1.00 | 10.95 | 1.39 | 10.48 | 1.92 |
| 3 | 10.62 | 1.50 | 10.53 | 1.18 | 10.13 | 1.18 |
| 4 | 5.64 | 1.92 | 10.47 | 1.37 | 6.77 | 3.58 |
| 5 | 5.50 | - | 10.35 | 1.29 | 6.70 | 1.05 |
| 6 | 5.35 | - | 5.55 | 1.03 | 6.20 | - |
| 7 | 4.94 | - | 5.52 | 1.31 | 5.95 | 1.21 |
| 8 | 4.80 | - | 4.90 | 1.55 | 5.27 | 1.23 |
| 9 | 4.73 | - | 4.32 | - | 5.17 | - |
| 10 | 4.25 | - | 4.25 | - | 4.97 | - |
| 11 | 4.04 | - | 3.52 | - | 4.73 | 1.19 |
| 12 | 3.83 | - | 3.45 | - | 4.41 | - |
| 13 | 3.41 | - | 3.43 | - | 4.25 | - |
| 14 | 3.41 | 1.10 | 2.53 | - | 2.85 | - |
| X | 12.98 | 1.20 | 6.54 | 1.95 | 9.15 | 1.09 |
| y | 1.80 | - | 2.05 | - | 1.98 | - |

## Discussion

The three analysed species of the *Acalles echinatus* group are characterised by having karyotypes with the same chromosome numbers and sex determination systems, of the formula $n♂=14+Xy_p$, however the morphology of their chromosomes is different. In the majority of chromosomes, *A. echinatus* possesses centromeres in a terminal position, in contrast to the autosomes of *A. fallax* and *A. petryszaki*, which show a tendency towards metacentry. Some differences were also observed on the sex chromosomes, the X

chromosome in *A. echinatus* and *A. petryszaki* being equal in size to the longest autosomes, while in *A. fallax* it is one of the medium- sized elements. In all species the y chromosomes are dot-like, as commonly found in a "parachute" association ($Xy_p$). The behaviour of the chromosomes during meiosis is also similar, the longest bivalents forming rings and the small bivalents presenting a rod-and-cross structure.

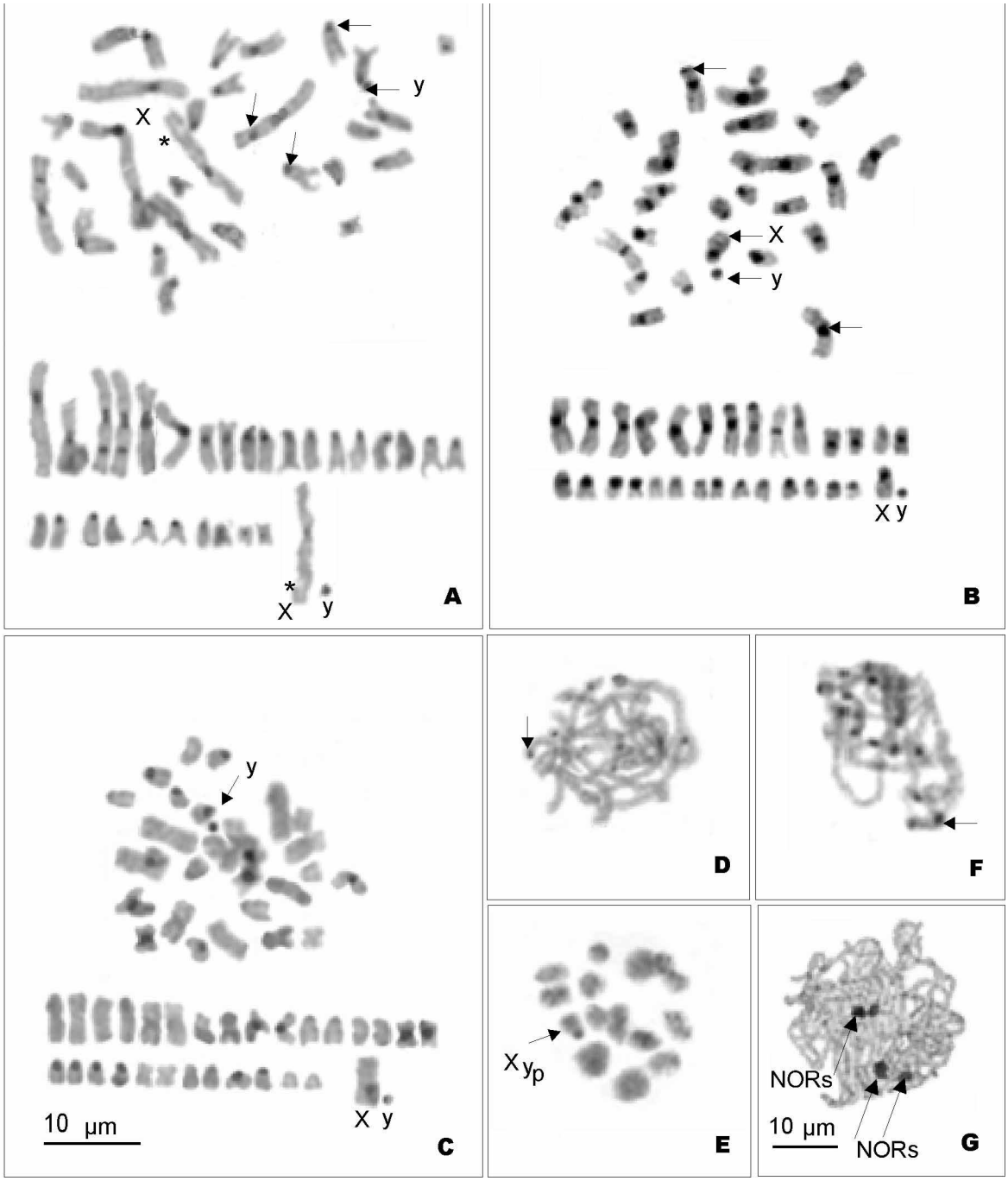


**FIGURE 1.** Mitotic and meiotic chromosomes of *Acalles echinatus*, *A. fallax* and *A. petryszaki* with C and AgNORs bands (stars indicating secondary constriction on X chromosome, unlabelled arrows C-bands). (A) Karyogram of *A. echinatus*, showing pericentromeric C-bands on all chromosomes and intercalary C-bands on second pair of autosomes. (B) Karyogram of *A. fallax*, showing pericentromeric C-bands on all chromosomes (lower arrow) and terminal C-bands on fourth pair of autosomes (upper arrow). (C) Karyogram of *A. petryszaki*, showing pericentromeric C-bands on five autosomal pairs. (D) Pachytene of *A. petryszaki*. (E) Meiotic metaphase I of male *A. petryszaki.* (F**)** Pachytene of male *A. fallax*, showing wide C-bands. (G) Mitotic prophase of *A. echinatus*, showing large nucleolar organizer regions.

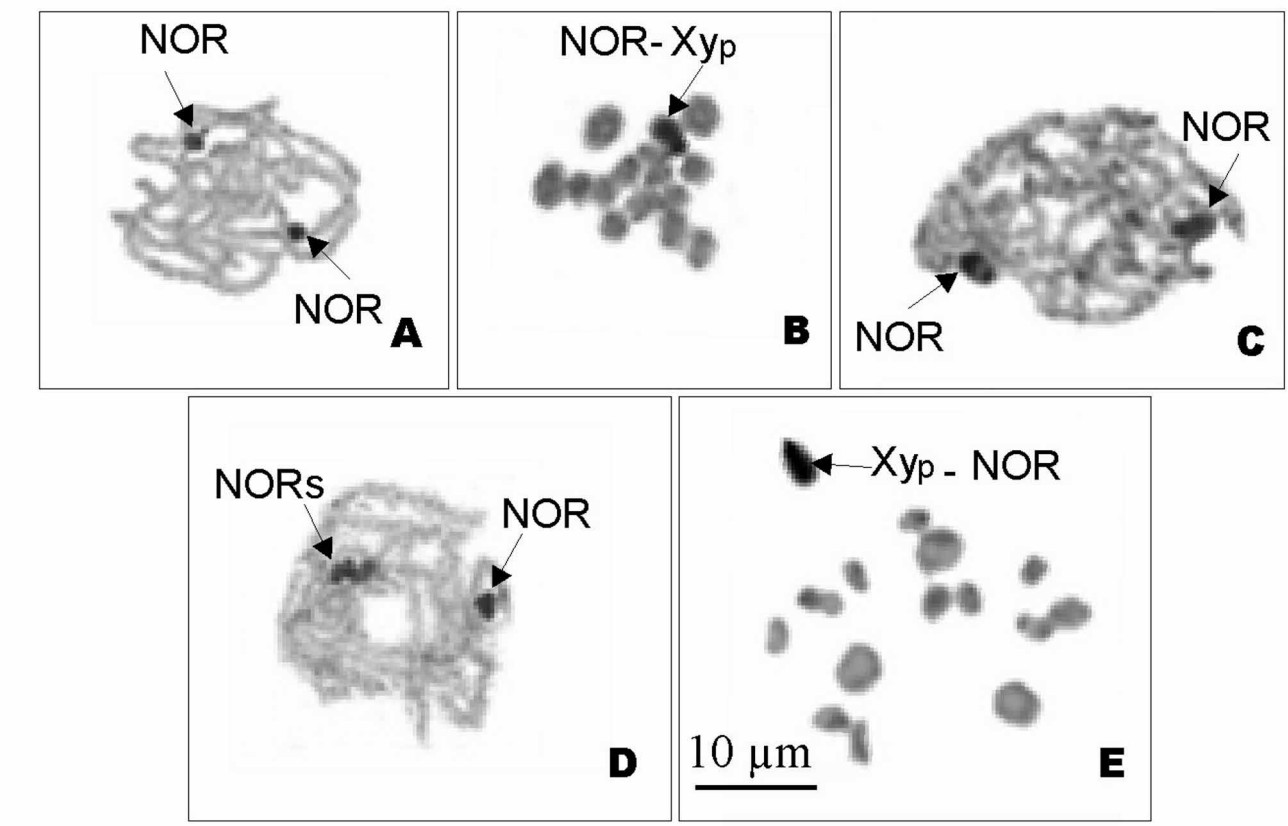


**FIGURE 2.** Nucleolar organizer region (NOR) pattern obtained after silver impregnation. (A) Pachytene of *A. echinatus*, with two NORs. (B) Metaphase I of *A. echinatus*. (C) Pachytene of *A. petryszaki*, with two NORs. (D) Pachytene of *A. fallax*, with three NORs. (E) Meiotic metaphase I of male *A. petryszaki*, showing impregnated sex bivalent ($Xy_p$).

In the family Curculionidae, 2n=22 is considered an ancestral karyotype (Smith & Virkki 1978), so that in *Acalles* the increase in chromosome number is likely to have arisen by centric fissions of metacentric elements, leading to acrocentry. In Curculionidae, C-heterochromatin mainly occurs in small proportions and is located in a centromeric position (Rożek *et al.* 2004, Lachowska *et al.* 2005). The examined species of *Acalles* possess different amounts of heterochromatin. Wider C-bands located centromerically and terminally are found in *A. fallax*, whereas in *A. echinatus* the heterochromatic segments are smaller and located centromerically and intercalary and *A. petryszaki* possesses small amounts of heterochromatin only on 11 chromosomes. C-banding karyotypes have occasionally been used for identification of closely related species in other coleopteran groups, e.g. Carabidae, Aphodiinae, Hydrophilidae etc., in which conventional staining techniques often provide insufficient information (Angus *et al.* 2000, Wilson & Angus 2004). These results confirm that the examined species of the *Acalles echinatus* group have a very similar external chromosomal structure but differ in their karyotype (e.g. the number of long metacentric chromosomes, length of X chromosomes, localisation and numbers of NORs) and the amount of heterochromatin and its position on individual chromosomes (pericentromeric, almost terminal-subterminal, intercalary). It is evident that the structure of the karyotype of *A. echinatus* is more similar to that of *A. petryszaki*, whereas that of *A. fallax* is divergent. A preliminary study of mitochondrial DNA data (fragments of cytochrome oxidase II gene) showed the greatest divergence to be between *A. echinatus* and *A. fallax* (14.4%) and the lowest between *A. fallax* and *A. petryszaki* (9%) (Lachowska, unpublished). In summary, all karyological evidence supports the placement of these three sibling species in one group, as suggested by taxonomic study (Stüben *et al.* 2003).

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