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Chromosomes and identification of the sibling species *Pterostichus nigrita* (Paykull) and *P. rhaeticus* Heer (Coleoptera: Carabidae)

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Abstract. The karyotypes of *Pterostichus nigrita* (Paykull) and *P. rhaeticus* Heer are described. Both species have eighteen pairs of autosomes, sex chromosomes which are XO (♂) and XX (♀), plus a variable number of totally heterochromatic B chromosomes. Males may be identified by the form of the inflated endophallus, but the shape of the right paramere is not a reliable character. Females may be identified by the form of the eighth abdominal sternite.

Introduction

Koch & Thiele (1980) reported laboratory crossing experiments on German populations of *Pterostichus nigrita* (Paykull) from Cologne and Rees (Niederrhein) which demonstrated their complete inability to cross, even after attempts at copulation, although both these populations could breed in the laboratory. They therefore concluded that two sibling species were involved. Later, Koch (1984) reported detailed morphological investigation of the two populations and of relevant type material and showed that the Cologne material was referable to *P. rhaeticus* Heer, whereas that from Rees was true *P. nigrita*. Koch described differences in the form of the male endophallus and right paramere of the two species and the eighth abdominal sternite of females. She described the female lectotype of *Carabus nigrita* Paykull (*Pterostichus nigrita*), including the form of the eighth abdominal sternite, as well as the male lectotype of *Pterostichus rhaeticus* Heer, including details of both the endophallus and the right paramere. The details of the eighth sternite and the endophallus show clearly that these type specimens belong to the two species as currently interpreted. Koch (1986) reviewed this work and drew attention to the different chromosome numbers in the two species, which had been reported by Nettmann (1976). Nettmann, using testis as his source of dividing cells, found that all '*P. nigrita*' from upland *Sphagnum* bogs had forty-six chromosomes, including two very small ones, whereas three beetles from a woodland swamp had only forty chromosomes and lacked the very small ones. In both forms Nettmann recorded the results as indicating XY sex chromosomes. Nettmann added that the upland material corresponded with Heer's *P. rhaeticus.* Koch (1986) illustrated nuclei from Rees and Cologne material, showing forty and forty-six chromosomes, respectively. The first indication that Nettmann's results did not give a complete account of the chromosomes of these species came from work by Serrano (1981) on Spanish *P. nigrita*, which showed males to have either forty-one or forty-three chromosomes, indicating an XO sex chromosome system. These results are amplified by those of Galián *et al*. (1992), who obtained values of forty, forty-six and fifty-one for Spanish *P. nigrita.* They interpreted the first two numbers as implying an XY system of sex chromosomes, while for the fifty-one chromosome karyotype they showed an unpaired X. They commented on differences in the size of the largest chromosome, which they suggested might be the X chromosome.

Initial attempts by the senior author to resolve the situation, using techniques found to be successful with aquatic Coleoptera (Angus, 1982; Shaarawi & Angus, 1991), resulted in failure. In no case was it possible to pair all the chromosomes in a nucleus (even allowing for an unpaired X chromosome in males), even though many of the chromosome clusters appeared to be the contents of single undamaged nuclei, with evenly rounded outlines and without other chromosomes (either singly or in clusters) nearby on the slide.

A student project done with Rebecca Brown in 1994 led to the discovery of heterochromatic, largely unpaired, B chromosomes and established the karyotype of *P. rhaeticus*. Similar work done with Leonie Bryant in 1997 established the karyotype of *P. nigrita*, and also demonstrated a degree of variation in that of *P. rhaeticus* sufficient to suggest that there might be no consistent difference in the chromosome numbers of the two species. The chromosomal variation encountered was sufficient to question the reliability of the morphological features used to separate the two species.

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The present study was conducted to establish which morphological features give reliable identification of males and females of the two species, to compare the basic karyotypes (without the B chromosomes) of the species and see whether there are any interspecific differences, and to compare the numbers and morphology of the B chromosomes of the two species.

Materials and methods

The sources of the material used in this investigation are given in Table 1. For British material the Watsonian vice-counties (V.C.) are used, as outlined by Dandy (1969). In all, about ninety specimens were processed, with clear karyotypes obtained from about half the material.

Morphological characters

Throughout this study males were identified by the form of the inflated endophallus, as this character gives a clear and consistent separation of the two species. Females were identified by the form of the sclerotized parts of abdominal sternite eight.

For males the aedeagophore was removed from the abdomen, and the parameres were separated from the aedeagus. Aedeagal endophalli were inflated by means of a glass microelectrode fixed over the needle of a hypodermic syringe and inserted into the hole at the base of the aedeagus. Initial inflation was by injection of distilled water, and this was followed by injection of absolute ethanol, with the aedeagus placed in a covered watch glass of 80% ethanol and kept for 12 h. This stage is critical if scanning electron micrographs are required. The endophallus must be inflated before it becomes fixed by the alcohol. The most usual problem is that the apical section, by the gonopore lips, remains partly retracted. Inflated endophalli were held on the microelectrode in the fixative for about 2 min, with occasional reinflation if required, until they turned white due to the fixation of the tissues. They were then released. Aedeagi were checked during the first 2 h and reinflated if necessary. After 12 h aedeagi were transferred to absolute ethanol (reinflated if necessary), before storage for a few days (at least) in a tube of absolute ethanol. They were then transferred to acetone before being critical-point dried and mounted on electron microscope stubs and coated with gold, using a sputter-coater.

Aedeagophores of older, dried preparations were softened by soaking in 1% sodium hydroxide solution. This was usually successful, but not suitable for scanning electron microscopy as the preparations generally collapsed on critical-point drying. However, these preparations sometimes show the apical section of the ductus ejaculatorius, extruded through the gonopore lips, in both species.

The right parameres have been traditionally used for identifying males of these species, and Luff (1990) devised a system of measurements to facilitate this. We refined and attempted to standardize Luff's system as follows. Parameres were glued to cards, as flat as possible, with the inner (concave) faces uppermost. Longitudinal orientation was determined by having the card horizontal in the longitudinal plane. Transverse orientation was determined by rotating the specimen transversely until the base and apex of the ventral margin of the apical section of the paramere were in line with its inner basal angle (Fig. 1A,B). There was some variation in the presentation as the concavity of the apical, expanded section varies so that the degree to which the apical and basal ends of the ventral margin are raised also varies. At this stage outline drawings of parameres were made, using a camera lucida. Linear measurements were made of the length from the apex of the apical section to its basal angle (Fig. 1A: L) and the median width (Fig. 1A: MW), taken along a line drawn at right angles to the L line, halfway down its length. The apical angle was also measured, which presented another problem. Luff (1990) noted that in *P. nigrita* the outer margin of the paramere is generally rounded before the apical angle, and established the angle by continuing the line of the margin as a straight line from a point before it becomes rounded, and measuring the

Table 1. Sources of material used.

Country		Species
England	V.C. 11 (South Hampshire): New Forest, Matley, Bog	*P. rhaeticus*
	V.C. 15 (East Kent): Maidstone, Mote Park	*P. nigrita*
	V.C. 15: Hothfield Common	*P. rhaeticus*
	V.C. 17 (Surrey): Ockham Common	*P. nigrita*; *P. rhaeticus*
	V.C. 17: Langham Pond, Runnymede	*P. nigrita*
	V.C. 22 (Berkshire): Cothill Nature Reserve	*P. nigrita*
	V.C. 27 (East Norfolk): Catfield Fen	*P. nigrita*; *P. rhaeticus*
	V.C. 61 (South-East York): Skipwith Common	*P. rhaeticus*
Scotland	V.C. 75 (Ayrshire): Hule Moss	*P. rhaeticus*
	V.C. 106 (East Ross): Strathconon	*P. nigrita*; *P. rhaeticus*
	V.C. 106: Moy Bridge	*P. rhaeticus*
Czech Republic	Jizerské Mts, Nova Louka	*P. rhaeticus*
Sweden	Ångermanland, Mullsjö	*P. rhaeticus*

angle at which it meets line L, which may be extended beyond the apex of the paramere (Fig. 1A,B). We standardized this procedure by defining the point on the outer margin that sets the angle as being at a distance of about two-thirds the length of L from the apical angle, and applied this to both species. Comparison of our data (Fig. 5) with Luff's (1990: Fig. 2) showed that the main result of this appears to be to reduce the maximum apical angle found in *P. rhaeticus*. Thus, our maximum value was 63°, whereas Luff reported values in excess of 90°. This may be checked by applying our system to Fig. 1d,e of Luff (1990). Our system gives values of about 70°, whereas direct measurement of the apical angle gives a value of about 90° in Luff's Fig. 1e, where the three points used to establish the transverse orientation are clearly not in line and the direct measurement would not use the line L at all. In support of our system, we felt that exclusion of at least some *rhaeticus*-type parameres and use of direct measurements instead, would prejudge their identity, making the measurements at best superfluous. Also, direct measurement of the apical angle is very sensitive to the orientation of the paramere, especially in the longitudinal plane. We prepared scanning electron micrographs of selected parameres, from specimens identified by the endophallus, by transferring the parameres to stubs and orientating them as described.

Females of the species traditionally have been recognized by the form of the sclerotized part of abdominal sternite eight. Luff (1990) set out a system of measurements for this character, and we followed this (Fig. 1C). There was no difficulty in obtaining these measurements as the sternite is virtually flat. Koch (1986) described differences in the bursa copulatrix of the two species, noting in particular the larger size of the bursa in *P. nigrita*, where the sclerotized part has longitudinal folds at each side. Abdomens of dried females were softened by boiling briefly in 1% sodium hydroxide solution, flesh was removed from the chitinous tissue and the bursa and gonocoxae were dissected out. The folds of the sclerotized part (where present) were readily visible in water, but the size was difficult to establish in these cleared preparations. We mounted some bursae and gonocoxae in water-soluble DMHF mountant (Steedman, 1958) on slides for photography, and found that use of the high-contrast chromosome film gave clear pictures.

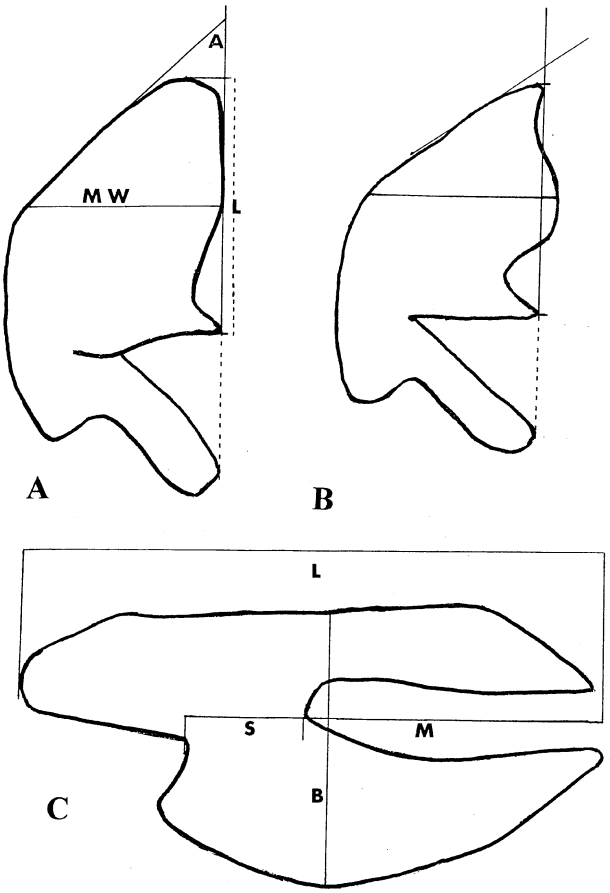


Fig. 1. Measurements of taxonomic characters. A, *P. nigrita*, right paramere; B, *P. rhaeticus*, right paramere; C, *P. rhaeticus*, left half of abdominal sternite 8 of female. A = apical angle; L = length (of apical portion of paramere: A, of half sclerite: C); MW = median width; B = breadth; M = length of membranous strip; S = distance from membranous strip to start of basal lobe.

Chromosomes

Chromosome preparations were obtained from mid-gut crypt, testis and ovary using the methods developed by Angus (1982) and Shaarawi & Angus (1991), with the

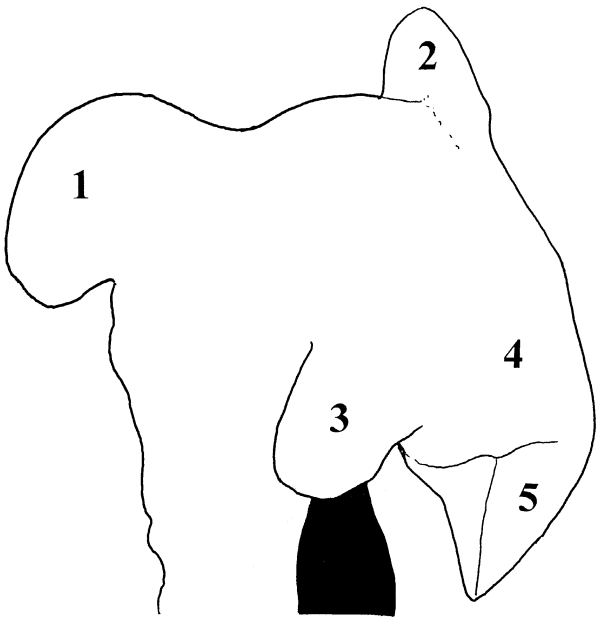


Fig. 2. Everted endophallus of *P. nigrita*, viewed from the left, to show the principal features. 1 = basal lobe; 2 = apical lobe; 3 = ventrolateral lobe; 4 = gonopore field; 5 = gonopore lips. Apex of aedeagus in black.

colchicine and hypotonic potassium chloride treatments both lasting 12.5 min. Following the discoveries made in the course of Rebecca Brown's project, all preparations were treated for C-banding (Sumner *et al*., 1971), using two-day old slides. We found that a 5-min treatment in barium hydroxide at 25–28°C gave the best results. If the C-banding had not developed, the chromosomes could be destained by immersion for 1 min in salt-sodium citrate (SSC) at 60°C, and giving an additional 2-min treatment in barium hydroxide followed by 1 h in SSC. Preparations were photographed under immersion oil (no cover slip), using an interference filter to give monochromatic green light, on Agfa Copex Rapid AHU microfilm. It was felt that the accuracy of pairing the chromosomes in the karyograms (Fig. 8) was sufficient to allow calculation of their Relative Chromosome Lengths (RCL). The centromere indices were not calculated because, with the exception of chromosome 1 (submetacentric), all the chromosomes are more or less metacentric.

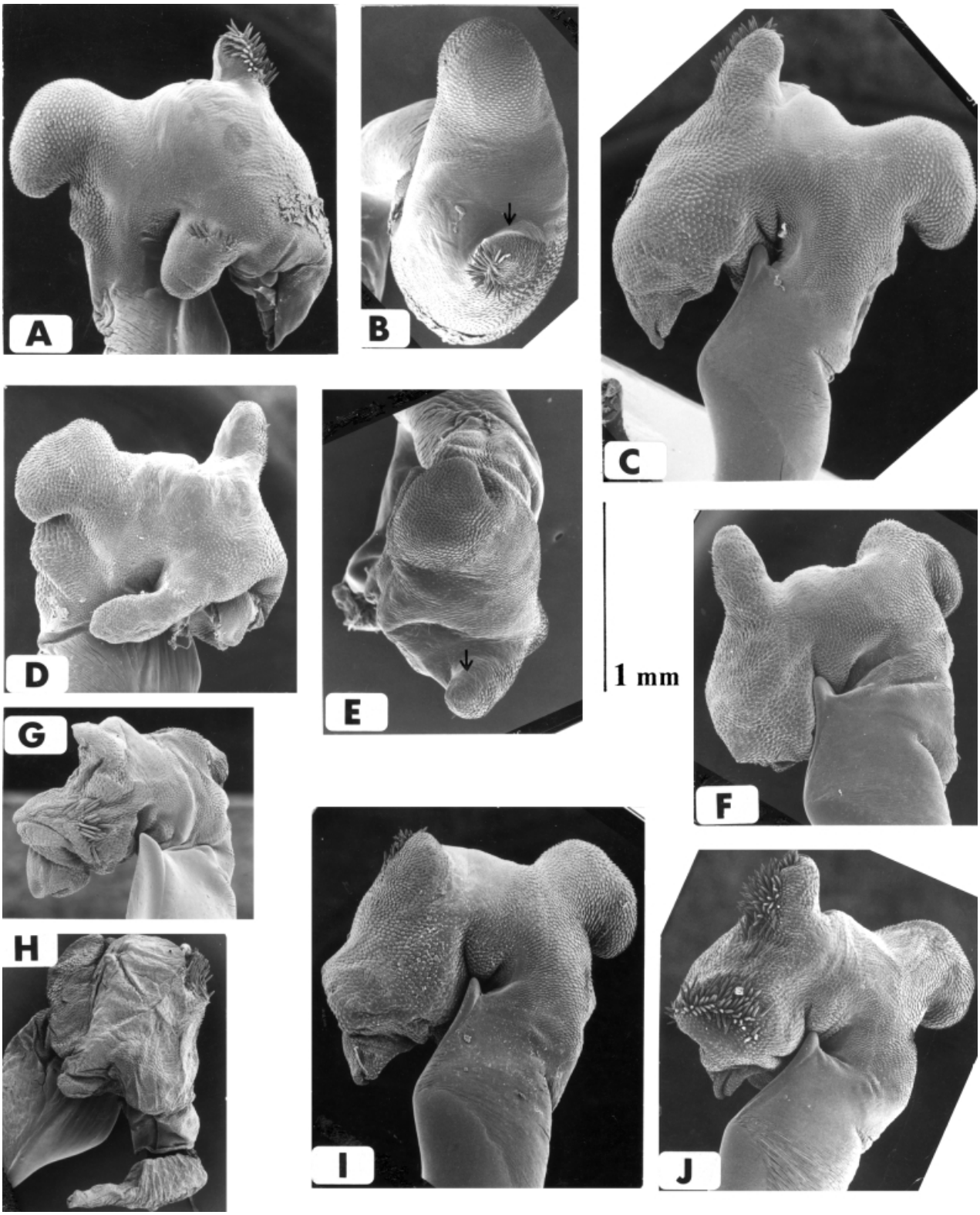
Results

Taxonomic characters

The basic structure of the endophallus of *P. nigrita* is shown in Fig. 2. The salient features are two dorsal lobes (basal and apical), one ventrolateral lobe (on the left side of the endophallus) and the gonopore field, i.e. the apical part approaching the somewhat sclerotized gonopore lips, which appear to consist of four main elements, although these are not clearly demarcated from one another. The apical section of the ductus ejaculatorius is shown in Figs 3H and 4E. This was not figured by Koch (1984, 1986), but would seem to be necessary for the apex to reach the entrance to the spermathecal duct (see the account of females). The endophallus of *P. nigrita* (Fig. 3) exhibits considerable variation in chaetotaxy. Chaetae may be absent (Fig. 3D,F), or they may be present on the apical lobe (Fig. 3A–C,H–J), the basal part of the ventrolateral lobe (Fig. 3A), either alone or in addition to the chaetae on the apical lobe, or they may be present on the right side of the gonopore field, either alone (Fig. 3G) or with chaetae on the apical lobe (Fig. 3J). The endophallus of *P. rhaeticus* is shown in Fig. 4A–F. As noted by Koch (1984, 1986), the principal differences between the endophallus of *P. rhaeticus* and *P. nigrita* are the smaller size and less prominent apical section (with the apical lobe and gonopore field) in the former. However, the two species also differ in the development of the upwardly directed apical lobe. In *P. nigrita* this lobe is much larger and is on the right side of the endophallus (very clear in dorsal view, Fig. 3B,E, arrow). *Pterostichus rhaeticus* has a much smaller apical lobe on the left side of the endophallus (Fig. 4B, arrow) as well as a small laterally directed protuberance on the right side of the gonopore field (Fig. 4B–D,F, double arrow). This protuberance occupies the same position on the endophallus as the apical lobe of *P. nigrita* and appears to be homologous with it. The endophallus of *P. nigrita* has no trace of a separate lateral protruberance on the right side of the gonopore field (Fig. 3C,F,I,J), nor any vestige of a projection on the left side, above the ventrolateral lobe (Fig. 3A,D). In *P. rhaeticus*, the northern material so far examined (Skipwith Common, Scotland, and Sweden) is completely without chaetae, but southern material may have chaetae on the right side of the gonopore field (Fig. 4D,F). The association of these chaetae with the protuberance at the base of this area supports the homology of this protuberance with the apical lobe of *P. nigrita*, rather than homology of the apical lobes of the two species. Thus, Fig. 4F shows *P. rhaeticus* with chaetae above the protuberance, suggesting comparison with the apical lobe of the *P. nigrita* shown in Fig. 3C,I, whereas Fig. 4D shows *P. rhaeticus* with chaetae in the area above the protuberance, and also extending down the right gonopore field, as shown for *P. nigrita* in Fig. 3J. Some New Forest material has a few chaetae on the apical lobe on the left side of the endophallus. One feature of these chaetae which must be emphasized is that they are very conspicuous in both species, i.e. dark brown (almost black) in sharp contrast to the rest of the endophallus, which is transparent (white when fixed) with, at most, a yellowish or brownish suffusion by the gonopore lips or on the fine scales near the basal lobe.

The length/median width of right parameres of specimens identified by their endophalli, plotted against the apical angle, is shown in Fig. 5, with the line separating the *P. nigrita* and *P. rhaeticus* zones taken from Luff (1990). Figure 4G–M illustrates some of the specimens. A number of specimens of *P. rhaeticus* fall within the *P. nigrita* zone (mostly fairly near the separation line), whereas one *P. nigrita*, plotted as point no. 2 in Fig. 5, falls well within the *P. rhaeticus* zone, having the third largest apical angle measured in the present study. This paramere (Fig. 4I) came from one of three males taken on Ockham Common and processed the same day. The immediate objective was study of the endophallus and parameres, so the form of both these characters was noted at the time, and the parameres were mounted on cards with the beetles until required for scanning electron micrography. At this stage all the *P. nigrita* parameres used were mounted on one stub, and all the *P. rhaeticus* parameres on another, avoiding any

Fig. 3. Scanning electron micrographs of everted endophalli of *P. nigrita.* A,D,H, Left side view; B,E, dorsal view (apex directed downwards, apical lobe indicated by an arrow); C,F,G,I,J, right side view; A–C, specimen from Cothill, with setae on the apical lobe and at the base of the ventrolateral lobe; D–F, specimen from Catfield Fen, with no setae and with the gonopore lips not fully everted; G, specimen from Strathconon, partially collapsed and with setae on the right side of the gonopore field; H, specimen from Ockham Common, everted following softening with sodium hydroxide, collapsed but with the apical section of the ejaculatory duct everted through the gonopore lips; I, specimen from Ockham Common, whose right paramere is shown in Fig. 4I; I, specimen from Catfield Fen, with setae on the apical lobe and right side of the gonopore field.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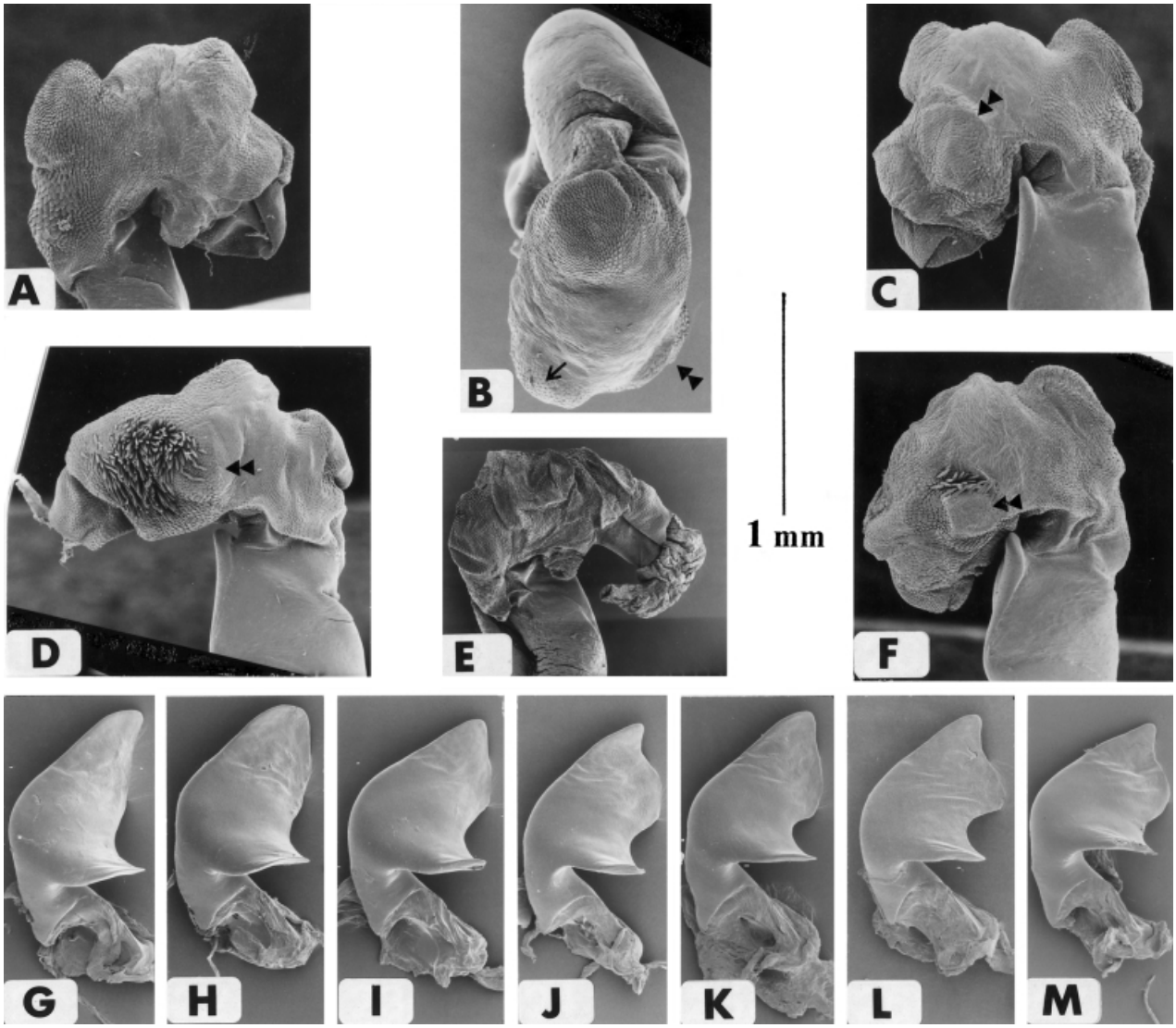

Fig. 4. Scanning electron micrographs of male genitalia. A–F, Everted endophalli of *Pterostichus rhaeticus*; G–M, right parameres of specimens identified by examination of the endophallus, inner view, apex directed upwards. A,E, Left side view; B, dorsal view (apex directed downwards, apical lobe indicated by an arrow, lateral protuberance by a double arrow); C,D,F, right side view, lateral protuberance indicated by a double arrow. A–C, Specimen from Ockham Common, with no setae; D, specimen from Ockham Common with well developed setal area on right gonopore field; E, specimen from Mullsjö, everted following softening with sodium hydroxide solution, with no setae, and collapsed but with the apical section of the ejaculatory duct everted through the gonopore lips; F, specimen from Ockham Common with a small setal area on the right gonopore field. G–I, *P. nigrita,* J–M, *P. rhaeticus.* G, From Catfield Fen (Fig. 5: point 8); H, from Cothill (Fig. 5: point 5); I, from Ockham Common (Fig. 5: point 2; endophallus: Fig. 3I); J, from Ockham Common (Fig. 5: point 11); K,L, from Moy Bridge (Fig. 5: points 17, 16); M, from Skipwith Common (Fig. 5: point 15).

confusion between the species. Figure 4K shows a *P. rhaeticus* paramere from Moy Bridge, which not only falls within the range of *P. nigrita* (Fig. 5, point 17), but also looks like a *P. nigrita* paramere because it has a narrow apical angle and less transverse apical section. Figure 4L shows a more 'typical' *P. rhaeticus* paramere from Moy Bridge, and this one falls within the range of *P. rhaeticus*, albeit near the line of separation (Fig. 5, point 16). The inescapable conclusion is that although there appear to be two forms of paramere (the α and β forms of Bucciarelli & Sopracordevolle, 1958), their separation is not always clear, and, even when clear, it does not always correspond to the separation of the two species. In short, in critical (mixed) populations, the right paramere is not a reliable means of identifying specimens of the two species.

The bursa copulatrix and gonocoxae of the two species are shown in Fig. 6A–C. Features to note are the sclerotized area of the bursa (scl), which lies on its ventral surface and, in *P. nigrita*, has conspicuous lateral longitudinal folds (Fig. 6A: lf). The sclerotized area of the bursa of *P. rhaeticus* (Fig. 6B) is smaller and lacks the lateral folds. The oviduct (Fig. 6B: od) runs into the dorsal face of the bursa more or less at its middle, and the spermathecal duct (Fig. 6A: sd) runs into the dorsal

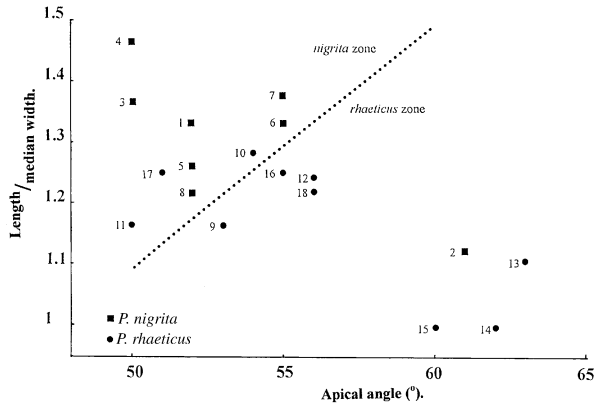

Fig. 5. Right paramere of *P. nigrita* and *rhaeticus*, specimens identified by the endophallus, length over median width plotted against the apical angle. Based on Luff (1990), with the measurements modified as described in this paper. The line separating the *P. nigrita* and *P. rhaeticus* zones is from Luff. 1–8, *P. nigrita*; 9–18, *P. rhaeticus.* 1, Ockham Common; 2, Ockham Common (Fig. 4I); 3, Langham Pond; 4, Maidstone; 5, Cothill (Fig. 4H); 6,7, Cothill; 8, Catfield Fen (Fig. 4G), 9,10, Ockham Common; 11, Ockham Common (Fig. 4J); 12, Catfield Fen; 13,14, Hothfield Common; 15, Skipwith Common (Fig. 4M); 16,17, Moy Bridge (Fig. 4L,K); 18, Mullsjö.

part of the oviduct just before its opening into the bursa. It therefore seems that the apical section of the male ductus ejaculatorius, protruded through the gonopore lips (Figs 3H, 4E), is necessary for contact to be made with the spermathecal duct. The spermathecal duct is sclerotized just before its connection with the oviduct and this sclerotized section (Fig. 6A: scls) is consistently smaller in *P. nigrita* than in *P. rhaeticus*, even though other features of the bursa copulatrix of *P. nigrita* are larger. In *P. rhaeticus*, the sclerotized section is about 0.35 mm long and about the same length as the apical segment of the gonocoxa (Fig. 6B: gcx), whereas in *P. nigrita* it is about 0.25 mm long, about half the length of the apical segment of the gonocoxa.

The left half of abdominal sternite eight of females identified from the bursa copulatrix is shown in Fig. 6D–K, and the L/B against M/S plots are shown in Fig. 7. In this case there is no disagreement between the results obtained from sternite eight and those from the bursa copulatrix. The only points to note are that some more deep-bodied sternites of *P. rhaeticus* (Fig. 7H,K) could at a glance be mistaken for those of *P. nigrita*, although not if measured; and in some specimens the inner part of the membranous strip is somewhat darkened and mottled (Fig. 7K). This mottled area belongs to the membrane.

Chromosomes

Mitotic chromosomes of *P. nigrita*, arranged as karyograms, are shown in Fig. 8 A–H, and those of *P. rhaeticus* are shown in Fig. 8(I–V). In both species, the karyotype comprises eighteen pairs of autosomes (A chromosomes), sex chromosomes that are XX (♀) and XO (♂) and a variable number of B chromosomes. The Relative Chromosome Lengths (RCL) of the autosomes and X chromosomes are listed in Table 2. The autosomes are arranged in order of decreasing RCL, with the centromeric C-bands used to help match up homologous chromosomes. There appear to be consistent differences in the sizes of the centromeric C-bands of different chromosomes, despite differences in the intensity of the banding in different preparations (Fig. 8M,N). Details of the apparent C-band position may differ in preparations from the same specimen. Thus, in the ovarian preparation shown in Fig. 8J, the C-bands of chromosomes 6, 9, 10, 12 and 15 are clearly not located symmetrically in the centromere constriction, but in a mid-gut preparation from the same specimen (Fig. 8K) they appear to coincide with the centromere constrictions. The C-bands are somewhat heavier in Fig. 8K, but the sequence of relative sizes of the bands along the rows of chromosomes is the same in both preparations.

The sequence of chromosomes is intended to be the same in both species, but the results must be treated with caution as some confusion between chromosome pairs of similar size must be expected. Because of this, differences between the RCL values obtained for the two species are only considered if the chromosome is sufficiently distinctive (chromosome 1) or the differences cannot be eliminated by rearranging the karyotypes (chromosomes 14–18). Chromosome 1 has a small centromeric C-band, and the long arm is generally darkened following C-banding, although not always (Fig. 8G,U). Unbanded preparations (Fig. 8L) suggest that this darkening is associated with a secondary constriction, perhaps the site of a nucleolus organiser. In some specimens there appears to be a size polymorphism in this chromosome (Fig. 8C,E,O,P,R), whereas in others the two replicates are the same length, either long (Fig. 8N) or shorter (Fig. 8V). It is not clear whether there is a polymorphism in the amount of C-banding material present that is associated with the secondary constriction, or whether the size variation merely reflects differences in the expansion and contraction of the constriction. In either case, chromosome 1 appears to have a significantly greater RCL in *P. nigrita* than in *P. rhaeticus*. Chromosomes 14–18 appear to have significantly lower RCL values in *P. nigrita* than in *P. rhaeticus*.

The heterochromatic B chromosomes are a striking and characteristic feature of the karyotypes of both species. In C-banded preparations of mitosis they appear conspicuously and more or less uniformly dark, in contrast to the pale autosomes and X chromosomes, where C-bands are confined to the centromere region and the secondary constriction of chromosome 1 (Fig. 9B,C). The number and form of the B chromosomes vary widely in both species. It should be stressed that the RCL values for the B chromosomes are consistent in different nuclei from the same specimen (e.g. Fig. 8J,K), so the observed differences in size are genuine, not artefacts. In *P. nigrita*, the number of B chromosomes so far encountered ranges from three (Fig. 8B,C,F), giving a male chromosome number of forty, to eight (Fig. 8E), giving a male chromosome number of forty-five. The RCL values of the B

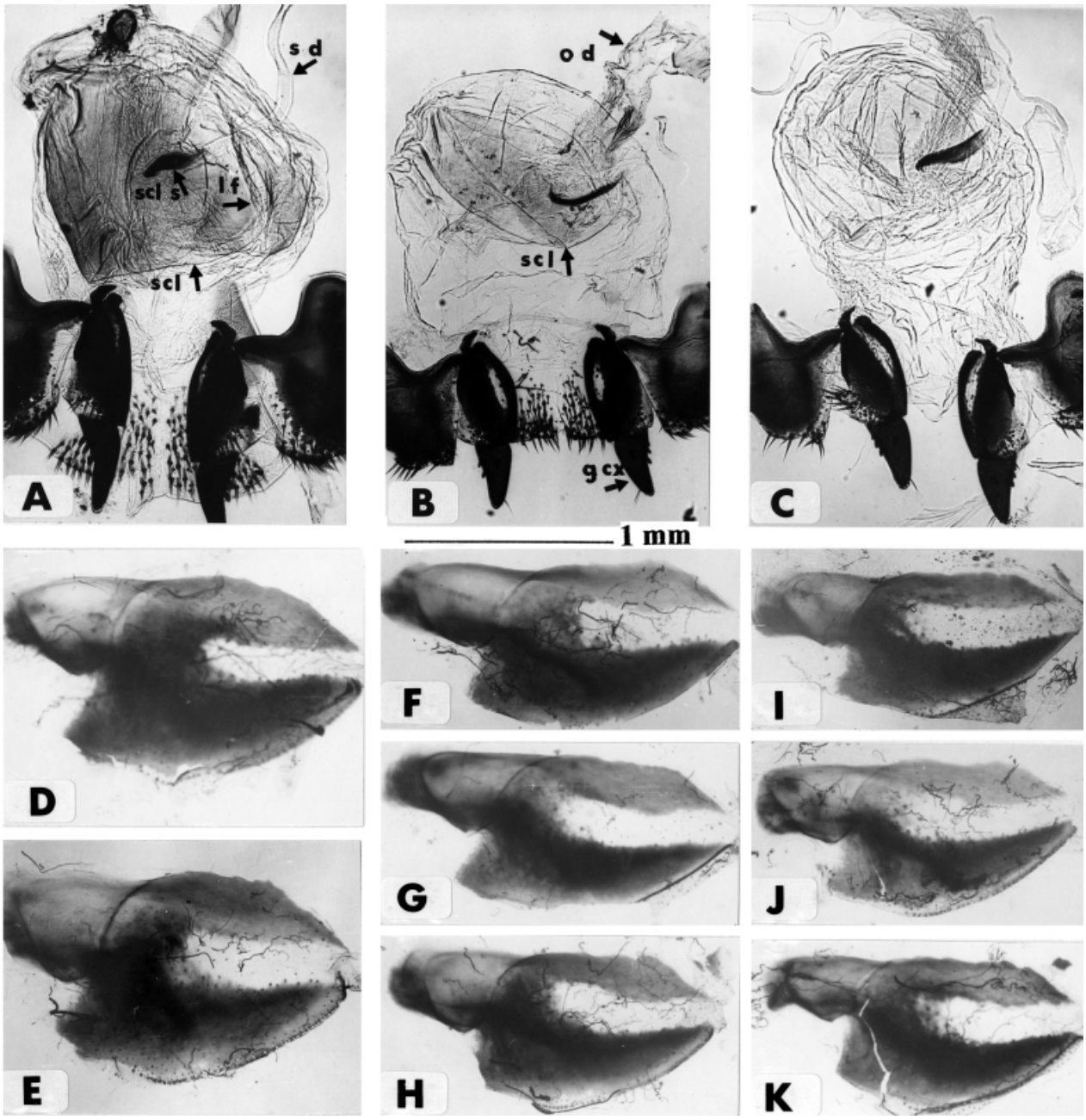

Fig. 6. Photomicrographs of female structures. A–C, Bursa copulatrix and gonocoxae; D–K, sternite 8, left half, from specimens whose bursa copulatrix has been checked. scl = sclerotized area; lf = lateral fold; sd = spermathecal duct; scl s = sclertotized section of spermathecal duct; od = oviduct; gcx = gonocoxa. A, *P. nigrita* from Catfield Fen (sternite 8: D); B,C, *P. rhaeticus* from Mullsjö (sternite 8: J, K). D,E, *P. nigrita*; F–K, *P. rhaeticus*. D, From Catfield Fen (Fig. 7: point 2); E, from Strathconon (Fig. 7: point 3); F, from Ockham Common (Fig. 7: point 5); G,H, from Nova Louka (Fig. 7: points 11, 10); I, from Skipwith Common (Fig. 7: point 7); J,K, from Mullsjö (Fig. 7: points 13, 12).

chromosomes vary from about 8–8.5 (the long B chromosomes in Fig. 8A,B,D,E) to about 1.8 (the shortest B chromosomes in Fig. 8E), whereas their combined RCL values range from about 6.6 (Fig. 8F) to about 31.6 (Fig. 8G). The B chromosomes may appear uniformly C-banded, sometimes with a narrow gap (Fig. 8B–E), or may appear to be composed of close-set dark bands with vague paler gaps between them (Fig. 8G). In some specimens one of the B chromosomes may be consistently darker at the two ends and rather paler between (Fig. 8A, the longest B chromosome). In one clear preparation of first metaphase of meiosis (Fig. 9A, from the same specimen shown in Fig. 8G) the seven B chromosomes all appear as separate, highly condensed elements.

The B chromosomes of *P. rhaeticus* are even more variable, in both number and size range, than are those of *P. nigrita*. The lowest number of B chromosomes so far encountered is two (Fig. 8T,U), whereas the highest number is twelve (Fig. 8V). The RCL values of the B chromosomes range from about 14 (the longest B chromosomes in Fig. 8J,K,U,V) to 0.7 (the dot-like B chromosomes in Fig. 8L,P,S), with values of about 2 being a more usual lower limit (the smallest B chromosomes in Fig. 8J,K,M,V). The combined RCL values of the B chromosomes vary from about 72 (Fig. 8V) to about 8.5 (Fig. 8T.) As in *P. nigrita*, the B chromosomes may be uniformly C-banded, with or without a narrow pale gap at some point in the length (Fig. 8J,K,M,N), or they may have a more obviously 'segmented' appearance, appearing to consist of a number of close-set dark bands separated by narrow paler gaps (Fig. 8I, the longest B chromosome, Fig. 8S,V). However, photographs of other nuclei from the same specimens show the B chromosomes uniformly dark so that it seems that these 'segmented' chromosomes have been caught in a slightly less condensed state than others, but this is not sufficient to alter their RCL values. Some specimens have a B chromosome that appears heavily banded at the ends but paler between (Fig. 8P,Q). In these chromosomes, the contrast between the dark ends and the paler median section is more marked than in *P. nigrita* (Fig. 8A), but in both specimens some preparations show the middle part of these chromosomes to be somewhat darker than the non-banding regions of the autosomes. These 'double ended' B chromosomes appear to be different in the two specimens: the one shown in Fig. 8P has a RCL value of about 4.7, whereas the one in Fig. 8Q has a RCL of about 6.9. In both cases, the RCL values are consistent in different nuclei from the two beetles. In one case (Fig. 8J), there are two very small B chromosomes which may not be heterochromatic, although they are so small that the C-banding reaction may have been lost. A mid-gut nucleus from the same specimen (Fig. 8K) lacks these small B chromosomes, although this nucleus appears to be undamaged (Fig. 9B,C). Figure 8L shows an unbanded testis preparation from Matley Bog, and this is the only specimen we have seen which corresponds to Nettmann's (1976) description of the *P. rhaeticus* karyotype of forty-six chromosomes, including two very small ones. No meiotic

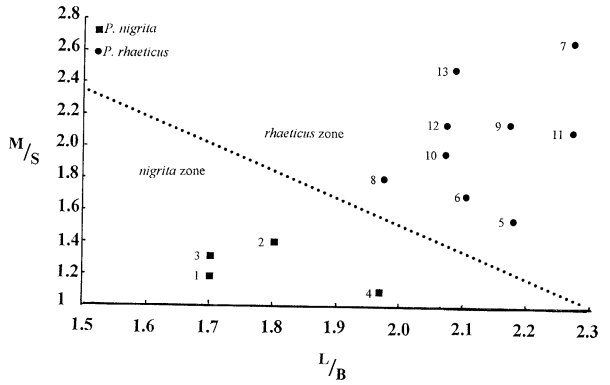

Fig. 7. Sternite 8 of *P. nigrita* and *P. rhaeticus*, L/B plotted against M/S. Measurements as in Fig. 1C. Plotted after Luff (1990). All specimens whose bursa copulatrix has been checked. 1–4, *P. nigrita*; 5–13, *P. rhaeticus*. 1, Catfield Fen; 2, Catfield Fen (Fig. 6D); 3, Strathconon (Fig. 6E); 4, Ockham Common; 5, Ockham Common (Fig. 6F); 6, Catfield Fen; 7, Skipwith Common (Fig. 6I); 8, Moy Bridge; 9, Strathconon; 10,11, Nova Louka (Fig. 6H,G); 12,13, Mullsjö (Fig. 6K,J).

Table 2. Relative Chromosome Length.

	P. nigrita			*P. rhaeticus*		
Chromosome no.	*n*	Mean	95% confidence limits (*t*-test)	*n*	Mean	95% confidence limits (*t*-test)
1	16	7.63	6.86–8.41	28	6.82	6.52–7.13
2	16	6.61	6.38–6.85	28	6.57	6.41–6.75
3	16	6.48	6.23–6.72	28	6.36	6.18–6.54
4	16	6.47	6.21–6.74	28	6.29	6.12–6.46
5	16	5.96	5.76–6.17	28	6.17	6.02–6.34
6	16	5.94	5.76–6.12	28	6.12	5.94–6.29
7	16	5.76	5.59–5.93	28	5.76	5.61–5.92
8	16	5.72	5.54–5.90	28	5.74	5.61–5.87
9	16	5.61	5.40–5.81	28	5.57	5.54–5.81
10	16	5.59	5.33–5.82	28	5.58	5.40–5.66
11	16	5.59	5.31–5.87	28	5.59	5.43–5.77
12	16	5.36	5.15–5.58	28	5.41	5.26–5.56
13	16	5.32	5.11–5.54	28	5.20	5.04–5.37
14	16	4.98	4.81–5.15	28	5.24	5.07–5.43
15	16	4.72	4.52–4.92	27	4.94	4.82–5.06
16	16	4.30	4.09–4.51	28	4.82	4.65–4.98
17	16	4.20	3.93–4.47	28	4.49	4.36–4.63
18	16	3.71	3.47–3.95	28	3.57	3.35–3.76
$\bar{x}$	9	5.16	4.83–5.49	18	4.84	4.57–5.10

Fig. 8. Karyograms of mitotic chromosomes of *P. nigrita* and *P. rhaeticus*. A–H, *P. nigrita*; I–V, *P. rhaeticus*. All except L are C-banded. A, Male, mid-gut, Strathconon; B, male, mid-gut, Langham Pond; C,D, males, mid-gut, Catfield Fen; E,F, males, mid-gut, Cothill; G, male, mid-gut, Ockham Common; H, female, mid-gut, Ockham Common; I, male, testis, Hule Moss; J, female, ovary, Ockham Common, with one replicate of autosome 15 missing; K, the same specimen as J, mid-gut, lacking the two smallest B-chromosomes; L, male, testis, Matley Bog; M, female, mid-gut, Mullsjö; N, male, mid-gut; Ockham Common; O, male, mid-gut, Hothfield Common; P, male, testis, Hothfield Common; Q, female, mid-gut, Nova Louka; R,S, males, mid-gut, Catfield Fen; T, male, mid-gut, Mullsjö; U, male, mid-gut, Moy Bridge; V, male, mid-gut, Skipwith Common, phase contrast.

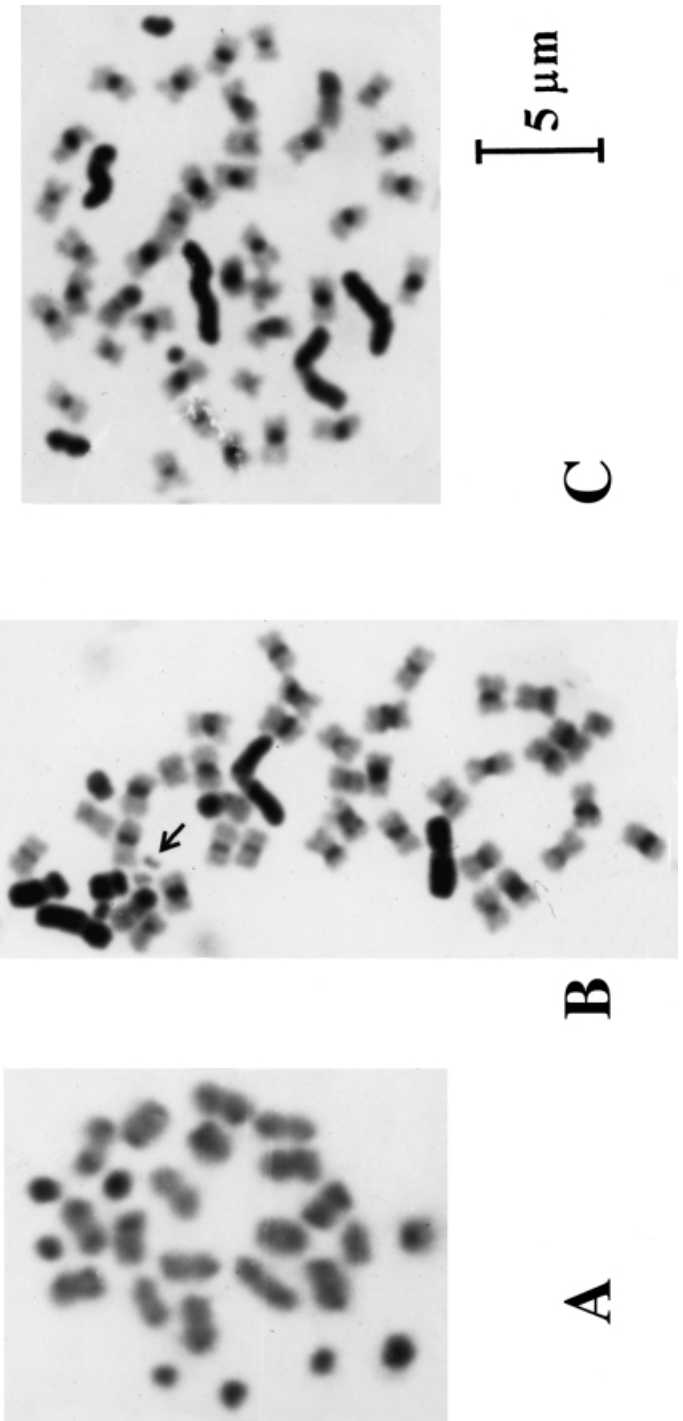

Fig. 9. Chromosomes of *P. nigrita* and *P. rhaeticus*, uncut nuclei. A, Meiotic chromosomes *P. nigrita*, metaphase 1 from testis, same specimen as Fig. 8G. The X chromosome is bottom centre, three B chromosomes are at the top (small and dark), while the other four B chromosomes are on the left; B,C, mitotic chromosomes of *P. rhaeticus*, from ovary (B) and mid-gut (C) of the same specimen, shown as karyograms in Fig. 8J,K. The smallest B chromosomes are arrowed in B.

preparations are shown for *P. rhaeticus* as none was obtained that appears sufficiently unambiguous. In some cases, dark B chromosomes can be distinguished and in general it appears that, as in the preparation of *P. nigrita* shown in Fig. 9A, the B chromosomes are largely unpaired.

There does not appear to be any obvious regional effect on the numbers of B chromosomes found in the two species. Thus, in *P. nigrita* the highest and lowest number of B chromosomes (three and eight) both occurred in specimens from Cothill, while as few as four occurred in the case of the Skipwith material of *P. rhaeticus,* which included the specimen with twelve B chromosomes (Fig. 8V). Moy Bridge material, which included a specimen with only two B chromosomes (Fig. 8U), also included material with eight B chromosomes. Swedish material had up to four B chromosomes. The range of size and form of the B chromosomes observed in both species means that B chromosome number alone is of very little interest.

Discussion

The discovery of the suites of B chromosomes in *P. nigrita* and *P. rhaeticus* not only resolves the conflicting data presented by Nettmann (1976), Serrano (1981) and Galián *et al*. (1992), but also establishes that both species have the same basic karyotype of eighteen pairs of autosomes plus XO/XX sex chromosomes, with the B chromosomes as a variable additional component. This karyotype (without the B chromosomes) is by far the most frequently recorded in *Pterostichus* by Serrano & Yadav (1984) and Serrano & Galián (1998).

The occurrence of B chromosomes appears to be unusual in the Adephaga. Thus, of more than 900 species of Carabidae whose karyotypes are listed by Serrano & Galián (1998), only twenty-two are listed as having B chromosomes. Most of these have only one B chromosome, although some have two. According to J. Galián (personal communication), the B chromosomes are almost without exception dot-like, and none has been subjected to C-banding. Only in *Princidium punctulatum* Drap. is the B chromosome of moderate size (Serrano, 1981). De la Rua *et al*. (1996) record six dot-like B chromosomes in *Cychrus caraboides* (L.). There are no records of B chromosomes among the Hydradephaga, but the senior author (personal observation) observed one long heterochromatic B chromosome in a female of *Agabus congener* (Thunberg) (Dytiscidae) from Sweden. Thus, the extensive suites of heterochromatic, frequently large, B chromosomes found in *P. nigrita* and *P. rhaeticus* are, based on present knowledge, unique in the Adephaga.

The status of *P. nigrita* and *P. rhaeticus* as separate, sibling species is not open to doubt. The breeding and hybridization data of Koch & Thiele (1980) make this quite clear. The discovery that the differences in chromosome number observed in these species result from the presence of B chromosomes means that the chromosome numbers do not constitute an interspecific difference. However, the small differences between the autosomes of the two species are entirely in accord with their status as separate, related species and suggest that there have been a number of translocations of chromosome material since they speciated. The overall similarity of their karyotypes, and in particular of their unusual suites of B chromosomes, reflects their status as closely related sibling species.

The investigation of the taxonomic characters reported here shows that the identification of males is problematic. The form of the inflated endophallus is entirely diagnostic of both species, despite the variation both show in its chaetotaxy. The unreliability of the form of the right paramere as a taxonomic character is unfortunate. Although the parameres are often of the forms hitherto regarded as characteristic of each species, the number and distribution of anomalous cases found in the present study mean that identification of males on the basis of paramere shape is not reliable. The fact that the differences in the form of the endophallus have not been observed to break down indicates that it is the paramere shape as a taxonomic character, rather than the integrity of the two species, that breaks down. The nature of the differences between the endophalli of these two species, involving the development of an apical lobe on the right side of the dorsal face in *P. nigrita* and its apparent reduction to a small protuberance on the right side of the gonopore field in *P. rhaeticus*, where a different, smaller apical lobe is present on the left side of the dorsal face, strongly suggests that a mechanical isolating mechanism is the principal means of speciation.

The characters of the eighth abdominal sternite that are used to identify females have been found to be reliable. However, these should be measured rather than merely glanced at, especially if occasional *P. nigrita* are suspected among populations of *P. rhaeticus.*

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