Comparative study of four known species of the genus Acrobeles von Linstow, 1877 (Nematoda, Cephalobidae) with ‘single’ and ‘double’ cuticle from coastal dunes in Spain

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Abstract

The nematode genus Acrobeles is composed of two morphological groups distinguished by the presence (‘double’ cuticle) or absence (‘single’ cuticle) of the refringent inner layer of the cuticle. In the present study, four species of this genus, two with ‘single’ cuticle (Acrobeles ciliatus and Acrobeles cylindricus) and two with ‘double’ (Acrobeles aenigmaticus and Acrobeles complexus) are studied from coastal dunes in Spain. This study provides detailed morphological and morphometrical analyses for the four species, while molecular analysis, based on 18S and 28S ribosomal DNA, is provided for A. complexus. The four species are studied with scanning electron microscopy, which is obtained for the first time for A. cylindricus. These analyses revealed morphological and molecular differentiations between both groups, appearing as two related monophyletic entities. The subgenera Acrobeles and Seleborca, formerly considered as separate genera, are proposed to accommodate both groups.

Introduction

The nematode genus Acrobeles von Linstow, 1877 (Rhabditida, Cephalobidae) was proposed by Otto F.B. von Linstow (1877) to include one species having oral expansions. Later, Thorne (1925) revised this genus and included 40 species, all of them with oral expansion (labial probolae) with variable morphology. Thorne (1937) revised again these species and divided the genera Acrobeles according to the morphology of the labial probolae and proposed five new genera, maintaining in Acrobeles only those species with long bifurcate labial probolae. Thus, this genus is mainly characterized by having a lip region with six asymmetrical triangular lips and an oral opening surrounded by three bifurcated labial probolae, all of which – lips and probolae – are bordered by triangular processes. Andràssy (1985) divided this genus in two genera: the genus Acrobeles sensu stricto containing species that have a cuticle lacking an inner refringent layer (known as ‘single’ cuticle), and the new genus Seleborca Andràssy, 1985 (the word ‘Seleborca’ comes from ‘Acrobeles’ inverted) containing species that have a cuticle with an inner refringent layer (known as ‘double’ cuticle). Rashid et al. (1990) examined species of both genera and, based on the structure of the cuticle and the variability of the lateral field, confirmed the validity of the genus Seleborca. However, in the same year, De Ley et al. (1990) considered that the separation of both genera based only on the morphology of the cuticle was not justified and, later, Shahina & De Ley (1997) considering Seleborca a junior synonym of Acrobeles. However, Andràssy (2005) maintained Seleborca as a valid genus.

On the other hand, molecular studies provided by several authors (Nadler et al., 2006; Mehdizadeh et al., 2013; Abolafia et al., 2014, 2019; Abolafia & Peña-Santiago, 2020) showed that both morphological groups appear in different clades. The genus Acrobeles sensu lato includes 34 valid species (Abolafia & Peña-Santiago, 2004; Boström & Holovachov, 2019), 21 of them having a ‘single’ cuticle and 13 of them having a ‘double’ cuticle. This paper redescibes four known species of the genus Acrobeles, two belonging to the ciliatus-group (Acrobeles ciliatus von Linstow, 1877 and Acrobeles cylindricus Ivanova, 1968) and two to the complexus-group (Acrobeles aenigmaticus Abolafia, Shokohi, Du Preez & Faurie, 2019 and Acrobeles complexus Thorne, 1925), all of which were collected from sand dunes on the Atlantic-Mediterranean coast of Spain. Each pair of species, easily confused, is characterized morphologically, morphometically and, for some, also molecularly.

Materials and methods

Sampling and nematode extraction

The specimens examined were extracted from the rhizosphere of xerophile plants from sand dunes in three coastal localities in the provinces of Alicante, Barcelona and Huelva (Spain).
Nematodes were extracted from soil samples using a modified Baermann’s (1917) funnel technique provided of a stainless-steel sieve (10 cm diameter, 100 μm mesh), killed by heat and fixed in a 4% formalin solution. Nematodes were processed to anhydrous glycerine according to Siddiqi’s (1964) method using lactophenol-glycerine solutions, and were then permanently mounted on glass microscope slides to enable species identification.

**Light microscopy (LM)**

Observations were made using a Nikon Eclipse 80i (Nikon, Tokyo, Japan) microscope. Measurements were taken using an ocular micrometer or a curvimeter after drawing the corresponding organ or structure attached to an Olympus BH-2 microscope (Olympus, Tokyo, Japan); Demanian indices (de Man, 1881) and other ratios were calculated. Micrographs were taken with a Nikon Eclipse 80i (Nikon, Tokyo, Japan) light microscope equipped with differential interference contrast optics and a Nikon Digital Sight DS-U1 camera. Micrographs were combined using Adobe Photoshop® CS (Adobe Inc., San José, USA) and figures mounted using Microsoft® PowerPoint (Microsoft Corporation, Redmond, USA). The terminology used for the morphology of stoma and spicules-gubernaculum follows the proposals by De Ley et al. (1995) and Abolafia & Peña-Santiago (2017), respectively.

**Scanning electron microscopy (SEM)**

Specimens preserved in glycerine were selected for observation under SEM according to Abolafia (2015). The nematode was cut into small pieces using a sterilized dental needle on a clean slide with 18 ml of TE (Tris-EDTA) buffer and observed with a Zeiss Merlin microscope (5 kV) (Zeiss, Oberkochen, Germany).

**DNA extraction, polymerase chain reaction (PCR) and sequencing**

Nematode DNA was extracted from single fresh individuals using the proteinase K protocol and PCR assays as described by Castillo et al. (2003), somewhat modified (Archidona-Yuste et al., 2016). The specimens were cut into small pieces using a sterilized dental needle on a clean slide with 18 ml of TE (Tris-EDTA) buffer (10 mM Tris-Cl (tris hydrochloride) + 0.5 mM EDTA (ethylene-diamine-tetraacetic acid); pH 8.5), transferred to a microtube and adding 2 μl proteinase K (700 μg/ml−1) (Roche, Basel, Switzerland), and stored to −80°C within 15 min (for several days). The microtubes were incubated at 65°C (1 h), then at 95°C (15 min). For DNA amplification, 3 μl of the extracted DNA was transferred to a microtube containing 0.6 μl of each primer (10 μM), 3 μl Master Mix Taq DNA Polymerase (5× Hot FirePol Blend Master Mix) and ddH2O to a final volume of 20 μl. The primers used for amplification of the region of 18S ribosomal RNA (rRNA) gene were the forward primer 988 F (5′-CTCTAAAGAT TAAAGCATC-3′) and the reverse primer 1912R (5′-TTTAT GGTCA GAACTAGGG-3′) (Holterman et al., 2006). The primers used for amplification of the D2–D3 region of 28S rRNA gene were the D2A (5′-ACAAGTACCGTGAGGG AAAGTAGT-3′) and the D3B (5′-TGGAAGAAACCAGCTAC TA-3′) primers (Nunn, 1992; De Ley et al., 1999). PCR cycle conditions were as follows: one cycle of 94°C for 15 min, followed by 35 cycles of 94°C for 45 s + annealing temperature of 55°C for 45 s + 72°C for 45 s and finally one cycle of 72°C for 5 min. After DNA amplification, 5μl of product was loaded on a 1% agarose gel in 0.5% Tris-acetate-EDTA (40 mM Tris, 20 mM glacial acetic acid and 2 mM EDTA; pH 8) to verify the amplification using an electrophoresis system (Labnet Gel XL Ultra V–2, Progen Scientific, London, UK). The bands were stained with RedSafe (20,000×) previously added to the agarose gel solution. The sequencing reactions of the PCR products were performed at Sistemas Genómicos (Paterna, Valencia, Spain) according the Sanger et al. (1977) method. The sequences obtained were submitted to the GenBank database.

**Phylogenetic analyses**

For phylogenetic relationships, analyses were based on 18S and 28S ribosomal DNA (rDNA) fragments. The newly obtained sequences were manually edited using BioEdit 7.2.6 (Hall, 1999) and aligned with another 18S or 28S rDNA sequences available in GenBank using ClustalW alignment tool implemented in the MEGA7 (Kumar et al., 2016). Poorly aligned regions at extremes were removed from the alignments using MEGA7. The best-fit model of nucleotide substitution used for the phylogenetic analysis was statistically selected using jModelTest 2.1.10 (Darriba et al., 2012). Phylogenetic trees were generated with the Bayesian inference method using MrBayes 3.2.6 (Ronquist et al., 2012). Driloecephalobus sp. (AY284680) for 18S rDNA and Teratolobus sp. (KJ652552) for 28S rDNA was chosen as out-group. Analysis under the General Time Reversible plus Invariant sites plus Gamma distribution (GTR + I + G) model was initiated with a random starting tree and run with the Markov Chain Monte Carlo method (Larget & Simon, 1999) for 1 × 10⁶ generations. The trees were visualized and saved with FigTree 1.4.4 (Rambaut, 2018).

**Results**

**Acrobeles ciliatus von Linstow, 1877**

**Material examined**

Three females and three males from sand dunes in L’Alfàs (province of Alicante, Spain), in good condition.

**Measurements**

For measurements, see table 1.

**Description**

Adult (figs 1A–E and 2A–F). Body fusiform, 0.48–0.53 mm long. Usually curved ventrad after fixation. Cuticle clearly annulated and ‘single’. Annulli 3 μm wide. Lateral fields with two longitudinal incisures that continue until phasmids, occupying 26–37% of mid-body diameter. Lip region very wide, continuous with body contour, having three pairs of asymmetrical lips, one dorsal and two ventrolateral and bearing six labial and four cephalic sensilla. Primary axes deep, U-shaped and bearing two elongate triangular processes originating from the incomplete first annulus. Secondary axes with one small and rounded guarding process. Lips asymmetrical, triangular, with dentate margin bearing triangular tines (pinnae) with elongate tip with similar morphology: eight pinnae at primary axes and seven pinnae at secondary axes and one longer acute apical pinna. Oral opening surrounded by three labial probolae; each probolae composed of a short basal part (stipe) and a longer and bifurcated distal part (furca) with...
very long and divergent prongs, bearing seven elongated and very
thin lateral pinnae in the outer and inner margin, and two very
thin elongated pinnae at distal or apical end (apex). Amphids situ-
ated at the base of each lateral lip, and rounded. Stoma cephalo-
boi.d. Pharynx also cephaloboid, differentiated in three parts:
pharyngeal corpus subcylindrical, 3.1–4.8 times isthmus length;
Isthmus narrower than metacorpus; basal bulb ovoid, with well-
developed valvular apparatus. Cardia conoid, surrounded by
intestinal tissue. Nerve ring at 74–78% of neck length at level of
the isthmus. Excretory pore anterior at 45–53% of neck length.

Fig. 1. LM. (A–E) Acrobeles ciliatus. (F–J) Acrobeles cylindricus; (K–O) Acrobeles aenigmaticus; (P–T) Acrobeles complexus.

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the isthmus. Excretory pore anterior at 45–53% of neck length.

Deirids at 83–91% of neck length, at level of bulb. Intestine with-
out distinct specializations.

Female. Reproductive system monodelphic–prodelpbic, cephaloboid, right side in relation to intestine. Ovary with flex-
ure, very short oviduct and well-developed spermatheca, 0.8
times the body diameter. Uterus length twice the body diameter.
Post-vulval uterine sac with length 0.9–1.1 times the body diam-
eter. Vagina short, extending inward 30–32% of body diameter.
Vulva not protruding. Rectum 0.8–1.0 times the anal body
diameter; three small gland-like cells are distinguishable around
the intestine–rectum junction. Tail conical. Phasmids located at 26–35% of tail length.

**Male.** Reproductive system monorchid, with well-developed testis reflexed ventrally, anteriorly. Spicules paired and symmetrical, 28–39 times longer than wide, slightly elongate and ventrally curved, having cylindrical calamus and ventrally curved with acute tip bent ventrally. Gubernaculum arcuate in lateral view. Three small gland-like cells are distinguishable around the cloaca. Two pairs of pre-cloacal genital papillae. Five pairs of post-cloacal genital papillae (two pairs at the middle of tail and three pairs near tail terminus). Tail conical, ventrally curved, with acute terminus. Phasmids located at 30–38% of tail length.

**Acrobeles cylindricus Ivanova, 1968**

**Material examined**
Ten females and ten males from sand dunes in Gavá (province of Barcelona, Spain), in good condition.
**Table 1. Morphometrics of Acrobeles species collected from coastal dunes in Spain. All measurements in μm.**

| Province | Acrobeles aenigmatus | Acrobeles ciliatus | Acrobeles complexus | Acrobeles cylindricus |
|----------|----------------------|-------------------|---------------------|----------------------|
| 1. **Species** | | | | |
| 2. **Habitat** | | | | |
| n | 8 | 3 | 10 | 10 |
| **Body length** | 689.4 ± 61.5 (600–795) | 723.9 ± 43.7 (670–800) | 752.0 ± 35.0 (484–553) | 466.6 ± 29.5 (416–528) |
| 3. **Labial probolae** | 15.0 ± 0.0 (15.0–15.0) | 15.7 ± 0.7 (15.0–15.7) | 15.7 ± 0.7 (15.0–15.7) | 15.7 ± 0.7 (15.0–15.7) |
| 4. **Stoma length** | 9.3 ± 0.7 (8–10) | 9.3 ± 0.7 (8–10) | 9.3 ± 0.7 (8–10) | 9.3 ± 0.7 (8–10) |
| 5. **Pharyngeal corpus length** | 105.0 ± 4.9 (97–114) | 110.7 ± 5.1 (104–118) | 109.7 ± 6.4 (101–119) | 109.7 ± 6.4 (101–119) |
| 6. **Isthmus length** | 28.8 ± 5.1 (20–35) | 31.3 ± 5.2 (21–35) | 24.7 ± 2.5 (22–26) | 24.7 ± 2.5 (22–26) |
| 7. **Bulbus length** | 30.1 ± 2.9 (26–35) | 31.3 ± 1.3 (23–33) | 24.7 ± 2.3 (22–26) | 24.7 ± 2.3 (22–26) |
| 8. **Pharynx length** | 163.9 ± 10.1 (150–184) | 169.1 ± 5.1 (161–174) | 173.9 ± 10.8 (159–190) | 173.9 ± 10.8 (159–190) |
| 9. **Nerve ring – anterior end** | 125.5 ± 9.9 (117–143) | 126.4 ± 8.4 (115–137) | 103.7 ± 2.1 (102–106) | 105.0 ± 3.6 (101–108) |
| 10. **Excretory pore – anterior end** | 125.9 ± 8.4 (113–141) | 120.0 ± 4.9 (115–130) | 66.7 ± 7.0 (60–74) | 70.7 ± 8.4 (61–76) |
| 11. **Deirid – anterior end** | 157.0 ± 12.9 (137–175) | 143.7 ± 10.7 (131–160) | 117.7 ± 2.5 (115–120) | 127.7 ± 6.8 (120–133) |
| 12. **Neck length** | 173.1 ± 10.3 (160–194) | 178.9 ± 4.9 (171–183) | 136.0 ± 3.6 (132–139) | 151.0 ± 5.3 (145–155) |
| 13. **Annumi width** | 3.0 ± 0.0 (3) | 2.9 ± 0.4 (2–3) | 3.0 ± 0.0 (3) | 3.0 ± 0.0 (3) |
| 14. **Cuticle at mid-body** | 3.1 ± 0.4 (3–4) | 2.7 ± 0.0 (2–3) | 3.0 ± 0.0 (3) | 2.9 ± 0.0 (3) |
| 15. **Body diameter at neck base** | 34.4 ± 1.9 (31–36) | 36.4 ± 2.8 (33–41) | 28.7 ± 0.6 (28–29) | 29.0 ± 1.7 (28–31) |
| 16. **Body diameter at mid-body** | 34.4 ± 2.3 (31–38) | 37.7 ± 4.6 (32–43) | 31.0 ± 2.0 (29–33) | 30.3 ± 0.6 (30–31) |
| 17. **Lateral field width** | 5.6 ± 0.9 (5–7) | 6.6 ± 0.5 (6–7) | ? | 7 (n=1) |

(Continued)
| Species                  | Acrobeles aenigmaticus | Acrobeles ciliatus | Acrobeles complexus | Acrobeles cylindricus |
|-------------------------|------------------------|-------------------|---------------------|-----------------------|
| Province                | Alicante               | Alicante          | Huelva              | Barcelona             |
| Habitat                 | Sand dune              | Sand dune         | Sand dune           | Sand dune             |
| n                       | 8 ♀♀                   | 7 ♂♂              | 3 ♀♀                | 10 ♀♀                 |
|                         | 8 ♂♂                   | 3 ♀♀              | 3 ♂♂                | 10 ♂♂                 |
|                         | 10 ♀♀                  | 10 ♂♂             | 10 ♀♀               | 10 ♂♂                 |
| Anterior ovary/ testis  | 201.0 ± 16.7 (177–225) | 177.2 ± 14.4 (162–193) | 149.5 ± 0.7 (149–150) | 129.7 ± 11.9 (120–143) |
|                         | 239.0 ± 24.7 (215–278) | 220.2 ± 3.5 (216–225) | 143.6 ± 15.7 (113–158) | 112.1 ± 10.1 (100–132) |
| Spermatheca length      | 34.0 ± 3.3 (30–40)     | –                 | 25.0 ± 1.4 (24–26)  | –                     |
|                         | 41.0 ± 8.4 (32–49)     | –                 | 26.1 ± 3.9 (20–31)  | –                     |
| Anterior uterus length  | 64.9 ± 4.6 (56–70)     | –                 | 63.5 ± 3.5 (61–66)  | –                     |
|                         | 105.5 ± 9.5 (90–118)   | –                 | 52.7 ± 6.0 (47–64)  | –                     |
| Post-vulval sac length  | 110.5 ± 12.4 (95–121)  | –                 | 32.5 ± 6.4 (28–37)  | –                     |
|                         | 81.4 ± 12.4 (75–109)   | –                 | 35.1 ± 2.9 (30–40)  | –                     |
| Vagina length           | 11.3 ± 1.0 (10–12)     | –                 | 9.7 ± 0.6 (9–10)    | –                     |
|                         | 14.8 ± 1.3 (14–17)     | –                 | 9.9 ± 1.2 (8–11)    | –                     |
| Vulva – anterior end    | 415.0 ± 40.5 (354–480) | –                 | 316.0 ± 19.1 (296–334) | – | 429.3 ± 31.5 (384–494) | – | 263.4 ± 18.4 (226–294) | – |
| Rectum length           | 22.9 ± 2.0 (19–26)     | 34.4 ± 2.9 (30–39) | 18.0 ± 1.0 (17–19) | 27.0 ± 3.0 (24–30) |
|                         | 16.0 ± 2.3 (13–19)     | 32.4 ± 1.9 (30–36) | 18.5 ± 2.6 (15–22) | 23.3 ± 1.4 (20–25) |
| Anal body diameter      | 21.8 ± 2.9 (18–25)     | 25.7 ± 2.2 (23–30) | 20.3 ± 1.5 (19–22) | 22.0 ± 1.0 (21–23) |
|                         | 22.6 ± 2.0 (21–28)     | 32.6 ± 1.3 (30–34) | 20.3 ± 2.7 (16–24) | 21.9 ± 1.7 (18–24) |
| Tail length             | 74.5 ± 4.0 (70–80)     | 59.6 ± 4.6 (53–67) | 68.3 ± 3.5 (65–72) | 53.0 ± 3.5 (51–57) |
|                         | 79.9 ± 5.0 (70–89)     | 61.6 ± 2.8 (56–66) | 64.2 ± 4.2 (60–70) | 44.5 ± 3.2 (40–48) |
| Phasmid–anus distance   | 21.0 ± 1.2 (20–23)     | 20.6 ± 1.3 (19–23) | 20.3 ± 3.2 (18–24) | 18.7 ± 2.5 (16–21) |
|                         | 21.1 ± 2.7 (18–24)     | 21.5 ± 3.0 (39–45) | 18.5 ± 2.4 (16–23) | 16.0 ± 2.3 (13–19) |
| Spicules length         | –                      | 40.6 ± 1.4 (38–42) | 25.0 ± 1.4 (24–26) | –                     |
|                         | –                      | 41.8 ± 2.3 (39–45) | –                    | 27.0 ± 1.3 (25–30) |
| Gubernaculum length     | –                      | 19.9 ± 1.9 (18–22) | 15.0 ± 1.4 (14–16) | –                     |
|                         | –                      | 22.5 ± 1.2 (21–25) | –                    | 14.9 ± 1.4 (13–18) |

Demanian indices (de Man, 1881): \(a = \) body length / body diameter; \(b = \) body length / neck length; \(c = \) body length / tail length; \(c' = \) tail length / anal body diameter; \(V = \) vulva – anterior end / body length × 100.
Measurements
For measurements, see table 1.

Description
Adult (figs 1F–J and 2G–M). Body fusiform, 0.4–0.5 mm long. Usually curved ventrad after fixation. Cuticle clearly annulated and ‘single’. Annuli 2 μm wide. Lateral fields with two longitudinal incisures that continue until phasmids, occupying 29–46% of mid-body diameter. Lip region narrower than the adjacent part of body, continuous with the body contour, having three pairs of asymmetrical lips, one dorsal and two ventrolateral, and bearing six labial and four cephalic sensilla. Primary axes deep, U-shaped and bearing two elongate triangular processes originating from the incomplete first annulus. Secondary axes bearing two elongated guarding processes. Lips asymmetrical, triangular, bordered by more or less triangular pinnae having elongate, almost filiform, terminus: six pinnae at primary axes, seven pinnae at secondary axes and apex with one longer acute pinna. Oral opening surrounded by three labial probolae provided by a short basal stipe and a longer and bifurcated distal furca bordered by pinnae similar to those from lips: seven pinnae at both outer and inner margin, and two very thin and elongated apical pinnae. Amphids situated at the base of each lateral lip, and rounded. Stoma cephaloboid. Pharynx also cephaloboid, differentiated in three parts: pharyngeal corpus subcylindrical, 2–4 times isthmus length; isthmus narrower than metacorpus; basal bulb ovoid, with well-developed valvular apparatus. Cardia conoid, surrounded by intestinal tissue. Nerve ring at 48–65% of neck length at level of pharyngeal corpus base. Excretory pore anterior at 23–31% of neck length. Deirids at 56–62% of neck length. Intestine without distinct specializations.

Female. Reproductive system monodelphic–prodelphic, cephaloboid, dextral side in relation to intestine. Ovary without flexure, oviduct very short and spermatheca one times the body diameter. Uterus length 1–2 times the body diameter. Post-vulval uterine sac length one times the body diameter. Vagina short, extending 20–33% of body diameter. Vulva not protruding. Rectum one times the anal body diameter; three small gland-like cells are distinguishable around the intestine–rectum junction. Tail conical with acute of finely rounded terminus. Phasmids located at 26–33% of tail length.

Male. Reproductive system monorchid, with well-developed testis reflexed ventrally, anteriorly. Spicules paired and symmetrical, 7–9 times longer than wide, slightly elongate and ventrally curved, having cylindrical calamus and ventrally curved lamina with acute tip bent ventrally. Gubernaculum arcuate in lateral view. Three small gland-like cells are distinguishable at rectum–cloaca junction. Two pairs of pre-cloacal genital papillae and five pairs of post-cloacal genital papillae (two pairs at the middle of tail and three pairs near tail terminus). Tail conical, ventrally curved, with acute terminus. Phasmids located at 29–46% of tail length.

Acrobeles aenigmaticus
Abolafia, Shokoohi, Du Preez & Fourie, 2019

Material examined
Eight females and seven males from sand dunes in L’Altet (province of Alicante, Spain), in good condition.

Measurements
For measurements, see table 1.

Description
Adult (figs 1K–O and 3A–H). Body fusiform, 0.6–0.7 mm long. Usually curved ventrad after fixation. Cuticle annulated and ‘double’; annuli with few and separated small pore-like structures located at the interannular space. Lateral fields with two longitudinal incisures that continue until phasmids, occupying 14–21% of mid-body diameter. Lip region continuous with body contour having three pairs of asymmetrical lips, one dorsal and two ventrolateral, and bearing six labial and four cephalic sensilla. Primary axes deep, U-shaped and bearing two elongate triangular processes originating from the incomplete first annulus. Secondary axes bearing two guarding processes, each one originating from each lip. Lips asymmetrical, triangular, with dentate margin bearing triangular pinnae with fine rounded or rhomboid terminus: 7–9 pinnae at primary axes, 6–7 pinnae at secondary axes, the third from base more elongated and one longer acute apical pinna. Oral opening surrounded by three labial probolae, each composed of a short basal stipe and a longer and bifurcated distal furca having very long and divergent prongs bearing lateral pinnae, thinner towards the apex: eight elongated with fine rounded terminus at outer margin, six rounded shorter with rounded terminus at inner margin and two or three thinner at apical end. Amphids situated at the base of each lateral lip, large and rounded. Stoma cephaloboid. Pharynx also cephaloboid, differentiated in three parts: pharyngeal corpus subcylindrical, 3–5 times isthmus length; isthmus slightly anteriorly wider; basal bulb ovoid, with well-developed valvular apparatus. Cardia conoid, surrounded by intestinal tissue. Nerve ring at 70–74% of neck length, at level of posterior part of metacorpus. Excretory pore at 67–78% of neck length, at level of posterior part of metacorpus. Deirids at 80–100% of neck length, at level of bulb. Intestine without distinct specializations.

Female. Reproductive system monodelphic–prodelphic, cephaloboid, dextral in relation to intestine. Ovary long, oviduct very short and spermatheca well developed, 1.0–1.1 times the body diameter. Uterus length twice the body diameter. Post-vulval uterine sac well developed, long, 3–4 times the body diameter. Vagina short, extending inward 29–36% of body diameter. Vulva very reduced and displaced to left side, close to lateral field and without protruding lips. Rectum one times the anal body diameter; three small gland-like cells are distinguishable around the intestine–rectum junction. Tail conoid-elongate, anteriorly slightly ventrad curved and posteriorly straight or slightly dorsal, curved, narrower after phasmids, especially on dorsal side. Phasmids located at 26–29% of tail length.

Male. Reproductive system monorchid, dextral in position, with underdeveloped testis reflexed ventrad anteriorly. Spicules paired and symmetrical, 10–11 times longer than wide, slightly elongate and ventrally curved, having rounded calamus and ventrally curved lamina with acute tip bent ventrally. Gubernaculum well developed, curved, about half the length of spicules, well-developed crura. Three small gland-like cells are distinguishable at rectum–cloaca junction. Genital papillae as follows: three pre-cloacal pairs and five post-cloacal pairs (two at middle part, one lateral pair at lateral field level and one subventral, and three pairs near tail terminus), one subdorsal, one lateral and one subventral. Tail conical, posteriorly ventrad curved, with acute tip. Phasmids located at 30–38% of tail length.

Acrobeles complexus
Thorne, 1925

Material examined
Ten females and ten males from sand dunes in Matalascañas (province of Huelva, Spain), in good condition.
Measurements
For measurements, see table 1.

Description
Adult (figs 1P–T and 3I–P). Body fusiform, 0.7–0.87 mm long. Usually curved ventrad after fixation. Cuticle annulated and ‘double’; annuli with few and separated small pore-like structures. Lateral fields with two longitudinal incisures that continue until phasmids, occupying 14–18% of mid-body diameter. Lip region continuous with body contour having three pairs of asymmetrical lips, one dorsal and two ventrolateral, bearing six labial and four cephalic sensilla. Primary axils deep, U-shaped, with two elongate triangular processes originating from the incomplete first annulus. Secondary axils bearing two guarding processes, each one originating from each lip. Lips asymmetrical, triangular, bordered by rounded pinnae: six pinnae at primary axils, seven pinnae at secondary axils, the third of them from the base having an elongate tip, and one longer acute pinna at apex. Oral opening surrounded by three labial probolae, each one provided by a short stipe and a longer and bifurcated distal furca with very long and convergent lips.

Fig. 3. SEM of Acrobeles species with ‘double’ cuticle. (A–H) Acrobeles aenigmaticus; (I–P) Acrobeles complexus.
prongs bordered by lateral pinnae: six rounded to almost triangular at outer margin, six almost triangular at inner margin and two very elongated at apical terminus. Amphids situated at the base of each lateral lip, clearly visible with circular opening. Stoma cephaloboid. Pharynx also cephaloboid, differentiated in three parts: pharyngeal corpus subcylindrical, 2.5–3.9 times isthmus length; isthmus slightly anteriorly wider; basal bulb ovoid, with well-developed valvular apparatus. Cardia conoid, surrounded by intestinal tissue. Nerve ring at 68–75% of neck length, at isthmus level. Excretory pore at 64–78% of neck length, at level of posterior part of metacorpus. Deirids at 65–82% of neck length, at level of bulb. Intestine without distinct specializations.

Female. Reproductive system monodelphic–prodelphic, cephaloboid, dextral in relation to intestine. Ovary long, oviduct very short and spermatheca well developed, 0.7–1.2 times the body diameter. Uterus length 2.3–2.7 times the body diameter. Post-vulval uterine sac well developed, long, 1.7–2.5 times the body diameter. Vagina well developed, extending inward 32–43% of body diameter. Vulva transverse. Rectum 0.6–0.9 times the anal body diameter; three small gland-like cells are distinguishable around the intestine–rectum junction. Tail conical with acute rounded terminus. Phasmids located at 22–31% of tail length.

Male. Reproductive system monorchid, dextral in position, with well-developed testis reflexed ventrally, anteriorly. Spicules paired and symmetrical, 7.3–10.5 times longer than wide, slightly elongate and ventrally curved, having rounded calamus and ventrally curved with acute tip bent ventrally. Gubernaculum well developed, curved, about half of spicule length, with well-developed crura. Three small gland-like cells are distinguishable at rectum–cloaca junction. Two pairs of pre-cloacal papillae and five pairs of post-cloacal genital papillae (two close to phasmid and three at tail terminus). Tail conical, posteriorly ventrad at rectum elongate and ventrally curved, having rounded calamus and ventrally curved.

Molecular characterization

Five 18S rDNA sequences of A. complexus were obtained, having 894 bp (MZ407234), 690 bp (MZ407235), 795 bp (MZ407236), 741 bp (MZ407237) and 708 bp (MZ407238), all of which were 100% similar in having a shared segment in common with 679 bp. Compared with other A. complexus sequences (AY284671, KU180671), the Spanish specimens showed 99.5% similarity (or 3 bp differences) in having a segment in common with 635 bp. On the other hand, one 28S rDNA sequence with 755 bp (MZ407239) maintained 3 bp differences with A. complexus from California (DQ145620).

Discussion

Morphological results

Each pair of species examined, all of which are very frequent in the xeric areas examined in southern Spain, appear together in the same samples with very similar morphology, thus accounting for why they could be easily confused. As a result of the present study, the following important morphological differences have been found:

**Acrobeles ciliatus vs. A. cylindricus**

Both species with ‘single’ cuticle are very similar, having similar body size (484–553 μm vs. 402–528 μm), but they can be distinguished by the width of lip region, similar at the adjacent part of the body in A. ciliatus (figs 1A and 2A) and visibly narrower in A. cylindricus (figs 1F and 2G), excretory pore located anteriorly (at metacorpus level (fig. 1A) vs. at procorpus (fig. 1F)). Both species present labial probolae with similar elongate pinnae (figs 2B, C and 4C for A. ciliatus; figs 2H, I and 4D for A. cylindricus).

**Acrobeles aenigmaticus vs. A. complexus**

Both species with ‘double’ cuticle were considered very similar by Abolafia et al. (2019), but these species present some clear differences. The body length of A. aenigmaticus is slightly smaller than in A. complexus (600–800 μm vs. 700–870 μm), labial probolae of the first species are also smaller and elongate than the second species (12–15 μm vs. 13–17 μm), having different morphology of the pinnae, triangular in A. aenigmaticus (figs 3B, C and 4A) vs. more or less rounded (figs 3J, K and 4B). However, a very important character that differentiates both species is the position of the vulva (ventrally centred in A. complexus (fig. 3L) vs. left sublateral in A. aenigmaticus (fig. 3D)). This characteristic appears in other cephalobid pairs of species, such as Acrobeoloides saeedi Siddiqi, De Ley & Khan, 1992 and Acroboloides longiuterus (Rashid & Heyns, 1990) Siddiqi, De Ley & Khan, 1992, Chiloplacus insularis Orselli & Vinciguerra, 2002 and Chiloplacus magnus Rashid & Heyns, 2000, Chiloplacus tenuis Rashid & Heyns, 2000 and Chiloplacus membranifer Holovachov, Boström, Mundo-Ocampo & Villenave, 2008, all very similar species to each other and mainly distinguishable by the position of the vulva (midventral vs. sublateral). In the material examined of A. aenigmaticus in this study, males have a very underdeveloped, small testis, while in A. complexus, testis is very well developed and large. Another difference between males is in the spicule morphology, where the manubrium is rounded in A. aenigmaticus and conoid in A. complexus.

**Ciliatus-group vs. complexus-group**

Both groups, distinguished by the presence of a ‘single’ and ‘double’ cuticle, respectively, are also distinguished by having several important differences: body size (402–553 μm vs. 600–870 μm), morphology of the pinnae at labial probolae (conoid with elongate tip vs. more rounded), absence vs. presence of pore-like cuticular processes and position of the excretory pore (at pharyngeal corpus level vs. at isthmus level). We can also consider other differences, such as the presence of three longitudinal incisures in the lateral field in the ciliatus-group and 2–4 incisures in the complexus-group. Also, in most species having a ‘double’ cuticle, the post-vulval sac is well developed and large (with the exceptions of Acrobeles iranicus Shokoohi, Abolafia & Zad, 2007, Acrobeles marianae Andrassy, 1968, Acrobeles oasiensis Böstrom, 1985 and Acrobeles timmi (Chaturvedi & Khera, 1979) Andrassy, 1985), while in species with a ‘single’ cuticle, the post-vulval sac is very small (with the exceptions of A. ciliatus, Acrobeles microstomus Iliev, Ilieva & Mitor, 2003, Acrobeles seychae Rashid, Heyns & Coomans, 1990 and Acrobeles sheasbyi Heyns & Hogewind, 1969).

Molecular results

Molecular analyses in the present paper and previous papers (Nadler et al., 2006; Meh dizadeh et al., 2013; Abolafia et al., 2014, 2019; Abolafia & Peña-Santiago, 2020) showed the phylogenetic separation of both complexus and ciliatus groups being both monophyletic groups. The trees based on 18S rDNA
(fig. 5) and 28S rDNA (fig. 6) segments show two clearly separated groups of species belonging to the complexus-group and ciliatus-group. Species of the genus Cervidellus Thorne, 1937 appear related with these two groups, especially with the complexus-group, as is observable in the 28S tree, while the 18S tree does not resolve this relationship.

The 18S sequences of \textit{A. complexus} compared with other species with a ‘double’ cuticle, in a segment in common with 635 bp, present 98.8% similarity (8 bp differences: insertions, deletions or substitutions) with \textit{A. aenigmaticus} (MH092911) and 97.6% (17 bp) with \textit{A. mariannae} (KC509907), while in comparison to species with a ‘single’ cuticle, such as \textit{A. ciliatus} (AF202148), they present 98.3% similarity (9 or 12 bp differences). The other two species, \textit{Acrobeles cephalatus} (Cobb, 1901) Thorne, 1925 and \textit{Acrobeles ctenocephalus} Thorne, 1925 (AB630972, AY630971, DQ080560) do not show any overlapped segment with \textit{A. complexus} sequenced in the present study; however, with other \textit{A. complexus} sequences (KU180671, AY284671) and \textit{A. ciliatus}, they maintain a shared segment with 591 bp, having 97.6% similarity (14 bp differences) with each other. Thus, \textit{A. ctenocephalus}, with a ‘double’ cuticle, presents 98.9% similarity (6 bp differences) with \textit{A. ciliatus}, 98.8%
(7 bp) with *A. cephalatus* and 98.6% (8–9 bp) with *A. complexus* (KU180671, AY284671). *Acrobeles cephalatus* with a 'single' cuticle has 99.2% similarity (2 bp and 7 bp differences) with *A. complexus* (KU180671, AY284671, respectively), and 98.3% (10 bp) with *A. ciliatus*.

The new 28S sequence obtained has been analysed and compared with other 28S rDNA sequences available in GenBank. From a shared segment with 674 bp, *A. complexus* maintains 97.6% similarity (15–18 bp differences) with *A. aenigmaticus* (MG200059), 91.5% (56–59 bp) with *Acrobeles cf. undulatus* Loof, 1964 (HM055387), 88.8% (73–75 bp) with *Acrobeles singulus* (DQ145622) and 88.7% (76 bp) with *A. ciliatus* (DQ14561).

On the other hand, *A. ciliatus* presents 92.9% similarity (48 bp differences) with *A. singulus*, 87.9% (81 bp) with *A. undulatus*, all of them with a 'single' cuticle, and 89.2% (73 bp) with *A. aenigmaticus* and 88.7% (76 bp) with *A. complexus*, both species with a 'double' cuticle.

With respect to other genera, the 18S sequences of the *complexus*-group and the *ciliatus*-group, comparing a shared segment with 637 bp, differ, respectively, in 7 vs. 10 bp with *Cervidellus*, 32 vs. 33 bp with *Acrobeloides* (Cobb, 1924) Thorne, 1937, 30 vs. 27 bp with *Pseudacrobeles* Steiner, 1938 and 34 vs. 33 bp with *Eucephalobus* Steiner, 1936. The 28S sequences differ, regarding the *complexus* and *ciliatus* groups, respectively, in 125 vs. 138 bp with *Nothacrobeles* Allen & Noffsinger, 1971, 150 vs. 186 bp with *Cervidellus*, 143 vs.

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**Fig. 5.** Bayesian inference tree from known and newly sequenced *Acrobeles complexus* based on sequences of the 18S rDNA region. Bayesian posterior probabilities (%) are given for each clade. Scale bar shows the number of substitutions per site.
203 bp with *Acrobeloides*, 179 vs. 220 bp with *Eucephalobus* and 213 vs. 232 bp with *Pseudacrobeles*. This shows that the species of the genera *Cervidellus* and *Nothacrobeles* are the most related with both *Acrobeles* groups, as is visible in both phylogenetic trees. Nevertheless, these molecular analyses show that these related genera are polyphyletic, as is evident in the morphology of the lip region of their species, which have great variability and are in need of a deep review.

**Integrative morphological and molecular results**

The morphological and molecular analyses show that both groups are very similar but have important differences – in particular, the presence or absence of an inner refringent cuticle. This could indicate that both groups of species are not closely related, as shown in the phylogenetic trees. The *ciliatus*-group contains 21 species but, unfortunately, only four of them have available 18S
or 28S sequences. The complexus-group includes 13 species, only four of them have available 18S or 28S sequences. Unfortunately, most specimens with sequences available in GenBank lack description and their identity cannot be confirmed. The present molecular analyses based on 18S and 28S DNA segments show that species with a ‘single’ cuticle appear together as well as species with a ‘double’ cuticle. This phylogenetic arrangement agrees, in general, with the morphological differences described previously. Thus, according to morphological and molecular observations, the ciliatus-group present plesiomorphic characters (‘single’ cuticle, absence of cuticular pore-like structures), while the complexus-group present apomorph characters (‘double’ cuticle, presence of cuticular pore-like structures).

According to these morphological and molecular differences, it should justify erecting both groups as separate genera, as proposed by Andrássy (1985, 2005). Both groups maintain few, albeit important, morphological differences, which are not enough to consider them as separate genera. On the other hand, three species were described with an ‘intermediate’ cuticle having an incomplete refringent inner cuticular layer (Acrobeles andalisicus Abolafia & Peña-Santiago, 2004, Acrobeles sparsus Heyns, 1969 and A. undulatus), provisionally included in the ciliatus-group; however, any sequences that may have been obtained for these species (only an unidentified species but having similarities with A. undulatus, HM053587) and their phylogenetic relationships remain unknown. In addition, most species belonging to both groups lack molecular analyses at the present, being premature to restate both taxa to the generic level. Thus, according to both morphological and molecular differences, we consider it more suitable to erect the separation of both groups in subgeneric levels, the subgenus Acrobeles for the ciliatus-group and the subgenus Seleborca for the complexus-group, maintaining the names proposed by Andrássy (1985, 2005) until sequences of more species are obtained and their phylogenetic position more reliably identified.

List of species

Subgenus Acrobeles (ciliatus-group)
- Acrobeles (Acrobeles) andalisicus Abolafia & Peña-Santiago, 2004
- Acrobeles (Acrobeles) annulatus Heyns, 1969
- Acrobeles (Acrobeles) bushmanicus Heyns, 1969
- Acrobeles (Acrobeles) canalis Andrássy, 1985
- Acrobeles (Acrobeles) chelatus Thomas & Allen, 1965
- Acrobeles (Acrobeles) ciliatus von Linstow, 1877
- Acrobeles (Acrobeles) cylindricus Ivanova, 1968
- Acrobeles (Acrobeles) elaboratus Thorne, 1925
- Acrobeles (Acrobeles) ensicaudatus Thomas & Allen, 1965 (species was transferred to the genus Seleborca by Andrássy (1985); however, it lacks a ‘double’ cuticle and is now maintained in Acrobeles as was originally proposed by Thomas & Allen (1965))
  = Acrobeles ensicaudatus Thomas & Allen, 1965
  = Seleborca ensicaudata (Thomas & Allen, 1965) Andrássy, 1985
- Acrobeles (Acrobeles) farzanae Heyns, 1995
- Acrobeles (Acrobeles) hokingtongus Yeates, 1967
- Acrobeles (Acrobeles) microstomus Iliev, Ilieva & Mitor, 2003
- Acrobeles (Acrobeles) seyleae Rashid et al., 1990
- Acrobeles (Acrobeles) serricornus Thorne, 1925
- Acrobeles (Acrobeles) sheasbyi Heyns & Hogewind, 1969
- Acrobeles (Acrobeles) singulus Heyns, 1969
- Acrobeles (Acrobeles) sparsus Heyns, 1969
- Acrobeles (Acrobeles) tarus Yeates, 1967
- Acrobeles (Acrobeles) thornei Heyns, 1962
- Acrobeles (Acrobeles) undulatus Loof, 1964
- Acrobeles (Acrobeles) zapatai Mundo-Ocampo, Baldwin, Durado-Ramírez & Morales-Ruiz, 2003

Subgenus Seleborca n. rank (complexus-group)
- Acrobeles (Seleborca) aenigmaticus Abolafia, Shokoohi, Du Preez & Fourie, 2019 (n. comb., n. rank)
- Acrobeles (Seleborca) complexus Thorne, 1925 (n. rank)
  = Acrobeles complexus Thorne, 1925
  = Seleborca complexa (Thorne, 1925) Andrássy, 1985
  = Acrobeles crossotus Steiner, 1929
- Acrobeles (Seleborca) etenocephalus Thorne, 1925 (n. rank)
  = Acrobeles etenocephalus Thorne, 1925
  = Seleborca etenocephala (Thorne, 1925) Andrássy, 1985
- Acrobeles (Seleborca) dimorphus Heyns & Hogewind, 1969 (n. rank)
  = Acrobeles dimorphus Heyns & Hogewind, 1969
  = Seleborca dimorpha (Heyns & Hogewind, 1969) Andrássy, 1985
- Acrobeles (Seleborca) emmatus Shahina & De Ley, 1997 (n. comb., n. rank)
- Acrobeles (Seleborca) geraerti Rashid, Heyns & Coomans, 1990
  = Seleborca geraerti Rashid, Heyns & Coomans, 1990
- Acrobeles (Seleborca) geraerti Rashid, Heyns & Coomans, 1990
  = Seleborca geraerti Rashid, Heyns & Coomans, 1990 (n. rank)
  = Shahina & De Ley, 1997
- Acrobeles (Seleborca) iranicus Shokoohi, Abolafia & Zad, 2007 (n. comb., n. rank)
- Acrobeles (Seleborca) mariannae Andrássy, 1968 (n. rank)
  = Acrobeles mariannae Andrássy, 1968
- Acrobeles (Seleborca) mariannae (Andrássy, 1968) Andrássy, 1985
- Acrobeles (Seleborca) capensis Heyns, 1969
- Acrobeles (Seleborca) oasiensis Böstrom, 1985 (n. rank)
  = Acrobeles oasiensis Böstrom, 1985
- Acrobeles (Seleborca) oasiensis (Böstrom, 1985) Rashid, Heyns & Coomans, 1990
- Acrobeles (Seleborca) ornatus Thorne, 1925 (n. rank)
  = Acrobeles ornatus Thorne, 1925
- Acrobeles (Seleborca) ornata (Thorne 1925) Andrássy, 1985
- Acrobeles (Seleborca) recurvus Heyns, 1969 (n. rank)
  = Acrobeles recurvus Heyns, 1969
- Acrobeles (Seleborca) recurvus (Heyns, 1969) Andrássy, 1985
- Acrobeles (Seleborca) timmi Chaturvedi & Khera, 1979 (n. rank)
  = Acrobeles timmi Chaturvedi & Khera, 1979
- Acrobeles (Seleborca) timmi (Chaturvedi & Khera, 1979) Andrássy, 1985
- Acrobeles (Seleborca) welwitschiae (Rashid, Heyns & Coomans, 1990) Shahina & De Ley (n. rank)
- Acrobeles (Seleborca) welwitschiae Rashid, Heyns & Coomans, 1990
  = Acrobeles welwitschiae Rashid, Heyns & Coomans, 1990
  = Shahina & De Ley, 1997

Species inquirenda vel incertae sedis
- Acrobeles cephalatus (Cobb, 1901) Thorne, 1925
- Acrobeles ilidzensis Paesler, 1941
- Acrobeles neocephalatus Kannan, 1961
- Acrobeles pachidinovae Atakhanov, 1958
- Acrobeles raoi Kannan, 1961
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Conflicts of interest. None

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