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Redescription and synonymization of *Oscheius citri* Tabassum, Shahina, Nasira and Erum, 2016 (Rhabditida, Rhabditidae) from India and its taxonomical consequences

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Research Paper

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18S rDNA; 28S rDNA; ITS rDNA; molecular analysis; morphology; *Oscheius citri*; *O. cobbi*; *O. cynodonti*; *O. esculentus*; *O. punctatus*; *O. sacchari*; taxonomy

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Abstract

A population of a nematode species belonging to the genus *Oscheius* was isolated in western Uttar Pradesh, India. Morphological and morphometrical studies on this species showed its high similarity with six species described previously from Pakistan (*Oscheius citri*, *O. cobbi*, *O. cynodonti*, *O. esculentus*, *O. punctatus* and *O. sacchari*). The molecular analysis of the ITS1-5.8S-ITS2 rDNA sequences of the Indian population and the six species described from Pakistan showed that all the sequences are almost identical. Thus, based on morphological and molecular characteristics, all of the six above-mentioned Pakistani species and Indian strain do not differ from each other, hence can be considered synonyms. The correct name for this taxon is the first described species *O. citri*. Additionally, the phylogenetic analysis of the 18S rDNA and the 28S rDNA sequences showed that *Oscheius citri* is sister to the clade formed by *O. chongmingensis* and *O. rugaoensis* from China. The high similarity of morphological and morphometric characteristics of *O. citri* and other species, *O. maqbooli*, *O. nadarajani*, *O. niazii*, *O. shamimi* and *O. siddiqii*, suggest their conspecificity; however, lack of molecular data for these species does not allow this hypothesis to be tested.

Introduction

The genus *Oscheius* was established by Andrássy (1976) and comprises free-living nematodes which feed on bacteria, some of them reported to display entomopathogenic (Liu *et al.*, 2012; Pervez *et al.*, 2013) or scavenging behaviour (Campos-Herrera *et al.*, 2015; Zhang *et al.*, 2019). Owing to potential entomopathogenicity, this genus received increased attention from the scientific community in recent years. Furthermore, *Oscheius* species display a remarkable phylogenetic pattern in mode of reproduction, gonad development, body size and vulva formation. This genus was revised recently by Abolafia & Peña-Santiago (2019), and includes two subgenera, *Oscheius* Andrássy, 1976, containing 26 species, and *Dolichorhabditis* Andrássy, 1983, containing 16 species, several of them with doubtful identity. Some of these doubtful species is the group composed of six species described by Tabassum *et al.* (2016) from Pakistan: *Oscheius citri*, *O. cobbi*, *O. cynodonti*, *O. esculentus*, *O. punctatus* and *O. sacchari*. These Pakistani species were described as having very similar morphology and with overlapping measurements. Unfortunately, line illustrations are inaccurate while LM illustrations lack the quality to distinguish these species correctly from each other. Furthermore, molecular support for these species is not satisfactory with only internal transcribed spacer (ITS) sequences of quite low quality being presented.

In the present study, during a survey carried out in agricultural fields of Meerut district, Uttar Pradesh, India, a white grub cadaver was recovered from sugarcane field from which one species of the genus *Oscheius* was isolated by White trap method (White, 1927). Morphological, morphometrical and molecular studies on this species showed important similarity with all of these six species described by Tabassum *et al.* (2016). Now, detailed redescription of this species based on morphological, morphometrical and molecular (18S, 28S and ITS rDNA gene sequences) data are provided including line, LM and SEM illustrations.

Materials and methods

Nematode source

During a survey of soil samples in different agricultural fields of district Meerut (28°98′N, 77°71′E, and elevation of 225 m asl) of western Uttar Pradesh, India, a dead larva of white

grub (*Holotrichia* sp.) was recovered from sugarcane fields (*Saccharum officinarum* L.). The cadaver was brought to the laboratory, washed with ddH_2O, disinfected with 1% NaOCl (Bhat *et al.*, 2017), placed in White's traps (White, 1927) and kept at 27°C till emergence of the juveniles. The emerged nematodes from the White's trap method were harvested, disinfected and finally stored in 250-ml culture flasks in the biological oxygen demand (BOD) incubator at 15°C as described by Bhat *et al.* (2019a).

Morphological and morphometrical characterization

For light microscopy and morphometric measurements, nematodes were reared on last instar larvae of *Galleria mellonella*. A total of ten larvae of *G. mellonella* were injected with sterilized third-stage juveniles (>2000) in sterile Petri plates using a 1-ml insulin syringe, which died within 36 to 48 h (Rana *et al.*, 2020; Bhat *et al.*, 2020). Adult generation (males and females) and freshly emerged third-stage juveniles were recovered from white traps within 7–10 days (Suman *et al.*, 2020). These were killed in hot water (60°C), fixed in triethanolamine formaline (TAF) (7 ml formalin, 2 ml triethanolamine, 91 ml distilled water) (Courtney *et al.*, 1955), processed to glycerine (Seinhorst, 1959) and mounted into a small drop of glycerine with extra amount of paraffin wax to prevent flattening of nematodes (Bhat *et al.*, 2019b).

Observations and measurements were performed with the help of inbuilt software (Nikon DS-L1) of phase contrast microscope (Nikon Eclipse 50i) in micrometres. The best-preserved specimens were also photographed using a Nikon Eclipse 80i (Nikon, Tokyo, Japan) light microscope provided with differential interference contrast optics (DIC) and a Nikon Digital Sight DS-U1 camera. Micrographs were edited using Adobe® Photoshop® CS. The terminology used for the morphology of stoma and spicules follows the proposals by De Ley *et al.* (1995) and Abolafia & Peña-Santiago (2017), respectively.

Scanning electron microscopy

For the scanning electron microscopy (SEM), specimens preserved in glycerine were selected for observation under SEM according to the Abolafia's (2015) protocol. The nematodes were hydrated in distilled water, dehydrated in a graded ethanol–acetone series, critical point dried with liquid carbon dioxide, mounted on SEM stubs, coated with gold and observed with a Zeiss Merlin microscope (5 kV) (Zeiss, Oberkochen, Germany).

Molecular characterization

DNA was extracted both from single females and from the pool of infective juveniles (IJs) (n >500). In first case, each female was transferred into a sterile Eppendorf tube (500 µl) with 10 µl of extraction buffer (8.85 µl of ddH_2O, 1 µl of 10 × polymerase chain reaction (PCR) buffer, 0.1 µl of 1% Tween and 0.05 µl of proteinase K). Buffer and nematode were frozen at −20°C for 20 min and then immediately incubated at 65°C for 1 h, followed by 10 min at 95°C. The lysates were cooled on ice, centrifuged (2 min, 9000 g) and 1 µl of supernatant was used for PCR (Bharti *et al.*, 2020; Bhat *et al.*, 2021a). The DNA from the IJs was extracted via Qiagen Blood and Tissue Analysis Kit (Hilden, Germany) following the manufacturer's protocol. For each fragment amplified (see below) the DNA extracts from five females and from pool of IJs were used. A fragment of rDNA containing the internal transcribed spacer regions (ITS1, 5.8S, ITS2) was amplified using primers 18S: 5′-TTGATTACGTCCCTGCCCTTT-3′ (forward), and 28S: 5′-TTTCACTCGCCGTTACTAAGG-3′ (reverse) (Vrain *et al.*, 1992). The other fragment containing D2-D3 expansion segments of the 28S rDNA gene was amplified using primers D2F: 5′-CCTTAGTAACGGCGAGTGAAA-3′ (forward) and 536: 5′-CAGCTATCCTGAGGAAAC-3′ (reverse) (Nguyen, 2007) and fragment containing 18S rDNA using two pairs of primers 18S-F: 5′-GATACCGCCCTAGTTCTGACC-3′ and 18S-R: 5′-ACCAACTAAGAACGGCCATG-3′ (Liu *et al.*, 1997) and G18S4: 5′-GCTTGTCTCAAAGATTAAGCC-3′ and 18P: 5′-TGATCCWMCRGCAGGTTCAC-3′ (Blaxter *et al.*, 1998).

The PCR master mix consisted of ddH_2O 7.25 µl, 10 × PCR buffer 1.25 µl, dNTPs 1 µl, 0.75 µl of each forward and reverse primers, polymerase 0.1 µl and 1 µl of DNA extract. The PCR profiles were used as follows for ITS: one cycle of 94°C for 7 min followed by 35 cycles of 94°C for 60 s, 50°C for 60 s, 72°C for 60 s and a final extension at 72°C for 7 min (Nguyen, 2007); for 28S rDNA: one cycle of 94°C for 7 min followed by 35 cycles of 94°C for 60 s, 55°C for 60 s, 72°C for 60 s and a final extension at 72°C for 10 min (Mráček *et al.*, 2014); and for 18S rDNA: one cycle of 94°C for 5 min followed by 37 cycles of 94°C for 60 s, 55°C for 90 s, 72°C for 2 min and a final extension at 72°C for 10 min (De Ley & Blaxter, 2002). PCR was followed by electrophoresis (45 min, 120 V) of 2 µl of PCR product in a 1% Tris-acetate-EDTA buffered agarose gel stained with ethidium bromide (20 µl per 100 ml of gel) (Bhat *et al.*, 2021b). For each fragment, five PCR products were sequenced by Eurofins Genomics (Ebersberg, Germany) and one by Bioserve PVT Ltd (Hyderabad, India). No variability was present among the product for each marker and one sequence for each fragment was deposited in GenBank under accession numbers MN137988 (ITS sequences), MK932087 (28S sequence) and MK932670 (18S sequence).

Sequence alignment and phylogenetic analyses

The sequences were edited and compared with those present in GenBank by means of a Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI) (Altschul *et al.*, 1990). An alignment of our samples with sequences of other *Oscheius* species was produced for each amplified rDNA region (ITS, SSU and LSU) using default ClustalW parameters in MEGA 7.0 (Kumar *et al.*, 2016) and optimized manually in BioEdit (Hall, 1999). In order to compare ITS region of the selected *Oscheius* species from the '*insectivorus*' group, pairwise distances were computed using MEGA 7.0 (Kumar *et al.*, 2016). Codon positions included were 1st + 2nd + 3rd + Noncoding.

The phylogenetic trees were inferred from the datasets using Bayesian inference (BI). All characters were treated as equally weighted and gaps as missing data. *Heterorhabditis downesi, H. bacteriophora* and *H. zealandica* were used as outgroup taxa. Bayesian phylogenetic reconstructions were performed using MrBayes 3.2.7 (Ronquist *et al.*, 2012). The best fit model was identified as the GTR + G model test using the MrModeltest 2.0 program (Nylander, 2004). Metropolis-coupled Markov chain Monte Carlo (MCMCMC) generations were run for 1×10^7 cycles and one tree was retained every 1000 generations.

Results

Systematics

Oscheius citri Tabassum, Shahina, Nasira and Erum, 2016 (figs 1–4, tables 1–4)

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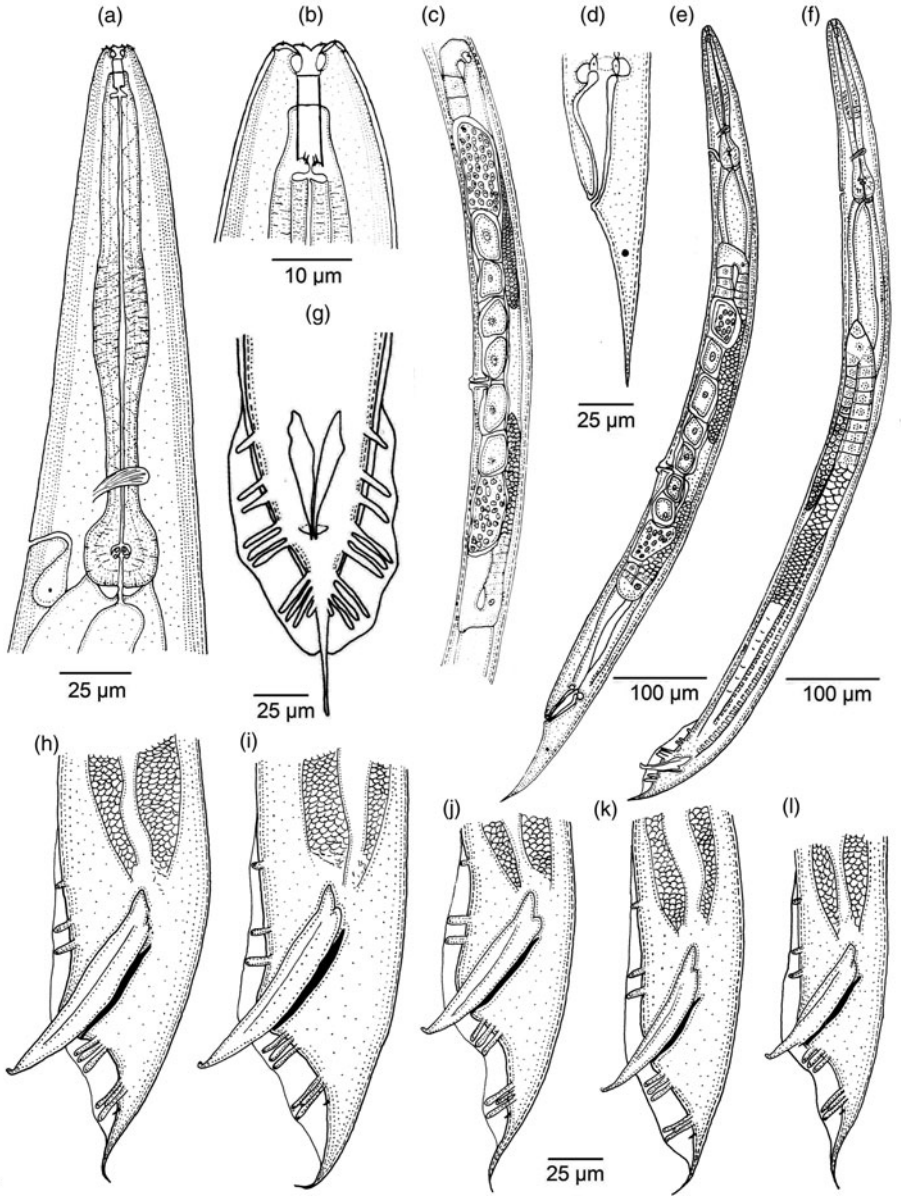

Fig. 1. *Oscheius citri* Tabassum, Shahina, Nasira and Erum, 2016 (line drawing). (a) neck; (b) anterior end; (c) female reproductive system; (d) female posterior end; (e) entire female; (f) entire male; (g) male posterior end at ventral view; (h–l) male posterior end at lateral views showing spicule variability.

Material examined: 20 females, 20 males and 20 L3 juveniles (obtained from *Galleria* specimens from agricultural soils).

Description

Adults: Body fusiform, 1.37–2.35 mm long in females and 1.02–1.43 mm long in males, straight to slightly arcuate tapering at both the ends. Cuticle somewhat transversely annulated. Lateral fields with eight distinct longitudinal incisures at the middle of the body, the more lateral scarcely deep. Lip region with six separate lips, bearing six inner labial papillae and four outer cephalic papillae; primary and secondary axils deep, with similar morphology. Amphidial openings reduced, ovoid, located frontally. Stoma rhabditoid, 4.2–4.6 times longer than wide or 1.6–3.3 times longer than the lip region wide, with tubular part slightly wider posteriorly; cheilostom conspicuous, with ovoid rhabdia that is poorly cuticularized; gymnostom cuticularized, straight; pro-mesostegostom with refringent rhabdia; metastegostom with isomorphic glottoid apparatus with each bearing two minute warts, telostegostom short, connected to pharyngeal lumen. Pharyngeal collar surrounding the stegostom, 25–39% of the stomatal tube length. Pharynx rhabditoid: pharyngeal corpus differentiated into cylindrical procorpus and robust cylindroid metacorpus, 1.7–2.0 times the procorpus length; isthmus robust, well differentiated from metacorpus, basal bulb spheroid to ovoid with conspicuous valvular apparatus. Nerve ring encircling isthmus usually in posterior half, at 61–81% of the neck length. Excretory pore located at the level of basal bulb or slightly posterior, at 84–104% of the neck length. Deirids located at the level of isthmus-bulb junction at 69–80% of the neck length. Cardia short conoid. Intestine without differentiation but having thinner walls at cardiac part.

Female: Reproductive system didelphic, amphidelphic. Ovaries well developed, dorsally reflexed often extending beyond vulva. Oviducts dilated, connected to sperm filled spermathecae. Uteri 9.3–17.4 times the corresponding body diameter, containing eggs in different stages of embryonation, 40–56 × 22–39 µm, ranging up to 23–56, in young females and numerous larvae in old females which develop *endotokia matricida*. Vagina short,

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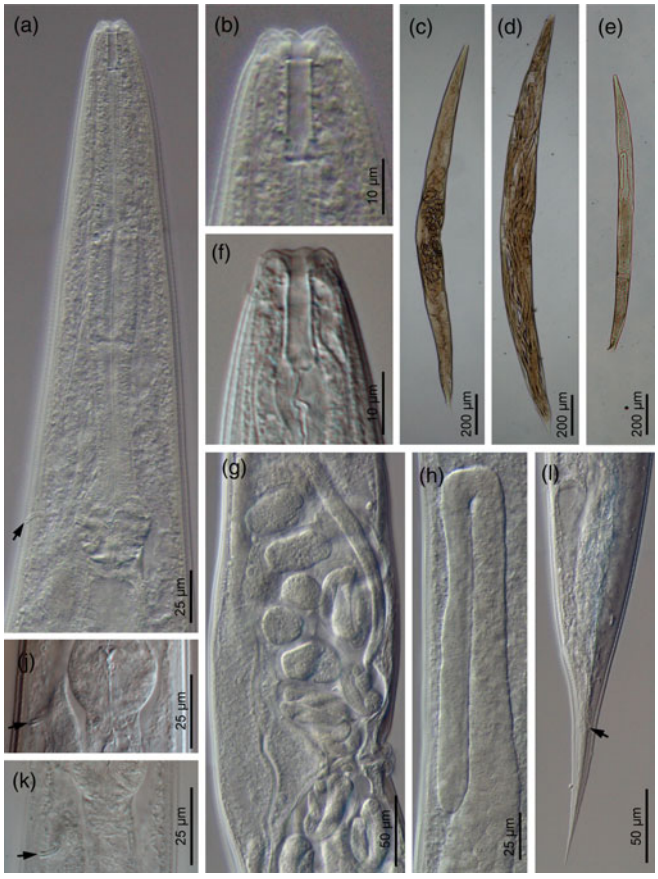

Fig. 2. *Oscheius citri* Tabassum, Shahina, Nasira and Erum, 2016 (light microscopy). (a) neck (arrow pointing the excretory pore); (b, f) anterior end; (c) young female; (d) Old female; (e) male; (g) uterine eggs; (h) testis; (i female posterior end (arrow pointing the phasmid); (j, k) excretory pore (arrow).

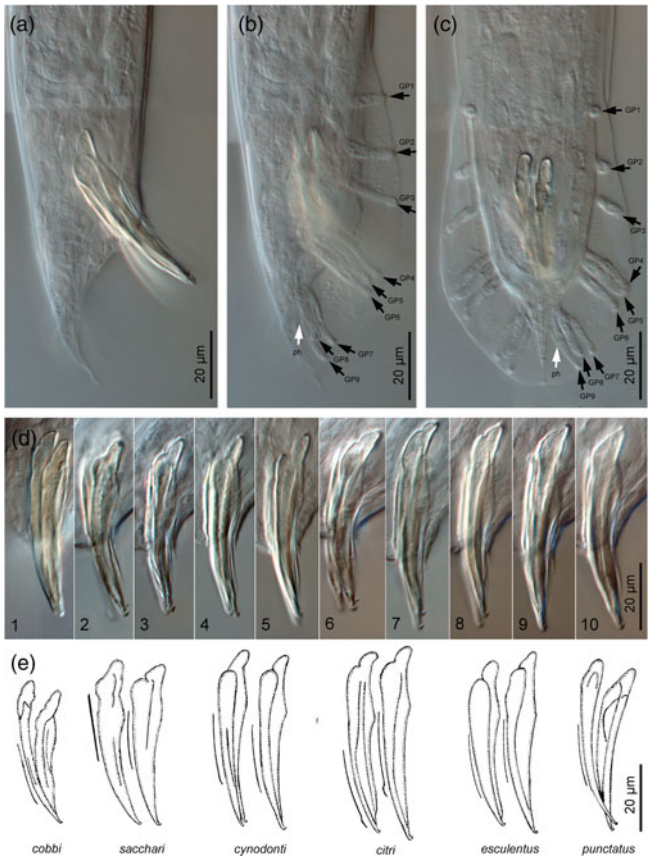

Fig. 3. *Oscheius citri* Tabassum, Shahina, Nasira and Erum, 2016 (light microscopy). (a, b) male posterior end at lateral view at spicules level and bursa level, respectively; (c) male posterior end at ventral view (black arrows pointing the genital papillae, GP, white arrow pointing the phasmid, ph). (d) spicules variability. (e) facsimile reproduction of the spicules of the six Pakistani species (obtained from Tabassum *et al.*, 2016).

straight, 0.2 times the corresponding body diameter. Vulva transversal, laterally limited by reduced epygtigma. Rectum elongate, 1.1–2.2 times the anal body width. Anus a crescent-shaped slit. Tail conoid, dorsally almost straight and ventrally slightly concaved posterior to anus, with pointed tip. Phasmids located 26–32% of tail length from anus.

Male: General morphology similar to female except smaller size. Reproductive system monorchic with testis ventrally reflexed anteriorly, located on the left side of intestine. Bursa present, leptoderan. Genital papillae nine pairs (1 + 2/3 + 3 + ph), three pairs pre-cloacal (GP1 and GP2 more spaced) and six pairs post-cloacal appearing three pairs at cloaca level and three pairs posteriorly. Phasmids well observed, located posterior to GP9. Spicules elongate, with variable morphology at proximal end, 2.0–2.4 times the gubernaculum length, with manubrium conoid and scarcely ventrally bent, calamus very short and lamina with dorsal hump variable in size, poorly developed ventral velum and very finely hooked tip. Gubernaculum slender, 0.4–0.5 times the spicules length, with manubrium ventrally arcuate and almost straight corpus.

Juvenile: J3 juvenile 0.50–0.72 µm long. Body straight, elongate, cuticle almost smooth, lip region similar to adult specimens. All morphological features were similar to adults except the absent reproductive system. Stoma tubular, 2.3–3.0 times the lip region width and 6.8–11 times longer than wide. Tail ending at a hyaline part, 22–31 µm long or 26–34% of tail length.

Molecular characterization

The *Oscheius* species examined in present study is molecularly characterized by the sequences of three genes, 18S rDNA (1661 bp), ITS1-5.8S-ITS2 rDNA (1036 bp) and the D2-D3 fragment of 28S rDNA (869 bp). The Blastn analysis of the ITS1-5.8S-ITS2 rDNA sequences of our sample showed the highest similarity with six ITS1-5.8S-ITS2 rDNA sequences of *Oscheius* (KT250509 *O. citri*; KT878513 *O. cobbi*; KT250510 *O. sacchari*; KU997024 *O. cynodonti*; KT878514 *O. esculentus* and KU284845 *O. punctatus*) from Pakistan described by Tabassum *et al.* (2016). The pairwise distances analysis have shown that the similarity ranges from 93% to 98% (table 4); however, the vast majority of the differences were present close to the 3′ and 5′ ends of the sequences, whereas in the middle part, the sequences are almost identical to the sequence of the *Oscheius* species examined now. It is thus likely that the sequences of the Pakistani species are inadequately edited or were not even edited at all before submission to the GenBank database. After deleting the *c.* 100 bp segment from both 3′ and 5′ ends of the Pakistani sequences and trimming the whole alignment accordingly, pairwise distance analysis shows 99–100% similarity of Pakistani sequences with each other and with the *Oscheius* species studied now, but the distances among other species remained very prominent (table 5). This clearly demonstrates that a vast majority of differences that were found in Pakistani sequences are present

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Table 1. Morphometrics of *Oscheius citri* Tabassum, Shahina, Nasira and Erum, 2016 from India.

Characters	Female	Male	3rd stage juvenile
n	20	20	25
Body length (L)	1826 ± 282 (1370–2348)	1184 ± 117 (1024–1433)	581 ± 42 (504–715)
a (L/BD)	17.6 ± 2.3 (14.5–23.2)	19 ± 1.6 (15.7–22.1)	22.7 ± 1.5 (20.1–27.4)
b (L/Neck)	8.5 ± 1.3 (6.4–11.0)	6.4 ± 0.7 (5.3–8.1)	4.3 ± 0.4 (3.6–5.0)
c (L/T)	16.5 ± 2.5 (12.3–23.7)	30 ± 4.4 (24.8–41.4)	6.6 ± 0.6 (5.5–8.0)
c′ (T/ABW)	3.4 ± 0.4 (2.7–4.2)	1.6 ± 0.2 (1.2–2.1)	5.3 ± 0.6 (4.4–6.6)
V% (VA/L×100)	50 ± 13 (48–52)	–	–
Lip region width	7.9 ± 1.1 (6–11)	6.6 ± 0.9 (5–8)	4.2 ± 0.6 (3–6)
Stoma length	18.2 ± 2.0 (14–21)	14.7 ± 1.6 (11–18)	13.1 ± 1.8 (10–17)
Stomatal tube width	4.0 ± 0.6 (3–5)	3.1 ± 0.6 (2–4)	1.7 ± 0.4 (1–3)
Procorpus length	61 ± 5.8 (53–74)	57 ± 5.5 (49–68)	49 ± 5.1 (39–51)
Metacorpus length	38 ± 2.8 (28–44)	31 ± 2.0 (27–33)	19.5 ± 2.3 (15–24)
Isthmus length	51 ± 5.0 (41–60)	47 ± 3.2 (40–53)	28 ± 2.7 (21–32)
Bulb length	30 ± 1.8 (27–34)	26 ± 1.9 (23–30)	15.6 ± 1.0 (14–17)
Pharynx length	194 ± 11.7 (167–224)	169 ± 10.8 (154–199)	123 ± 6.3 (108–134)
Nerve ring–ant. end (NR)	151 ± 16.5 (130–201)	116 ± 13 (96–150)	89 ± 14 (62–113)
Excretory pore–ant. end (EP)	195 ± 21 (144–223)	171 ± 15 (149–211)	86 ± 10 (64–100)
Deirid–ant. end	167 ± 12.8 (152–198)	117 ± 20 (86–163)	?
Neck length (stoma + pharynx)	216 ± 12.3 (190–250)	187 ± 11.1 (170–217)	136 ± 7.6 (122–155)
Body width at neck base	63 ± 4.3 (57–72)	42 ± 3.6 (37–53)	22 ± 4.1 (20–24)
Body diameter at midbody (BD)	103 ± 8.4 (91–118)	63 ± 5.8 (54–73)	26 ± 1.4 (22–28)
Anterior ovary or Testis	237 ± 34.3 (174–311)	576 ± 87 (473–779)	–
Anterior spermatheca length	51 ± 10.4 (28–74)	–	–
Anterior genital branch	237 ± 23 (199–292)	–	–
Posterior ovary	192 ± 38 (115–289)	–	–
Posterior spermatheca length	43 ± 7.2 (25–57)	–	–
Posterior genital branch	222 ± 27 (182–290)	–	–
Vagina length	19.7 ± 3.6 (14-27)	–	–
Vulva – ant. end (VA)	967 ± 258 (678–1883)	–	–
Rectum length	52 ± 15.3 (34–86)	22 ± 4.1 (17–35)	26 ± 3.7 (18–32)
Anal body diam. (ABD)	33 ± 3.3 (28–40)	25 ± 2.5 (20–31)	17 ± 1.5 (13–19)
Tail length (T)	111 ± 14 (87–151)	39 ± 4.2 (32–51)	88 ± 7.6 (71–102)
Phasmid to anus distance	33 ± 6.4 (23–48)	39 ± 6.3 (31–53)	?
Spicule length	–	58 ± 7.6 (42–75)	–
Gubernaculum length	–	25 ± 3.0 (21–31)	–

All measurements are in µm (except n, ratios and percentages) and in the form: mean ± SD (range).
?, Measurement unknown; –, Character absent.

in the conservative parts of the sequences and thus are most likely the result of inadequate sequence editing.

There is no record of 18S rDNA and D2-D3 fragments of 28S rDNA of these six Pakistani species. The Blastn analysis of 18S rDNA and D2-D3 fragment of 28S rDNA sequences of present specimens showed the highest 98.4% and 97% similarity, respectively, with the 18S rDNA (EF503692, MT548590, MT548589, MT548588, EU273597, JQ002566) and D2-D3 fragment of 28S rDNA (EU273599, MT548594-MT548600) sequences of *O. chongmingensis* (Zhang *et al.*, 2008) Ye *et al.*, 2010 from China.

Diagnosis (based on the Indian and Pakistani populations)

Oscheius citri from India is characterized by having 1.37–2.35 mm long females and 1.02–1.43 mm long males, cuticle with very fine transverse striation, lateral field with eight distinct lines, lip region

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Table 2. Comparative morphometrics of females of *Oscheius citri* Tabassum, Shahina, Nasira and Erum, 2016 from India and its related species.

Species	*citri*	*citri*	*cobbi*	*cynodonti*	*esculentus*	*punctatus*	*sacchari*	*Andrassyi*	*indicus*	*maqbooli*	*nadarajani*	*niazii*	*shamimi*	*siddiqii*
Reference	Present study	Tabassum *et al.* (2016)	Tabassum *et al.* (2016)	Tabassum *et al.* (2016)	Tabassum *et al.* (2016)	Tabassum *et al.* (2016)	Tabassum *et al.* (2016)	Tabassum & Shahina (2008)	Kumar *et al.* (2019)	Tabassum & Shahina (2002)	Ali *et al.* (2011)	Tabassum & Shahina (2010)	Tahseen & Nisa (2006)	Tabassum & Shahina (2010)
Country	India	Pakistan	Pakistan	Pakistan	Pakistan	Pakistan	Pakistan	Pakistan	India	Pakistan	India	Pakistan	India	Pakistan
Locality	Meerut	Gadap	Karachi	Islamabad	?	Karachi	Nawabshah	Jhang	Cachar	Chatha Goth	Kurmikhera	Niaz Manzil	Aligarh	Niaz Manzil
Province/State	Uttar Pradesh	Sindh	Sindh	Islamabad	Azad Kashmir	Sindh	Sindh	Punjab	Assam	Balochistan	Uttar Pradesh	Sindh	Uttar Pradesh	Sindh
Habitat	Sucarcane	Citrus sp.	Grass	Grass	Okra	Grass	Dead moth	Sugarcane	Fallow fields	Sugarcane	Lentil	Coco palm	Flowerbeds	Coco palm
L	1370–2348	1173–2015	950–1712	1252-1662	1249–1798	1217–1680	1362–2015	1322–1962	1072–1480	942–1342	1358–1606	837–1487	1360–2420	1130–1390
a	14.5–23.2	17.7–28.5	12.0–21.6	16.0–20.0	15.6–19.5	12.5–18.4	16.0–19.8	15.0–21.0	16.8–20.6	14.1–18.0	17.5–20.1	14.6–17.3	12.0-20.0	15.0–18.0
b	6.4–11.0	4.8–7.9	6.6–8.8	8.5–10.7	6.8–8.9	7.0–9.6	6.7–10.3	6.7–9.4	5.7–7.1	4.8–5.6	5.0–5.2	8.8–11.5	4.2–6.3	6.0–6.9
c	12.3–23.7	9.0–13.0	9.0–14.8	13.0–24.0	13.0–15.3	8.2–16.8	7.6–16.8	11.0–17.9	7.5–10.4	6.7–11.0	11.1–13.0	13.5–20.0	6.8–13.8	10.0–13.0
c′	2.7–4.2	4.0–5.9	2.8–4.3	4.0–6.7	2.9–4.2	3.3–5.7	3.5–5.9	3.0–4.3	5.0–6.6	4.6–5.9	5.1–5.2	2.8–4.0	4.1–5.8	3.5–5.4
V	48–52	47–51	42–57	41-53	49–55	46–61	45–52	50–53	45–50	49–53	53*	48–54	45-51	40–52
Lip region width	6–11	10*	10–12	12–18	14–20	12–15	12–14	8*	11–15	13–16	10*	6	11–14	10*
Stoma length	14–21	17–20	14–22	12–18	13–18	12–15	16–18	14–18	13–18	19*	19	15–25	19–23	13–17
Isthmus length	41–60	50–54	29–50	35–45	33–48	28–45	42*	35–50	37–47	58*	27*	40–45	58*	45–50
Bulb length	27–34	34–36	27.6*	28–32	32.5*	34–40	32–36	30–40	33–41	32–40	33*	35–40	26–30	30–38
NR–ant. end	130–201	120–190	130–240	100–125	129–240	117–150	128–175	130–165	118–162	128–164	165–201	140–170	112–165	130–160
EP–ant. end	144–223	187–265	130–280	130–160	140–240	132–185	170–200	160–225	154–201	150–200	175–194	130–200	137–190	155–197
EP %	79–105	80*	86*	85*	93*	85*	87*	109*	91*	78*	65*	82*	74*	80*
EP position	Bulb to intestine	Bulb	Bulb	Bulb to intestine	Bulb to intestine	Bulb	Bulb to intestine	Bulb	Bulb to intestine	Isthmus	Isthmus	Bulb	Isthmus	Bulb
Pharynx length	167–224	234–273	134–282	135–159	165–213	157–190	165–232	175–215	181–210	182–237	256–267	180–200	181-241	185–204
Mid-body diam.	91–118	52–105	59–115	65–100	70–115	70–112	72–125	70–122	58–82	53–92	78–79	50–100	58–97	73–90
Anal body diam.	28–40	22–28	20–38	15–30	26–35	14–35	21–33	25–40	23–28	20–29	23–26	20–35	21–32	24–31
Rectum length	34–86	40–56	?	?	?	?	?	75–120	58–76	60–118	42–63	50–85	56–85	48–93
Rectum/ABD	1.2–2.2	1.8–2.0	?	?	?	?	?	3.0–4.0	2.6–2.9	3.0–4.0	1.8–2.4	2.5–3.4	2.2–2.6	2.0–3.0
Tail length	87–151	120–160	80–170	60-108	90–120	80–170	100–135	100–130	131–156	112–148	121–123	75–175	110–132	100–132

All measurements are in μm (except ratios and percentages) and in the form of range.
*Measurements taken from line drawings; ?, measurements not known.

Table 3. Comparative morphometrics of males of *Oscheius citri* Tabassum, Shahina, Nasira and Erum, 2016 from India and its related species.

Species	*citri*	*citri*	*cobbi*	*cynodonti*	*esculentus*	*punctatus*	*sacchari*	*andrassyi*	*indicus*	*maqbooli*	*nadarajani*	*niazii*	*shamimi*	*siddiqii*
Reference	Present study	Tabassum *et al.* (2016)	Tabassum *et al.* (2016)	Tabassum *et al.* (2016)	Tabassum *et al.* (2016)	Tabassum *et al.* (2016)	Tabassum *et al.* (2016)	Tabassum & Shahina (2008)	Kumar *et al.* (2019)	Tabassum & Shahina (2002)	Ali *et al.* (2011)	Tabassum & Shahina (2010)	Tahseen & Nisa (2006)	Tabassum & Shahina (2010)
Country	India	Pakistan	Pakistan	Pakistan	Pakistan	Pakistan	Pakistan	Pakistan	India	Pakistan	India	Pakistan	India	Pakistan
Body length	1024–1433	1097–1464	789–1110	863–1560	875–1370	800–1118	760–1390	1025–1032	886–1209	720–1165	1191–1395	715–1020	938–1118	828–998
a	15.7–22.1	18.0–28.0	17.0–21.0	17.4–43.6	19.0–27.7	12.2–18.6	16.3–24.0	20.0–25.0	17.4–22.4	12.8–19.0	23.1–27.8	15.6–17.0	17.0–20.0	18.0–21.0
b	5.3–8.1	5.0–6.1	5.8–7.4	4.5–6.8	5.6–7.5	5.2–7.7	6.2–8.0	6.2–7.0	4.9–6.9	3.9–5.3	4.5–5.3	4.4–5.8	5.1–5.5	4.3–5.6
c	24.8–41.4	25.0–36.0	22.0–38.0	19.3–34.0	18.9–34.0	19.5–36.0	28.0–36.0	17.9–24.9	26.0–36.0	18.6–27.0	34.2–37.9	18.0–25.5	25.0–31.0	20.0–24.0
c′	1.2–2.1	1.2–1.6	1.2–2.5	1.1–1.8	1.3–1.9	1.0–1.7	1.0–1.6	1.1–2.1	1.2–1.4	1.3–1.6	1.3–1.8	1.0–1.8	1.1–1.5	1.3–1.5
Lip region width	5–8	8*	8–12	11–18	11–12	11–12	12–16	?	11–13	12–16	9*	?	12–14	10*
Stoma length	11–18	15–20	13–16	14–17	14–17	10–17	15–21	15–17	14–17	10–18	19	?	18–22	14–15
Isthmus length	40–53	45*	?	39*	39*	?	42*	28–40	31–41	50*	48–64	30–50	50*	40–52
Bulb length	23–30	32*	?	26*	28*	?	30*	25–30	27–32	32–40	29–33	30–40	31*	28–37
NR–ant. end	96–150	140–190	120–220	130–170	110–140	95–140	110–135	123–160	122–146	124–176	160–168	130–165	124–148	120–140
EP–ant. end	149–211	165–215	130–170	153–210	135–185	105–160	142–200	167–198	158–187	144–177	166–175	130–160	166–192	150–172
EP (%)	71–104	82*	93*	96*	81*	84*	84*	102*	101*	80*	71*	83*	94*	72*
EP position	Bulb to intestine	Bulb	Bulb	Bulb to intestine	Bulb to intestine	Bulb	Bulb to intestine	Bulb	Bulb to intestine	Isthmus	Isthmus	Bulb	Isthmus	Bulb
Pharynx length	154–199	200–250	130–170	168–208	155–200	125–169	154–178	158–178	159–181	184–248	242–256	160–175	181–203	165–180
Mid-body diam.	54–73	43–68	36–58	43–58	46–64	53–72	53–75	45–51	46–64	40–69	41–47	45–57	49–57	43–53
Anal body diam.	20–31	28–35	10–25	25–33	28–31	20–30	24–30	30–40	24–28	28–36	23–29	20–37	25–31	28–30
Tail length	32–51	35–50	20–32	32–50	30–60	26–45	26–40	45–63	32–39	32–48	32–36	35–40	29–38	38–45
Spicule length (SL)	42–75	57–70	47–62	54–60	45–57	50–60	47–55	45–51	54–66	48–60	58–63	42–52	53–67	50–55
Gubernaculum (GL)	21–31	25–30	20–35	20–27	20–24	20–25	18–22	20–25	24–28	19–32	32*	18–30	22–28	20–25
GS (GL/SL)	3.3–5.2	3.4–5.0	3.8–6.0	3.8–4.5	3.8–4.6	3.6–4.7	2.5–4.4	4.8*	2.4*	5.1*	5.6*	4.3*	4.3*	4.9*

All measurements are in μm (except ratio and percentage) and in the form of range.
*Measurements taken from line drawings; ?, measurements not known.

Table 4. Pairwise distances of the internal transcribed spacer ((ITS1-5.8S-ITS2)) region of the rDNA among species of the '*insectivorus*' group.

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	MN137988 *O. citri* (present study)		97	93	98	96	94	92	89	89	89	89	90	71	72	76	76	71	71
2	KU284845 *O. punctatus*	23		95	97	97	96	96	86	87	86	86	89	70	72	72	72	71	67
3	KT878514 *O. esculentus*	59	35		96	94	93	92	83	83	82	82	84	69	68	68	68	68	64
4	KT250509 *O. citri*	16	22	30		98	98	96	87	87	87	87	89	72	72	71	71	71	67
5	KT250510 *O. sacchari*	37	22	51	15		95	93	84	84	84	84	86	69	70	70	70	71	67
6	KT878513 *O. cobbi*	49	25	60	17	44		91	84	84	84	83	86	70	70	69	69	71	66
7	KU997024 *O. cynodonti*	66	29	69	33	59	75		83	83	83	83	86	68	69	67	68	69	65
8	EU273598 *O. chongmingensis*	105	94	142	100	131	138	143		100	99	99	95	71	72	75	75	71	69
9	EF503690 *O. chongmingensis*	104	93	141	99	130	137	142	1		99	99	95	71	72	75	75	71	70
10	MT548591 *O. chongmingensis*	110	98	148	105	136	139	144	15	14		98	95	71	72	75	75	71	70
11	JQ002565 *O. rugaoensis*	109	95	145	103	135	142	144	13	9	20		95	71	71	75	75	70	70
12	MF441494 *O. indicus*	87	75	124	80	115	118	118	43	42	46	46		72	72	72	72	71	67
13	KF684370 *O. safricana*	229	191	239	202	240	239	247	226	225	226	228	215		83	83	83	84	67
14	MF372144 *O. myriophilus*	226	184	249	206	237	241	251	224	223	221	226	221	132		99	100	86	67
15	KP792651 *O. myriophilus*	232	185	250	214	239	256	266	239	235	233	238	226	137	9		99	84	70
16	KT825914 *O. microvilli*	230	184	248	212	237	254	264	239	235	233	238	226	135	4	7		85	70
17	KM492926 *O. basothovii*	227	189	236	213	218	228	239	226	225	220	227	222	119	108	124	120		68
18	FJ547241 *O. carolinensis*	279	233	294	256	280	292	298	299	292	297	298	279	266	266	288	287	253	

Below diagonal: total character differences, above diagonal: percentage similarity.

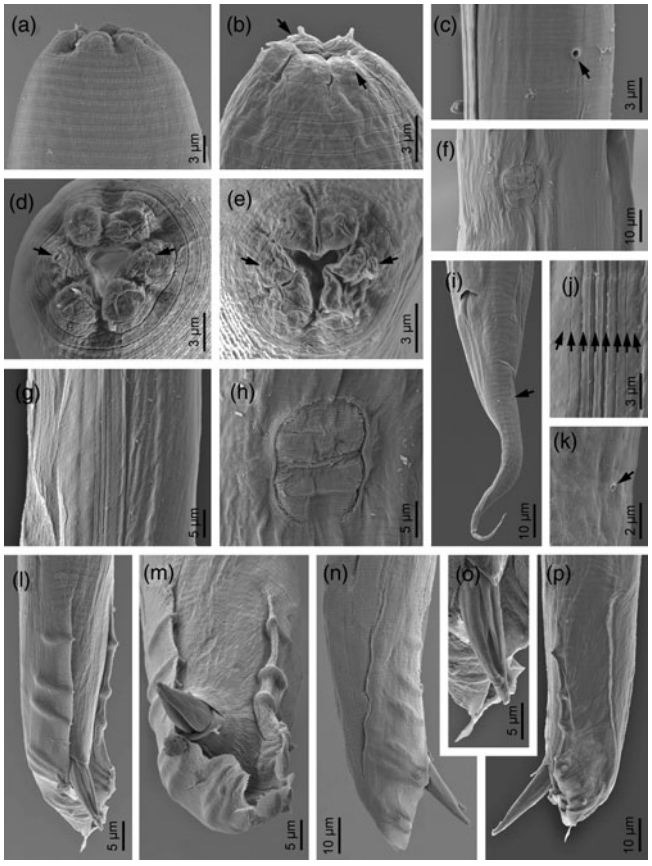

Fig. 4. *Oscheius citri* Tabassum, Shahina, Nasira and Erum, 2016 (scanning electron microscopy). (a, b, d, e) Lip region in lateral (a, b) and frontal (d, e) views (arrows pointing the amphids); (c) excretory pore (arrow); (f, h) vulva; (g, j) lateral field (arrows pointing the longitudinal incisures); (i) female posterior end (arrow pointing the phasmid); (k) female phasmid (arrow); (l, m, n, p) male posterior end in subventral, ventral, right lateral and left lateral views, respectively; (o) spicules tip.

bearing six separate lips, pharynx with metacorpus cylindroid, nerve ring surrounding isthmus posteriorly, excretory pore located at basal bulb level, female reproductive system didelphic-amphidelphic, rectum 34–86 µm long or 1.2–2.2 times the anal body width, female tail conical (60–170 µm long, c = 7.6–24.0, c' = 2.7–6.7) with hooked tip, male reproductive system monorchid, tail conoid (20–60 µm long, c = 18.9–41.4, c' = 1.0–2.5), bursa peloderan bearing nine pairs of genital papillae (1 + 2/3 + 3 + ph) and phasmids posterior to 9GP, spicules 42–75 µm long with variable morphology and gubernaculum 18–35 µm long.

Phylogenetic analysis

The phylogenetic analysis based on the ITS region of the rDNA (fig. 5) shows the *Oscheius* species studied now as a member of a well-supported clade with *O. citri, O. cobbi, O. sacchari, O. cynodonti, O. esculentus* and *O. punctatus* from Pakistan (Tabassum *et al.*, 2016). This further supports conspecificity of these nematodes, and therefore, the members of this clade most likely represent the Pakistani and Indian strains of a single species *O. citri*. All phylogenetic analyses based on ITS (fig. 5), 18S (fig. 6) and 28S (fig. 7) rDNA fragments show *O. citri* as a sister group to the clade formed by *O. chongmingensis, O. rugaoensis* (Zhang et al., 2012) Darsouei, Karimi & Shokoohi, 2014 and *O. indicus* Kumar, Jamal, Somvanshi, Chauban & Mumtaz, 2019.

Discussion

The *Oscheius* species examined now from India agrees very well with the six species described (*O. citri*, *O. cynodonti*, *O. cobbi*, *O. esculentus*, *O. punctatus* and *O. sacchari*) by Tabassum *et al.* (2016) from Pakistan. Unfortunately, despite several attempts, we were unable to obtain paratypes of the Pakistani species to re-examine their morphology and to confirm their identity.

Morphologically, the *Oscheius* species described now from India resembles *O. citri* in the shape of spicules at proximal end (fig. 3d; 6, 7), presence of six separate lips in lip region, presence of phasmids posterior to anus, shape of gubernaculum, tail conical with a small part protruding beyond bursa, bursa leptoderan bearing nine pairs of genital papillae of different lengths with GP1 and GP2 more spaced than GP2 and GP3. Morphometrically, most important morphometric measurements including body length, ratios and percentages were in close vicinity to each other or have overlapping measurements except pharynx length in females (167–224 vs. 234–273 µm) and males (154–199 vs. 200–250 µm) (tables 2 and 3). Molecularly, ITS sequence of WGN does not show any nucleotide difference with the relevant part of the sequence of *O. citri* (table 5), however, gaps and nucleotide differences are observed at 5′ end only which may be due to inadequate editing of the sequence.

With respect to *O. cynodonti*, it resembles in shape of spicules bearing hooked tips with rounded spicule head (fig. 3d; 4, 5), boat shaped gubernaculum, bursa leptoderan type with a short part of tail protruding beyond bursa, nine pairs of genital papillae with GP1 and GP2 more spaced than GP2 and GP3, same reproductive system in males and females. Most of the important morphometric measurements including ratios and percentages were in close proximity to each other and some have overlapping measurements, except variation in body length in adults and in characters like lip region width in males (5–8 vs. 11–18 µm) and females (6–11 vs. 12–18 µm); anal body width (28–40 vs. 15–30 µm), pharynx length (167–224 vs. 135–159 µm) and nerve ring to anterior end (130–201 vs. 100–125 µm) in females (tables 2 and 3). Molecularly, ITS rRNA sequence of the Indian *Oscheius* showed six nucleotide differences (table 5) and three gaps with *O. cynodonti* in the middle of the sequence. Large gaps and nucleotide differences at 3′ and 5′ prime ends are obviously a result of sequencing errors and inadequate sequence editing.

With *O. cobbi*, it resembles lip region bearing six rounded lips, metastegostom bearing small denticles, position of nerve ring and excretory pore, reproductive system in adults, shape of the spicules bearing hooked tips and rounded head (fig. 3d; 1), boat shaped gubernaculum, bursa leptoderan type with a short part of tail protruding beyond bursa, nine pairs of bursal papillae at different lengths with GP1 and GP2 more spaced than GP2 and GP3, juvenile tail elongate conical tapering to a hyaline part. Morphometric measurements of adults including demanian indexes and percentages were in close similitude to each other or show overlapping morphometry; however, variations were observed in their body length and tail length (32–51 vs. 20–32 µm) and lip region (5–8 vs. 8–12 µm) width in males. The alignment file of ITS rDNA sequence of the Indian species with *O. cobbi* showed 1 nucleotide difference (table 5), 2 ambiguous positions and 3 gaps in the middle part of the sequences, however, at 3′ and 5′ ends large gaps and nucleotide differences are observed which are obviously a result of sequencing errors and inadequate sequence editing.

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Table 5. Pairwise distances of the internal transcribed spacer ((ITS1-5.8S-ITS2)) region of the rDNA among species of the '*insectivorus*' group with the trimmed 3′ and 5′ ends.

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	MN137988 *O. citri* (present study)		100	99	100	100	100	99	90	90	89	89	90	78	77	80	80	76	74
2	KU284845 *O. punctatus*	1		99	100	100	100	99	89	90	88	88	91	83	84	83	84	82	79
3	KT878514 *O. esculentus*	6	5		99	99	99	100	89	89	88	88	90	78	76	76	77	76	71
4	KT250509 *O. citri*	0	1	6		100	100	99	89	89	88	88	90	78	76	76	76	76	70
5	KT250510 *O. sacchari*	1	0	5	1		100	99	89	89	88	88	90	78	76	76	77	77	71
6	KT878513 *O. cobbi*	1	0	5	1	0		99	89	89	87	88	90	78	76	76	77	76	71
7	KU997024 *O. cynodonti*	6	5	0	6	5	5		89	90	88	88	91	78	77	77	77	77	72
8	EU273598 *O. chongmingensis*	99	46	78	80	79	85	74		99	98	98	95	77	76	79	79	74	72
9	EF503690 *O. chongmingensis*	98	44	75	79	78	84	71	6		98	98	96	77	77	79	79	74	73
10	MT548591 *O. chongmingensis*	110	53	87	90	89	95	82	16	19		97	95	77	76	79	79	74	73
11	JQ002565 *O. rugaoensis*	109	51	86	88	87	94	82	20	16	25		95	76	76	79	79	74	73
12	MF441494 *O. indicus*	88	34	66	70	69	76	61	41	37	40	46		78	77	77	77	74	71
13	KF684370 *O. safricana*	177	70	142	148	145	157	137	180	176	181	188	169		85	85	85	84	70
14	MF372144 *O. myriophilus*	181	65	155	161	158	167	146	185	183	186	186	176	110		99	99	87	71
15	KP792651 *O. myriophilus*	187	67	156	161	158	167	146	200	195	198	198	181	117	8		99	86	73
16	KT825914 *O. microvilli*	184	65	152	158	155	164	144	197	192	195	195	178	113	8	10		87	74
17	KM492926 *O. basothovii*	188	69	152	156	152	168	144	196	197	195	194	190	112	96	111	102		71
18	FJ547241 *O. carolinensis*	252	89	205	214	209	222	193	265	253	262	267	237	238	231	255	247	224	

Below diagonal: total character differences, above diagonal: percentage similarity.

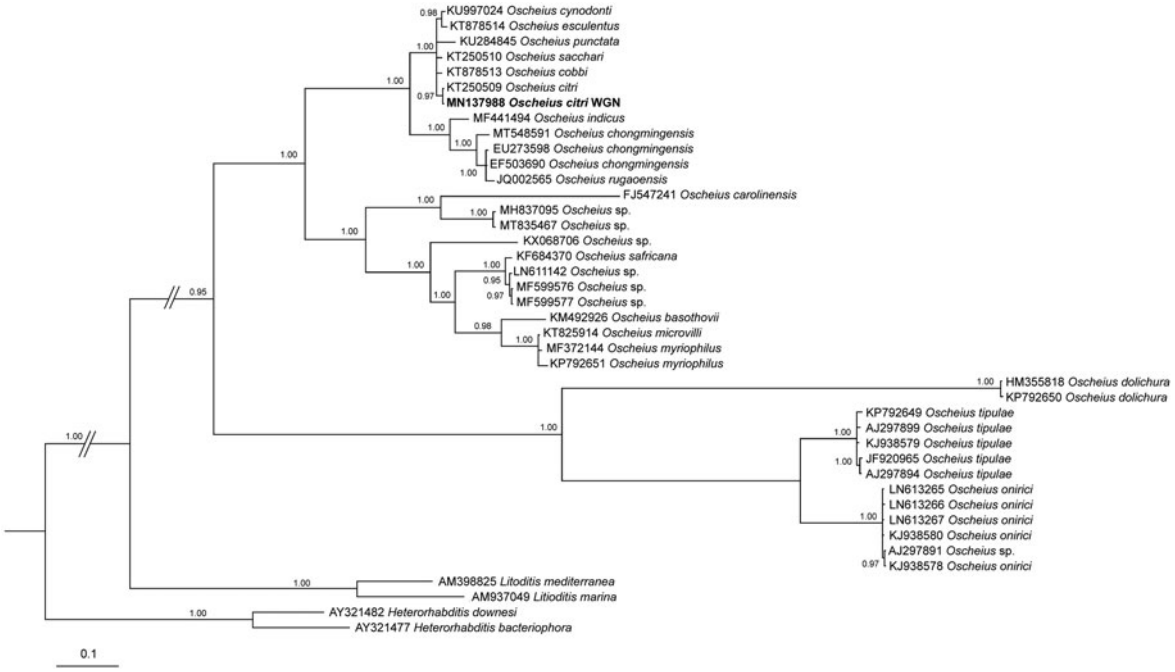

Fig. 5. Phylogenetic relationships of the Indian and Pakistani strains of *Oscheius citri* and other closely related species as inferred from Bayesian analysis of sequences of the Internal Transcribed Spacer (ITS1-5.8S-ITS2) rDNA region. Bayesian posterior probabilities (%) equal to or more than 60% are given for appropriate clades. The scale bar shows the number of substitutions per site.

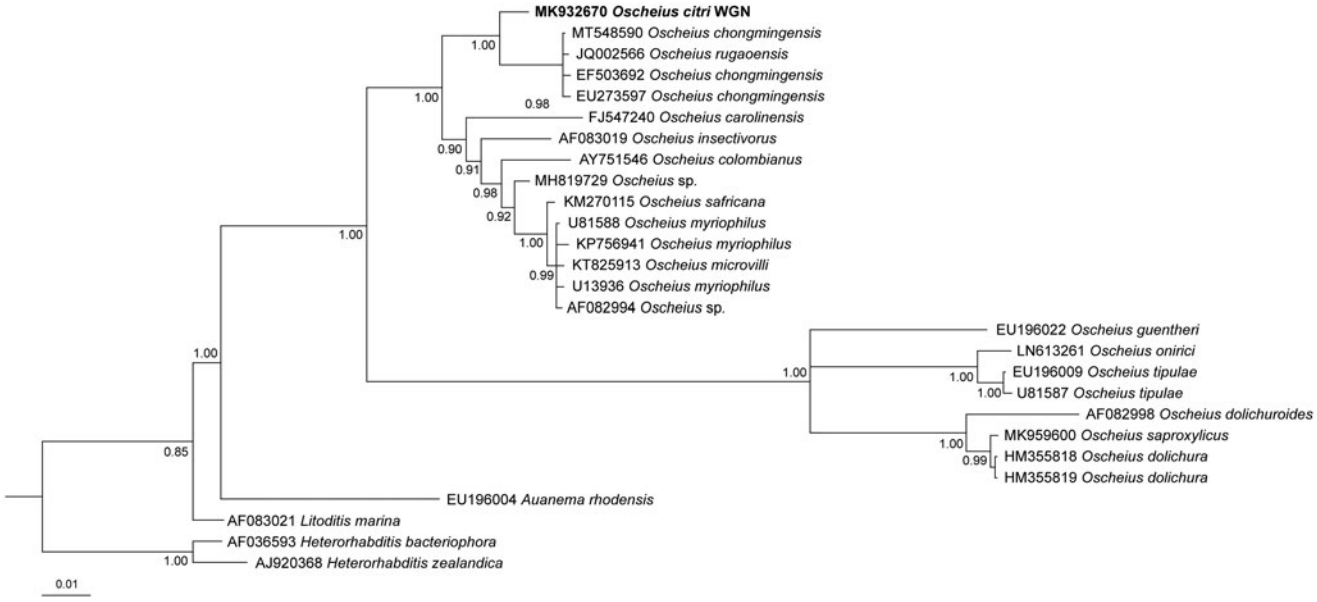

Fig. 6. Phylogenetic relationships of the Indian strain of *Oscheius citri* and other closely related species as inferred from Bayesian analysis of sequences of the small subunit (18S) of rDNA region. Bayesian posterior probabilities (%) equal to or more than 60% are given for each appropriate clade. The scale bar shows the number of substitutions per site.

Compared with *O. esculentus*, it shows similar lip shape having six rounded lips, finely cuticularized cheilostom, metastegostom with small denticles, reproductive system in adults, position of vulva, shape of the spicules and gubernaculum (fig. 3d; 8, 9), bursa leptoderan type, nine pairs of bursal papillae of different lengths with more spacing between GP1 and GP2 than GP2 and GP3, juvenile tail elongate conical tapering to a hyaline part. Further, most important morphometric measurements including body length, percentages and ratios show overlapping morphometry or were quite close to each other except lip region width in females (6–11 vs. 14–20 µm) and males (5–8 vs. 11–12 µm). The alignment file ITS rDNA sequence of WGN with *O. esculentus* showed 6 nucleotide differences (table 5) and 2 gaps in the middle part of the sequences, however, at 3′ and 5′

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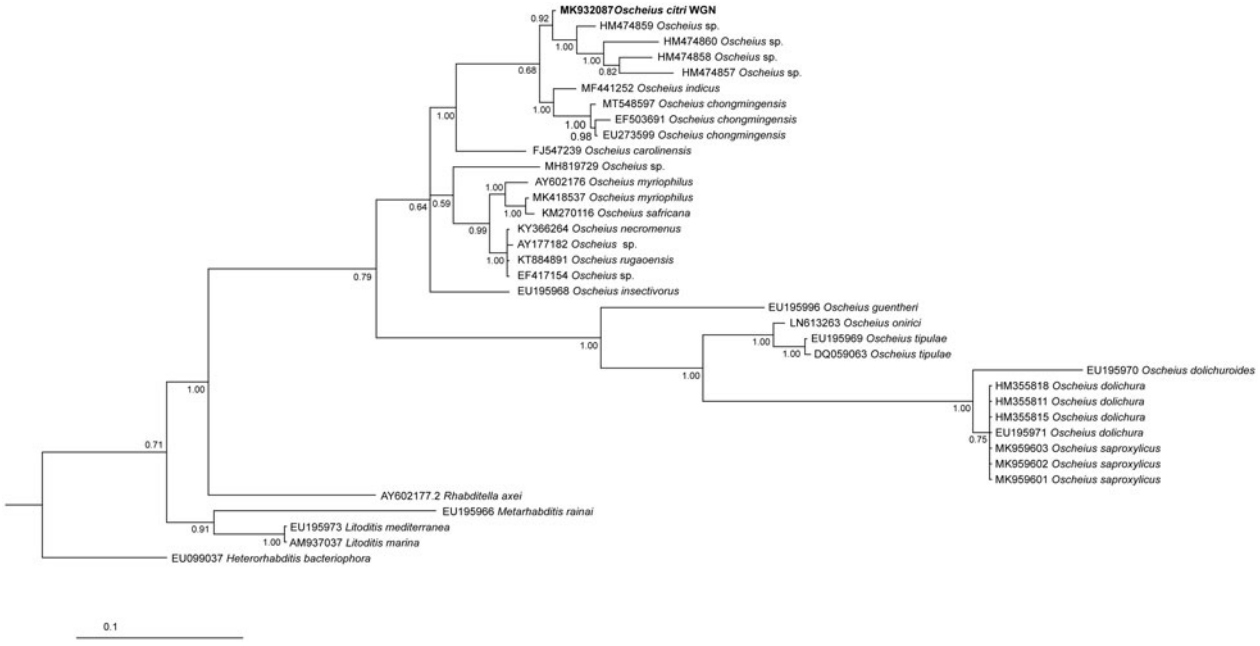

Fig. 7. Phylogenetic relationships of the Indian strain of *Oscheius citri* and other closely related species as inferred from Bayesian analysis of sequences of the D2-D3 domain of large subunit (28S) of rDNA region. Bayesian posterior probabilities (%) equal to or more than 60% are given for each appropriate clade. The scale bar shows the number of substitutions per site.

ends large gaps and nucleotide differences are observed which are obviously a result of sequencing errors and inadequate sequence editing.

When compared with *O. punctatus*, it shares same morphology like lip region continuous with six rounded lips, finely cuticularized cheilostom, short gymnostom, metastegostom with small denticles, amphimictic reproduction, reproductive system in adults, spicule shape with hooked tips and rounded head (fig. 3d; 10), boat shaped gubernaculum, bursa leptoderan type, nine pairs of bursal papillae of different lengths with more spacing between GP1 and GP2 than GP2 and GP3, juvenile tail elongate and conical tapering to a hyaline part. The important morphometric measurements, percentages and ratios of the two are in close range to each other or with somewhat overlapping morphometry except lip region width (6–11 vs. 12–15 µm) and stoma length (14–21 vs. 12–15 µm) in females and lip region width (5–8 vs. 11–12 µm) in males. Molecularly, ITS rDNA sequence of WGN showed one nucleotide difference (table 5), one ambiguous sequence and ten gaps with *O. punctatus* in the middle of the alignment file, however, more gaps and nucleotide differences are observed at 3′ and 5′ ends only which are obviously a result of sequencing errors and inadequate sequence editing.

With *O. sacchari* also, it showed similar morphology as with above Pakistani strains like shape of spicules and gubernaculum (fig. 3d; 2, 3), amphimictic reproduction, nine pairs of genital papillae, juvenile tail elongate, conical and tapering to a hyaline part. Here also, most morphometric parameters, percentages and ratios were in close proximity to each other or display overlapping morphometry except lip region width in females (6–11 vs. 12–14 µm) and males (5–8 vs. 11–16 µm). The alignment file ITS rDNA sequence of WGN with *O. sacchari* showed one nucleotide difference (table 5) and three gaps in the middle part of the sequences, however, at 3′ and 5′ ends 13 gaps and 2 nucleotide differences are observed which are obviously a result of sequencing errors and inadequate sequence editing.

Other very similar species, also described from India and Pakistan, are *O. andrassyi* Tabassum & Shahina, 2008, *O. maqbooli* Tabassum & Shahina, 2002, *O. nadarajani* Ali, Asif & Shaheen, 2011, *O. niazii* Tabassum & Shahina, 2010, *O. shamimi* Tahseen & Nisa, 2006 and *O. siddiqii* Tabassum & Shahina, 2010. These species show morphological and morphometric similarity (tables 2 and 3), existing the probability that some of them is/are synonym(s) of *O. citri*. However, *O. magbooli*, *O. nadarajani* and *O. shamimi* have more anterior excretory pore, at isthmus level, while in *O. citri* it appears at basal bulb or more posterior, and additionally *O. magbooli* presents visibly shorter female rectum (slightly longer than anal body width according the light microscopy picture of the fig. 2d). *O. andrassyi*, *O. indicus*, *O. niazii* and *O. siddiqii* are very similar each other, distinguishing of the *O. citri* from India by having narrower stomatal tube, while of the *O. citri* from Pakistan and its synonyms show similar stomatal morphology. Between them, *O. andrassyi*, *O. niazii* and *O. siddiqii* presents shorter female rectum than *O. indicus* (*ca.* 2 vs. 3 times anal body diam.). Unfortunately, the most of these species lacks molecular data to confirm their relationship, except for *O. indicus* which show slightly differences at its rDNA sequences with respect to *O. citri* from India and Pakistan.

Thus, based on similar morphology and overlapping morphometrics the Indian material and the six Pakistani species described by Tabassum *et al.* (2016) agree very well each other, being found two of them (*O. cobbi* and *O. punctatus*) in the same sample. Additionally, based on molecular data, all the six above mentioned Pakistani species and the material examined now from India do not differ each other. According to this, the Indian material and the six Pakistani species are conspecific taxa. With respect to the valid name, according to International Code of Zoological Nomenclature (ICZN), *O. citri* was described the

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first in the paper published by Tabassum *et al.* (2016) being the type population, while the rest of the five species should be considered as their junior synonyms.

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