Comparison of the cryptic nematode species *Caenorhabditis brenneri* sp. n. and *C. remanei* (Nematoda: Rhabditidae) with the stem species pattern of the *Caenorhabditis Elegans* group

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Abstract

The new gonochoristic member of the *Caenorhabditis Elegans* group, *C. brenneri* sp. n., is described. This species is reproductively isolated at the postmating level from its sibling species, *C. remanei*. Between these species, only minute morphological differences are found, but there are substantial genetic differences. The stem species pattern of the *Elegans* group is reconstructed. *C. brenneri* sp. n. deviates from this character pattern only in small diagnostic characters. In mating tests of *C. brenneri* sp. n. females with *C. remanei* males, fertilization takes place and juveniles occasionally hatch. In the reverse combination, no offspring were observed. Individuals from widely separated populations of each species can be crossed successfully (e.g. *C. brenneri* sp. n. populations from Guadeloupe and Sumatra, or *C. remanei* populations from Japan and Germany). Both species have been isolated only from anthropogenic habitats, rich in decomposing organic material. *C. brenneri* sp. n. is distributed circumtropically, *C. remanei* is only found in northern temperate regions. To date, no overlap of the ranges was found. Hypotheses to explain the allopatric distribution of the two species are discussed. One suggests that the speciation center for the *Elegans* group was in East Asia, and globally distributed members dispersed from there.

Key words: *Caenorhabditis*, biogeography, biological species concept, circumtropical, mating tests, Nematoda, *remanei,* stem species pattern

Introduction

Starting with the investigations by Nigon & Dougherty (1949, 1950) and the exceptionally successful studies by Brenner (1974), *Caenorhabditis elegans* (Maupas, 1899) and *C. briggsae* (Dougherty & Nigon, 1949) have become important model systems for basic biological studies. In the last 10 years, the interest of the scientific community has broadened to include comparative research, aimed at understanding the evolution of developmental processes. Therefore, other *Caenorhabditis* species have come into focus, in particular those most closely related to *C. elegans* which are members of the *Elegans* group (Sudhaus & Kiontke 1996, Kiontke et al. 2002). The *Elegans* group is a complex of sibling and complementary species that are difficult to differentiate except by the mode of reproduction (gonochoristic versus self-fertilizing hermaphroditic). At present, this group comprises eight species. Five of them are studied in different laboratories, even though two of them are not yet described and named. The three other members of the *Elegans* group are dubious and were never isolated after the initial description. The six named species are (well known species marked with *): **Caenorhabditis briggsae* (Dougherty & Nigon, 1949), *C. clavopapillata* (Kreis & Faust, 1933), **C. elegans* (Maupas, 1899), *C. formosana* (Yokoo & Okabe, 1968), *C. oncomelaniae* (Yokoo & Okabe, 1968), and **C.*

remanei (Sudhaus, 1974). Here, we describe one of the two new species, which was previously referred to as *C.* n. sp. 4 (Sudhaus & Kiontke 1996, Kiontke & Sudhaus 2006 and at www.wormbase.org). The new species was discovered in 1975 and distinguished from *C. remanei* by mating tests (W. S.). Later, the strain CB5161 of this new species was erroneously identified as *C. remanei* (e.g. Fodor & Timar 1989, Fodor et al. 1989). Failing mating tests between strain CB5161 with a new *Caenorhabditis* isolate from New York (Baird et al. 1990, 1992, 1994) lead to the description of *C. vulgaris* (Baird et al. 1994) which later turned out to be synonymous with *C. remanei*. Thus, *C. remanei* was described twice, and *C.* sp. 4 remained undescribed even 30 years after its first discovery.

There were two difficulties with describing this species. Foremost, it appeared to be morphologically identical with its sibling species (but not sister species) *C. remanei*. In addition, it could not be excluded that this isolate was one of two sketchily described species from East Asia associated with *Oncomelania* snails, *C. formosana* and *C. oncomelaniae*. Several new efforts to reisolate one or both of these Asian species failed, and their status remains dubious. We now rule out that *C.* sp. 4 is conspecific with either *C. formosana* or *C. oncomelaniae*. Numerous mating tests between different strains of *C.* sp. 4 and *C. remanei* have demonstrated beyond doubt that the two species are reproductively isolated. Even though an extensive comparative study has yielded only two small morphological differences between *C.* sp. 4 and *C. remanei*, molecular data show a considerable divergence between these species and provide numerous diagnostic characters for distinguishing them. The amount of this kind of data is growing daily since the whole genome of the new species is in the process of being sequenced (http://genome.wustl.edu/genome.cgi?GENOME=Caenorhabditis%20n.%20sp.%20PB2801), and sequencing of the *C. remanei* genome is finished (http://genome.wustl.edu/genome.cgi?GENOME=Caenorhabditis%20remanei). We thus describe this new species as *C. brenneri* sp. n.

Material and methods

Morphology and morphometrics

Specimens of several strains of *C. brenneri* sp. n. and *C. remanei* were studied alive with bright field and differential interference contrast (DIC) microscopy. The nematodes were immobilized by gentle heat. Drawings were made with the help of a camera lucida mounted on a Leitz Diaplan. Measurements were taken with a micrometer plate inserted into the eyepiece of the microscope.

For microphotography, worms were placed in a drop of M9 buffer on 5 % agar pads and studied at 1000× magnification with a Zeiss Axioskop set up with DIC. Images were recorded with a model C4742-95 "Orca" Hamamatsu digital camera and Openlab software, ver. 3.0.9 (Improvision).

Cross-mating experiments

Mating tests were conducted in small droplets of a liquid freshly prepared from raw potato immersed in tap water, or dried algae (*Fucus*) soaked in water overnight. The "algae-juice" is an excellent and always transparent culture medium. Seven droplets were placed in plastic Petri dishes, and one dauerlarva was put into each droplet. After they had matured, virgin females were put together with three males of a different strain in a new droplet and observed daily under a dissecting microscope. The behavior of males and females was recorded as well as whether eggs were laid and juveniles hatched. Controls were performed in parallel with males of the same strain. Dead males were replaced in all experiments. Only those experiments were assessed, in which males lived with the female for several days. A cross was counted as negative if it produced no progeny or only single juveniles that died early or were sterile. Mating tests were counted as positive if viable progeny were produced and a fertile second or third generation was observed. If there were no offspring after about a week, some of the females were paired with males of their own strain and were observed until they died. In most of those cases, offspring resulted immediately.

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Results

Geographic distribution of C. brenneri *sp. n. and* C. remanei

Isolates of C. brenneri *sp. n.:*

The new species was collected repeatedly. In each case, its species status was established by cross mating experiments. Four different strains of *C. brenneri* sp. n. are currently kept in laboratories:

– SB129 was isolated in Sumatra (Bohorok) from compost-like material, mostly banana leaves (June 1975, W. S.).

– CB5161 was isolated in Trinidad from a sugar cane field (spring 1980, David J. Hunt) and cultivated by him and since 1982 by Jonathan Hodgkin in Cambridge (England), before it was sent to the CGC in April 1988.

– SB280 is from Guadeloupe, found in rotting banana leaves in a plantation (July 1996, W. S.).

– LKC28 stems from roots of *Liriope* (Convallariaceae) grown in a nursery in Costa Rica (Provincia de Alajuela) (isolated 2003 by Hernan Ruiz).

– In addition, this species was sampled from dry chaff as well as from rich soil in Bali (near Denpasar), from dry water buffalo dung in Penang (Malaysia, both June 1975, W. S.), and from soil of the Botanical garden in Bangalore (Karnataka, India, 1986, András Fodor 1986 and pers. comm.). Judging from these records, the distribution of *C. brenneri* sp. n. is circumtropical (Fig. 1). There is no unequivocal record from Africa yet. Perhaps the saprobiotic nematode from Bukawu (Lake Kivu), depicted by Schuurmans Stekhoven (1951, Fig. 41) under the wrong name *Rhabditis teres,* was in fact this species.

Isolates of C. remanei*:*

At the current state of knowledge, the distribution of *C. remanei* is confined to a belt around the globe between the 31st and 53rd degree of latitude of the northern hemisphere (Fig. 1). *C. remanei* was found regularly in different places in Germany and in the northern part of the USA. In all but one case (the Hungarian record) the species identification was confirmed by mating tests. In Germany, *C. remanei* was first isolated in 1969 in Freiburg-Zähringen (type locality). Strain SB146 was isolated 1994 at the type locality. The same species was found by W. S. and his students near Tübingen, in Berlin (SB111), near Freiburg in the Kaiserstuhl, and in Basel (Switzerland). Obernai (Alsace, JU825), the only place in France where Marie-Anne Félix (pers. comm.) and her team could sample this species during their survey of *Caenorhabditis* species, is also located close to Freiburg. A record from Hungary was reported by Andrássy (2005). In the USA, *C. remanei* was isolated in 1990 by Baird et al. (1990) in New York (EM464), and by William Fixsen in Connecticut (VT733). Later it was recorded repeatedly in Dayton, Ohio (PB206–PB229) (Baird 1999), in Gloucester, Massachusetts (CR1014, CR1415, CR2124, Graustein et al. 2002), Bloomington, Indiana (PB276, Scott E. Baird pers. comm.), and Seattle, Washington (James H. Thomas pers. comm.). Recently *C. remanei* was sampled four times in the Far East: in China in Zhouzhuang, Jiangsu Province by Marie-Anne Félix (JU724), in Japan on Kyushu (near Nobeoka, Miyazaki Prefecture) by Toyoshi Yoshiga, and on Honshu (Hiratsuka and Kamakura, Kanagawa Prefecture) by Chiharu Kato.

Cross-breeding experiments

Mating experiments are a powerful tool for determining biological species status. The degree to which different species and strains are able to copulate and produce offspring is an important diagnostic character. Cross-mating tests were already performed in other investigations on *Caenorhabditis* species (Nigon & Dougherty 1949, Friedman et al. 1977, Fodor 1986, Baird et al. 1992, Baird & Yen 2000). We performed mating tests with *C. brenneri* sp. n. strains from 6 locations and *C. remanei* strains from 5 locations. The results of our cross-mating experiments are presented in Table 1. In addition, crosses were performed with a *C. remanei* strain from Berlin (Germany) and *C. remanei* strains from Basel (Switzerland), Kaiserstuhl and Tübingen (Germany), and between isolates from Kamakura (Japan) and Kaiserstuhl (data not shown).
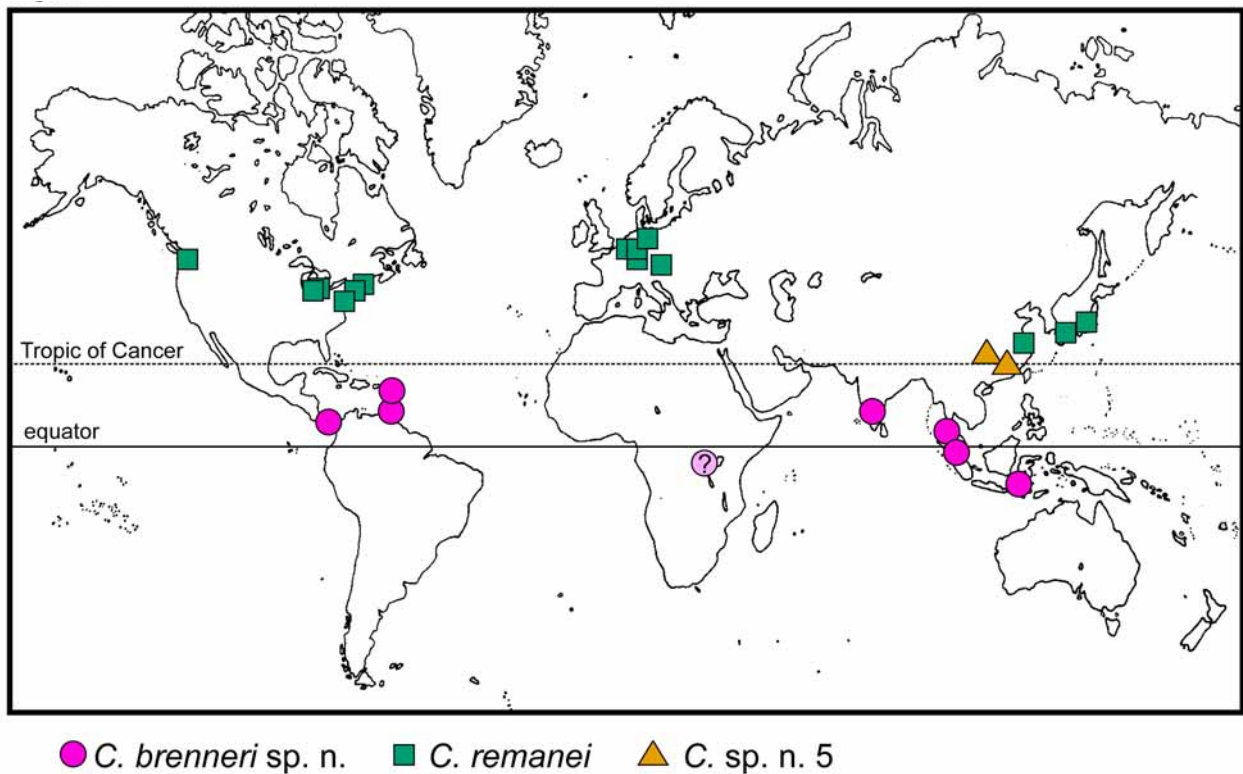
FIGURE 1. Distribution map for three *Caenorhabditis* species. Records from west to east: for *C. brenneri* sp. n. (circles): Costa Rica, Trinidad, Guadeloupe, (? Bukawu), Bangalore, Penang, Sumatra, Bali; for *C. remanei* (squares) North America (Washington, Ohio, Indiana, New York, Connecticut, Massachusetts), Europe (North-France, Switzerland, Germany, Hungary), East Asia (Shanghai, Kyushu, Honshu); for the recently discovered gonochoristic *C.* sp. n. 5 in China (triangles): Sanjiang, Guangxi Province (JU727); Guangzhou, Guangdong Province (SB378). Details in text.

With rare exceptions, intraspecific crosses were positive (fertile F2 generation), even between geographically widely separated populations like those of *C. brenneri* sp. n. from Guadeloupe and Sumatra or those of *C. remanei* from Japan and Germany. On the other hand, all attempts to hybridize *C. brenneri* sp. n. and *C. remanei* strains failed. In these crosses males were attracted to the female of the sibling species and displayed vigorous searching behavior. Mating plugs indicated that copulations had occurred, and occasionally copulations were observed directly in both combinations. Whereas virgin females did not lay unfertilized eggs, oviposition often occurred in interspecific crosses, apparently stimulated by mating. Usually such eggs were laid within the first day, rarely over a period of several days. It was not checked if some of these eggs were fertilized or whether cleavages occurred. These eggs or embryos degenerated very soon. Development never reached the juvenile stage when *C. remanei* females were crossed with *C. brenneri* sp. n. males. In contrast, some juveniles hatched in 5 crosses of *C. brenneri* sp. n. females with *C. remanei* males. From a total of 15 J1, 8 moved sluggishly and died within a day. Seven reached adulthood: 5 males and 2 females. These adults appeared to be infertile. Four hybrid males lived with their *C. brenneri* sp. n. mother for several days without producing offspring. One of the two hybrid females was vulva-less. Similarly, Baird et al. (1992) and Baird & Yen (2000) found that crosses of *C. brenneri* sp. n. females with *C. remanei* males resulted in arresting embryos, whereas in reciprocal crosses with *C. remanei* females and *C. brenneri* sp. n. males only unfertilized eggs were laid.

Our experiments are complemented by mating tests conducted by other investigators: Successful mating tests were performed between strains of *C. brenneri* sp. n. from Bangalore (BA4002) and Sumatra (SB129), which "produced a 100 % normal progeny" (András Fodor, pers. comm.), and between *C. remanei* strains

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from Germany (SB146) and Ohio (PB227–PB229) (Baird 1999). Mating tests which did not yield viable progeny were performed between *C. brenneri* sp. n. CB5161 and *C. remanei* VT733 (Jonathan Hodgkin, pers. comm.), *C. brenneri* sp. n. CB5161 and *C. remanei* EM464 (Baird et al. 1990, 1992, 1994) and *C. brenneri* sp. n. CB5161 and *C. remanei* SB146 (Baird & Yen 2000).

Molecular differences

With the two genome sequencing projects for *C. brenneri* sp. n. and *C. remanei*, a huge amount of DNA and protein sequence data will soon be available for both species. As of today, 6 publications specifically report molecular differences between *C. brenneri* sp. n. and *C. remanei* (Lee et al. 1993, Moss et al. 1997, Cho et al. 2004, Kiontke et al. 2004, Roy & Penny 2006, Stothard & Pilgrim 2006).

Differences between the two species are large in genes of the sex determination pathway. The protein sequence identity of FOG-1 is only 49 %, that of FOG-3 64 % (Cho et al. 2004) and that of FEM-2 58 % (Stothard & Pilgrim 2006). Other genes are more highly conserved, but still show considerable divergence: The protein sequences of LIN-28 are 81 % identical in the two species. Conservation level of the small noncoding temporal RNA *lin-4* is 72 %, that of the gene for SSU rRNA is 98 % and of LSU rRNA 98.7 %. It should be noted that generally, only few polymorphisms are observed in the latter two highly conserved genes. Therefore, most or all of the observed differences are likely to be fixed differences between the two species. The part of the largest subunit of RNA polymerase II (RNAP2, *ama-1* in *C. elegans*) sequenced by Kiontke et al. (2004) is 96 % identical at the protein level. At the nucleotide level, 99 synonymous positions are polymorphic between, and partially within, the four strains of *C. brenneri* sp. n. (CB5161, LKC28, SB129 and SB280). This amounts to 5.5 % of all 1800 sites. However, there are still 265 differences (14.7 %) between *C. remanei* (SB146) and all *C. brenneri* sp. n. strains (K. K. unpublished). The level of divergence at the nucleotide level between the two species (strains CB5161 and SB146) in RNAP2 and also in the gene for SSU rRNA is thus larger than that between human and mouse (Kiontke et al. 2004).

Differences were also found in the presence and absence of introns: *C. brenneri* sp. n. contains 3 fewer introns in *fog-3,* but one more intron in *fog-1* than *C. remanei* (Cho at al. 2004). *C. brenneri* sp. n. also possesses one intron in RNAP2 which is not found in any of 45 other nematode species, including all *Caenorhabditis* species in culture, for which this region of the gene was sequenced (K. K. unpublished, intron m in Kiontke et al. 2004). Roy & Penny (2006) report differences between the two species in the presence/absence for 25 further introns. Whereas intronic sequences differ between strains of *C. brenneri* sp. n. and even between alleles within the same strain (data for RNAP2, K. K. unpublished), the presence of introns is usually species-specific. At present, there is only one known case of an intron presence/absence polymorphism (in the *jingwei* gene of *Drosophila teissieri*: Llopart et al. 2002).

Why are C. brenneri *sp. n. and* C. remanei *different species?*

We conclude that *C. brenneri* sp. n. and *C. remanei* are two different species based on evidence from cross-breeding experiments, biogeography and DNA sequences, even though we have found almost no differences in morphology, physiology or ecology. The results of the mating experiments clearly favor two reproductively isolated species. Regarding biogeography, we found that *C. brenneri* sp. n. populations are geographically separated from *C. remanei* populations (Fig. 1). The two species are thus allopatrically distributed—*C. remanei* in temperate regions north of the Tropic of Cancer (~23.5°N), and *C. brenneri* sp. n. in the tropics south of the Tropic of Cancer. As far as we know, there is no contact or overlap of their geographic ranges. Consequently, they are to be classified as allospecies. The ecological niches of both species, but in particular of *C. brenneri* sp. n., are insufficiently known. *C. brenneri* sp. n. and *C. remanei* represent historically distinct evolutionary lineages. Phylogenetic analyses with molecular data (Cho et al. 2004, Kiontke et al. 2004) have shown that the two species are not sister taxa (Fig. 5). However, as they do not show any significant divergence in morphology or mate recognition, we can designate them as sibling species.

Stem species pattern of the Caenorhabditis Elegans *group*

The stem species pattern—often called ground plan or ground pattern—is the set of all apomorphic and plesiomorphic characters in the stem species of a taxon, here of the *Elegans* group. Many features of the *Elegans* group stem species can be reconstructed by comparison of extant *Caenorhabditis* species. We collected 140 morphological and biological/ecological characters for the species of this taxon (unpublished) and established the plesiomorphic state in each case for the eight species of the *Elegans* group by ingroup and outgroup comparison. From this we conclude that the last common ancestor of the *Elegans* group was gonochoristic, lived in decaying organic material and had waving dauerlarvae that were phoretically associated with different arthropods and/or gastropods of the same habitat (Kiontke & Sudhaus 2006).

The stem species had the following morphological and biological characters:

Adult: Body of medium size, relatively slender. Cuticle with fine transverse striae, three longitudinal ridges on the lateral surface. Six lips closed, not offset, each with one external apical sensillum; only in males a second circle of four cephalic sensilla at level with anterior end of gymnostom. Apertures of amphids on lateral lips (anterior level of cephalic sensilla in males). Buccal tube longer than lip region is wide, triangular in cross section; cheilostom more or less conspicuous; length of stegostom (enveloped by pharyngeal sleeve) half or more of total stoma length; stegostom cuticle with delicate striation. Three sectors of glottoid apparatus isomorphic and almost isotopic, each bearing a triangular, distally rounded projection (flap). The pharynx exhibited a strong, oval median bulb and a rounded terminal bulb with duplex haustrulum posterior of the valves; the open cardia was well developed. Position of nerve ring around isthmus variable. Excretory pore and deirids at level with anterior end of terminal bulb; deirid eccentric in dorsal-most ridge of the lateral field. Posterior deirids slightly dorsal of lateral ridges. Lateral canals of secretory-excretory system from about two stoma lengths behind anterior end to about level with anus or cloaca; two secretory-excretory cells or one cell with two separate cell bodies present.

Female: Vulva midbody, a transverse slit with slightly protruding lips, the corners covered with longitudinal cuticular flaps. A mating plug was deposited by the male. Amphidelphic gonads with dorsal flexures within the growth zone of the ovary (homodromous), the anterior branch right and the posterior branch left of intestine. Spermatheca present at transition of oviduct and uterus, separated from uterus by a sphincter. Each uterus carried one to six embryos at different stages of development (oviparous to ovoviviparous). Two pseudocoelomocytes each positioned ventrally at the anterior flexure, and dorsally at the posterior flexure of the ovary. Rectum nearly as long as anal body width (= ABW). Tail conical, about 5–7 ABW long, tip filiform; phasmid opening about two ABW behind anus.

Male: Gonad right of intestine and ventrally reflexed to about one fifth or one fourth of the length of the genital tract. Two pseudocoelomocytes situated ventrally at level of flexure. Bursa ("fan") peloderan, heart-shaped in ventral view, the velum anteriorly closed. The bursa edge was serrated; the "saw teeth" diminished posteriorly until, at a point slightly posterior to the 6th genital papilla (GP, "ray"), the edge of the velum was smooth. There were about 30 teeth along the anterior margin of the closed bursa between the left and right first genital papillae, and on each side 10–14 teeth between GP1 and GP3, 12–16 between GP3 and GP6, and 5–7 very small teeth posterior GP6. There was a terminal notch. Posterior part of velum transversely striated (washboard pattern). Nine pairs of GPs arranged as (2/1+3+3), or more precisely [v1^{dors}, v2]/v3 [v4, ad, v5][pd, v6, v7, ph]; thus, there were three clusters of papillae (in brackets). Indistinct phasmids (ph) opened ventrally at the base of GP9; GP1, GP5 and GP7 terminated on the dorsal (= d) side of the velum, the other GPs on the ventral side (= v), except for GP6 which had no free sensillum tip. GP2 and GP4 did not extend to near the velum edge. GP2, GP3 and GP6 basally thickened or bottle shaped, GP6 tapering. A small, club-shaped tail tip extended beyond the base of GP9, but was embedded in the bursal velum. Precloacal lip formed an anteriorly projecting hook, shaped like an arrowhead in ventral view, with a basal bulge bearing the precloacal sensillum. Spicules yellowish-brown, separate, slightly curved ventrally, differentiated in a truncated head, a narrowed shaft exhibiting a transverse seam, and a pointed blade with a thinner dorsal lamella

(velum). The long gubernaculum (about three-fourth the spicule length) was ventrally curved, laterally narrow and highly bendable, so that it was strongly arched during copulation. Distal part of gubernaculum protruded outside of the cloaca and carried two lateral processes (ears) and a dorsally bent forked terminus. The tiny rod shaped postcloacal sensilla were positioned to either side of this part of the gubernaculum just posterior of the lateral ears.

Dauerlarva: Not ensheathed, with a broad lateral field consisting of four ridges with a broadened median furrow; deirids and postdeirids conspicuous; the proximal excretory duct pulsating; waving.

Caenorhabditis brenneri **sp. n.**[1]
(Figs 2–4)

= *Caenorhabditis remanei* auct.[2]
= *C.* n. sp. 4 in Sudhaus & Kiontke (1996) and Kiontke & Sudhaus (2006)
= ? *Rhabditis teres* apud Schuurmans Stekhoven, 1951, nec (Schneider, 1866)

Measurements see Table 2.

With the characters of the stem species pattern of the *Elegans* group. The following details were observed in strain SB129 and might be of diagnostic value.

Adult: Stoma length about 1.5–2 times the diameter in the lip region. Stoma diameter sexually dimorphic; the ratio of stoma length to width is about 5 in the ♀ and about 7 in the ♂. Corpus intima finely transversely striated. Nerve ring at anterior third of isthmus. Position of deirids variable, slightly anterior or posterior to excretory pore. Position of postdeirids at approximately 66 % of body length in the ♀, and 81 % in the ♂. Width of lateral field about 3 µm or 1/7 to 1/12 of body width; the longitudinal cuticular ridges extend from level of median pharynx bulb to posterior the anus in the ♀, and nearly to the distal end of the spicules in the ♂. Chromosome number 1n=6.

Female: Lateral canals of secretory-excretory system extend to one ABW posterior of the anus. The position of the vulva appears exactly in the middle between pharynx end and anus; the distance between vulva and anus is 92–124 (104) % the distance from pharynx end to vulva. The posterior gonad arm comprises 88–118 (101) % of the anterior one. At maximum 7 embryos in each uterus, sometimes developed to J1 ready to hatch.

Male: The distance between the pharynx end and flexure of the testis is 56–159 (101) µm or 71–154 (116) % of testis flexure length. Bursa as in the stem species. In degenerating specimens GP7 becomes slightly bottle shaped like GP2, GP3 and GP6. GP4 a little shorter than GP5. Spicules can differ in length by about 3 %. The dorsal velum on the spicule terminates one fifth of a spicule length from distal tip. Proximal end of gubernaculum appears slightly swollen where muscles insert, the distal projections (ears) are curved like claws or hooks, weakly sclerotized, whereas the forked terminal piece is heavily sclerotized, visible as two conspicuous points in ventral view of the intact ♂.

Aberrations: One ♂ with testis left of intestine. In one case both spicules and the gubernaculum were stunted (a phenotype which is observed in *C. elegans* when the muscles required for morphogenesis of the

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[1.] This species is dedicated to Sydney Brenner, who, 40 years ago, initiated the fundamental work on *C. elegans.*

[2.] In papers published before 1995/96 (Binder et al. 1992, Cangiano & La Volpe 1993, Felsenstein & Emmons 1988, Fitch et al. 1995, Fodor & Timar 1989, Fodor et al. 1989, Hekimi 1990, Hodgkin 1984, Jones & Schedl 1995, Kloek et al. 1996, Lee et al. 1993, Sedensky et al. 1994, Thomas & Wilson 1991, Youngman et al. 1996) this nematode was designated as "*C. remanei*" referring to strain CB5161 isolated in Trinidad and deposited at the CGC in 1988. Of the sequences deposited at GenBank under the name *C. remanei,* six belong to *C. brenneri* sp. n.. These sequences have the accession numbers U75913, U01838 and U48292–5. Some *C. remanei* sequences are deposited under the synonym *C. vulgaris.*

proctodeum are damaged or absent; Sulston et al. 1980); in another case, the right spicule was abnormally short and broad, measuring 21 µm whereas the left spicule was 36 µm long (Fig. 3E, F). In one ♂ GP4 missing on the left hand side. In another ♂ the arrangement of GPs as usual on the left side (GP1 and GP2 clustered), but on the right side GP1 was separated from GP2 and shifted to the anterior margin of the closed velum (Fig. 3I). Once, both GP1 were positioned anterior of the closed bursa velum, standing slightly asymmetrically (Fig. 3G, H). This aberration is of some evolutionary significance. It demonstrates that a bursal papilla which is integrated in a proximally closed velum can disassociate from the velum and secondarily adopt a prebursal position. Such a scenario was discussed for the evolution of the prebursal genital papillae in *Pelodera kolbi* (Sachs, 1950) within the *Coarctata*-group (Sudhaus 1976, p. 25; Sudhaus & Fitch 2001, p. 23).
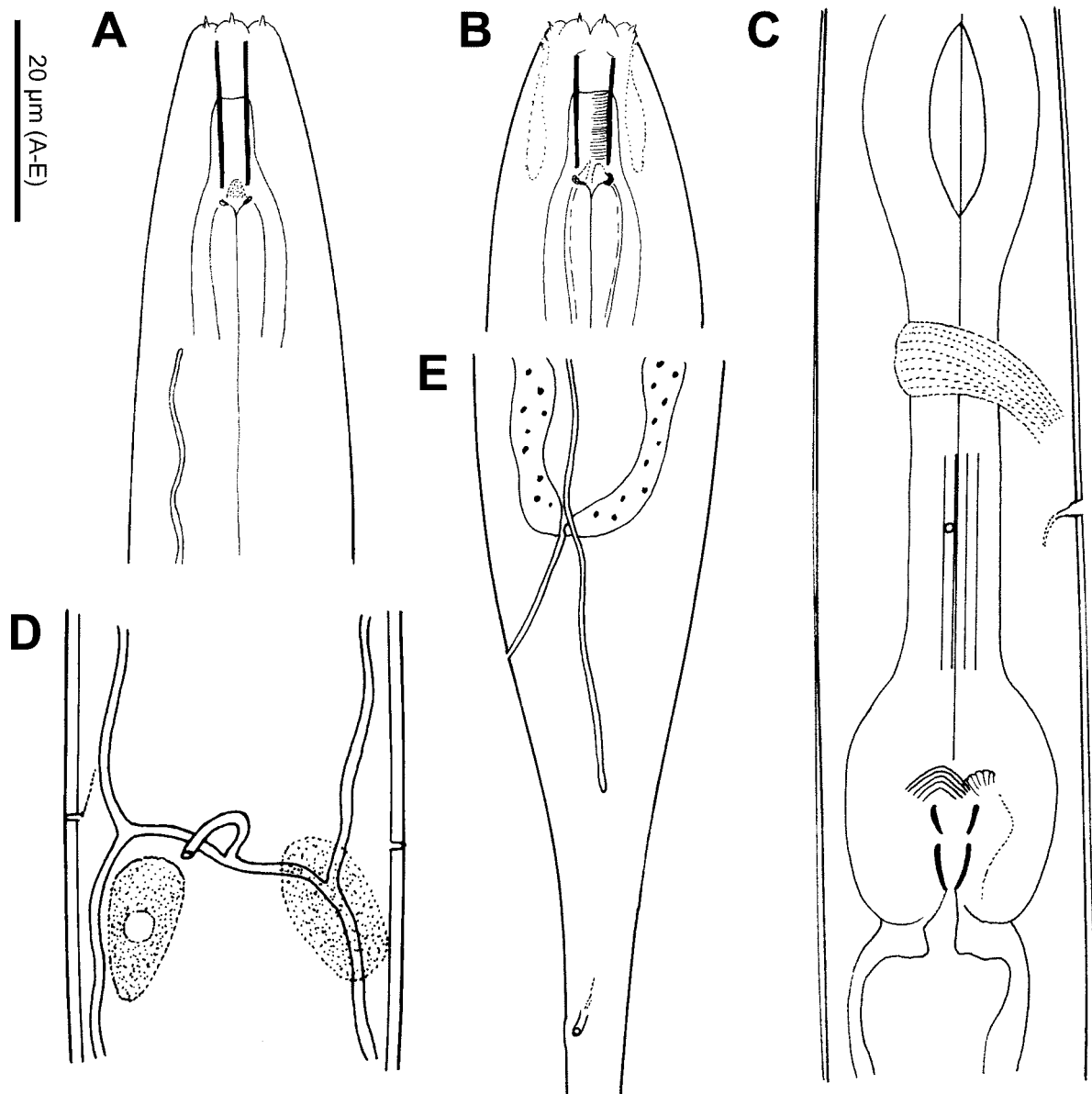

FIGURE 2. *Caenorhabditis brenneri* sp. n. female. A: anterior end subventral, with anterior end of lateral canal; B: anterior end ventral; C: pharynx region with lateral field and deirid; D: secretory-excretory system at the level of the deirid ventral; E: anus region lateral, with posterior end of lateral canal and phasmid.

Type locality and habitat

Bohorok in Sumatra (SB129). Compost-like material, mostly banana leaves.
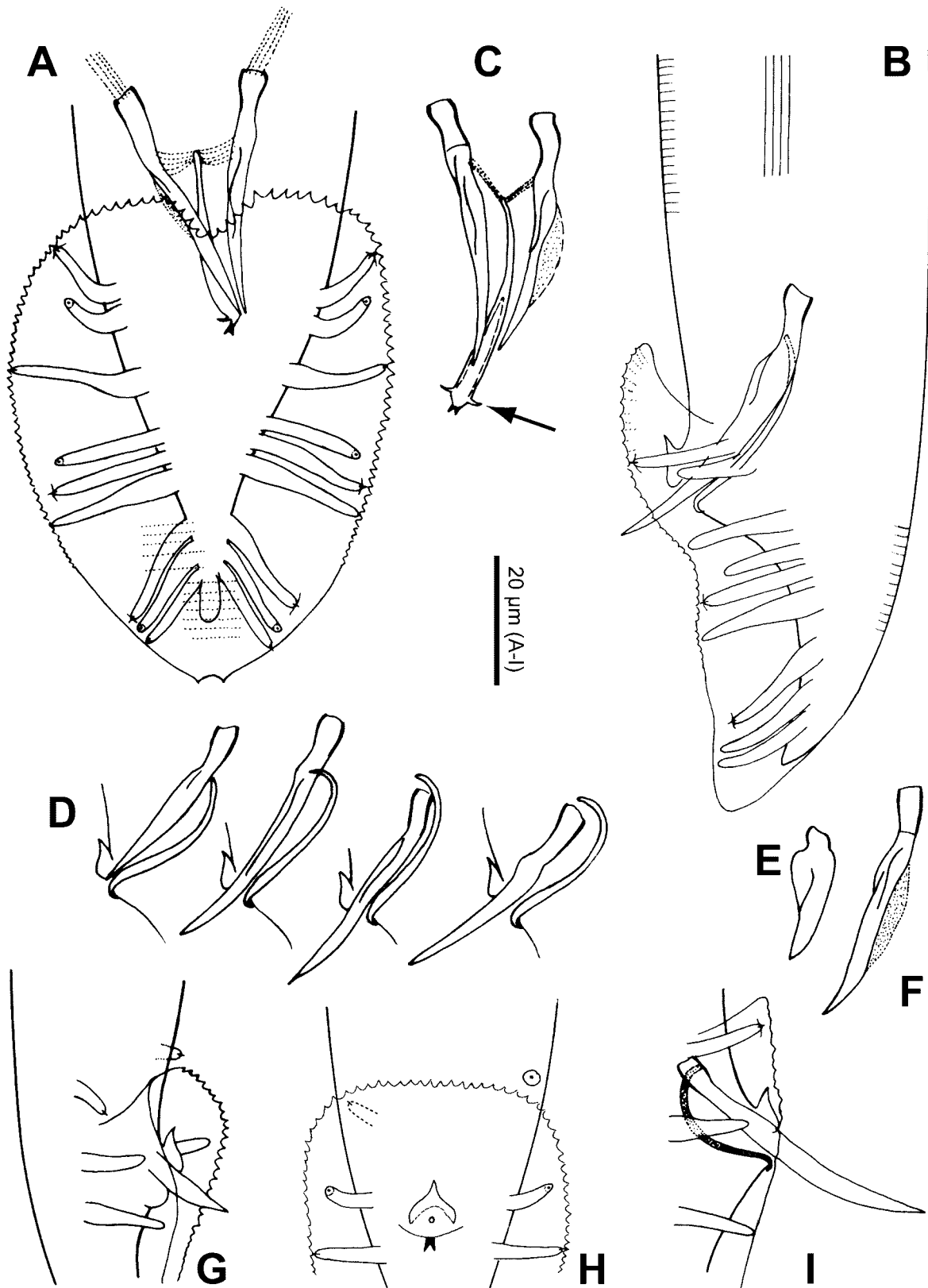
FIGURE 3. *Caenorhabditis brenneri* sp. n. male. A: posterior end ventral showing bursa, spicules, gubernaculum; note the striped pattern of the cuticle in the posterior part of the tail; B: posterior end lateral; C: spicules and gubernaculum subventral; arrow points to the conspicuous lateral projections on the gubernaculum ("ears"); D: series demonstrating the bending of the gubernaculum when the spicules are extruded, lateral view, also showing the hook; E–F: aberrant right (E) and normal left spicule (F) of the same specimen lateral; G–I: aberrant positions of genital papillae: both GP1 positioned anterior of the bursa (G, H), right GP1 shifted to the anterior margin of the bursa (I).
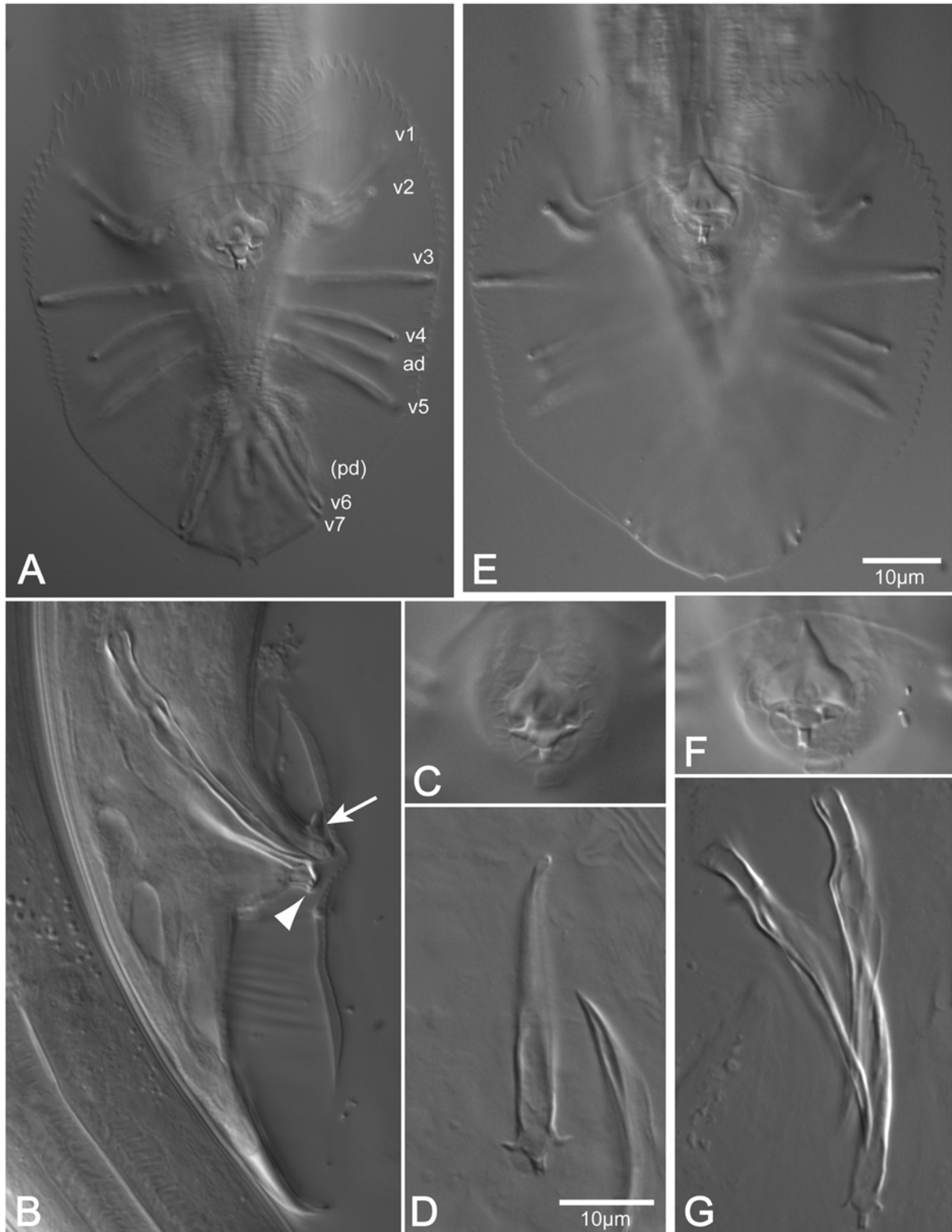
FIGURE 4. Aspects of male copulatory structures in *C. brenneri* sp. n. (A–D) and *C. remanei* (E–G). A and E: bursa in ventral view showing precloacal hook, distal part of gubernaculum and genital papillae (GP). In A the GPs are numbered (v = ventral GPs, ad = anterior dorsal GP, pd = posterior dorsal GP slightly out of focus). The differences in size and shape of the bursa in A and E are individual differences and do not mark differences between the species. B: male tail in lateral view. Arrow points to precloacal hook, arrowhead points to distal part of gubernaculum which is bent dorsally in living animals. C and F: cloacal region with precloacal hook and distal part of gubernaculum in situ. D and G: isolated gubernaculum in ventral view and spicules (G). Note that the two lateral processes (ears) are larger and more strongly refractive in *C. brenneri* sp. n. than in *C. remanei*. Scale is the same in Figs. A, B, E; and in Figs. C, D, F, G, respectively.

Type material

Holotype (male, no. 11232) and paratypes (no. 11233) deposited in the collection of the Museum für Naturkunde der Humboldt-Universität zu Berlin, Germany; further paratypes in Naturhistoriska Riksmuseet Stockholm, Sweden; Laboratorium voor Nematologie, Landbouwhogeschool, Wageningen, the Netherlands; USDA, Nematology Laboratory, Beltsville, Maryland, USA; collection of Prof. W. Sudhaus, Institut für Biologie, FU Berlin, Germany.

Comparison and diagnosis

From a comparison with *C. brenneri* sp. n., we can exclude *C. briggsae* and *C. elegans*, which are self-fertilizing protandrous hermaphroditic species with a very low percentage of residual males. *C. brenneri* sp. n. differs from *C. clavopapillata*, which exhibits a group of only three (instead of 1+3 = 4) genital papillae (GP) immediately posterior of the cloaca. Since the first and third GP of this group are basally swollen, they can be identified as GP3 and GP6. Therefore, a likely interpretation is that GP4 disappeared by fusion with GP3, as it is often observed in *C. briggsae*. Despite thorough analyses, no clear difference could be established in morphometric data between *C. brenneri* sp. n. and *C. remanei* which are isolated post mating. Moreover, there are almost no differences in morphology. During an extensive survey of morphological characters we found slight differences in a number of characters between and within isolates of the two species. However, these differences do not constitute clear differences between the two species. The characters include the location of deirids in relation to the cervical pore, the number of teeth along the bursa margin between GP1 and GP3 and between GP3 and GP6, and the conspicuousness of the forked process at the distal end of the gubernaculum. An exception is the shape of the lateral processes at the distal part of the gubernaculum which we call ears. These projections are shaped like small thorns in *C. remanei* (Fig. 4F) but like elongate curved claws in *C. brenneri* sp. n. (Fig. 3C and 4C). The sibling species are further distinguished by the length of GP4 relative to GP5: GP4 is slightly shorter than GP5 in *C. brenneri* sp. n., whereas both GPs are of almost equal length in *C. remanei*. GP4 is longer than GP5 in *C. briggsae* and *C. elegans*.

C. brenneri sp. n. must be compared with two further species, *C. formosana* from Taiwan (Changhua Country) and *C. oncomelaniae* from Japan (Kyushu). Both were described as associates of the snail *Oncomelania hupensis* Gredler. Based on the descriptions, we would not hesitate to synonymize these two species, were they not studied by the same authors at the same time (Yokoo & Okabe 1968). Unfortunately, the authors did not conduct cross mating experiments. Neither species was found again even though a large body of field work was done on the snail *Oncomelania hupensis,* it being the intermediate host of *Schistosoma*. Unfortunately, *Oncomelania* snails can not be examined anymore at or near the type locality of *C. oncomelaniae* in the prefecture Saga of Kyushu because some years ago the snails were eradicated in this area (Toyoshi Yoshiga, pers. comm.). We know of only three isolates of *Caenorhabditis* from *Oncomelania* in Taiwan (PS1185 and PS 1186 W. Kelley Thomas, pers. comm.) and Japan (Hokkaido, sampled 1995 by Mitsuhiko Asakawa for W. S.), and all were identified as *C. briggsae*. These records are thus incompatible with the gonochoristic *C. formosana* and *C. oncomelaniae*. Since there is thus no new material of these gonochoristic *Caenorhabditis* species from East Asia, we rely on the original descriptions by Yokoo & Okabe (1968) to argue that *C. brenneri* sp. n. is different from *C. formosana* and *C. oncomelaniae*. Most characters and measurements are overlapping, except for the length of the spicules (<38 μm in *C. brenneri* sp. n. as opposed to >40 μm in *C. oncomelaniae* and >45 μm in *C. formosana*). *C. formosana* differs from *C. brenneri* sp. n. in possibly basally fused GP4 and GP5, and in the more anterior position of the phasmids in the female (1.5 times ABW posterior anus vs. >2 times ABW in *C. brenneri* sp. n.), assuming that this is not an artifact in the photograph (Fig. 6.4) or an error in the drawing (Fig. 5.4) in Yokoo & Okabe (1968). *C. oncomelaniae* is larger than *C. brenneri* sp. n. (♂ body length 1020–1430 μm vs. <950 μm; b = 5.7–7.8 vs. <5.6; gubernaculum length 30–37.5 μm vs. <30 μm), the anterior gonad of the *C. oncomelaniae* female is always shorter than the posterior gonad, whereas both are of almost equal length in *C. brenneri* sp. n., the anterior ovary comprises 14–21 % of body

length in *C. oncomelaniae* vs. 18–31 % in *C. brenneri* sp. n.; finally, the nerve ring of *C. oncomelaniae* is located in the posterior part instead of the anterior part of the isthmus. As a potential additional difference, *C. formosana* and *C. oncomelaniae* were both isolated from temperate locations north of and at the tropic of cancer, whereas *C. brenneri* sp. n. is only known from tropical locations. Even though Okabe & Shiraishi (1971) were highly successful in infesting snails with *C. oncomelaniae* in the laboratory, there are two reasons not to consider the association of *C. formosana* and *C. oncomelaniae* with *Oncomelania* as an ecological difference to *C. brenneri* sp. n.. First, the records of *C. formosana* and *C. oncomelaniae* are unique and could not be repeated as yet; second, preliminary results in our laboratory show that it is easy to infest snails and slugs with different rhabditids, including *Caenorhabditis*. So far there is no evidence for a specific association of *Caenorhabditis* species with snails in Eastern Asia.

Ecology and biology

C. brenneri sp. n. was only found in anthropogenic habitats (plantations, agricultural land, plant nurseries, gardens) in rotting plant material, rich soil and dung. The species can be cultured on organic material of various kinds, and on nutrient agar. Because of the waving behavior of the dauerlarvae—single and in plaits with up to 50 individuals—an unspecialized phoretic association is expected. In the laboratory, phoretic transport by the predatory mite *Hypoaspis miles* (Berlese) was observed. Exceptional within *Caenorhabditis* is the desiccation tolerance of this species. Dauerlarvae can be kept in dry substrate for 11.5 months at 8°C, for 7.5 months at room temperature, and for 4.5 months at 32°C. In tap water dauer larvae lived for 8 months at 8°C and for 160 days at 16°C.

Gonochoristic; on average 48.1 % of adult progeny of single females in drops of nutrients were males (n = 5288), spanning between 40.3 and 54.9 % within the progeny of one female (n = 25). Copulation can occur in liquid medium. Initially, the male coils around the female with its posterior end. Later, mating males and females adopt a parallel position, equally frequently of the λ- or Y-type (14 : 15). At maximum, 7 embryos were observed in each uterus. Mostly oviparous, sometimes juveniles hatch within the uterus. The progeny of one female is between 103 and 309 (187 ± 62; n = 29). Once 415 offspring were counted (females with fewer than 100 progeny were disregarded). One *C. brenneri* sp. n. ♀ which was mated with *C. remanei* ♂♂ laid 234 degenerating eggs, and one *C. remanei* ♀ laid approximately 100 eggs after mating with *C. brenneri* sp. n. ♂♂ (Table 1). Virgin females lived 3–40 (14.3 ± 8.1) days (n = 30), and reproducing females lived 3–43 (12.5 ± 7.4) days (n = 55). Males lived 3–25 (11 ± 4.7) days (n = 139).

Discussion

Cross-breeding experiments

In mating experiments, males of *C. brenneri* sp. n. copulated with *C. remanei* females and vice versa. They also mate with hermaphrodites of *C. elegans* and *C. briggsae*, but not with females of *Pelodera teres* Schneider or *Oscheius myriophila* (Poinar) (*R.* sp. in Baird et al. 1992, David Fitch, pers. comm.). Thus, males are attracted to females or hermaphrodites from other closely related *Caenorhabditis* species, but they do not recognize females of distantly related species as mates (Baird et al. 1992). Whether *Caenorhabditis* males are able to distinguish between conspecific females and females of a related species remains to be tested in choice experiments.

A postmating compatibility barrier prevented gene flow between *C. brenneri* sp. n. and *C. remanei*. Interspecific copulations stimulated deposition of some eggs, usually in small numbers (less than 35, but occasionally more than 100, see Table 1). These eggs were not fertilized when *C. remanei* females were mated with *C. brenneri* sp. n. males (degenerating eggs in Table 1 and data by Baird et al. 1992). When *C. brenneri* sp. n. females were mated with *C. remanei* males, fertilization occurred at least sometimes. Baird et al. (1992)

recorded an average of 70 fertilized eggs. Such embryos arrested during gastrulation or post gastrulation during embryonic compaction in the matings performed by Baird et al. (1992) and Baird & Yen (2000). We obtained occasionally nonviable juveniles and 7 apparently infertile adults.

TABLE 1: Results of intra- and interspecific crosses between populations of *C. brenneri* sp. n. and *C. remanei*. + = fertile F[2]-generation. − = no offspring, or rarely juveniles which only exceptionally reached adulthood without being fertile. deg. eggs = eggs or embryos which degenerated without hatching. Crosses between *C. brenneri* sp. n and *C. remanei* which resulted in juvenile progeny are highlighted. Included are some unpublished crosses between strains of *C. remanei* conducted by Angelika Weber (1995). The numbers for eggs laid by the females are minimum numbers; some eggs may have been overlooked.

males \ females	*C. brenneri*						*C. remanei*			
	Costa Rica LKC28	Trinidad CB5161	Guadeloupe SB280	Penang	Sumatra SB129	Bali	Freiburg [a] SB146 [b] isolate 1975	Berlin SB111	New York EM464	Japan Kyushu or Kanagawa
Costa Rica LKC28	X				8 + / 4 −					
Trinidad CB5161		X				13 +			10 − / 3x deg. eggs	[1]
Guadeloupe SB280			X		6 + / 1 −		[a] 7 −			
Penang				X		9 +	[b] 7 −			
Sumatra SB129	6 + / 4 −		4 + / 1 − / 109 deg. eggs, 1 juv.	2 +	X		[a,b] 31 − / 7x deg. eggs [2]	15 −	13 − / 1x 100 deg. eggs	4 −
Bali		18 +		7 +	4 +	X	[b] 10 − / 2x deg. eggs			
Freiburg [a] SB146 [b] isolate 1975	[a] 4 − / 1x 2 juv. [3]	[a] 17 − / 10x deg. eggs [4,5] / 2x juv.	[a] 7 − / 1x 54 deg. eggs / 1x 1 juv. [6]	[b] 7 − / 6x deg. eggs / 1x 1 juv. [7]	[a,b] 22 − / 14x deg. eggs [8]	[b] 11 − / 3x deg. eggs [9]	X	[a] 14 + / [a] 2 −	[a] 19 + / [a] 3 −	[a] 15 + / [a] 1 − / (1 juv.)
Berlin SB111					3 − / 2x deg. eggs / 1x 1 juv. [10]		[a] 25 +	X	14 +	
New York EM464		[11]			7 − / 1x 2 deg. eggs	3 − / 1x 6 deg. eggs	[a] 19 + / [a] 2 −	27 +	X	
Japan Kyushu & Kanagawa							[a] 15 + / [a] 1 −			X

[1] Baird et al. (1992) observed only unfertilized eggs in 8 crosses.

[2] 3, 3, 4, 4, 5, 5, 9 degenerating eggs were counted.

[3] Of the two juveniles one died at once, one lived for 3–4 days as J1.

[4] One female produced 5 juveniles, 2 of which developed into adults (1male, 1female). The second female produced 5 juveniles; one died early and 4 became males, which lived with their mother for 5 days without backcrossing.

[5] Baird & Yen (2000) observed embryonic arrest in crosses of CB5161 females with SB146 males. No juveniles hatched.

[6] This juvenile developed very slowly; after 8 days it became an adult vulvaless female with only short gonads. It lived for 12–13 days.

[7] 15, 32, 32, 58, 66, 67 degenerating eggs were counted over a period of 1–3 days; only 1 J1 hatched, which died very soon.

[8] 10–51 degenerating eggs were counted.

[9] 6–26 degenerating eggs were counted.

[10] 84 and 234 degenerating eggs were laid within 3–4 days; only one J1 hatched which died very soon.

[11] Baird et al. (1992) observed embryonic arrest, mostly prior to gastrulation.

There is a notable difference in the infertility of these interspecific crosses, depending on whether the female belongs to *C. brenneri* sp. n. or to *C. remanei*. Crosses with *C. remanei* females and *C. brenneri* sp. n. males were fertilization defective, whereas in crosses of *C. brenneri* sp. n. females with *C. remanei* males fertilization occurred and embryonic development was defective. This dissymmetry in hybrid development suggests dissymmetry in effects of maternal factors or parental imprinting.

TABLE 2: Ratios and measurements (in μm) of heat relaxed specimens of *Caenorhabditis brenneri* n. sp. (strain SB129). Emphasis is on the variability of the population as expressed as the range of distances. Mean and standard deviation in parentheses.

	15 females	10 males
body length	744–1770 (1457±296)	562–953 (741±124)
body width	37–94 (75± 16)	26–38 (32±3.5)
lip region diameter	10–16 (13±1.5)	8.5–11 (10±0.8)
stoma length	17–24 (21±2.3)	16–21.5 (18±2)
stoma diameter	2.9–5.1 (4±0.6)	1.9–2.8 (2.2±0.4)
pharyngeal sleeve as % stoma length	44–62 (53±5)	45–55 (51±2.5)
stoma length as % pharynx length	9–11 (10±0.6)	10–12 (11±0.8)
pharynx length	164–251 (213± 21)	156–199 (172±12)
corpus length	96–138 (122±11)	91–113 (99±6)
corpus as % pharynx length	53–61 (57±1.7)	57–59 (58±1)
median bulb (MB) diameter	20–40 (31±5)	17–21 (19±1)
terminal bulb (TB) diameter	24–48 (38±7)	18–25 (21±1.7)
MB diam. as % TB diam.	75–90.5 (84±4)	88–95.5 (93±2.7)
anterior end to excretory pore	150–182 (162±14)	122–167 (134±15)
excr. pore position as % pharynx length	75–83 (80±3.5)	72–87 (79±4.4)
tail length	188–260 (230±24)	33–40 (37±2.5)
anal (cloacal) body width (ABW)	19–42 (31±6.5)	20–22 (21±0.7)
gonad length[1]	277–910 (641±189)	293–617 (437±112)
gonad length as % body length	37–58 (45±7)	52–65 (58±5)
gonad length as % intestine length[2]	54–81 (66±9)	77–88 (81±4)
anterior gonad branch length	134–483 (325±102)	–
posterior branch length	143–458 (319±89)	–
testis flexure length	–	76–126 (95±14)
anterior flexure as % of ant. branch	57–94 (71±16)	19–26 (22±2.7)
posterior flexure as % of post. branch	52–83 (67±17)	–
sperm diameter	n.d.	4.5–6.3 (5±0.6)
egg length	48–57 (52±3)	–
egg diameter	26–34 (30±2.3)	–
rectum length	25–35 (30±4)	n.d.
rectum length/ABW	0.7–1.2 (0.9±0.2)	n.d.
anus to phasmid distance	44–89 (72±13)	n.d.
anus to phasmid distance/ABW	2–2.7 (2.3±0.2)	n.d.
position phasmid as % tail length	27–37 (32±3)	–
spicule length	–	27–38 (32±2.9)
gubernaculum length	–	21–30 (25±3.2)
gubernac. length as % spicule length	–	67–87 (77±5.4)
a	17.5–21.9 (19.8±1.3)	20.4–29.8 (24.3±2.7)
b	4.5–8.2 (6.7±1)	3.6–5.6 (4.3±0.6)
c	4.8–7.5 (6.5±0.6)	17.1–25.1 (20.2±3)
d (= tail length/ABW)	5.8–8.7 (7.2±0.8)	1.6–1.9 (1.7±0.1)
V (vulva position in % body length	46.5–51 (49±1.3)	–

[1] from anterior to posterior flexure in the female; from cloaca to flexure in the male

[2] distance from pharynx end to anus or cloaca, respectively.

SUDHAUS & KIONTKE

Sibling species

The species *C. brenneri* sp. n. and *C. remanei* show a striking disparity between an extensive genetic divergence and the almost complete absence of morphological differences. To which extent the species differ ecologically is currently difficult to judge because ecological data are still sparse. Both species were isolated from putrefying organic matter and can be cultured in the laboratory with the same methods. *C. brenneri* sp. n. dauerlarvae survive desiccation better than *C. remanei* dauerlarvae (data for *C. remanei* in Sudhaus 1974). *C. remanei* dauerlarvae have been found to embark on isopods (Baird 1999, Weber 1995), and they are able to survive the passage through the gut of slugs and snails (unpublished data). There is currently nothing known about phoretic associates of *C. brenneri* sp. n.

Distribution

Although our knowledge on the distribution of *C. brenneri* sp. n. and *C. remanei* is insufficient, the current data suggest that their ranges are completely allopatric, possibly separated by a broad gap along the Tropic of Cancer (Fig. 1). This distribution pattern might be explained historically by an origin of these species in a more tropical (*C. brenneri* sp. n.) or more temperate region (*C. remanei*) and a subsequent colonization of further regions with the adequate climate. Future comprehensive collections in the region of the possible distribution gap near the Tropic of Cancer are required to confirm that there is no contact zone or overlap of the ranges of *C. brenneri* sp. n. and *C. remanei*. So far, collections of suitable material were performed in Taiwan (Asher D. Cutter, pers. comm.) and southern China (Marie-Anne Félix, pers. comm.). Neither yielded *C. brenneri* sp. n. or *C. remanei*. Instead, the Chinese samples contained another gonochoristic species of the *Elegans* group, *C.* sp. n. 5, which could be the sister species of *C. briggsae*. Thus it is possible that these three species replace each other in similar habitats at different latitudes.

Comments on speciation

Considering the present knowledge about the geographical distribution of *Caenorhabditis* species, we cautiously propose that the species of the *Elegans* group originated in Asia and colonized their current ranges from there. The rationale is based on the observation that Asia is the only geographic region where all *Elegans* group species were found so far. At least one species of the *Elegans* group—*C.* sp. n. 5, disregarding the dubious *C. formosana* and *C. oncomelaniae*—is only known from Asia. The sister species of the *Elegans* group, *C. japonica* Kiontke, Hironaka & Sudhaus, 2002, is Asiatic, as well as three out of the four next closely related clades (*C. anthobia* (Schneider), *C. auriculariae* Tsuda & Futai, *C. avicola* Schmidt & Kuntz, see Fig. 5). If they originated in Asia, *C. briggsae, C. brenneri* sp. n., *C. elegans* and *C. remanei* must have spread across the globe. Considering their anthropogenic habitats, it is possible that the dispersal of all four species was aided by human activities. It is reasonable to propose that *C. remanei* invaded Europe postglacially, aided by human agricultural activities. The zonal distribution of *C. brenneri* sp. n. and *C. remanei* might then be caused by physiological or ecological incompatibilities of the species with temperate or tropical habitats, respectively.

Alternatively, it can not be fully ruled out that the zonal distribution of *C. brenneri* sp. n. and *C. remanei* reflects a very old separation event, as it is reminiscent of distributions resulting from the breakup of Pangaea and the formation of the Tethys ocean. However, it follows from this hypothesis that the stem species of *C. brenneri* sp. n. and *C. remanei* existed before the formation of the Tethys ocean, which leads to an unlikely age estimate for the *Elegans* group of more than 200 MYA.

Recent dispersal by humans would yield a pattern consistent with the global distribution of *C. brenneri* sp. n. and *C. remanei*. It would also be consistent with the results of population genetics studies in *C. elegans* and *C. remanei* which suggest that long-distance dispersal is a regular occurrence in these species (Cutter 2006 and references therein, Cutter et al. 2006). I.e. genetic data from *C. elegans* showed no signature of geographic structure, and the study of North American and some European samples of *C. remanei* did not find evidence for deviation from panmixis. However, as has been noted by Sudhaus (1976), a global distribution is

also observed in other saprobiotic rhabditid species (including *Caenorhabditis plicata* (Völk)) for which an association with humans is unlikely. Mating experiments with these species have demonstrated that here, too, individuals from geographically very distant populations remain interfertile, even though the motility of rhabditid nematodes is small, and the separation of the populations should thus be old. Currently, this paradox remains unexplained. Future population genetic investigations on species of the *Elegans* group may shed light on this issue.
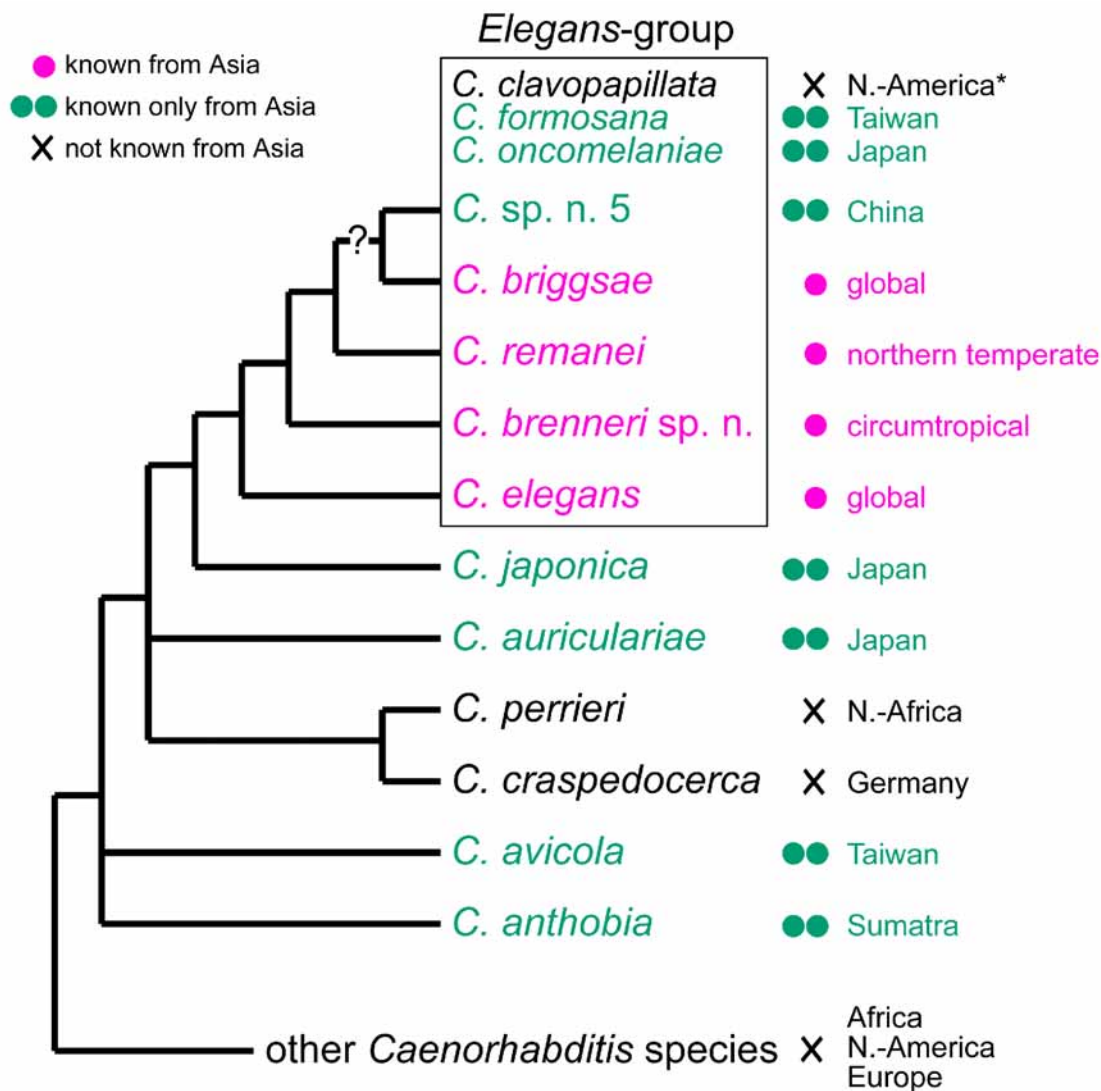

FIGURE 5. Phylogenetic tree (after Kiontke & Sudhaus 2006) and geographic distribution of the *Elegans* group and related species. With the exception of *C. craspedocerca* (Völk) and *C. perrieri* (Maupas), all species were found in Asia. Species highlighted in red are so far only known from Asia. *C. clavopapillata* (Kreis & Faust) was isolated from captive dogs and monkeys in the USA. Its natural range is unknown.

Acknowledgement

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