Whole genome sequencing and morphological analysis of the human-infecting schistosome emerging in Europe reveals a complex admixture between *Schistosoma haematobium* and *Schistosoma bovis* parasites.

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Abstract

Background: Schistosomes cause schistosomiasis, the world's second most important parasitic disease of public health importance after malaria. A peculiar feature of schistosomes is their ability to hybridize. So far, individual hybrid schistosomes are considered as such when they display incongruent genetic signatures at a nuclear (ITS-2) and a mitochondrial (COI) gene. Nevertheless, the introgression levels of any *Schistosoma* hybrid have never been assessed at the whole genome level. Here we have analysed both the morphological characteristics and the genomic composition of the schistosome that infected over one hundred humans in Corsica during summer 2013. This parasite was determined as a hybrid between the ruminant-infective (*Schistosoma bovis*) and the human-infective (*Schistosoma haematobium*) schistosomes. Characterizing precisely the genomic introgression of these hybrids is essential to better understand their life history traits, and the disease dynamics and epidemiology *in natura*.

Results: For this work, we first performed genome assembly improvement and annotation of *S. bovis*. Whole genome sequencing of the European hybrid revealed that this parasite in strongly introgressed and composed of 77% of sequences of *S. haematobium* origin, and 23% of S. *bovis* origin. We also present the morphological characterization of adult hybrids, and compatibility with their potential vector snails.

Conclusions: For the first time since the discovery of hybrid schistosomes, these results reveal at the whole genomic level a complex admixture of parental genomes highlighting (i) the high permeability of schistosomes to other species' alleles, and (ii) the importance of hybrid formation for pushing species boundaries not only conceptionally but also geographically.

Keywords: *Schistosoma haematobium*, *Schistosoma bovis*, Hybridization, Genomic Introgression, Whole Genome Next Generation Sequencing.

Background

Hybridization is where the genomes of two distinct species are brought together in one individual. By definition, hybridization between individuals from two previously reproductively isolated species is expected to produce offspring less fit than the parents, sometimes infertile or even sterile. In some cases, however, hybridization leads to new non-detrimental phenotypes [1–3]. First-generation fertile hybrids have 50% of each parental genome and successive reproduction events between the resulting hybrids, or between hybrids and individuals from the parental species, progressively lead to a widespread invasion of one genome by the other. Such genomic introgression might have important consequences on the evolutionary outcomes depending on the adaptive value of the resulting new genomic combinations [4]. When hybrid individuals display greater fitness than individuals from the parental species, a phenomenon known as hybrid vigour (or heterosis; [5–7]), some species can be supplanted by the emerging hybrids and ultimately become extinct [8]. Alternatively, although non-exclusively, some advantageous combinations of parental genes in the hybrid offspring may allow progeny to switch and adapt to new environments compared to the parental species. Such niche shifts together with selection against reproduction between hybrids and the parental species can ultimately lead to speciation, a process called "speciation by hybridization" [9–12].

Global changes, both anthropogenic and environmental modifications, may contribute to modifications in the geographical distribution of species and expand their potential ecological niches [13,14]. Populations of genetically distinct species may thus acquire a new capacity to interact, and enhanced contact between them may allow them to hybridize and subsequently introgress their genomes by backcrossing with parental species or other hybrids. This phenomenon called "hybrid swarm" has mainly been evidenced in plant species [15]. Generally, hybridization has been well described in plants, and for a long time it was considered a rare "mistake" in the reproductive isolation mechanisms of animals [16]. Today, with the advances in the development of molecular tools, and an integrative view of genetics and ecology (molecular ecology), scientist have revealed to a larger extent new cases of hybridization and introgression, both in plants and animals [4,17–24]. Surprisingly, very little attention has been given to pathogens, and particularly to metazoan parasites, that do not deviate from the rule that hybridization is more frequent than previously thought [18,21,25]. Hybridizations of

human parasites are not scarce and are a real concern in terms of parasite transmission, epidemiology and disease [18,25–27]. For instance, hybridization is evident in the parasitic worms–*Schistosoma*, *Fasciola*, *Ascaris* and *Trichinella*–and protozoa–*Plasmodium*, *Leishmania*, *Toxoplasma* and *Trypanosoma* [25,26].

Schistosomes are parasitic flatworms, responsible for the major tropical disease schistosomiasis, and known to hybridize; natural hybrids are increasingly been detected in human hosts [28]. The epidemiological statistics associated with the disease are sobering: 800 million people are at risk in 78 countries, mostly concentrated in sub-Saharan Africa; 230 million are infected and the disease causes more than 200 000 deaths each year as well as between 1.7 and 4.5 million Disability Adjusted Life Years (DALYs) [29]. The most exposed groups are children and young adults that have predominant activities linked to contaminated freshwater environments. In addition to humans, schistosomiasis severely impacts livestock in Africa and Asia where an estimated 165 million animals are infected [30].

Schistosomes have a complex life cycle that includes passage through a freshwater snail intermediate host and a final vertebrate definitive host. Hybridizations between schistosomes have been identified: (i) between different human-specific schistosome species, (ii) between different animal-specific schistosome species, (iii) and between human-specific and animal-specific schistosomes species [28]. These latter hybrid forms are particularly alarming because they raise the possibility of the emergence of new zoonotic parasitic strains, introducing an animal reservoir in the disease, and therefore greatly hampering our ability to properly control the transmission.

In 2013, schistosomiasis reached Southern Europe (Corsica, France). Since then, endemic infections were recurrently identified in 2015 and 2016, clearly indicating that the parasite has settled and established locally [31]. Using two molecular markers (the nuclear Internal Transcribed Spacer II (ITS-2) and the mitochondrial Cytochrome C Oxidase subunit I (COI), we had previously demonstrated that the parasitic strain now well established in Corsica is a hybrid between *Schistosoma haematobium* and *S. bovis* that are known to infect humans and livestock, respectively [32,33]. The emergence of a hybrid strain out of endemic areas of both parental species strongly suggest a role of hybridization in the emergence success of schistosomes in Corsica. Among the hundreds of schistosome eggs recovered from patients infected in Corsica, almost all had a classical *S. haematobium* shape [32], but using two genetic markers (ITS-2 and COI) we

showed that 42% of eggs were considered hybrids, and only 58% were considered pure *S. haematobium*. Wherever in Africa [34] or in Corsica, a clear discrepancy exists between the phenotypes usually used for species identification (egg shape) and the genotypes ("hybrid" or "pure"). Therefore, egg shape cannot be considered a good indicator for hybrid detection.

PCR amplification of the two abovementioned genes ITS-2 and the mitochondrial COI is the current method for schistosome molecular identification. Individuals having both nuclear and mitochondrial haplotypes of a unique species are considered as "pure". Conversely, individuals that display a nuclear haplotype characteristic of one species and a mitochondrial haplotype characteristic of another species are defined as hybrid parasites. This simple diagnosis procedure may clearly lead to identification of false "pure" individuals notably because recombination and successive reproduction events between hybrids and/or hybrids and "pure" individuals may lead to a congruency between these two genes despite introgression at other genomic locations. Moreover, this method does not indicate the genomic introgression levels of hybrids (i.e., the relative hybrid genomic composition between each parental genomes). Other particular cases of hybridization can be observed with mitochondrial introgression that in extreme cases may lead to the replacement of the mitochondrial genome of one species with that of another (mitochondrial capture) [35–38]. The precise characterization of the introgression levels of hybrid populations is essential for a diagnostics purpose but is also necessary to better understand the parasite life history traits, the disease dynamics and the epidemiology in the field. Next generation whole-genome sequencing is now the tool of choice for a deeper insight into the genomic composition of natural hybrids.

In this work, our aim was to fully characterize schistosome hybrids that have recently emerged in Europe. More particularly we first describe hybrids based on morphological traits (on eggs and adults) as well as parasite compatibility for snail intermediate host. On the genome level, we characterized the extent of introgression in Corsican hybrids at the whole genome scale. To do so, we first re-sequenced the *Schistosoma bovis* genome using long reads, as the previous assembly was not sufficient for robust introgression analysis. We then developed an analytical pipeline that we validated on experimentally produced hybrids (F1) between *S. haematobium* and *S. bovis*. We found that *Schistosoma* hybrids established in Corsica display *S. haematobium* biased morphology and life history traits. These characteristics are congruent with the

genome background of hybrids that was found to be composed of 77% of *S. haematobium* and 23% of *S. bovis* genome, respectively. To our knowledge this is the first study that provides a precise whole-genome scale evaluation of the introgression levels in a parasitic hybrid strain.

Results:

- **Morphological description of the eggs and adult worms**

A total of 44 eggs were examined for morphological characterization. The length and width were assessed on all eggs, but the spine length have been measured on a subset of 36 that had a spine distinctive enough to allow for proper estimation of their sizes. The results are presented in Table 1. Hybrid eggs had a mean length of 126.4 µm (±22.9 µm SD), and a mean width of 60.8 µm (±13.0 µm SD). The mean spine length was of 8.2 µm (±2.1 µm SD). The minimum-maximum and number of eggs analyzed for each variable are presented in Table 1.

Table 1: Morphological measurements results of the Corsican hybrid schistosome eggs (SD = Standard deviation).

	Length (including spine)	**Width**	**Spine length**
No. of eggs	**44**	**44**	**36**
Mean (± SD) in **µm**	126.4 (±22.9)	60.8 (±13.0)	8.2 (±2.1)
Minimum in **µm**	73.9	40.9	3.95
Maximum in **µm**	170.9	92.5	13.6

Most of the eggs displayed a representative elliptical morphotypes and were characterized by a terminal spine, reminiscent of *S. haematobium* infection in humans. Nevertheless, not all eggs had a typical *S. haematobium* morphotype and in some cases they seemed intermediate with *S. bovis*-type eggs (Fig 1).

Figure 1: Egg morphologies of the pure parental species and the Corsican *S. haematobium X S. bovis* hybrid. Eggs 1 and 2 show typical morphologies of *S. haematobium* (elliptical with a terminal spine) and *S. bovis* (spindle shape with a terminal spine), respectively. Eggs 3-8 show the egg morphology of the Corsican hybrid schistosome. While most eggs were typical to *S. haematobium* (3-7), a high variability of morphotypes was observed (8-9).

Like other species of the genus *Schistosoma*, male and female adults of the schistosome hybrids exhibited a characteristic marked sexual dimorphism and females living in the gynaecophoric canal of male worms (Additional Fig S1). The detailed morphological characteristic measurements are presented in Table 2.

Table 2: Morphological measurements of adult Corsican hybrid male and female schistosomes

	Hybrid schistosomes from Corsica			
	Male		**Female**	
	Mean (±SD)	**Range**	**Mean (±SD)**	**Range**
Total length (mm)	5.3 (±1.0)	3.3 – 6.6	9.6 (±1.2)	8.6 – 14.1
Largest body width (mm)	0.3 (±0.07)	0.2 – 0.5	0.2 (±0.04)	0.1 – 0.3
Oral sucker length (mm)	0.2 (±0.01)	0.2 – 0.2	0.05 (±0.004)	0.04 – 0.06
Oral sucker width (mm)	0.2 (±0.006)	0.1 – 0.2	0.05 (±0.002)	0.05 – 0.06
Ventral sucker length (mm)	0.3 (±0.008)	0.2 – 0.3	0.06 (±0.001)	0.06 – 0.07
Ventral sucker width (mm)	0.3 (±0.01)	0.2 – 0.3	0.06 (±0.001)	0.06 – 0.07
Testes number	4.2 (±0.5)	4 –5	-	-
Ovary length (mm)	-	-	0.3 (±0.01)	0.3 – 0.3
Ovary width (mm)	-	-	0.09 (±0.01)	0.08 – 0.1
Extension of the vitellaria (mm)	-	-	5.0 (±0.7)	4 – 5.9

The adult male parasites had an elongated body measuring 5.3 (±1.0) mm long and 0.3 (±0.07) mm wide at largest part of the body. The male body was dorso-ventrally flattened with folds in the ventral axis forming the gynaecophoral canal where the paired females are commonly found (Fig 2a). The anterior region was narrower than the rest of the body and presented a sub-terminal mouth formed into the oral sucker (length: 0.2±0.01 mm; width: 0.2±0.006 mm) and a robust ventral sucker or acetabulum (length: 0.3±0.008 mm; width: 0.3±0.01 mm) near the top of the gynaecophoral canal (Fig 2b). The oesophagus began at the oral sucker and was extended posteriorly to the acetabulum where it bifurcates into the gut caeca until it reunites at the posterior end of the body.

Figure 2: Morphological observations of male hybrid schistosomes from Corsica: (A) Whole parasite body locating oral sucker, acetabulum (ventral sucker) and gynaecophoral canal; (B) Frontal view of anterior region showing oral sucker, acetabulum (ventral sucker) in detail; (C) Dorso-lateral view of spines and tubercle over parasite surface in detail.

The testes, situated dorsally posterior to the ventral sucker, were round to ovoid and we found 4 to 5 in number in adult males (4.2±0.5). Posterior to the acetabulum, in the dorsal region, tubercles with a round extremity began to appear at the level of the gynaecophorial canal and occurred to all the posterior region of the body (Fig 2c). The presence of tegument projections on the tubercles where identified with several apical spines which decreased in distribution and size towards the back and sides of the male's bodies (Fig 2c). The female's bodies were elongated and filiform and measured 9.6 (±1.2) mm long and 0.2 (±0.04) mm wide, with the posterior half of body expanded (Fig 3a). The anterior regions were smaller compared to males, and females had a small oral sucker (length: 0.05±0.004 mm; width: 0.05±0.002 mm) and acetabulum (length: 0.06±0.001 mm; width: 0.06±0.001 mm) (Fig 3b).

The oesophagus started near the oral sucker and bifurcated immediately after the acetabulum. The genital pore was situated at the posterior of the ventral sucker (dorsally) and some eggs could be observed in the uterus (Fig.3c). A single ovary measuring 0.3 (±0.01) long and 0.09 (±0.01) wide was situated in the posterior third of the female's body (Fig 3a). The vitelline glands, also called vitellaria was extensive, occupying roughly 50% of the posterior part of the worm and extending further

posteriorly than the intestine. The females' tegument was smooth and uniform without significant projections. The posterior extremity was tapered and rounded.

Figure 3: Morphological observations of female hybrid schistosomes from Corsica: (A) Lateral view of whole parasite body showing oral sucker, acetabulum (ventral sucker), ovary and vitellaria; (B) Frontal view of anterior region showing oral sucker, acetabulum (ventral sucker) in detail; (C) Dorso-lateral view of two eggs inside female uterus in detail.

- **Hybrid parasite compatibility with the natural hosts of the parental species, *Bulinus truncatus* and *Planorbarius metidjensis***

The susceptibility of the parental snail hosts was inferred at 35 days after exposition to 5 parasite larvae (miracidia). The prevalence in *Bulinus truncatus* was 24% (9 infected snails out of 37 alive). In *Planorbarius metidjensis,* which is a specific host to *S. bovis,* the prevalence was 0% (0 infected snails out of 29 alive).

- **Whole genome sequencing introgression level analyses**

As the previous *S. bovis* assembly was highly fragmented (111 328 scaffolds, N50 7kb), we resequenced the strain from Spain using PacBio long reads. This produced 4,102,584 filtered subreads of a total of 48,987,175,429 bases. Genome assembly led to a new genome version of 486 scaffolds (N50 3.1Mb). Gene prediction led to identification of 14,104 protein-coding genes of 18,725 bp average length (Table 3). Protein-coding exons represented 5.3% of the genome length. These results are consistent with the known characteristics of *Schistosoma* genomes [39].

From 10 male and 40 female worms from the natural Corsican hybrid, a total of 93,395,155 and 96,181,625 clusters (2x100 bp paired-end reads) were produced respectively. A total of 289,873,531 reads (76.5%) were mapped against the 681.2 Mb concatenated genomes of *S. haematobium* [39] and *S. bovis* (this work), representing 42X mean coverage. Details on the number of reads and mapping results are presented in Table 4. We also sequenced F1 males from experimental first generation cross between male *S. haematobium x* female *S. bovis* as a control to validate our analytical pipeline. A total of 2,658,864 clusters (2x150 bp paired-end reads) were produced. Of these,

4,910,354 reads (92.3%) were mapped against the concatenate of *S. haematobium* and *S. bovis* genomes (Table 4).

Table 3: Assembly and gene prediction metrics of *S.bovis.*

Proportion of genome that is coding (%)	5.27
Number of genes	14,104
Average gene length (bp)	18,725 ± 21,625
Average number of exons per gene	7.9
Average exon length (bp)	299
Average intron length (bp)	2,389
Repeat rate (%)	49.5%

Table 4: Summary of the introgression level analysis of the experimental F1 hybrids (control) and the Corsican natural hybrid strain.

	Experimental F1 genome (males)	**Corsican hybrid genome**	
		Males	Females
mapped reads (% of total number)	4,910,354 (92.3%)	150,994,161 (80.8%)	138,879,370 (72.2%)
reads mapped against *S. haematobium* genome	2,417,196 (49.2%)	116,052,836 (76.9%)	106,969,327 (77%)
reads mapped against *S. bovis* genome	2,493,158 (50.8%)	34,941,325 (23.1%)	31,910,043 (23%)
reads mapped against *S. haematobium* mitochondrion	168 (1.7%)	50,436 (2.2%)	33,454 (2.2%)
reads mapped against *S. bovis* mitochondrion	9,825 (98.3%)	2,229,096 (97.8%)	1,455,815 (97.8%)

As expected, the mapping of the F1 reads to *S. haematobium* and *S. bovis* genomes gave a proportion that perfectly matched the expected genomic proportions of a first generation hybrid (~50% of each of the parental species genome; Table 4). This control was crucial to validate our analytical pipeline and thus the results obtained for the Corsican natural hybrid parasite. Moreover, aligning reads to the mitochondrial genomes of both species revealed a strong similarity to *S. bovis* (98.3% of mapped reads), which is fully consistent with the direction of the F1 cross (female *S. bovis* X male *S. haematobium*) and maternal inheritance of the mitochondrial genome (Table 4).

Interestingly, the mapping of the Corsican hybrid reads against *S. haematobium* and *S. bovis* reference genomes revealed a complex admixture between the parental genomes with a proportion of 76.9% of sequences mapping on *S. haematobium*, and 23.1% mapping on *S. bovis* genomes for both male and female parasites (Table 4). Aligning reads to the COI genes of both parents also showed results concordant with the previous Sanger sequencing data for this marker, with 97.8% of reads being mapped on *S. bovis* mitochondrial genome, and 2.2% on *S. haematobium* mitochondrial genome [32]. To figure out the divergence level between the two pure species genomes, we identified the orthologous regions between *S. haematobium* and *S. bovis* using CACTUS. A total of 234,5 Mb sequences aligned between the parental species, which represents 64% of the S. haematobium genome length. The mean similarity of these shared sequences was 95.88 %.

Discussion

The importance and the frequency of hybridization in parasitic species are certainly underestimated, and very little attention has been given so far to the role of introgression in pathogens and the evolution of parasites [25]. The fact that parasites are often hidden inside a host and have morphological characters that are shared with closely related species may explain the lack of evidences on free-living animal species compared to animal parasites. Hybridization has been evidenced in digenean helminths (*Schistosoma*, *Fasciola*, *Paragonimus*, and *Diplostomum*), two cestodes (*Taenia* and *Echinococcus*), one monogenean (*Gyrodactylus*), and five nematodes (*Ascaris*, *Contracaecum*, *Parascaris*, *Pseudoterranova*, and *Paramacropostrongylus,* see [25] for review). In the majority of these studies a genetic diagnosis approach was used to detect hybrids using either a parental specific marker, nuclear-mitochondrial discordance, or

multi-locus microsatellite markers associated with model-based Bayesian clustering methods.

To our knowledge, our work provides for the first time, a full genome level estimation of the introgression in a helminth parasite. Concerning the genus *Schistosoma,* latest reports have revealed that hybrids are frequent throughout Africa, and are a real concern in terms of parasite transmission, disease control and human and animal health [26–28,40]. Interestingly, it is the first time that a hybrid schistosome is involved in a large-scale outbreak in Europe [32,33,41]. The hybrid status of the parasite might have increased its invasive capacity. In this work we investigated both the genomic composition as well as several life history traits of the hybrid schistosome that emerged in Europe in 2013. We believe that these results are important not only to understand the evolutionary outcomes but also how it may impact epidemiology through pathogen infectivity, virulence, transmission, or host specificity.

In humans, schistosome eggs that are partly retained in the tissues are the cause for the disease and induced pathology, but are also classical tools for diagnosis and species identification. At first sight, egg morphology and localisation in the urine of infected patients in Corsica strongly suggested an *S. haematobium* infection [32]. Indeed *S. haematobium* eggs that are usually voided by the urine have a typical round to oval shape (elliptical or elongated) with a terminal spine. According to previous studies, *S. haematobium* eggs measure between 100-156 µm long and 40-50 µm wide with usual length between 115-135 µm long [42–47]. A previous analysis of this Corsican hybrid schistosome eggs revealed smaller eggs (n=15) with a mean length of 106.5 µm and width of 42.8 µm, and with a spine length of 10.4 µm [33]. According to our results the eggs generally show an ovoid shape measuring 126.4 x 60.8 µm (Table 1) more similar to *S. haematobium* eggs. This is also consistent with the introgression levels that show a predominance of S. *haematobium*-type sequences (Table 4). Sometimes eggs were intermediate with spindle or diamond shapes, which are characteristic of *S. bovis* eggs (usually bigger and measuring between 170-223.9 µm long and 55-66.0 µm wide and have an elongated spindle or diamond shapes [43,47,48] (Fig 1). Consequently, our results confirm earlier publications showing that it is impossible detecting hybridization in African schistosome species using egg morphology alone [34].

We performed for the first time morphological analysis of the adult male and female European hybrid. Surprisingly, very few morphological data except for the length

and width is available for the parental species and in particular for *S. haematobium,* thus limiting our potential for comparing with the pure. Moreover, there is no consensus for adult morphology suitable for diagnostic, as the description of the worms may vary due to different sources of biological and technical variation (time of infection, parasite strain and genetics, and host species, but also methods to collect worms and fixation procedure for measurements) [49,50]. We show that the Corsican male and female hybrids are generally smaller (in length and width) when compared to both parental species [50–54], but this may also reflect the difference in the host from which they have been recovered. The most interesting element in this hybrid morphology was the presence of spines on the tubercles of the males. Indeed, this trait can be useful for taxonomic purpose as it is specific to *S. haematobium* [55–57].

Because the schistosome that emerged in Corsica is a hybrid between *S. haematobium* and *S. bovis*, i.e. two species with different host specificities, it was important to evaluate the compatibility of this hybrid parasite towards the intermediate hosts of both parental species. Indeed, human-infective *S. haematobium*, is specific to *Bulinus* snails as intermediate hosts, while livestock-infective *S. bovis*, has a broader intermediate host range and is able to use both *Bulinus* and *Planorbarius* snails as vector. Interestingly the Corsican hybrid schistosome population that we had recovered was not able to infect our laboratory strain of *Planorbarius metidjensis*, but displayed high levels of compatibility with *Bulinus truncatus* from Corsica (24% of prevalence). This result is consistent with the previous compatibility assessments for the hybrid parasite recovered directly from an infected patient in 2014 [32]. This also goes in line with the predominance of *S. haematobium* type genome in this hybrid. The remaining question is to know if such introgressed schistosomes are still capable of infecting livestock or other reservoir hosts in the field. A recent study suggests that rodents may be the most probable hosts in which hybridization occurs [58]. Indeed, wild rodents in Senegal harboured pure species (*S. bovis, S. haematobium*, and *S. mansoni*) as well as hybrids between *S. bovis* and *S. haematobium* [58].

The genome-wide sequencing approach used in this work revealed a complex admixture of the parental genomes, with many hybridization and backcrossing events between pure and hybrid parasites. Our results exclude a simple mitochondrial capture event or a first generation hybrid (for which 50% of sequences from each parental species would be expected). We show here that the genome of the Corsican hybrid presents a

predominance of *S. haematobium*-type genome (76.9% of sequences of *S. haematobium* origin) compared to *S. bovis* (23.1% of sequences of *S. bovis* origin). This also suggests that hybridization between these two species is an "old story", and that the hybrids detected today are the result of ancient crossing events. Nevertheless, it reveals the high permeability of schistosomes to other species' alleles, with the initial production of a viable offspring that was able to subsequently cross together or backcross with one, or both parental species. Two non-exclusive hypotheses may explain the direction of the cross and the predominance of *S. haematobium's* alleles in this hybrid. Firstly, it has been shown that *S. haematobium* (particularly males) are better sexual competitors than males of other species. In mating interaction experiments, it is evident that *S. haematobium* is better in pairing than *S. intercalatum* [59], S. *mattheii* [60] or *S. mansoni* [61]. Even if the competitiveness with *S. bovis* has never been tested, this could explain the direction of the cross, with a *S. bovis* mitochondrial type genome inherited from the mother. Second, we can hypothesize that this could result from host selective pressure as in humans; hybrid populations are more likely to be in contact and backcross with *S. haematobium* pure strains while *S. bovis* is not able to infect humans.

These two species belong to the *haematobium* clade and have been defined based on their host specificity (*S. haematobium* specific to humans as definitive host, and *Bulinus* as intermediate host, while *S. bovis* specific to cattle), but also their living site inside the definitive hosts (in the veins surrounding the bladder for *S. haematobium* or in the mesenteric veins for *S. bovis*). It was traditionally thought that this tropism would prevent heterospecific pairings to occur. One of the remaining questions highlighted with the detection of hybrids and the introgression levels found in this work is to know what the extent and frequency of hybridization events in the field is. Briefly, if hybridization is a rare event and occurred only a few times a long time ago because of host specificity "breakdown", then the genetics and introgression levels of the hybrid populations in the field should be homogeneous. Conversely, if crossing events are underestimated in the field and prove to be frequent, then distinct hybrids populations should exist with various levels of genomic introgression and different genetic signatures. Thus, it would be necessary to investigate the introgression levels of other hybrids across Africa. This will definitely help to highlight how much these two species of schistosomes are still permeable to gene flow.

Without considering many of the important pre-zygotic barriers that condition the hybridization events, the time of divergence and the genetic divergence between the species is strongly correlated with the viability and possible number of generations that may arise from the crosses. Indeed, incompatibilities between populations are strongly correlated with genetic distance and time of divergence [62], but also the selection operated on the populations that might undergo purifying or diversifying selection. Nevertheless, some studies reported hybridization events between very divergent species, and others suggested that this may be promoted by global changes [63–66]. The maximum divergence between mammalian species to produce viable offspring after independent evolution has been estimated about 2 million years, while successful hybridization of separated species may still occur after dozens of million years in fish, birds, and turtles [65,66]. The mutation rates, and therefore the genetic divergence between various organisms may differ; therefore it is difficult to predict the hybridization success in other organisms that may not be under the same selective pressures. For example, parasitic species may use different hosts as vector and/or for their reproduction; this may affect the mating systems of these parasites species, and may allow them to hybridize at higher rates than free-living organisms because the reproductive isolation between these distinct parasite populations is expected to be higher. For example, this has been evidenced in two very divergent *Leishmania* species [63].

In schistosomes this may also be the case, with schistosomes species of distinct hosts capable of hybridizing, while others from very divergent geographical areas produce poorly viable offsprings (see, [28] Table 2 for review). The genus *Schistosoma* comprises 23 known species categorized into four major groups based on their distribution, host specificity and egg morphology [67,68]. The *S. japonicum* group and the *S. indicum* groups are found in Asia, whereas both *S. mansoni* and *S. haematobium* groups are found in Africa. The African and the Asian schistosome groups are therefore the most divergent and never overlap in endemic areas, but some examples of interaction and experimental hybridization have been shown to occur between relatively distant schistosomes such as *S. mansoni* and *S. japonicum* [69] and for *S. mansoni* and *S. haematobium* [61,70], but seem poorly successful. The most viable and successful hybrids occur between less divergent species, whatever their differences in host specificity, and are predominantly, but not exclusively found within species of the

same clades and especially the *S. haematobium* clade [28] frequently involved in hybridization.

Because no fossil records are available for schistosomes, the origin of the genus and the divergence time estimation between species is not an easy task and remains controversial. Two theories on the origin of these parasites have been discussed with arguments of an African or Asian origin, thus affecting divergence time estimations [67,71–75]. According to the African origin suggested by George Davis [71,72], the African ancestor arrived to Asia during continental drift of Gondwanaland between 70-148 MYA and diverged to form the *S. indicum* and *S. japonicum* groups. This also suggests that the *Schistosoma* ancestors in Africa diverged over 120 MYA, giving rise to the *S. mansoni* and *S. haematobium* groups. According to the theory of an Asian origin of schistosomes [68,76], divergence is more recent with the Asian ancestor schistosome that arrived in Africa around 12-19 MYA via widespread mammal migration. The remaining schistosomes in Asia formed the *S. japonicum* group, and the African schistosome diverged 1-4 MYA, giving rise to the *S. mansoni* and *S. haematobium* groups (see [73,77] for review). The radiation of schistosomes in the *S. haematobium* and *S. mansoni* groups is another still open question but seems closely associated to human migration and the interaction with other suitable definitive hosts [75]. Whatever theory is true, the divergence time is expected to be less than 4 million years, which is in the range of hybridization success in other species [66]. Furthermore, hybridization within and amongst these clades indicates that speciation may be relatively recent [78].

The karyotype of species is also important regarding the success of hybridization. One of the first causes of hybrid incompatibilities and sterility is attributed to the differences in number of chromosomes, and the incorrect pairing of alleles from two or more genes, especially if they are sex-linked alleles. Introgression of sex chromosomes is generally more limited than that of autosomal regions [79] and this may be detrimental to the heterogametic sex as suggest by Haldane's Rule [80], because it only has a single copy of the deleterious allele. Asian and African schistosomes (including the terminal spined *S. haematobium* group) have the same karyotype with n=8 chromosome pairs including sex chromosomes [81]. This would allow chromosome pairing and recombination during the meiosis even with relatively modest levels of similarity between the two species genomes (mean 95.88 % identity over 64% of the genome length for *S. haematobium* and *S. bovis* in our analysis).

Conclusions

This work provides new insight into the study of schistosome hybridization and reveals that their genomes are highly permeable to other species' alleles. Our results suggest that the hybrids between *S. haematobium* and *S. bovis* that emerged in Europe are the result of several backcrosses and not a single crossing event. Similar conclusion has been proposed using microsatellites on *S. rodhaini x S. mansoni* hybrids in Kenya [82]. It is now essential to precisely characterize the impact of hybridization and introgression on the life history traits of the parasites and assess the molecular mechanisms underlying these phenotypic changes. Schistosomes with contrasted phenotypes, as it is the case for *S. haematobium* and *S. bovis,* may combine their alleles eventually allowing producing "fittest" or more "adapted" parasites compared to the parental species. These hybrids may have the capacities to take over the geographical distribution of the parental species, but also rise out of endemic areas, and specifically in the close future with ongoing climate change, up north and towards Europe, making of these hybrid parasites an emerging global threat.

Methods

Parasite / snail strains and experimental infections

The Corsican hybrid parasite (*S. haematobium x S. bovis*) was recovered from eggs found in the urine of a single infected patient and maintained in the laboratory in sympatric *Bulinus truncatus* snails as intermediate hosts and *Mesocricetus auratus* hamsters as definitive hosts [32]. All eggs genotyped using the classic two molecular markers (COI and ITS2) from this patient were diagnosed as hybrids with the ITS2 sequence typical of *S. haematobium* species and the COI sequence typical of *S. bovis* species. Adult worms produced in hamsters were recovered after portal perfusion and male/female couples were manually separated for subsequent analysis. Detailed methods employed for mollusc and rodent infections as well as portal perfusion were described previously [83–85]. *Schistosoma bovis* (Spain) and *Schistosoma haematobium* (Cameroon) were also maintained in the lab using *Planorbarius metidjensis* and *Bulinus truncatus* as intermediate hosts, respectively, and *Mesocricetus auratus* hamsters as definitive hosts [32,86]. Experimental crosses were conducted in the laboratory to produce F1 adult hybrids in hamsters between male *S. haematobium* and female *S. bovis*. For that, we

infected intermediate host molluscs with a single miracidium of the parental species in order to obtain male or female clonal populations of *cercariae*. The later were sexed by using molecular markers efficient on both parental species that we had developed previously [86]. We simultaneously exposed hamsters to 300 *cercariae* of male *S. haematobium* and 300 *cercariae* of male *S. bovis.* Three months after infection, hamsters were sacrificed, F1 hybrid eggs collected and used to infect molluscs. The infected molluscs were then used to infest hamsters (pools of 600 cercariae) and adult male worms were collected 3 months after exposition. These adult worms were used as a control for the genomic introgression level estimation (expectation of 50% of the genome from each parent).

Hybrid parasite compatibility with snail hosts

Bulinus truncatus as well as *Planorbarius metidjensis* snails that are the natural hosts of *S. haematobium* and *S. bovis* respectively are maintained in the laboratory and were used to infer their susceptibility to the hybrid strain from Corsica. *P. metidjensis* (n=40) and *B. truncatus* (n=40) snails were individually exposed overnight to 5 miracidiae of the parasite in well plate's. Molluscs were individually checked at 35 days after infection for parasite emission of the *cercariae* after light stimulation.

Morphological analysis of the Corsican schistosome hybrid eggs and adult worms

Hamsters infected with the Corsican hybrid schistosomes were euthanized. Twenty adult couples of worms (20 males and 20 females) were collected after hepatic perfusion as well as we recovered encysted eggs from hamster livers. Adult worms and eggs were washed in 8.5% w/v Tris-NaCl solution for subsequent morphological analysis. Male and female worms were manually separated and stored in 70% ethanol. After whole-mounted on glass slides [87] adult worms and eggs were viewed under light microscopy and photographed on a Wild Heerbrugg M400 ZOOM Makroskop (Leica, Germany) or Dialux20 (Leitz, Germany) coupled to Nikon digital sight DS – Fi1 digital camera and all measurements were produced with ImageJ version 1.51 [88,89] and drawings were done by image overlay in Adobe Photoshop CS2 version 9.0.1.

For eggs, objective lens magnification was set to x10 and we measured the length (including the spine), the width (at its larger point), and the size of the terminal spine of the eggs. Concerning adult worms, the following characters were measured in worms of

both sexes: worm length and width, area of the oral and ventral suckers, sucker's ratio, sucker ratio per worm length, distance from genital opening to anterior region. In female worms the area of the ovary area and extension of the vitellarium were measured. In males we also recorded the number of testes and the presence or not of tubercles, and characteristic spines on the tubercles.

DNA extraction and sequencing for *Schistosoma bovis* assembly

200 clonal adult male worms were used to prepare high molecular weight genomic DNA using CHEF Genomic DNA Plug Kits (BioRad). The DNA was quantified on a FEMTO Pulse®, qubit and nanodrop. A total of 8.1 µg genomic DNA was used to generate a size selected PacBio library. First the DNA was sheared to an average fragment size of 45 kb by gently passing the DNA sample through a 2" long, 26 gauge needle, 4 times and then concentrated using Ampure PB (Pacific Biosciences 100-265-900) before the library was prepared following the standard PacBio size selected library preparation protocol (Procedure & Checklist - 20 kb Template Preparation Using BluePippin™ Size-Selection System). The library was size selected at 15kb, and run on 6 SMRT cells on the Sequel platform, generating 47.9 Gb of data.

RNA extraction and sequencing for *Schistosoma bovis* annotation

Pools of 10-12 adult male and female worms were frozen with liquid nitrogen and grounded using Retsch MM400 cryobrush (2 pulses at 300Hz for 15s). Total RNA was extracted using TRIzoltm Thermo (Fisher Scientific) using manufacturer's protocol followed by DNase treatment with Turbo DNA-free kit. RNA was then purified using the RNeasy mini kit (Qiagen). The TruSeq stranded mRNA library construction kit (Illumina Inc., USA) was used according to the manufacturer's recommendations on 300 ng of total RNA per condition. Library preparation and sequencing was performed at the McGill University in the Génome Québec Innovation Centre, Montréal, Canada on a Illumina HiSeq 4000 (100 bp paired-end reads).

***Schistosoma bovis* genome assembly and annotation**

A total of 48 Gb PacBio reads were assembled using HGAP4 to generate a 446 Mb assembly in 486 contigs, with an N50 of 3.1 Mb in 38 contigs.

Gene prediction was carried out with AUGUSTUS 3.3.1. No new training was performed for our data but the parameter set "schistosoma2" of the AUGUSTUS distribution was used. RepeatMasker 4.0.7 and RepeatScout 1.0.5 were used for repeat masking. RNAseq data from male and female adult worms was employed as external hints. This RNA-seq data was aligned with STAR version 020201. The results of the gene prediction are available as track data hub of the UCSC genome browser under the following URL: http://ihpe.univ-perp.fr/IHPE_tracks/S.bovis/hub.txt.

DNA extraction and sequencing for the Corsican hybrid strain and experimental *S. haematobium x S. bovis* F1

Genomic DNA of the Corsican schistosome hybrids was recovered from one pool of 10 adult males and one pool of 40 adult females separately, while DNA of the experimental F1 progeny was recovered from a pool of 10 adult males. DNA was extracted using Qiamp DNA microkit tissue kit from Qiagen following manufacturer's protocol. After the lysis step, we used 4 µl (100 mg/ml) of RNase A (Qiagen) for 2 min at room temperature to digest RNA. The final elution step was performed twice with 35 µl of Tris HCl 5 mM pH 8.5. DNA quantification was performed using a Qubit fluorometer with the dsDNA HS Assay Kit (Invitrogen), and concentrations were estimated at 12.7 ng/µl for males and 12.8 ng/µl for females (Corsican parasites) and 4.24 ng/µl for the experimental F1 males. Genomic DNA of the Corsican hybrid worms was then sent to Genome Quebec for library construction using the Illumina TruSeq kit starting from 200 ng of genomic DNA (for each sex), and sequencing was performed on a Illumina HiSeq 2000 (100 bp paired-end reads). For the experimental F1 hybrid males, library construction was performed using the Nextera XT kit starting from 1 ng and sequenced on a Illumina NextSeq 550 (150 bp paired-end reads) on the Bio-Environment NGS platform at University of Perpignan.

Estimation of the genomic introgression levels for the hybrid strain

The sequencing reads were checked for quality and had PHRED score was > 30 over 90% of read length with no adapter contamination. All reads were retained for further analysis and aligned to a chimeric concatenate of *S. haematobium* and *S. bovis* genomes using the short alignment tool Bowtie 2 [90]. We used the SchistoDB *S. haematobium*

genome [39] and the *S. bovis* SBOS_v1.1 assembly genome produced for this study and available at ftp://ftp.sanger.ac.uk/pub/project/pathogens/Schistosoma/bovis/SBOS_v1.1_pilon.fasta. To avoid mapping bias due to differences in assembly size between the two genomes, only scaffolds > 1Mb were retained for further analysis. The genome size after concatenation of *S. haematobium* and *S. bovis* was 681.2 Mb. Mapping was thus performed by allowing each read, depending on its origin, to map against the more similar location in one or the other' species genome. Simple descriptive statistics were then performed on the BAM mapping files using SAMtools [91]. After aligning high quality sequencing reads to the concatenate of both *S. haematobium* and *S. bovis* genomes we counted the proportion of best location of aligned reads (*S. bovis* or *S. haematobium* genomes) in the SAM files.

We were also willing to explore the introgression levels of the mitochondrial genome of these hybrid schistosomes. The scaffold 000439F contained the mitochondrial genome of *S. bovis*, and we also downloaded the *S. haematobium* mitochondrion genome from GenBank accession NC_008074 [92]. The two genomes were concatenated and used as reference to map the Corsican genome reads, again by restricting to best mapping location reads, and counting the proportion of reads aligned to each of the two organelle genomes.

Similarity analysis between *S. haematobium* and *S. bovis* genomes

The two species genomes were aligned with CACTUS [93] using the pre-release version msca_1508_Progressive_Cactus (https://github.com/glennhickey/progressiveCactus/releases). We then processed the CACTUS output, identifying all alignments blocks composed of exactly one genome part of S. bovis and exactly one genome part of *S. haematobium*.

Declarations

Ethics approval

Housing, feeding and animal care, including experiments on animals were carried out according to the national ethical standards established in the writ of 1 February 2013 (NOR: AGRG1238753A) setting the conditions for approval, planning and operation of establishments, breeders and suppliers of animals used for scientific purposes and

controls. The Direction Départementale de la Cohésion Sociale et de la Protection des Populations (DDSCPP) provided the permit N°C66 – 136 -01 to our laboratory for experiments on animals. The investigator possesses the official certificate for animal experimentation (Decree n° 87–848 du 19 octobre 1987; number of the authorization 007083).

Consent for publication

Not applicable

Availability of data and material

The Illumina datasets for the Corsican hybdrid strain are available in the SRA repository under submission number SUB4330327 (to be released upon publication). The *S. bovis* PacBio data are available under accession number ERS2549235. The *Schistosoma bovis* assembly SBOS_v1.1 is available at ftp://ftp.sanger.ac.uk/pub/project/pathogens/Schistosoma/bovis/SBOS_v1.1_pilon.fasta. Annotations are available as trackhub at http://ihpe.univ-perp.fr/IHPE_tracks/S.bovis/hub.txt.

Competing interests

The authors have no competing interests to declare.

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Authors' contributions

ET and JB conceived and designed the study. JB, AO and SMC procured the parasite and snail strains. JKS, AR, and JB performed the experiments. RA conducted the morphological analysis. NH and JFA prepared the DNA for sequencing. NH coordinated the long read DNA sequencing experiments. JKS, AT, IB, MB and ET conducted the computational data processing and analysis with significant assistance and input from

OR, CC and CG. JKS and ET wrote the paper. All authors read and approved the final manuscript.

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Additional files

Additional Figure S1: Morphology of natural hybrid from Corsican strain. Sexual dimorphism of the hybrid schistosomes with adult females living in the gynaecophoric canal of adult male parasites.

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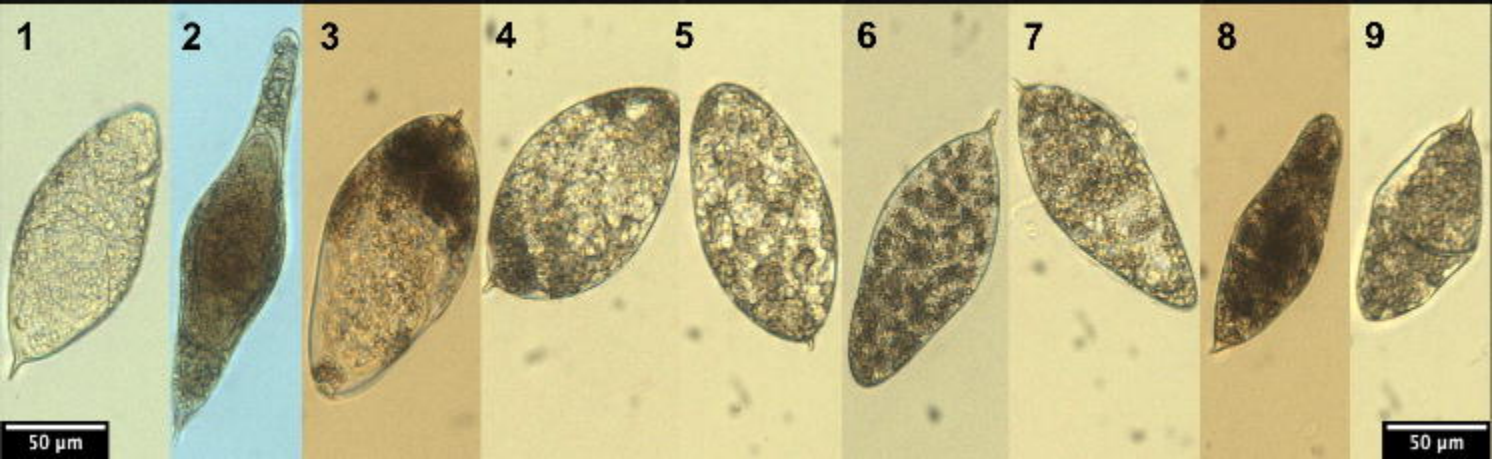
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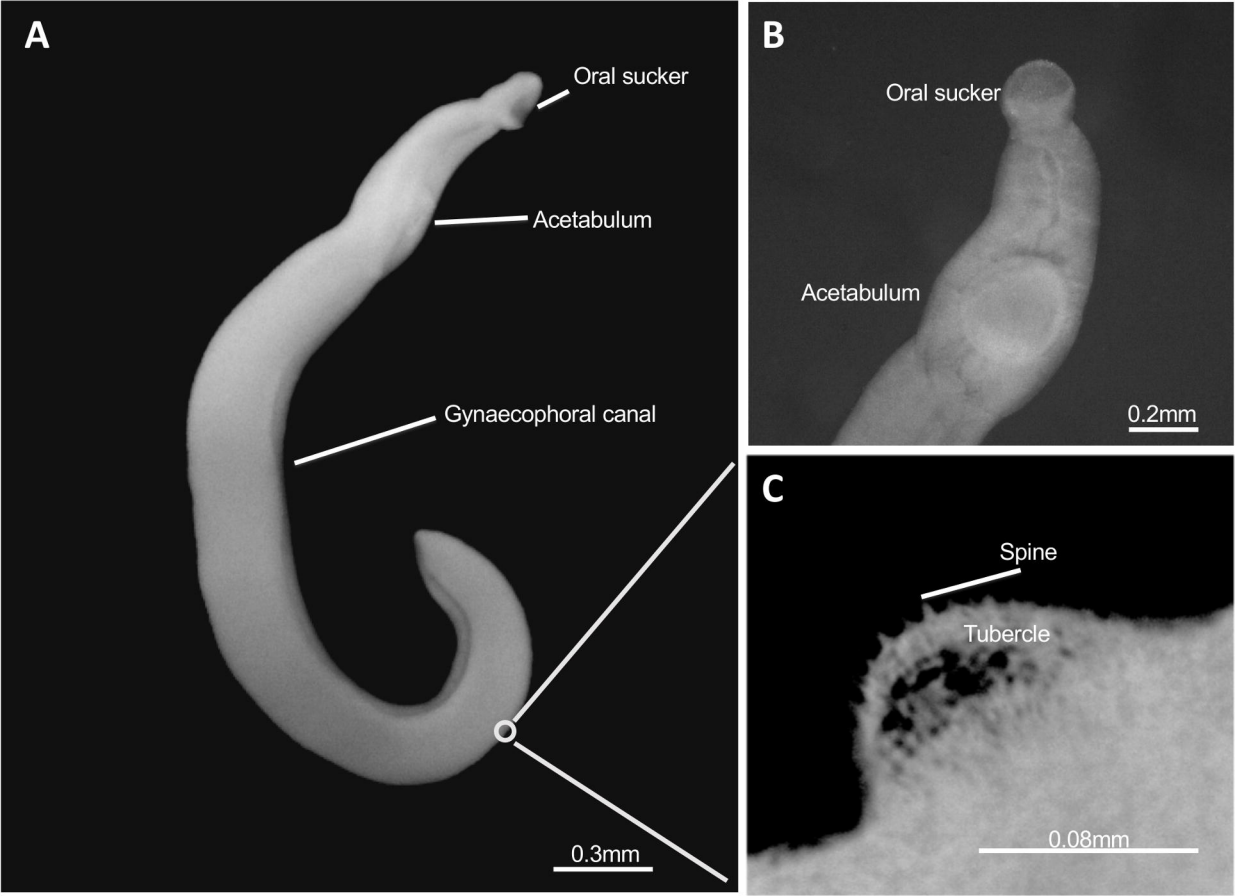
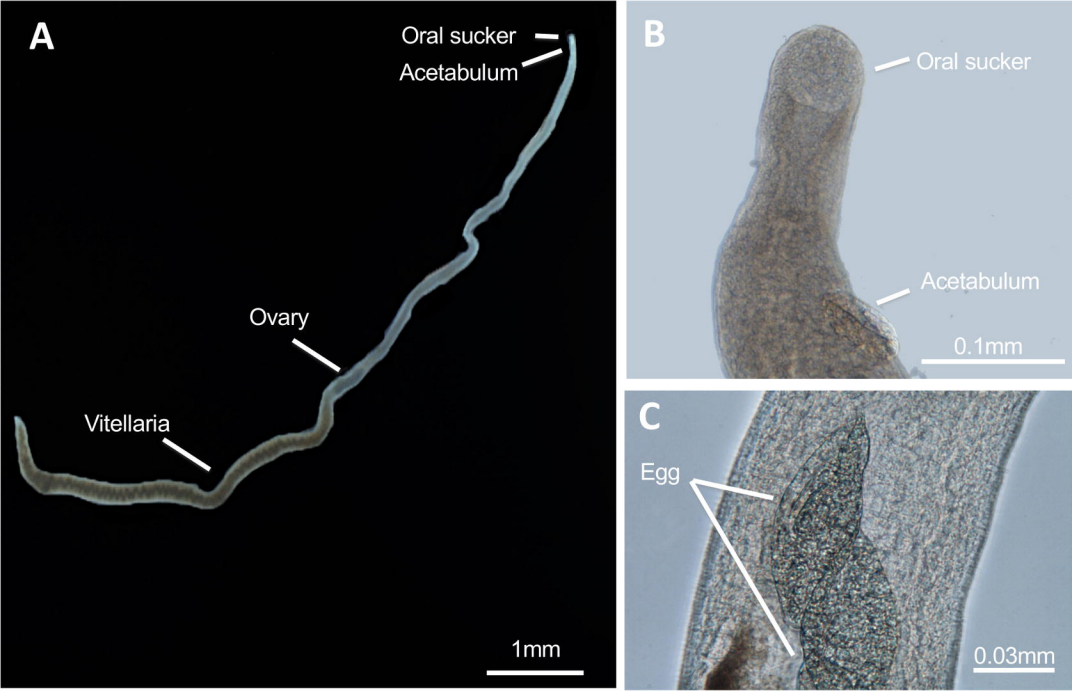

A
Oral sucker
Acetabulum
Gynaecophoral canal
0.3mm
B
Oral sucker
Acetabulum
0.2mm
C
Spine
Tubercle
0.08mm


A
Oral sucker
Acetabulum
Ovary
Vitellaria
1mm
B
Oral sucker
Acetabulum
0.1mm
C
Egg
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