

Research Article

Reproduction without cross-fertilisation in the invasive Asian leech *Barbronia weberi* (Blanchard, 1897) (Hirudinea: Arhynchobdellida)

Roy T. Sawyer

Medical Leech Museum, 2 Bryngwili Road, Hendy, Pontarddulais, Swansea SA4 0XT, UK

E-mail: leechmuseum@compuserve.com

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Abstract

Evidence is presented in this paper for the first time that reproduction without cross-fertilisation can occur in the arhynchobdellid leech *Barbronia weberi* (Blanchard, 1897). The discovery of “virgin birth” in this invasive Asian species was serendipitous in that a single unmated hatchling reared in isolation to maturity unexpectedly laid viable cocoons. In order to confirm unequivocally that this reproduction was indeed non-sexual the experiment was repeated rigorously for five successive generations. The formal possibility that cross-fertilisation may have occurred in the cocoon prior to hatching was specifically investigated by isolating embryos at the earliest stages of viability, well before reproductive structures developed. The cocoons and eggs of each successive generation were carefully quantified. No evidence of diminution of fertility was found in spite of severe inbreeding by the fifth generation. In fact fecundity of the inbred laboratory leeches was comparable to that of the original wild population. A total of 1,147 cocoons containing 4,455 eggs were laid during this year-long study. This was undoubtedly an underestimate of reproductive potential in that a number of healthy individuals had to be culled for pragmatic reasons. That a single individual is capable of producing thousands of progeny in one year, without mating, is sufficient to explain the success of *B. weberi* as an invasive species. In recent years this alien leech has become established on every inhabited continent. The mechanism of reproduction without cross-fertilisation in *B. weberi* is unknown but is probably attributable to self-fertilisation linked to its hermaphroditism. Taxonomic and phylogenetic implications of self-fertilisation in *B. weberi* are discussed.

Key words: alien species, non-native species, virgin birth, self-fertilisation, parthenogenesis, uniparental reproduction, model species in developmental biology

Introduction

In recent years the alien leech *Barbronia weberi* (Blanchard, 1897) has become established on every continent, except Antarctica (Govedich et al. 2003; Genoni and Fazzone 2008; Pamplin and Rocha 2000; Pavluk et al. 2011; Garduño-Mantes et al. 2016; Ludányi et al. 2019). In this paper a mechanism is presented to explain, at least in part, the remarkable invasive capability of this species. Specifically, in an ongoing study of a population of *B. weberi* in North Carolina (Sawyer and Sawyer 2018), it was discovered that it is able to reproduce without cross-fertilisation for over five successive generations. In fact, reproduction of this species reared in isolation in the

laboratory was extremely prolific on a diet only of tubifex. Non-sexual reproduction in *B. weberi* is probably attributable to self-fertilisation somehow linked to its hermaphroditism. The biological and taxonomic implications of a self-fertile leech are addressed.

Materials and methods

This study was carried out from May 2018 through April 2019. Some of the methods employed in this paper were similar to those presented in Sawyer and Sawyer (2018). The cocoons and embryos were examined and manipulated under a Wild M7A stereomicroscope. Images were taken with a digital camera (Conrad Electrics, Model DP-M14) under a Volpi Intralux 4000 light source, and/or a Lapsun Spot Point LED lamp. When appropriate cocoons, embryos and mature specimens were relaxed by slowly adding 70% ethanol, and subsequently preserved in 5% formalin.

Succinctly, all embryos and leeches of all generations were maintained in isolation in individual containers at ambient light and temperature (predominantly 18–22 °C). Cocoon deposition was essentially uniform throughout the year of the study, being only slightly less in winter in spite of its relatively cooler temperatures and shorter light cycle.

In order to facilitate replication of this research the methodology is given in detail with potential difficulties specifically highlighted. The plastic containers were manufactured by Jokey (JETB 365, Germany) and met food safety standards (ISO 9001:2008, ISO 22000:2005). These were transparent and cylindrical, approximately 8 cm in diameter and height. Each was fitted with a tight-fitting lid with about 15 equally spaced air holes (1.5 mm). Each container was filled two-thirds with HMA filtered water, about half of which was changed approximately every week as required.

At all stages the leeches were fed exclusively on tubifex worms obtained from a commercial supplier. Importantly, experimental early embryos required special care by feeding them very small hand-picked tubifex commensurate with size of their mouthparts. Larger juveniles and adults were voracious eaters, and consumed 3–5 medium-sized tubifex virtually every day. As a rule, these leeches were rather messy in their eating and defecation habits, requiring more or less daily cleaning with an eye dropper. In order to minimize contamination with potentially deleterious microorganisms during the feeding process best practice necessitated the use of fine forceps, rather than an eye dropper, to transfer individual tubifex into a clean intermediate container prior to final transference to the leech container. Each container holding an isolate was inspected daily for feeding and for monitoring cocoon deposition.

During the course of this study it was discovered empirically that adding a simple stone to each container could trigger cocoon deposition, and conversely the absence of a stone could significantly retard or even prevent cocoon deposition. This “stone effect” was serendipitous in that such a

stone was added initially because a “barren” individual was having difficulty removing its sloughed cuticle. Within a day or so this particular individual abruptly commenced depositing a total of 40 cocoons approximately every two days. This observation prompted a side study focused on the effect of the presence or absence of stones. It demonstrated without question that a stone (roughly $3 \times 2 \times 1$ cm), regardless of geological composition, significantly optimised cocoon deposition. The mechanism of this “stone effect” remains speculative, but future researchers are encouraged to add a small stone even from the hatchling stage.

To minimize the formal possibility of cross-fertilisation within a cocoon prior to hatching, embryos were systematically isolated well before they would normally hatch (approximately two-three weeks of age). Toward this end it was prerequisite to determine the earliest survivability outside the cocoon (Figure 1). Selected cocoons of known stages of development were opened as described below and each extracted embryo was immediately photographed, uniquely labelled and isolated. This was repeated for each successive generation. The complete genealogy of a representative fifth generation individual is documented in Figure 2.

Embryos were isolated prior to hatching using the following technique performed under water in a Petri dish. Using a binocular microscope a cocoon of known age was held securely with fine forceps with one hand while the other used light pressure from unopened fine scissors to gently encourage all the embryos away from one end of the cocoon. This yielded a safe area sufficient for a single transverse cut at right angle about one-fifth from the end. Once the cocoon is severed completely, the embryos and accompanying albumen could be teased out by patiently applying gentle pressure at the uncut end. Each extracted embryo was transferred singly to its designated container by means of a cotton bud (never with forceps). Henceforth, it was reared in total isolation with absolutely no opportunity for mating throughout its life.

The taxonomy used throughout this paper follows that of Sawyer (1986).

Results

The data generated in this study are presented below as straightforward arithmetic means and percentages. Overall, 25% of the isolated embryos failed to thrive ($N = 84$ embryos). This was due primarily to abnormal development and/or unsuccessful feeding. Moreover, approximately one-third of the overall cocoons laid ($N = 1147$) were misshapen or distorted, and typically devoid of eggs. The high frequency of abnormal cocoons in the laboratory was approximately the same for each generation: F1 (30.8% abnormal, $N = 13$ cocoons), F2 (39.0%, $N = 675$), F3 (32.1%, $N = 356$) and F4 (23.2%, $N = 103$). These abnormal cocoons were not included in the following results.

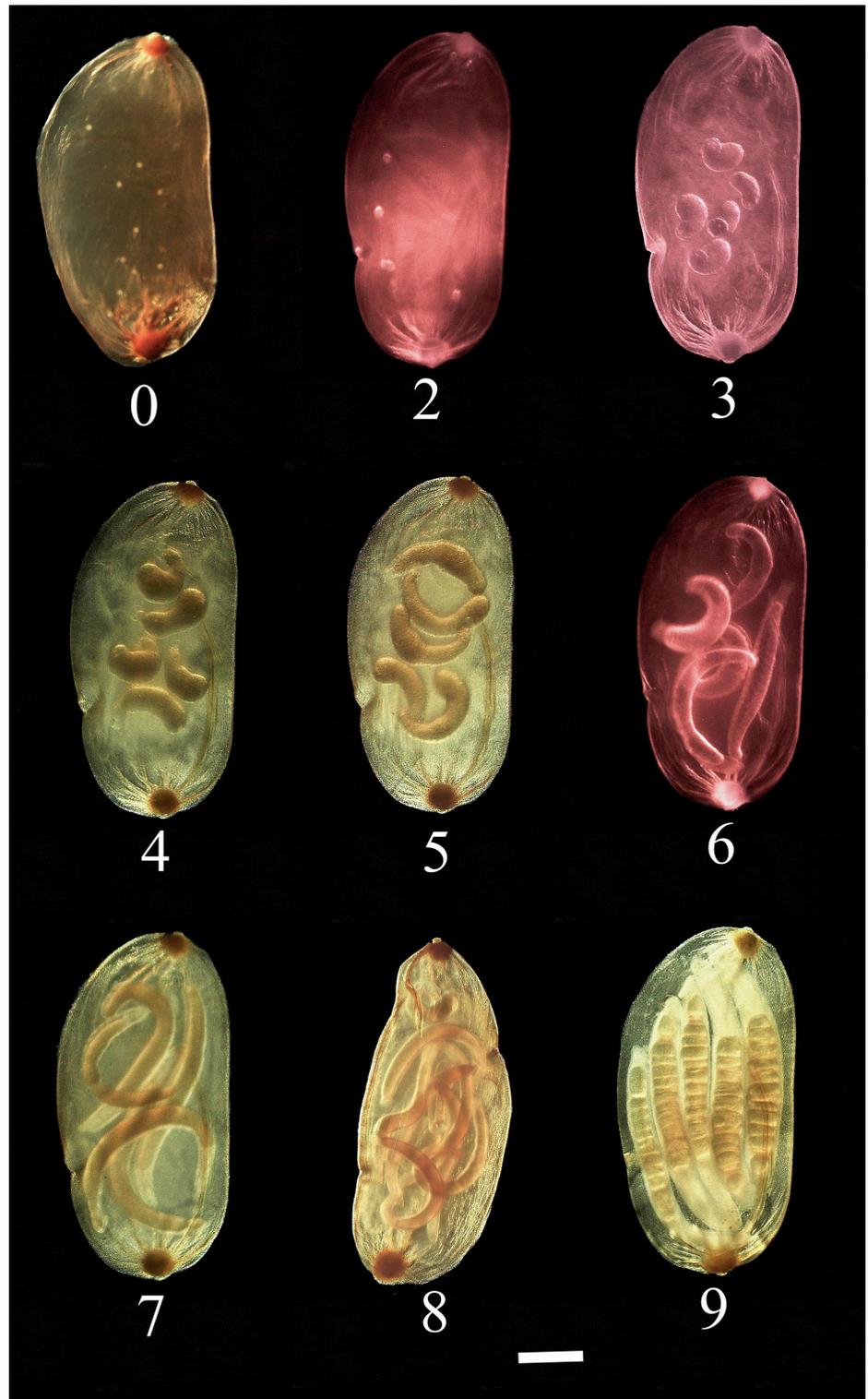


Figure 1. Developmental stages of *Barbronia weberi*, based on days at 20 °C. In this study the earliest stage to survive outside the cocoon was Day 4, and then for only 2–3 days. For experimental purposes Day 7 was the earliest stage embryos could be reared reliably (60%) to maturity, resulting in deposition of viable cocoons. Useful developmental landmarks for this and future studies are: Day 4, ganglia with connectives; Day 6, caudal sucker terminal; Day 8, caudal sucker ventral, attaching; Day 9, three pairs of eyes; Day 10, crawling; Day 11, sustained swimming; Day 12, first feeding. There was no indication of gonadal development. Scale bar, 1.0 mm. Images by author.

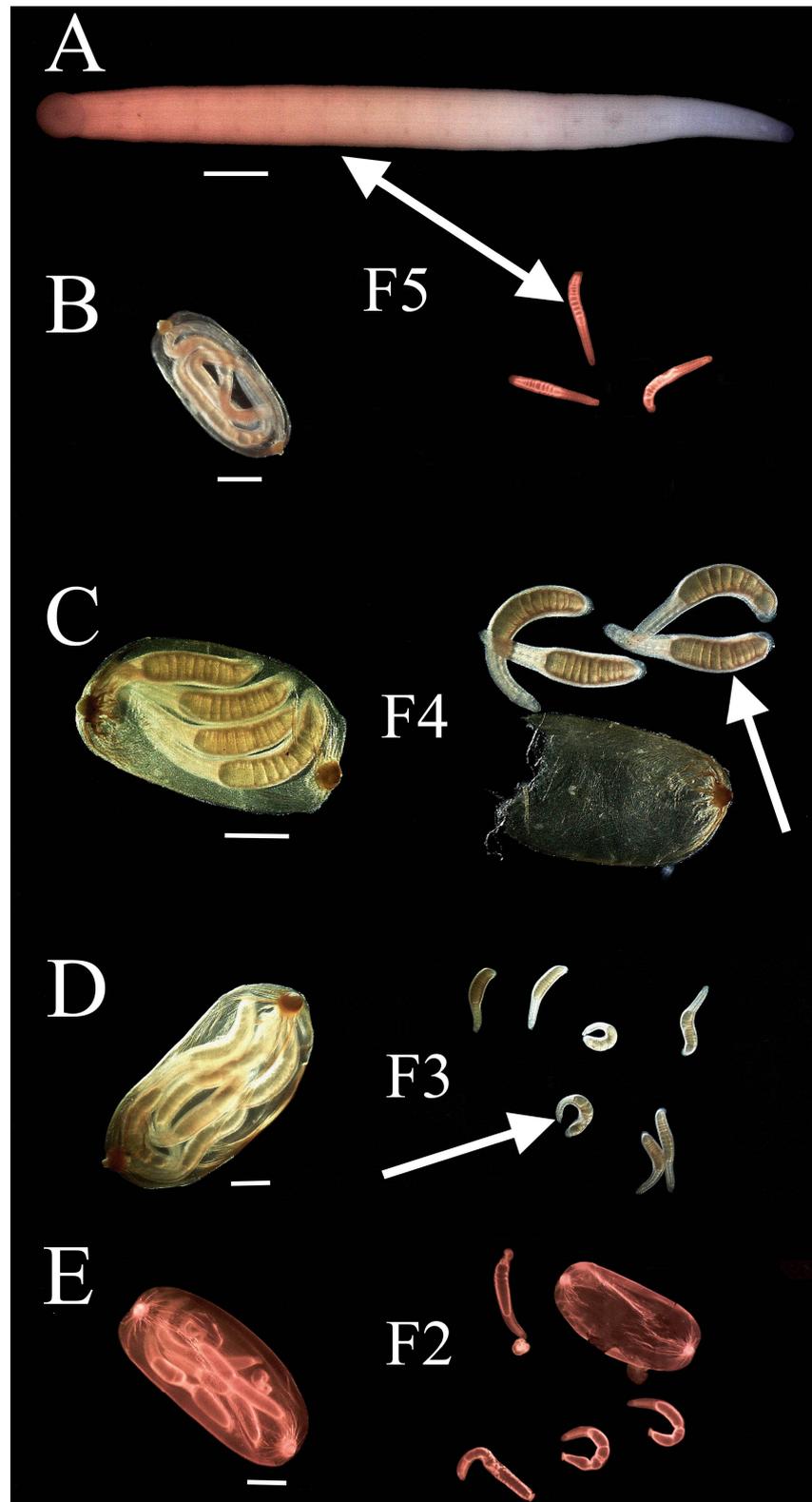


Figure 2. Direct lineal descent of *Barbronia weberi* isolated as pre-hatching embryos for five consecutive generations F1–F5. (A) Mature F5 individual grown in isolation from the embryo identified by the arrow in B. In life this individual had a faint clitellum in anticipation of an F6 generation (culled age 40 days; weight 33 mg). Anterior is right. (B–E) Left, unopened cocoons of F2–F5. Right, opened cocoons displaying the respective embryos which were immediately isolated. The arrows in B–D indicate the specific embryo which upon maturity gave rise to the next generation in this lineage (not recorded in E). (E), cocoon deposited by the unmated founder individual (F1) (see Introduction), opened Day 7; (B, D), opened Day 10; (C), opened Day 12. Scale bars: A, 5.0 mm; B–E, 1.0 mm. Images by author.

First generation, F1

The original, unmated **F1** individual thrived into maturity and lived for 166 days, having attained a weight of 202 mg. During this period of isolation it deposited 13 cocoons, 9 of which were viable and contained a total of 53 embryos (Figure 2E). The first cocoon, laid at age 101 days, was relatively small (4.2×1.9 mm) and contained only 4 embryos. As the individual grew the cocoons became correspondingly larger (up to 7.5×3.0 mm). The average length of cocoons produced by the **F1** individual was 5.2 mm ($N = 7$), and the average number of eggs per cocoon was 5.9 ($N = 9$). The largest number of eggs in a single cocoon was 13.

Successive generations, F2 to F5

Longevity

In the 12 months allocated for this study, only the second generation **F2** had sufficient time to grow fully to senescence. The average life span of **F2** individuals was 122 days ($N = 38$), and one individual lived 216 days. Subsequent generations overlapped and at the end of the study these had to be culled prematurely: **F3** (at 172–176 days, $N = 18$ culled), **F4** (at 102–111 days, $N = 13$) and **F5** (at 40–43 days, $N = 6$).

Maturation

The average size at maturation (defined empirically as weight at age 60–89 days) for each successive generation was: **F2** (141 mg, $N = 7$), **F3** (74 mg, $N = 11$) and **F4** (58 mg, $N = 19$). The earliest clitellum was noted in successive generations as follows: **F2** (day 47), **F3** (day 61) and **F4** (day 61), and this preceded laying of the first cocoon a few days later: **F2** (day 52), **F3** (day 64) and **F4** (day 68). By inference, the first cocoon corresponded to the shortest time from egg to egg in the respective generations. For all generations the average time from egg to egg was 80.7 days (spread 52–117 days, $N = 66$).

Fecundity

The largest number of eggs laid by a single individual in each successive generation was: **F2** (229 eggs, $N = 47$), **F3** (155, $N = 13$) and **F4** (58, $N = 13$). The average number of eggs laid by individuals was: **F2** (62.0 eggs, $N = 47$), **F3** (76.9, $N = 18$), and **F4** (27.4, $N = 13$). The largest number of cocoons laid by a single individual in each successive generation was: **F2** (47 cocoons, $N = 47$), **F3** (37, $N = 18$) and **F4** (12, $N = 13$). The average number of cocoons laid by individuals was: **F2** (14.5 cocoons, $N = 47$), **F3** (19.8, $N = 18$) and **F4** (7.9, $N = 13$).

The average length of cocoon in each successive generation was: **F2** (6.1 mm, $N = 8$), **F3** (5.9 mm, $N = 29$) and **F4** (4.9 mm, $N = 41$). The average

number of eggs per cocoon was: **F2** (7.0 eggs, N = 417), **F3** (5.7, N = 242) and **F4** (4.5, N = 79). The maximum number of eggs in a cocoon in each generation was: **F2** (15 eggs); **F3** (10) and **F4** (8). The average number of eggs in the first cocoon laid by each individual in each generation was: **F2** (6.7 eggs, N = 11), **F3** (4.9, N = 15) and **F4** (3.7, N = 13). The number of eggs per cocoon increased with the order laid in all generations. For example, in generation **F4** the average number of eggs per cocoon in order laid was: Cocoon 1 (3.7 eggs); cocoon 2 (3.8); cocoon 3 (4.3); cocoon 4 (4.4); cocoon 5 (4.8); cocoon 6 (5.0); cocoon 7 (5.5); and cocoon 8 (5.8) (N = 13 cocoons in each case).

Comparison with the wild population

The fecundity in the laboratory compared favorably with their natural counterparts (Sawyer and Sawyer 2018; see also Govedich et al. 2003), with two notable exceptions. Cocoons were collected on alligatorweed *Alternanthera philoxeroides* (Mart.) Griseb at the original site in successive years. The average length of cocoons collected 3 May 2017, 1 May 2018 and 26 May 2019 was 4.4 mm (N = 11), 3.8 mm (N = 13) and 4.9 mm (N = 20), respectively, generally smaller than those produced in the laboratory. Cocoons collected on 13 May 2019 contained an average of 6.7 eggs (N = 10), and the largest number of eggs in a cocoon was 10.

In contrast to laboratory-laid cocoons, none of the 20 cocoons collected at the original site on 13 May 2019 was abnormal. The high frequency of abnormal cocoons in the laboratory was presumably attributable to artificiality of conditions compared to nature.

Discussion

The foregoing study documents that *B. weberi* is indeed capable of reproducing without cross-fertilisation. It is not clear, however, if this capability is facultative or obligatory. In fact, on current knowledge it is not known if *B. weberi* ever reproduces sexually.

Assessment of overall fertility

It can be easily inferred from the above data that in general older (larger) individuals of each generation deposited larger cocoons containing more eggs. However, the significance, if any, of apparent diminution in size or number of certain parameters with successive generations could not be explained satisfactorily within the framework of this study. In any case taken together the foregoing summary of fertility clearly indicates that, in spite of presumed inbreeding over five successive generations, the leeches still maintained considerable reproductive potential.

Mechanism of uniparental reproduction

The mechanism by which *B. weberi* is capable of reproducing without cross-fertilisation is unclear. External self-“copulation” or self-impregnation

by means of spermatophores is very unlikely. Based on hundreds of daily observations throughout the year of this study not once was spermatophore production and/or self-impregnation detected. In other words, if self-fertilisation is occurring in *B. weberi* it may be an internal phenomenon. In this context it may be significant that *B. weberi* and congeners are anatomically unique in possessing two mid-ventral supernumerary openings anterior and posterior to the true gonopores, respectively (Sawyer 1986). In this study such accessory pores occurred in all mature specimens examined, including the F5 individual illustrated in Figure 2A (see also figures 1, 2 in Sawyer and Sawyer 2018). On current evidence *B. weberi* is unique among erpobdelliform species in displaying uniparental reproduction. In an ongoing parallel study a related species *Mooreobdella tetragon* Sawyer and Shelley, 1976, from the same geographic region has also been reared in isolation from a hatching under identical conditions but has failed to produce offspring after 225 days (489 mg). A similar result was reported for *Erpobdella octoculata* (Linnaeus, 1758) (Kutschera and Wirtz 1986).

Genetic and taxonomic implications of self-fertile reproduction in B. weberi

Self-fertilisation is the ultimate form of inbreeding such that heterozygosity of alleles is halved with each generation. Accordingly, in the current study the fifth generation would be virtually homozygous and in theory genetically very different from the original wild population. Thus, in principle if this phenomenon occurred in nature, a founder vagrant in a new locality could result in a quickly established population which is genetically distinct not from gradual adaptation but from random assortment of alleles.

The genetics and evolution of self-fertilisation is complex (see Wright et al. 2013) and lies outside the scope of this paper. However, in the case of *B. weberi* the genetics of self-fertilisation may be relevant toward understanding the recent proliferation in various parts of the world of several new “species” very closely allied to *B. weberi* (Hussein and El-Shimy 1982; Kutschera 2004; Westergren and Siddall 2004; Grosser and Trontelj 2008). In other words, if these new “species” are actually self-fertile vagrants, *B. weberi* (sensu lato) may be even more invasive than traditional taxonomy would suggest.

As discussed for *B. weberi* (sensu lato), differences between populations derived from self-fertilising founder vagrants do not necessarily reflect true phylogenetic relationships, as required for traditional taxonomy, especially at the species level. More broadly, this may explain the recent proliferation of new “species” derived from the glossiphoniids *Helobdella triserialis* (E. Blanchard, 1849) and *H. stagnalis* (Linnaeus, 1758) (see Iwama et al. 2019), both of which are self-fertilising, widely distributed and arguably also invasive species. Extrapolating from the current study, it is suggested that differences in very closely related self-fertilising leeches are generally not at the species level. The taxonomic distortion in recognising these as “species” is explored further in the following section.

Phylogeny of self-fertile reproduction in the Hirudinea

This study is the first demonstration of uniparental reproduction in the leech order Arhynchobdellida, a major taxonomic group which includes the medicinal leech *Hirudo medicinalis* Linnaeus, 1758 (Sawyer 1986). However, it is not generally known that “self-fertilisation” has been reported recently in several species of the order Rhynchobdellida, a group of proboscis leeches which normally reproduces by means of hypodermic impregnation of spermatophores (Whitman 1891). Thus, this paper greatly extends the known phylogenetic distribution of uniparental reproduction within the Hirudinea, the evolutionary significance of which is discussed below.

Glossiphoniidae. Whether some leeches can reproduce without cross-fertilisation has been mooted for nearly two hundred years. As early as 1839 the Italian zoologist Filippo de Filippi claimed he isolated an unidentified glossiphoniid leech (“Clepsine”) which later gave rise “to eggs which later developed”, but he gave no further details (Filippi 1839).

Four decades later, C.O. Whitman reared five isolated embryos of the glossiphoniid fish leech *Hemiclepsis marginata* (O.F. Müller, 1774) which later laid viable eggs. At the time this careful worker stated this “is an unquestioned case of self-fructification or of parthenogenesis...” (Whitman 1878). However, he later retracted this conclusion because he had feed the isolates on the same fish on 10 to 12 occasions, confessing in retrospect he was unaware of the possibility of impregnation by spermatophores during the feeding process (Whitman 1891).

The first convincing demonstration of reproduction without cross-fertilisation in a rhynchobdellid leech was in the 1980s in the glossiphoniid *Helobdella triserialis* (sensu lato), a widespread species complex comprised of an array of closely related polymorphic and molecularly distinct “varieties”, a term used here for brevity (Siddall and Borda 2003; Ocegüera-Figueroa et al. 2010). Kutschera and Wirtz (1986) reared 10 hatchlings of *H. triserialis* variety *papillornata* Govedich and Davies, 1998, in isolation. All 10 produced offspring which were isolated in turn and they also produced offspring. Reproduction in isolated individuals of *papillornata* was later affirmed in another laboratory (Tan et al. 2004).

In the late 1980s, David Weisblat and colleagues reared the eponymous *H. triserialis* variety *triserialis*, in isolation for seven successive generations over a period of 1.5 years (Wedeen et al. 1990). This same group of developmental biologists later showed that *H. triserialis* variety *robusta* Shankland et al., 1992, was also capable of uniparental reproduction (Iyer et al. 2019).

In 2019, the Weisblat laboratory extended this phenomenon to the cosmopolitan *H. stagnalis* (sensu lato) complex by showing that *H. stagnalis* variety *octatestisaca* Lai and Chang, 2009, is capable of both self- and cross-fertilisation (Iyer et al. 2019).

Piscicolidae. Kua et al. (2010) isolated hatchlings of the Malaysian marine fish leech *Zeylanicobdella arugamensis* de Silva, 1963. They were fed on individual fish fry and reared to maturity when they proceeded to deposit cocoons, 34% of which hatched normally.

Implications for clitellate hermaphroditism

All leeches are hermaphrodites and the overwhelming majority of species reproduces by non-reciprocal or reciprocal sex between individuals (Sawyer 1986). It is now known from the foregoing evidence that a few leech species can reproduce without cross-fertilisation. Of considerable phylogenetic significance is the observation herein that this phenomenon is not confined to one taxonomic group but is widely distributed in three very disparate taxa, comprising two unrelated families in the Rhynchobdellida, and now the Arhynchobdellida. This raises the evolutionary question whether reproduction without cross-fertilisation arose independently several times within the Hirudinea or, more likely, does the Hirudinea have a genetic predisposition to reproduce without cross-fertilisation. The latter implies some sort of evolutionary safeguard for maximizing reproductive success of isolated vagrants.

This presumptive predisposition to reproduce without cross-fertilisation in leeches may be linked to their hermaphroditism. For example, it is reasonable to presuppose that isolated individuals possessing both male and female gametes have the theoretical mechanism of self-fertilisation. While it is likely that reproduction without cross-fertilisation in leeches is a result of self-fertilisation, by way of caution this mechanism, as opposed to parthenogenesis for example, has not been proven for *B. weberi* nor any other uniparental leech species. Regardless of the mechanism, reproduction without cross-fertilisation undoubtedly contributes significantly to the capability of species to invade new habitats under optimal circumstances.

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