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Parthenogenesis in two Taiwanese mountain earthworms *Amynthas catenus* Tsai et al., 2001 and *Amynthas hohuanmontis* Tsai et al., 2002 (Oligochaeta, Megascolecidae) revealed by AFLP

Huei-Ping Shen [a,b], Hon-Tsen Yu [a,*], Jiun-Hong Chen [a]

[a] *Institute of Zoology, National Taiwan University, 1 Roosevelt Road, Section 4, Taipei 106, Taiwan*
[b] *Habitat and Ecosystem Division, Endemic Species Research Institute, 1 Ming-shen East Road, Jiji, Nantou 552, Taiwan*

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A B S T R A C T

Parthenogenesis has been known in a variety of animal taxa and is commonly found in oligochaetes. Many pheretimoid earthworms in Megascolecidae have been known to be or suspected to be parthenogenetic, but there has not been any genetic investigation on them. *Amynthas catenus* and *Amynthas hohuanmontis* are earthworms belonging to the *Pheretima* complex of the family Megascolecidae. Both are endemic to Mt. Hohuan at an elevation of about 3000 m in central Taiwan, and both have spermathecae from three pairs in segments VI–VIII (sexthecal) to absence (athecal) and reproductive organs in different degrees of degeneration. Here we present the genetic evidence revealed by amplified fragment length polymorphism (AFLP) markers to confirm the parthenogenetic mode of reproduction in *A. catenus* and *A. hohuanmontis*. Sixty-two selective primer combinations were used to generate a total of 4593 and 4812 bands for *A. catenus* and *A. hohuanmontis*, respectively. We found that the two earthworms are automictic (meiotic) parthenogens with offsprings showing slightly reduced number of AFLP markers compared with their parents. There was no sperm in any of the spermathecae examined. Selection and reproductive success among different genotypes of the two earthworms will be an important topic for future investigation.

1. Introduction

Parthenogenesis has been reported in a broad range of animal taxa with more than half of the animal phyla, including Vertebrata, containing parthenogens [6]. Parthenogens originated from their sexual ancestors and often occupy different geographical ranges and/or ecological niches compared with their sexual conspecifics [45]. The term "parthénogenèse géographique" was first coined by Vandel [62]. Generally it means that the distributions of parthenogens are often biased towards particular ecological settings including high latitudes, high altitudes, xeric environments and islands [27,35].

In oligochaetes, parthenogenesis has been established in Tubificidae, Enchytraeidae and Lumbricidae [13], and is a common mode of reproduction among the Lumbricidae [58]. Some species in Acanthodrilidae, Glossoscolecidae, Ocnerodrilidae, and many pheretimoid earthworms in Megascolecidae have been known to be or possibly to be parthenogenetic with signs of male sterility and degeneration or absence of reproductive organs [24,50]. Parthenogenetic reproduction in oligochaetes has been confirmed through isolation experiments [37,25,21,29,49,17,4,38,33,22], cytological studies [40,43,44,11,12] and electrophoretic analyses [31,4].

Amplified fragment length polymorphism (AFLP) requires comparatively short start-up time in most species and generates numerous loci without prior knowledge about the genomic makeup of the organism [39,7]. It is comparable to microsatellites when a sufficient number of loci were used [26,8,7]. Parthenogenesis in the Burmese python [28] and hammerhead sharks [9] was confirmed through AFLP analysis. To date in earthworm research, the AFLP technique has only been used in detecting interbreeding when defining species boundaries [36], and individual genotyping prior to ecotoxicological assays [3].

Amynthas catenus Tsai et al., 2001 and *Amynthas hohuanmontis* Tsai et al., 2002 are earthworms belonging to the *Pheretima* complex of the family Megascolecidae. Both are endemic to Mt. Hohuan at an elevation of about 3000 m in central Taiwan. In winter snow often covers this mountainous area in this subtropical island. Individuals of the two earthworms can be found from roadside ditches and slopes. *A. catenus* is a small to medium-sized earthworm with a body length of 61–106 mm and a clitellum width of 2.7–4.2 mm [60]. It has spermathecae from three pairs in segments VI–VIII

* Corresponding author. Tel.: +886 2 33662456; fax: +886 2 23638179.
E-mail address: ayu@ntu.edu.tw (H.-T. Yu).

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(sexthecal) to absence (athecal) and reproductive organs in different degrees of degeneration [60,55]. Three DNA ploidy levels, diploid, triploid and tetraploid were found for the athecals but only diploid for the sexthecals. The intermediates consisted of diploids and triploids [55]. Spermatogenesis occurred throughout the year with the highest activity in summer for athecals as well as sexthecals, and number of mature ova produced was higher from summer to winter for the athecals and from winter to spring for the sexthecals [55]. As for *A. hohuanmontis*, its body size approximates to that of *A. catenus* with 73–113 mm in length and 3.41–4.4 mm in width [61]. It was originally described as an athecal earthworm belonging to the *sheni* species-subgroup of the *illotus* species-group of the genus *Amynthas* [61]. However, examination of specimens from subsequent collections showed that this species had spermathecae from three pairs in segments VI–VIII to absence, and the structure of its prostate glands varied from large, asymmetrical, small, to absent. Only diploid was found for both athecal and thecal *A. hohuanmontis* individuals with chromosome number $2n = 126$ (somatic DNA contents (mean ± SD): 2.92 ± 0.27 pg/cell; gametic DNA contents (mean ± SD): 1.44 ± 0.12 pg/cell) [unpublished data]. Spermatogenesis of this species occurred all the year round, with highest activity in summer and spring for athecal and thecal individuals, respectively. Number of mature ova produced by athecal individuals was higher from summer to winter, whereas that of thecal individuals from autumn to winter [unpublished data].

It is possible that the variable number and structure of reproductive organs of *A. catenus* and *A. hohuanmontis* might be due to parthenogenetic degeneration. Seasonal collections of the two species have been made for two years prior to this study, and a total of 112 spermathecae belonging to 23 *A. catenus* individuals plus 57 spermathecae belonging to 14 *A. hohuanmontis* individuals were examined. No sperm was found in the serial-section (5 μm) slides of the spermathecae of either *A. catenus* or *A. hohuanmontis* [55 and unpublished data]. Here we present the genetic evidence revealed by AFLP markers to confirm the parthenogenetic mode of reproduction in *A. catenus* and *A. hohuanmontis*. We assume that each genetic marker appeared in the offspring should also be found in the parent if parthenogenetic reproduction is adopted in the two species.

2. Materials and methods

2.1. Study area

The study site was along the road sides and around two parking lots within 200 m from the High Altitude Experimental Station (24°9′48″N, 121°16′43″E) of the Taiwan Endemic Species Research Institute at elevations of 2985–3004 m of Mt. Hohuan in central Taiwan. Since November, 1986, this area has been a part of Taroko National Park. The vegetation was typical alpine forest and grassland. The site has a temperate climate with snow in winter and cool summer. Average annual precipitation was 4019 mm. Mean annual air temperature was 7.1 °C [55].

2.2. Earthworm collection and DNA extraction

According to our collecting experiences, the field populations of the two endemic mountain earthworms, *A. catenus* and *A. hohuanmontis*, were not large. Generally it took one person 1 h to obtain one or two individuals by digging into the soil at a depth of 20–30 cm. Sometimes individuals were found in the upper 20 cm of the soil. We have tried for two years prior to the present study to rear them in the lab, but none survived over one month. Therefore, we decided to keep them in their natural habitat where the temperature and humidity were best suited for these worms. As both athecal and sexthecal individuals of *A. catenus* and *A. hohuanmontis* had generally higher numbers of mature ova in winter [55 and unpublished data], sexually mature clitellate individuals of the two species were collected by hand sorting from Mt. Hohuan, central Taiwan in December, 2009. About 1 cm of the tails of the earthworms were amputated and placed immediately into 95% ethanol for DNA extraction. After amputation, single or paired

Table 1
Number of AFLP markers generated by each of the 62 primer combinations.

Primers	*Amynthas catenus*		*Amynthas hohuanmontis*	
	Total (polymorphic)	Polymorphism (%)	Total (polymorphic)	Polymorphism (%)
E32-T32	52 (7)	13.5	63 (23)	36.5
E32-T33	62 (15)	24.2	64 (8)	12.5
E32-T35	67 (11)	16.4	86 (7)	8.1
E32-T38	53 (11)	20.8	73 (10)	13.7
E32-T48	52 (9)	17.3	84 (21)	25
E32-T49	48 (12)	25	71 (9)	12.7
E32-T50	56 (19)	33.9	59 (14)	23.7
E32-T51	67 (13)	19.4	113 (32)	28.3
E33-T32	68 (13)	19.1	107 (15)	14
E33-T33	82 (23)	28	133 (47)	35.3
E33-T35	61 (9)	14.8	76 (16)	21.1
E33-T48	69 (20)	29	86 (37)	43
E33-T49	63 (21)	33.3	78 (6)	7.7
E33-T50	94 (31)	33	95 (11)	11.6
E33-T51	51 (21)	41.2	40 (7)	17.5
E35-T32	103 (29)	28.2	107 (68)	63.6
E35-T33	98 (30)	30.6	88 (38)	43.2
E35-T35	62 (20)	32.3	53 (6)	11.3
E35-T38	61 (19)	31.1	113 (40)	35.4
E35-T48	95 (29)	30.5	66 (18)	27.3
E35-T49	83 (20)	24.1	58 (17)	29.3
E35-T50	107 (32)	29.9	93 (38)	40.9
E35-T51	25 (5)	20	37 (6)	16.2
E38-T32	77 (20)	26	59 (13)	22
E38-T33	114 (10)	8.8	94 (12)	12.8
E38-T35	75 (21)	28	63 (9)	14.3
E38-T38	56 (14)	25	89 (15)	16.9
E38-T48	92 (29)	31.5	95 (36)	37.9
E38-T49	74 (16)	21.6	69 (18)	26.1
E38-T50	64 (26)	40.6	64 (20)	31.3
E38-T51	65 (17)	26.2	42 (7)	16.7
E39-T32	73 (17)	23.3	77 (18)	23.4
E39-T33	91 (12)	13.2	81 (17)	21
E39-T35	85 (19)	22.4	86 (20)	23.3
E39-T38	103 (36)	35	97 (23)	23.7
E39-T48	88 (23)	26.1	83 (13)	15.7
E39-T49	75 (20)	26.7	82 (16)	19.5
E39-T50	106 (22)	20.8	101 (26)	25.7
E39-T51	80 (27)	33.8	43 (11)	25.6
E42-T32	94 (17)	18.1	105 (19)	18.1
E42-T33	73 (19)	26	75 (23)	30.7
E42-T35	88 (16)	18.2	91 (14)	15.4
E42-T38	86 (27)	31.4	66 (18)	27.3
E42-T48	92 (24)	26.1	116 (26)	22.4
E42-T49	86 (18)	20.9	75 (7)	9.3
E42-T50	102 (20)	19.6	119 (10)	8.4
E42-T51	69 (23)	33.3	84 (20)	23.8
E44-T32	53 (10)	18.9	60 (11)	18.3
E44-T33	91 (13)	14.3	94 (12)	12.8
E44-T35	89 (21)	23.6	58 (19)	32.8
E44-T38	48 (11)	22.9	61 (9)	14.8
E44-T48	84 (16)	19	90 (29)	32.2
E44-T49	89 (23)	25.8	76 (9)	11.8
E44-T50	97 (23)	23.7	92 (16)	17.4
E44-T51	63 (13)	20.6	60 (16)	26.7
E45-T32	87 (29)	33.3	90 (5)	5.6
E45-T33	48 (11)	22.9	67 (6)	9
E45-T38	72 (14)	19.4	65 (16)	24.6
E45-T48	48 (15)	31.3	37 (9)	24.3
E45-T49	30 (7)	23.3	68 (39)	57.4
E45-T50	63 (7)	11.1	56 (8)	14.3
E45-T51	44 (12)	27.3	39 (10)	25.6
Total	4593 (1137)	24.8	4812 (1119)	23.3