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GENETIC STRUCTURE OF TWO INVASIVE EARTHWORMS, *AMYNTHAS AGRESTIS* AND *AMYNTHAS TOKIOENSIS* (OLIGOCHAETA, MEGASCOLECIDAE), AND A MOLECULAR METHOD FOR SPECIES IDENTIFICATION.

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ABSTRACT

Asian pheretimoid earthworms (genus *Amynthas*) are particularly aggressive invasive species in the northern United States. These earthworms disrupt both the physical and chemical structure of invaded soils as well as the resident biotic community. Understanding the factors that favor invasion by *Amynthas* earthworms is critical for control efforts. One factor previously unexplored is the population genetic structure of the earthworms. We scored 44 and 36 RAPD (Random Amplification of Polymorphic DNA) genomic markers for two species, *Amynthas agrestis* and *Amynthas tokioensis,* at three sites in Vermont, USA, and found substantial genetic variation for both species. This variation was partitioned into groups of worms of each species with identical genotypes at each site (clones), but there were also many singleton genotypes. This pattern could be a result of multiple introductions of genetically-distinct parthenogenetic lines, a repeated switch from sexual to parthenogenetic reproduction, or a mixed reproductive system of sexual and asexual worms. The mixed system would allow the worms to benefit from both rapid clonal reproduction as well as occasional generation of genetic diversity by sexual reproduction. Genetic structure did not differ for either species among sites, suggesting a single source population. However, there were no identical genotypes among sites. Last, a new method is described for rapidly identifying even hatchling worms with a PCR-based molecular marker.

Key words: Invasive species, *Amynthas* earthworms, DNA barcoding, RAPD markers, genetic structure.

RÉSUMÉ

Les vers de terre pheretimoides asiatiques (genre *Amynthas*) sont des espèces envahissantes particulièrement agressives dans le nord des États-Unis. Ces vers de terre perturbent à la fois la structure physique et chimique des sols envahis ainsi que la communauté biotique résidente. Comprendre les facteurs qui favorisent l'invasion par les vers de terre *Amynthas* est essentiel pour les efforts de contrôle. Un facteur précédemment inexploré est la structure génétique des populations de vers de terre. Nous avons noté 44 et 36 marqueurs génomiques RAPD (amplification aléatoire de l'ADN polymorphe) pour deux espèces, *Amynthas agrestis* et *Amynthas tokioensis*, dans trois sites du Vermont, États-Unis, et avons constaté une variation génétique substantielle pour les deux espèces. Cette variation est répartie en groupes de vers de chaque espèce avec des génotypes identiques à chaque site (clones), mais il y avait aussi de nombreux génotypes isolés. Cette observation pourrait résulter de multiples introductions de lignes parthénogénétiques génétiquement distinctes, d'un changement répété de la reproduction sexuelle à la parthénogénétique ou d'un système reproducteur mixte de vers sexuel et asexué. Le système mixte permettrait aux vers de profiter tant de la reproduction clonale rapide que de la génération occasionnelle de la diversité génétique par reproduction sexuelle. La structure génétique ne diffère pas pour l'une ou l'autre espèce parmi les sites, ce qui suggère une population source unique. Cependant, il n'y a pas de génotypes identiques parmi les sites. Enfin, une nouvelle méthode est décrite pour identifier rapidement même des vers récemment éclos avec un marqueur moléculaire à l'aide de l'amplification en chaîne par polymérase (PCR).

Mots clés: Espèces envahissantes, vers de terre *Amynthas*, codes à barres d'ADN, marqueurs RAPD, structure génétique.

RESUMEN

Las lombrices feretimoideas asiáticas (género *Amynthas*) son especies invasoras particularmente agresivas en el norte de los Estados Unidos. Estas lombrices alteran la estructura física y química de los suelos invadidos, así como la comunidad biótica edáfica residente. El entendimiento de los factores que favorecen la invasión por las lombrices *Amynthas* es crítica para ejercer un control adecuado. Un factor no explorado aún, es la estructura genética de la población de las lombrices de tierra. Se obtuvieron 44 y 36 marcadores genómicos RAPD (Amplificación aleatoria de ADN polimórfico) para dos especies, *Amynthas agrestis* y *Amynthas tokioensis*, en tres sitios de Vermont, EE.UU., y se encontró una variación genética sustancial para ambas especies. Para estudiar esta variación, se dividió en grupos a los gusanos de cada especie con genotipos idénticos en cada sitio (clones) además también hubo muchos genotipos simples. Este patrón podría ser el resultado de múltiples introducciones de líneas partenogenéticas, genéticamente diferentes, es decir, un cambio repetido de la reproducción sexual a la reproducción partenogenética, o un sistema reproductivo mixto de gusanos sexuales y asexuales. El sistema mixto permitiría a los gusanos beneficiarse tanto de la reproducción clonal rápida como de la generación ocasional con diversidad genética mediante la reproducción sexual. No encontramos evidencia en las condiciones geográficas entre los sitios para ninguna de las dos especies, lo que sugiere una sola fuente de población. Por último, se describe un nuevo método para identificar rápidamente gusanos recién nacidos usando un marcador PCR con base molecular.

Palabras clave: Especies invasoras, lombrices de *Amynthas*, códigos de barras de ADN, marcadores RAPD.

ZUSAMMENFASSUNG

Aus Asien stammende pheretimoide Regenwürmer (Megascolecidae: *Amynthas*) sind parthenogenetische Organismen und besonders destruktive Neozooten im Norden der Vereinigten Staaten. Diese Regenwürmer stören nicht nur Stoffkreisläufe im Boden, sondern verändern die ansässige Pflanzen- und Tiergesellschaften. Ein besseres Verständnis der Ökologie dieser Regenwürmer ist eine Vorrausetzung für eventuelle Kontrolmaßnahmen. Die genetische Struktur von phetetimoiden Populationen kõnnte dabei von besonderer Bedeutung sein. Wir haben 44 und 36 RAPD (Randon Amplification of Polymorphic DNA) genetische Marker von zwei Arten, *Amynthas agrestis* beziehungsweise *A. tokioensis*, an drei Waldstandorten analysiert. Erhebliche genetische Variationen existierten innerhalb und zwischen den Standorten. Obwohl es genetisch identische Individuen an jedem Standort gab (clones), kamen auch viele genetische Unikate vor. Dieses Muster könnte durch mehrfache Einführungen von genetisch unterschiedlichen parthenogenetischen Absstammungslinien hervorgerufen worden sein. Die genetische Vielfalt kann auch durch ein gemischtes Fortpflanzungssystem mit sexuellen und asexuellen Würmern erklärt werden. Ein solches System hätte die Vorteile beider Fortpflanzungsmodi: Schnelles pathenogenetisches Kopieren von erfolgreichen Klonen und das Hervorbringen von genetischer Vielfalt durch gelegentlichen Sex. Das Fehlen von Unterschieden in der genetischen Struktur der Populationen läßt auf einen einzigen rezenten Ursprung schließen obwohl die Genotypen an den Standorten nicht identisch waren. Außerdem wird eine PCR Methode beschrieben, die Arten sogar von Kokons und Körperflüssigkeiten identifiziert.

Schlüsselworte: Neozooten, *Amynthas* Arten; DNA Barcoding, RAPD Markers, genetische Struktur.

INTRODUCTION

The Asian *Amynthas* earthworms (Megascolecidae) are particularly aggressive and disruptive invaders of soil communities on the warm continents and even oceanic islands (Novo *et al.*, 2015; Hendrix *et al.*, 2008; Chang *et al.*, 2016a, b). Only 15 species of *Amynthas* of the large family Megascolecidae are invasive (Hendrix *et al.*, 2008). Introduced *Amynthas* were first reported in the USA from California in the 1860's, but have become widespread and locally abundant only in the past two decades (Snyder *et al.*, 2011). For example, *Amynthas agrestis* were first found in Vermont ten years ago, but ongoing surveys now find them throughout the state (Görres and Melnichuk, 2012, Reynolds *et al.*, 2015; Reynolds, 2012) posing a major conservation issue for some habitats in the region.

Amynthas spp. are epi-endogeic and their feeding behavior leads to depletion of leaf litter and accelerated decomposition of organic matter, with consequences for microbial communities (Chang *et al.*, 2016a), elevated soil mineral nitrogen and phosphorus (Greiner *et al.*, 2012), elimination of larger invertebrates (Chang *et al.*, 2016b; Hendrix *et al.*, 2008; Snyder *et al.*, 2011; Zhang *et al.*, 2010), and remobilizing of heavy metals into the food web (Richardson *et al.*, 2015). In our region, wherever *Amynthas* spp. are established, often all other earthworms are eliminated and *Amynthas* reach high densities, briefly exceeding 200/m^2 in the summer (Görres *et al.*, 2014a).

Life history traits that seem central to the success of invasive species is their genetic diversity and reproductive system. Although earthworms are typically hermaphroditic, with obligate mutual mating between two individuals, some, including *A. agrestis* and *A. tokioensis*, are considered parthenogenetic (Reynolds, 1974), and others may use a mixed reproductive system including both parthenogenetic and sexual reproduction (Terhivuo and Saura, 2006), which may be linked to successful invasive earthworms (Hendrix *et al.*, 2008). Asexual reproduction allows even a very small inoculum of an introduced species to become established and to recover from population crashes in newly invaded environment. The shortcoming of parthenogenesis is the clonal production of eggs (both meiotic or mitotic production of eggs yield offspring genetically identical to the mother worm [Dupont, 2009]). Unpredictable environmental conditions at the time of invasion, and environmental changes over time, would be problematic for clonal worms.

Amynthas offer a valuable system to focus on the life history traits that could facilitate their entry into soil communities that already include a diverse array of earthworm species. We studied the genetic population structure of two *Amynthas* species at three sites in Vermont, USA that are becoming widespread in the northern USA, *A. agrestis* (Goto and Hatai, 1899) and *A. tokioensis* (Beddard, 1892). Such data can cast light on the overall diversity of the invasive species (including number of introduction events) and the reproductive system of the earthworms.

Our look at the genetic structure of *Amynthas* had three goals: First, we determined the genetic diversity of the worms using a large number of RAPD markers (Random Amplification of Polymorphic DNA). We asked if the overall genetic diversity and structure in Vermont reflects sexual or asexual reproduction. Second, we examined the across-site population structure and asked if there is a single population of each species (and thus a likely single introduction into the study region), or are the sites genetically distinct (reflecting multiple introductions). Last, to overcome the difficulties in morphologically diagnosing *Amynthas* individuals to species, we developed a rapid and inexpensive molecular method to identify our specimens to species.

METHODS

Field site description and sampling

Three sites were sampled (Fig. 1). University of Vermont Horticulture and Education Center (hence HF) is a 39 ha experimental agricultural site (76 m elevation, 44.431489° N, -73.199211° W), and the collection area at the eastern edge is a deciduous forest with a canopy of mainly sugar maple (*Acer saccharum*). The University of Vermont Centennial Woods Natural Area (CW) is a 28 ha hilly diverse woodland of hardwoods, conifers, wetlands, and streams. Earthworm specimens were collected along frequently used paths (80 m elevation, 44.475701° N, -73.187088° W). The Green Mountain Audubon Center (AU) is a 100 ha complex hilly habitat of mixed forest, fields, wetlands, and streams located in the rural town of Huntington (190 m elevation, 44.346774° N, -72.996216° W). Permission to sample earthworms was granted for each site by the responsible authorities.

We sampled from 7 May 2015, and ended 24 June 2015. A total of 188 worms were collected by manually sifting through the upper 10 cm of soil and leaf debris. Sampling took place over approximately 5 m^2 at each site. During the sample period, the size differentiation between the adults of the two species (*A. agrestis* > *A. tokioensis*) was not yet expressed and our sample likely represents a random sample unbiased by species. Thus, sample sizes differed between species. We also collected adults later in the season from the same sites to help with verifying DNA based identification to species.

Fig. 1. Satellite image showing the locations of the three study sites, Audubon Center, Hort Farm, and Centennial Woods in northern Vermont, USA. Distance (km) between each site is labeled as well as Nei's unbiased genetic distance for *Amynthas agrestis* between sites. The details on locations is given in text.

Identification of species

The presence of *A. agrestis and A. tokioensis* species at our sites was confirmed by C. H. Chang with a key of pheretimoids that are established in North America (Chang *et al.*, 2016b). Adult worms of the two species differed clearly by size, with *A. agrestis* a maximum of 140 mm vs. *A. tokioensis* a maximum 80 mm in length. Using adults identifiable by size, we developed a PCR-based method (Polymerase Chain Reaction) to identify the worms to species. In brief, worms were killed in 50% ethanol, rinsed in water, dried, and the first three segments removed with a fresh razor blade (to eliminate contamination). DNA was extracted using the DNeasy blood and tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. We sequenced the mitochondrial cytochrome-c oxidase I (COI) gene used in barcoding earthworms (Huang *et al.*, 2007) with conserved primers for animals (Folmer *et al.*, 1994). The sequences were identical to those for worms identified by morphology from sites in Japan for the two species (Genbank accession numbers AB542601 for *A. agrestis* and AB542557 for *A. tokioensis*).

The COI sequences for each species were aligned and we chose a common forward primer and two reverse primers that differed by species. A multiplex PCR included all three primers and resulted in an amplicon length of 268 bp for *A. agrestis* or 386 bp for *A. tokioensis* when the product was run on a 1% agarose gel. The primers were Multi-A&T (primer in common) 5'-ATAATTGGAGCAGGAATAAGA-3', Multi-A (*A. agrestis* reverse primer) 5'-GCCGAGGACACTAATAAAAT-3', and Multi-T (*A. tokioensis* reverse primer) 5'-CCTGCTAAGTGGAGTGAG-3'. The multiplex PCR used program 94° for 1 min, followed by 32 cycles of 49° for 30 sec, 72° for 1 min, and a final extension of 72° for 2 min. Each juvenile worm used in the study was identified to species using this method.

Molecular genotyping

We genotyped earthworms using RAPD markers. This PCR method uses short random primers and low annealing temperatures in the PCR program that allows amplification of random locations on the genome (Williams *et al.*, 1990). RAPD markers are considered to be dominant; therefore, the presence of a band on an agarose gel is due to the worm being homozygous or heterozygous for the two alleles (Williams *et al.*, 1990). RAPD-PCR has been successfully used to evaluate genetic diversity in other earthworm species (Dyer *et al.*, 1998; Lentzsch and Golldack, 2006; Kautenburger, 2006).

We selected three RAPD primers after testing to ensure reproducibility and easily identifiable bands on an agarose gel: OPG12 (5'-CAGCTCACGA-3'), OPP 04 (5'-GTGTCTCAGG-3) and OPP06 (5'-GTGGGCTGAC-3'). DNA was extracted as described above for each worm. RAPD markers were then amplified in a 25 □l PCR that included a Ready-to-Go PCR bead (GE Health Care, Piscataway, NJ, USA) which includes a complete dNTP mix, polymerase, and buffer mix, 1 □l of one 10 □M primer, and 2 □l of the DNA extraction product, and using the program 94° for 2 min, then 34 cycles of 94° for 7 min at the primer-specific annealing temperature, 72° for 2 min, and a final extension of 72° for 10 min. Annealing temperatures were 32.3° (OPG12), 34.3° (OPP04), and 41.5° (OPP06).

The PCR products from each RAPD primer were run on a 1% agarose gel at 100V for 30 minutes; a 100 base pair ladder was included on each gel, in the first and last lanes, and pictures were taken of each gel (see Figure 2 for example of gel). A total of 564 gel lanes were examined (188 worms × 3 primers) to determine the total number of bands ever observed for each primer). The predetermined bands were selected by observing positions of bands from each primer and marking every position where a band was observed. Band scoring of the gels was then done by marking whether those predetermined bands were present or absent for each sample and each primer. A total of 44 RAPD markers for *A. agrestis* and 36 RAPD markers for *A. tokioensis*, regarded as loci with two alleles that were characterized by the presence or absence of a band, were scored (6,096 scores).

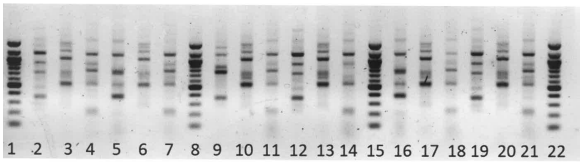


Fig 2. Example of RAPD primer OPG12 ran out on 1% agarose gel using 3µL of PCR product and running at 100V for 30 minutes. Lanes 1, 8, 15, and 22 are 100 base pair ladder, lanes 2-7 are Audubon worms, followed by Centennial Woods worms in lanes 9-14, and Hort Farm worms in lanes 16-21.

Statistical analyses

Band presence was coded "1", absence of a

band at a locus was noted as "0". The binary data were analyzed using the GenAlEx package (Peakall and Smouse 2006). Several measures of genetic diversity were evaluated: (1) The percent of polymorphic bands at each site calculated across all loci. (2) Shannon's Information Index (I) includes the number of possible alleles (for RAPD markers this is 2) and their relative abundance, where I = 0 if only one allele is present, and I = 0.693 if the two alleles are equally abundant. An advantage of I is that a confidence interval can be calculated (Peakall and Smouse, 2006). (3) The number of unique genotypes for each site was determined by comparisons of all worms across all loci. (4) Genetic distance based on number of band differences was calculated for each pairwise comparison of individuals for each species at each site. Worms that had identical band patterns for all 44 or 36 markers were considered as genetically identical.

Unique genotypes for each site were then used to calculate Nei's identity (Nei, 1972; Tajima and Nei, 1984) for each pairwise comparison of sites. The range for this measure is 0 for completely isolated populations and 1 if worms at the sites are drawn from the same population. An Analysis of Molecular Variance in GenAlEx was calculated for each species across all populations to determine the percentage of the total genetic variation accounted for by within population and among population genetic variation (Excoffier *et al.*, 1992; Peakall and Smouse, 2012).

RESULTS

We tested the multiplex PCR method against adult worms, picking sets of large (> 100 mm) and small worms (< 80 mm) that should be *A. agrestis* and *A. tokioensis* respectively. The method unambiguously identified each specimen to species, demonstrating the value of this technique. Sample sizes varied by species across sites (Table 1) because earthworms were randomly sampled, without knowledge of species status. Overall, *A. agrestis* represented 30% of worms sampled at HF, 100% at CW, and 80% at AU.

Measures of genetic diversity are presented in Table 1. All loci were polymorphic at each site, demonstrating the RAPD loci provided useful data for identifying identical vs. distinct genotypes and genetic isolation vs. uniformity across sites. Genetic diversity, measured by the Shannon Index, was similar for the samples except for higher measures for *A. agrestis* at AU and CW. Pairwise comparisons of individuals of the same species in a population yielded frequency of band differences (Fig. 3). Genetically identical individuals, or clone-mates, were common at each site but worms with unique genotypes for all loci were also common at each site (Table 1), with 45 - 100% of the worms across species and sites being singleton genotypes. The pairwise comparison between individuals showed that 21/33 (63.6%) *A. tokioensis* at HF had at least one clone-mate (identical genotype), and none of the eight *A. tokioensis* at AU had a clone-mate. For *A. agrestis*, 4/14 (28.6%) had at least one clone-mate at HF, 6/43 (13.9%) worms had at least one clone-mate at AU, and 24/48 (50%) worms had at least one clone-mate at CW. We expect that scoring errors for one or two loci would lead to apparent differences for some genetically identical worms (low number of band differences shown in Fig. 3). Thus, the number of genetically identical worms may be substantially higher than these values; however, using the stringent criterion of no band differences reveals identical groups of 2-9 worms with no identical genotypes occurring between sites (Table 2).

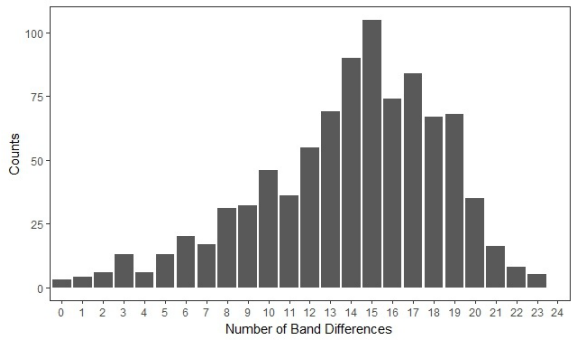
Nei's unbiased genetic distance measures were low for both species and all sites for *A. agrestis* (Fig. 1). The AMOVA showed that only 9% of total variance was accounted for by site for *A. agrestis* and 37% for *A. tokioensis*. Thus, both measures argue that the worms come from a single source population and have experienced little differentiation since the time of introduction.

Table 1. Measures of genetic diversity for two species of *Amynthas* earthworms at three sites in Vermont, USA (locations detailed in text).

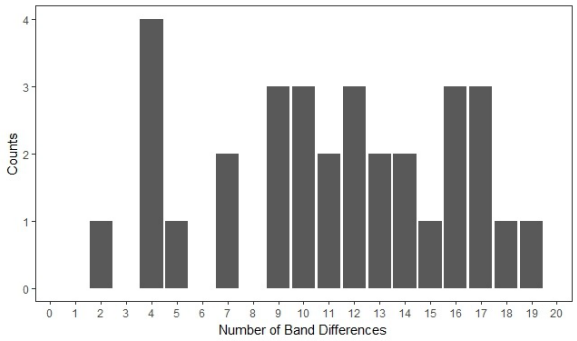
Species	Site	Sample Size	Number of unique genotypes	Shannon's Information Index (I)	± 1 S.E. of I	Polymorphic Loci %
Amynthas agrestis	CW	49	24	0.455	0.422-0.489	97.7
	HF	14	10	0.359	0.318-0.400	77.3
	AU	43	37	0.411	0.377-0.445	93.2
Amynthas tokioensis	HF	33	12	0.380	0.332-0.427	77.8
	AU	8	8	0.383	0.348-0.419	88.9

Table 2. The number of groups of clones at three sites in Vermont, USA (locations detailed in text) for two species of *Amynthas* earthworms, where doublet refers to two clonal individuals, triplet refers to three clonal individuals, *etc*.

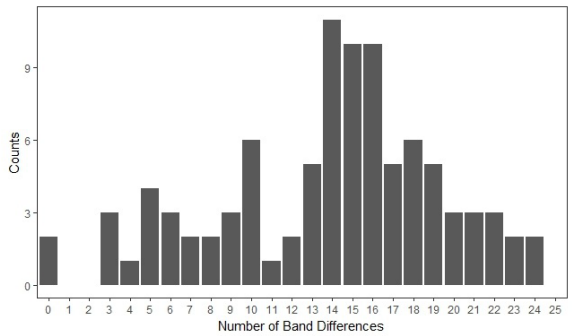
Species	Site	Doublet	Triplet	Quadruplet	Quintuplet	Sextuplet	Heptuplet	Octuplet	Nontuplet
Amynthas	CW	3	1	1	1	1	0	0	0
agrestis	AU	3	0	0	0	0	0	0	0
	HF	2	0	0	0	0	0	0	0
Amynthas	AU	0	0	0	0	0	0	0	0
tokioensis	HF	3	2	0	0	0	0	0	1



a. (*Amynthas agrestis* AU)

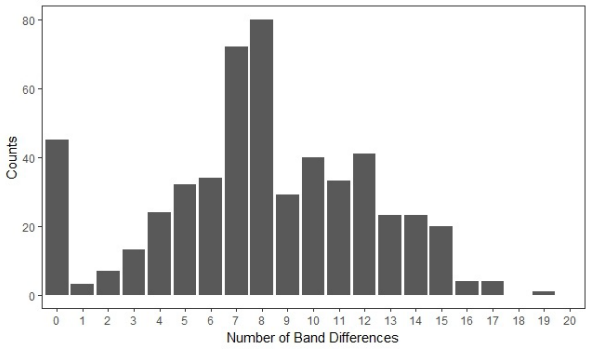


b. (*Amynthas tokioensis* AU)

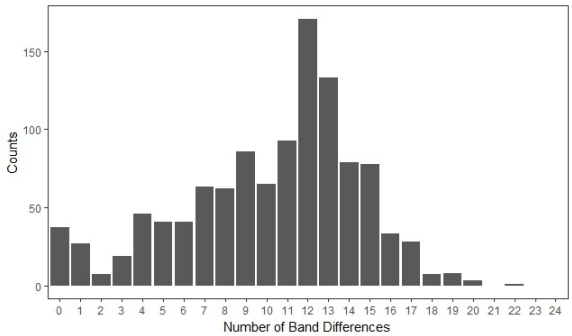


c. (*Amynthas agrestis* HF)

Fig. 3. Number of pairwise band differences between (**a**) *Amynthas. agrestis* at Audubon; (**b**) *Amynthas tokioensis* at Audubon; (**c**) *Amynthas agrestis* at Hort Farm; (**d**) *Amynthas tokioensis* at Hort Farm; (**e**) *Amynthas agrestis* at Centennial Woods. Sites are described in detail in text.



d. (*Amynthas tokioensis* HF)



e. (*Amynthas agrestis* CW)

Nei's unbiased genetic distance measures were low for both species and all sites for *A. agrestis* (Fig. 1). The AMOVA showed that only 9% of total variance was accounted for by site for *A. agrestis* and 37% for *A. tokioensis*. Thus, both measures argue that the worms come from a single source population and have experienced little differentiation since the time of introduction.

DISCUSSION

In our study of *A. agrestis* and *A. tokioensis*, at three sites in Vermont, we found both substantial genetic diversity with many singleton genotypes and clusters of individuals with identical genotypes. For example, at one site (HF), in a sample of 33 *A.*

tokioensis earthworms, there were 9 worms with a single identical genotype. Worms with very similar or identical banding patterns could also be the result of inbreeding between close relatives rather than parthenogenesis. However, the large number of apparent clones, especially at HF where 63.6% of *A. tokioensis* were clones-mates and at CW where 50% of *A. agrestis* were clones-mates, is significantly greater than the number of individuals expected to share an identical genotype in a population with a strictly sexual mating system. The likelihood of finding two full sibs with identical genotypes can be estimated as $p = (0.25)^n$, where n is the number of loci being compared and 0.25 as the probability of sharing the same genotype given two heterozygous parents. In this study, the likelihood of finding identical individuals is 3×10^{-27} and 2×10^{-22}, respectively, if loci are not linked. If they were linked assuming 14 chromosomes (Sharma *et al.*, 2016) the probability of two identical individuals with the same genotype would still be very low, 8×10^{-9}. Our results thus confirm the presence of parthenogenetic reproduction considered to be the norm for these species (Reynolds, 1974).

Even though clones were present, 83 distinct genotypes were found in the sample of 105 *A. agrestis* (12 in clonal worms and 71 found only once), and 26 genotypes found in a sample of 41 *A. tokioensis* (6 in clonal worms and 20 found once). The earthworms cannot be entirely sexual because of the presence of genetically identical groups, but can the two species be entirely parthenogenetic and still show such genetic diversity within each population?

The earthworms could follow a mixed reproductive system of parthenogenetic and hermaphroditic reproduction. The male reproductive structures are typically absent in both species, but a small proportion develop the male pores (Gates 1956; Minamiya *et al.*, 2011). This variation in male structures is common in *Amynthas* (Shen *et al.*, 2011; Plisko and Nxele, 2015) and we observed infrequent possession of male pores. A mixed strategy, asexual reproduction with occasional sexual reproduction may generate clusters of new, genetically unique clonal earthworms and be the mark of many successful invasive species (Shirk and Hamrick, 2014) providing the best of both systems. Although the genetic data are suggestive, the reproductive system of the *Amynthas* species would best be confirmed with cytological studies on chromosome complement.

Hermaphroditic reproduction as the ancestral condition in earthworms, can gradually be replaced by parthenogenesis in habitats favourable to asexual reproduction, specifically epi-endogeic habitats which experience variable and stressful environmental conditions (Jaenike and Selander, 1979; Jaenike *et al.*, 1982). Thus, the genetic patterns we observed would be seen in populations that have not yet completed this transition. The likelihood of two species and three sites all being caught during this transition, however, seems slight.

These *Amynthas* species could be entirely parthenogenetic. The populations we studied were derived from multiple invasions of distinct clones over a short period of time (just a few years). *A. vittatus* in Japan occurs in geographically separated populations with male pores present or absent Minamiya *et al.* (2011). The model of Jaenike and colleagues also results in diverse clonal populations across the landscape. But how could the Vermont populations derive from multiple parent populations that differ genetically? Worms imported with plants from multiple geographic sites may aggregate at large commercial nurseries and be redistributed to smaller producers. We have found both of these *Amynthas* species in commercial nurseries and gardens in Vermont. However, the sites sampled did not share genetically-identical earthworms; if the worms were introduced from a common source, clone-mates should have reached each site.

Sequencing of the barcoding (Hebert *et al.* 2003) fragment of the COI gene is clearly useful, though not practical for large number of worms used in ecological studies (Huang *et al.*, 2007). In addition to identifying field specimens, we have also used the method to identify worms from body fluid (and thus live worms can be processed) and even individual cocoons. The two species of *Amynthas* we studied are among the most widespread and important for ecosystem conservation. The method could be extended to include other species widespread in the northern USA, such as *A. hilgendorfi*. Future ecological studies, including assessment of the reproductive system by genetic techniques, are therefore possible for pheretimoid earthworms.

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