European Journal of Soil Biology 88 (2018) 41–47

Contents lists available at ScienceDirect

European Journal of Soil Biology

journal homepage: www.elsevier.com/locate/ejsobi

Hybridisation and species delimitation of Scandinavian *Eisenia* spp. (Clitellata: Lumbricidae)

Svante Martinsson, Christer Erséus*

Department of Biological & Environmental Sciences, University of Gothenburg, Box 463, SE-405 30, Göteborg, Sweden

ARTICLE INFO

Handling editor: Stefan Schrader

Keywords:
Earthworms
COI
28S
Histone 3
DNA barcoding
Oligochaeta

ABSTRACT

The earthworms *Eisenia fetida* and *E. andrei* are closely related and can hybridise under laboratory conditions, but it is not known if they hybridise under more natural conditions. The two species are genetically well separated, but within *E. fetida* there is also a deep split forming two well separated mitochondrial lineages. In the present study, 69 *Eisenia* worms from 23 outdoor (or indoor) composts or other human affected habitats in Sweden and Norway are analysed, using three molecular markers, Cytochrome C Oxidase subunit I (COI), the large 28S ribosomal subunit (28S), and Histone 3 (H3). We confirmed that *E. fetida* and *E. andrei* are separate species and, in most cases, are separated by both mitochondrial and nuclear markers, and that the two lineages of *E. fetida* indeed comprise a single, panmictic species despite the deep mitochondrial divergence. We did find evidence of historical hybridisation between *E. andrei* and *E. fetida*, but only in four of the 69 specimens studied.

1. Introduction

The two closely related earthworms *Eisenia fetida* (Savigny, 1826 [1]) and *E. andrei* Bouché, 1972 [2] (family Lumbricidae) are commonly used as models in ecotoxicology and physiology [e.g., 3,4] as well as for vermicomposting [e.g.,5]. The species are genetically well separated [6–10], and differ in colouration; *E. fetida* is striped with pale bands around the intersegmental furrows, whereas *E. andrei* is more uniformly reddish [11]. However, Latif et al. [9] found that some *E. andrei* in Iran, identified by DNA-barcoding, were striped like *E. fetida*, questioning the usefulness of this character for separation of the two species. Within *E. fetida*, mitochondrial markers provide evidence for two well separated clades, suggesting further speciation [e.g., 7], whereas in laboratory cultures and more natural European populations of *Eisenia*, no such clear subdivision has been found in *E. andrei* [7,8,12]. In Iran, however, there are several distinct mitochondrial lineages within *E. andrei*, although the genetic distances between them are smaller than between the two lineages of *E. fetida* [9].

Hybridisation between *E. fetida* and *E. andrei* has been found under laboratory conditions [6,12], and Plytycz et al. [12] even found that some of the hybrids were fertile, when back-crossed with non-hybrid specimens. Domínguez et al. [13], on the other hand, failed to produce hybrids between wild caught individuals of the two species, and it is not known how common hybridisation is in more natural habitats.

Growth rates and cocoon production are generally higher in *E. andrei* than in *E. fetida* [12,14], and in mixed populations *E. andrei* dominates when food is abundant, whereas *E. fetida* dominates when food is scarce [13]. In Scandinavia, *E. andrei* is the more common of the two species [6; CE unpublished data].

The aims of this study are to examine possible hybridisation between *E. fetida* and *E. andrei* in Scandinavian populations and to test if the two distinct lineages within *E. fetida* are separate species or not.

2. Material and methods

2.1. Specimens, DNA extraction and amplification

In total, 69 specimens of *Eisenia* spp. from 23 localities in Norway and Sweden were included in the study (Table 1). As colour and stripe patterns do not clearly separate the species [see 9], the specimens were grouped based on their COI sequences; for details, see below.

DNA was extracted from a small piece of the body wall taken from the posterior part of each specimen. The DNA was extracted either using Epicentre's QuickExtract DNA Extraction Solution 1.0 or Qiagen's DNeasyBlood & Tissue Kit. Three genetic markers, the mitochondrial Cytochrome C Oxidase subunit I (COI), and the nuclear Large 28S Ribosomal Subunit (28S) and Histone 3 (H3), were amplified using the primers and programs listed in Table S1; for amplification of 28S two alternative primer pairs were used. PCR was carried out using Red Taq DNA Polymerase Master Mix (VWR, Haasrode, Belgium) in 25 µL reactions. To confirm amplification, the PCR products were run on a 1% agarose gel, and purified using ExoTAP (Exonuclease I and FastAP

* Corresponding author.
E-mail address: christer.erseus@bioenv.gu.se (C. Erséus).

https://doi.org/10.1016/j.ejsobi.2018.06.003
Received 7 May 2018; Received in revised form 15 June 2018; Accepted 15 June 2018

Table 1
List of material included in this study, with specimen identification numbers, museum voucher numbers, GenBank accession numbers, collection locality data, and GPS coordinates. Specimens in **bold** are of hybrid origin, the number after the species name (*E. fetida*) refers to the mt-lineage. Note that many specimens have allelic variation in the 28S and H3 loci; hence double GenBank nos. for these specimens.

Spm no.	Museum voucher no.	Species	Accession nos.			Collection locality	Coordinates		Coll. date	Leg.
			COI	28S	H3		N	E		
CE2325	SMNH169208	*Eisenia andrei*	MH475664	MH475725/ MH475726	MH475840/ MH475841	SWE: Västergötland, Vårgårda, Fly	57.9969	12.5867	7 June 2003	C. Erséus
CE2873	**SMNH169209**	***E. andrei* x *fetida***	MH475669	MH475737	MH475858/ MH475859	SWE: Södermanland, Vingåker, Österåker	59.0864	16.0546	31 July 2007	C. Erséus
CE2874	SMNH169210	*E. andrei*	MH475670	MH475738	MH475860/ MH475861	SWE: Södermanland, Vingåker, Österåker	59.0864	16.0546	31 July 2007	C. Erséus
CE3045	SMNH169211	*E. andrei*	MH475671	MH475739	MH475862/ MH475863	SWE: Södermanland, Vingåker, Österåker	59.0875	16.0872	31 July 2007	C. Erséus
CE3046	SMNH169212	*E. andrei*	MH475672	MH475740/ MH475741	MH475864/ MH475865	SWE: Södermanland, Vingåker, Österåker	59.0875	16.0872	31 July 2007	C. Erséus
CE5141	SMNH169213	*E. andrei*	MH475673	MH475751	MH475878/ MH475879	SWE: Bohuslän, Strömstad, Tjärnö	58.8869	11.1442	6 Oct 2008	C. Erséus
CE6342	**SMNH169214**	***E. andrei* x *fetida***	MH475674	MH475757	MH475889/ MH475890	SWE: Uppland, Uppsala, Uppsala Botanical Garden	59.8495	17.6287	4 June 2009	C. Erséus
CE6343	**SMNH169215**	***E. andrei* x *fetida***	MH475675	MH475758	MH475891/ MH475892	SWE: Uppland, Uppsala, Uppsala Botanical Garden	59.8495	17.6287	4 June 2009	C. Erséus
CE12505	SMNH169216	*E. andrei*	MH475641	MH475682/ MH475683	MH475777/ MH475778	SWE: Västergötland, Göteborg, Göteborg Botanical Garden	57.6775	11.9544	25 Jun 2011	C. Erséus
CE13032	ZMBN109358	*E. andrei*	MH475642	MH475686	MH475781/ MH475782	NOR: Møre og Romsdal, Volda, Volda	62.1469	06.0716	25 Aug 2011	E. Willassen & C. Erséus
CE13034	ZMBN109359	*E. andrei*	MH475643	MH475687	MH475783/ MH475784	NOR: Møre og Romsdal, Volda, Volda	62.1469	06.0716	25 Aug 2011	E. Willassen & C. Erséus
CE13557	SMNH169217	*E. andrei*	MH475644	MH475695	MH475793/ MH475794	SWE: Västergötland, Göteborg, Kviberg Cemetery	57.7439	12.0120	14 Oct 2011	C. Erséus
CE13558	SMNH169218	*E. andrei*	MH475645	MH475696	MH475795/ MH475796	SWE: Västergötland, Göteborg, Kviberg Cemetery	57.7439	12.0120	14 Oct 2011	C. Erséus
CE13612	SMNH169219	*E. andrei*	MH475646	MH475697	MH475797/ MH475798	SWE: Västergötland, Vårgårda, Nårunga	57.9253	12.7983	22 Oct 2011	C. Erséus
CE13945	SMNH161292	*E. andrei*	MH475647	MH475698	MH475799/ MH475800	SWE: Södermanland, Vingåker, Österåker	59.0864	16.0544	1 Jan 2012	E. Boräng
CE13946	SMNH169221	*E. andrei*	MH475648	–	MH475801/ MH475802	SWE: Södermanland, Vingåker, Österåker	59.0864	16.0544	1 Jan 2012	E. Boräng
CE13947	SMNH169222	*E. andrei*	MH475649	MH475699/ MH475700	MH475803/ MH475804	SWE: Södermanland, Vingåker, Österåker	59.0864	16.0544	1 Jan 2012	E. Boräng
CE13949	SMNH169223	*E. andrei*	MH475650	MH475702	MH475807/ MH475808	SWE: Södermanland, Vingåker, Österåker	59.0864	16.0544	1 Jan 2012	E. Boräng
CE13950	SMNH169224	*E. andrei*	MH475651	MH475703	MH475809/ MH475810	SWE: Södermanland, Vingåker, Österåker	59.0864	16.0544	1 Jan 2012	E. Boräng
CE16397	ZMBN109361	*E. andrei*	MH475652	MH475706/ MH475707	MH475813/ MH475814	NOR: Akershus, Nittedal, Slattum	60.0278	10.8944	20 Aug 2012	C. Erséus
CE16398	ZMBN109362	*E. andrei*	MH475653	MH475708/ MH475709	MH475815/ MH475816	NOR: Akershus, Nittedal, Slattum	60.0278	10.8944	20 Aug 2012	C. Erséus
CE16399	ZMBN109363	*E. andrei*	MH475654	MH475710	MH475817/ MH475818	NOR: Akershus, Nittedal, Slattum	60.0278	10.8944	20 Aug 2012	C. Erséus
CE16452	SMNH169225	*E. andrei*	MH475655	–	–	SWE: Öland, Mörbylånga, N. Kvinneby	56.5378	16.6073	11 Oct 2012	S. Martinsson
CE16453	SMNH169226	*E. andrei*	MH475656	MH475711	MH475819/ MH475820	SWE: Öland, Mörbylånga, N. Kvinneby	56.5378	16.6073	11 Oct 2012	S. Martinsson
CE16539	ZMBN109364	*E. andrei*	MH475657	MH475712	MH475821/ MH475822	NOR: Oslo, Tøyen, University Botanical Garden	59.921	10.771	10 Oct 2012	C. Erséus, S. Martinsson & Y. Liu
CE16540	ZMBN109365	*E. andrei*	MH475658	MH475713/ MH475714	MH475823/ MH475824	NOR: Oslo, Tøyen, University Botanical Garden	59.921	10.771	10 Oct 2012	C. Erséus, S. Martinsson & Y. Liu

(*continued on next page*)

Table 1 (*continued*)

Spm no.	Museum voucher no.	Species	Accession nos.			Collection locality	Coordinates		Coll. date	Leg.
			COI	28S	H3		N	E		
CE16541	ZMBN109366	*E. andrei*	MH475659	MH475715	MH475825/ MH475826	NOR: Oslo, Tøyen, University Botanical Garden	59.921	10.771	10 Oct 2012	C. Erséus, S. Martinsson & Y. Liu
CE16542	ZMBN109367	*E. andrei*	MH475660	MH475716	MH475827/ MH475828	NOR: Oslo, Tøyen, University Botanical Garden	59.921	10.771	10 Oct 2012	C. Erséus, S. Martinsson & Y. Liu
CE16556	ZMBN109368	*E. andrei*	MH475661	MH475717/ MH475718	MH475829/ MH475830	NOR: Oslo, Tøyen, University Botanical Garden	59.921	10.771	10 Oct 2012	C. Erséus, S. Martinsson & Y. Liu
CE21288	ZMBN125737	*E. andrei*	MH475662	MH475723	MH475836/ MH475837	NOR:Vest-Agder, Lyngdal, Lene	58.1355	7.1816	12 May 2014	C. Erséus & M. Klinth
CE21289	ZMBN125738	*E. andrei*	MH475663	MH475724	MH475838/ MH475839	NOR:Vest-Agder, Lyngdal, Lene	58.1355	7.1816	12 May 2014	C. Erséus & M. Klinth
CE25872	ZMBN125739	*E. andrei*	MH475665	MH475727	MH475842/ MH475843	NOR: Sør-Trøndelag, Trondheim, Nordre Hoem	63.405	10.383	18 Nov 2014	C. Erséus
CE25879	ZMBN125740	*E. andrei*	MH475666	MH475728	MH475844/ MH475845	NOR: Sør-Trøndelag, Trondheim, Nordre Hoem	63.405	10.383	18 Nov 2014	C. Erséus
CE26534	ZMBN125741	*E. andrei*	MH475667	MH475734	MH475854/ MH475855	NOR: Sogn og Fjordane, Sogndal, Nagløyri	61.2666	7.1617	14 Aug 2015	C. Erséus
CE26535	ZMBN125742	*E. andrei*	MH475668	MH475735/ MH475736	MH475856/ MH475857	NOR: Sogn og Fjordane, Sogndal, Nagløyri	61.2666	7.1617	14 Aug 2015	C. Erséus
CE5189	SMNH169227	*E. fetida* 1	MH475623	MH475752	MH475880/ MH475881	SWE: Bohuslän, Strömstad, Tjärnö	58.8778	11.1475	8 Oct 2008	C. Erséus
CE5192	SMNH169228	*E. fetida* 1	MH475624	MH475753	MH475882	SWE: Bohuslän, Strömstad, Tjärnö	58.8778	11.1475	8 Oct 2008	C. Erséus
CE5193	SMNH169229	*E. fetida* 1	MH475625	MH475754	MH475883/ MH475884	SWE: Bohuslän, Strömstad, Tjärnö	58.8778	11.1475	8 Oct 2008	C. Erséus
CE5194	SMNH169230	*E. fetida* 1	MH475626	MH475755	MH475885/ MH475886	SWE: Bohuslän, Strömstad, Tjärnö	58.8778	11.1475	8 Oct 2008	C. Erséus
CE5195	SMNH160231	*E. fetida* 1	MH475627	MH475756	MH475887/ MH475888	SWE: Bohuslän, Strömstad, Tjärnö	58.8778	11.1475	8 Oct 2008	C. Erséus
CE12339	SMNH169232	*E. fetida* 1	MH475607	MH475676	MH475765/ MH475766	SWE: Värmland, Arvika, Gunnarskog	59.7612	12.6174	29 July 2011	C. Erséus
CE12340	SMNH169233	*E. fetida* 1	MH475608	MH475677	MH475767/ MH475768	SWE: Värmland, Arvika, Gunnarskog	59.7612	12.6174	29 July 2011	C. Erséus
CE12341	SMNH169234	*E. fetida* 1	MH475609	MH475678	MH475769/ MH475770	SWE: Värmland, Arvika, Gunnarskog	59.7612	12.6174	29 July 2011	C. Erséus
CE12342	SMNH169235	*E. fetida* 1	MH475610	MH475679	MH475771/ MH475772	SWE: Värmland, Arvika, Gunnarskog	59.7612	12.6174	29 July 2011	C. Erséus
CE12343	SMNH169236	*E. fetida* 1	MH475611	MH475680	MH475773/ MH475774	SWE: Värmland, Arvika, Gunnarskog	59.7612	12.6174	29 July 2011	C. Erséus
CE12344	SMNH169237	*E. fetida* 1	MH475612	MH475681	MH475775/ MH475776	SWE: Värmland, Arvika, Gunnarskog	59.7612	12.6174	29 July 2011	C. Erséus
CE13211	ZMBN125743	*E. fetida* 1	MH475613	MH475688/ MH475689	MH475785/ MH475786	NOR: Buskerud, Hol, Sudndalen	60.6696	07.9939	26 Aug 2011	C. Erséus
CE13212	ZMBN125744	*E. fetida* 1	MH475614	MH475690	MH475787/ MH475788	NOR: Buskerud, Hol, Sudndalen	60.6696	07.9939	26 Aug 2011	C. Erséus
CE13214	ZMBN125745	*E. fetida* 1	MH475615	MH475693/ MH475694	MH475791/ MH475792	NOR: Buskerud, Hol, Sudndalen	60.6696	07.9939	26 Aug 2011	C. Erséus
CE16396	ZMBN109373	*E. fetida* 1	MH475616	MH475704/ MH475705	MH475811/ MH475812	NOR: Akershus, Nittedal, Slattum	60.0278	10.8944	20 Aug 2012	C. Erséus
CE19158	ZMBN125746	*E. fetida* 1	MH475617	MH475719	MH475831	NOR: Buskerud, Hol, Sudndalen	60.6696	07.9939	26 Aug 2011	C. Erséus
CE19159	ZMBN125747	*E. fetida* 1	MH475618	MH475720	MH475832/ MH475833	NOR: Buskerud, Hol, Sudndalen	60.6696	07.9939	26 Aug 2011	C. Erséus
CE26043	SMNH169238	*E. fetida* 1	MH475619	MH475729	MH475846/ MH475847	Västergötland, Ale, Alafors	57.9310	12.1042	7 May 2015	C. Rhodén

(*continued on next page*)

Table 1 (*continued*)

Spm no.	Museum voucher no.	Species	Accession nos.			Collection locality	Coordinates		Coll. date	Leg.
			COI	28S	H3		N	E		
CE26044	SMNH169239	*E. fetida* 1	MH475620	MH475730	MH475848/ MH475849	Västergötland, Ale, Alafors	57.9310	12.1042	7 May 2015	C. Rhodén
CE26045	SMNH169240	*E. fetida* 1	MH475621	MH475731	MH475850/ MH475851	Västergötland, Ale, Alafors	57.9310	12.1042	7 May 2015	C. Rhodén
CE26533	ZMBN125748	*E. fetida* 1	MH475622	MH475732/ MH475733	MH475852/ MH475853	NOR: Sogn og Fjordane, Sogndal, Nagløyri	61.2666	7.1617	14 Aug 2015	C. Erséus
CE4785	**SMNH169241**	***E. fetida* 2 x *andrei***	MH475631	MH475742	MH475866/ MH475867	SWE: Norrbotten, Överkalix, Södra Sandsjärv	66.3367	22.7158	2 July 2008	E-B. Elinsdotter & O. Adolfsson
CE4786	SMNH169242	*E. fetida* 2	MH475635	MH475743/ MH475744	MH475868/ MH475869	SWE: Norrbotten, Överkalix, Södra Sandsjärv	66.3367	22.7158	2 July 2008	E-B. Elinsdotter & O. Adolfsson
CE4787	SMNH169243	*E. fetida* 2	MH475636	MH475745	MH475870/ MH475871	SWE: Norrbotten, Överkalix, Södra Sandsjärv	66.3367	22.7158	2 July 2008	E-B. Elinsdotter & O. Adolfsson
CE4788	SMNH169244	*E. fetida* 2	MH475637	MH475746	MH475872/ MH475873	SWE: Norrbotten, Överkalix, Södra Sandsjärv	66.3367	22.7158	2 July 2008	E-B. Elinsdotter & O. Adolfsson
CE4790	SMNH169245	*E. fetida* 2	MH475638	MH475747/ MH475748	MH475874/ MH475875	SWE: Norrbotten, Överkalix, Södra Sandsjärv	66.3367	22.7158	2 July 2008	E-B. Elinsdotter & O. Adolfsson
CE4792	SMNH169246	*E. fetida* 2	MH475639	MH475749/ MH475750	MH475876/ MH475877	SWE: Norrbotten, Överkalix, Södra Sandsjärv	66.3367	22.7158	2 July 2008	E-B. Elinsdotter & O. Adolfsson
CE8347	SMNH169247	*E. fetida* 2	MH475628	MH475759/ MH475760	MH475893/ MH475894	SWE: Jämtland, Strömsund, Stora Blåsjön	64.8268	14.1278	16 Jun 2010	C. Erséus
CE8348	SMNH169248	*E. fetida* 2	MH475630	MH475761/ MH475762	MH475895/ MH475896	SWE: Jämtland, Strömsund, Stora Blåsjön	64.8268	14.1278	16 Jun 2010	C. Erséus
CE8349	SMNH169249	*E. fetida* 2	MH475640	MH475763/ MH475764	MH475897/ MH475898	SWE: Jämtland, Strömsund, Stora Blåsjön	64.8268	14.1278	16 Jun 2010	C. Erséus
CE12844	ZMBN109369	*E. fetida* 2	MH475633	MH475684/ MH475685	MH475779/ MH475780	NOR: Sogn og Fjordane, Luster, Gaupne	61.4026	07.2995	23 Aug 2011	E. Willassen & C. Erséus
CE13213	ZMBN125749	*E. fetida* 2	MH475629	MH475691/ MH475692	MH475789/ MH475790	NOR: Buskerud, Hol, Sudndalen	60.6696	07.9939	26 Aug 2011	C. Erséus
CE13948	SMNH169250	*E. fetida* 2	MH475634	MH475701	MH475805/ MH475806	SWE: Södermanland, Vingåker, Österåker	59.0864	16.0544	1 Jan 2012	E. Boräng
CE19160	ZMBN125750	*E. fetida* 2	MH475632	MH475721/ MH475722	MH475834/ MH475835	NOR: Buskerud, Hol, Sudndalen	60.6696	07.9939	26 Aug 2011	C. Erséus

Thermosensitive Alkaline Phosphatase) [15]. Sequencing was carried out by Eurofins MWG Operon (Ebersberg, Germany) or Macrogen (Geumcheon-Gu, Seoul, Korea). Sequences were assembled into consensus sequences using Geneious v.8.1.9 (Biomatters Ltd., Auckland, New Zealand). The sequences of each marker were aligned using MAFFT v7.017 [16] as implemented in Geneious. In the H3 and 28S datasets, several individuals showed clear signs of heterozygosity, i.e., distinct double peaks at certain positions in the chromatograms. Due to this, we separated the H3, and 28S alleles using the PHASE algorithm [17,18] as implemented in DNAsp v.5.10 [19], the phasing was run for 100 iterations after 100 initial burn-in iterations, with a thinning interval of 1 using default settings. For homozygous specimens only one of the two identical alleles was kept. The phased datasets were used in all subsequent analyses. The alignments of the protein coding COI and H3 were translated into amino acids and checked for stop-codons. All sequences are deposited in GenBank; see Table 1 for accession numbers.

2.2. Distance analysis and clustering of specimens

COI is the recommended barcoding gene for the identification of animal species [20], and was used to divide the specimens into barcoding clusters (=putative species). Uncorrected genetic p-distances were calculated for the COI dataset in MEGA 6 [21]. The specimens were divided based on the existence of a barcoding-gap, i.e., when the COI distances within a group are clearly smaller than the distances between this group and the closest other group. This was done by visual inspection of the distances, as there was only one large (> 0.01) clear gap in the dataset. In total, three groups were found, one corresponding with *E. andrei*, and two within *E. fetida* (*fetida* 1, *fetida* 2); these clusters were named in accordance with Römbke et al. [8]. These groups were used as input species in the analyses accounted for in 2.4.

2.3. Haplotype networks

To visualize haplotype diversity, haplotype networks were constructed for all three markers in PopART v1 [22] using statistical parsimony [23,24].

2.4. Multi-locus species delimitation

The two nuclear markers (28S and H3) were included in a multilocus species delimitation analysis using BPP v.3.3 [25]. The COI dataset was not included as it was used to divide the dataset into groups, and therefore matches the groups found by default. Joint Bayesian species delimitations and species tree estimations were conducted, a method using the multispecies coalescent model to compare different arrangements of species delimitation and species phylogeny in a Bayesian framework, accounting for incomplete lineage sorting due to ancestral polymorphism and gene tree-species tree conflicts [26–28]. Three analyses (A-C) with different population size (θs) and divergence time (τ0) priors, were preformed, using the same settings and priors as in Martinsson and Erséus [29] (A: θ 2,400, τ0 2200; B: θ 2,1000, τ0 2200; C: θ 2,2000, τ0 2200). All analyses were performed three times to confirm consistency between runs. We considered species delimited with a PP > 0.90 in all analyses to be well supported. For clusters with a PP < 0.90, we accepted the best-supported more inclusive species.

2.5. Testing for hybridisation

The posterior predictive checking method [30] was used to test if the discordance between H3 and the other two markers in the placement of six sequences from four of our 69 specimens (see 3.2) was caused by hybridisation or incomplete lineage sorting (ILS). The method compares the pairwise genetic distances to gene trees simulated in species trees, to test the probability that the distances observed are caused by ILS alone; i.e., if ILS can be excluded as the cause of discordance, hybridisation is the main source of this kind of differences between trees. This method is implemented in the software JML [31], and uses the posterior distribution of species trees estimated in *BEAST [32], as implemented in the BEAST software [33,34]. Species trees were estimated with the mismatched sequences included. The sequences were divided into the species *E. fetida* and *E. andrei* as delimited by BPP (see 3.3). Each marker was given its own HKY + Γ substitution model, and empirical base frequencies were used. The Yule process speciation prior, and the piecewise linear with constant root population size prior were used, and the population size (ploidy level) of COI was set to half of that of H3 and 28S. Strict clocks were used, the rate was estimated for all markers, using normal distributed priors with a mean of 0.1 and SD of 0.05 for COI, mean 0.01, SD 0.05 for 28S, and mean 0.02 and SD 0.05 for H3 for the clock rate. The length of the species tree was set to one using a strong normally distributed prior (mean 1, SD 0.01) for the tmrca (time to most recent common ancestor) for all taxa. For species population mean and mean growth rate priors, an exponential distribution with mean 1 was used. For all other priors, default settings were used. The analysis was run for 100 million generations, sampling every 10,000 generations. Tracer v1.6 was used for examining effective sample size (ESS) for parameters and determining burn-in.

We compared the genetic distances from 1000 gene trees, simulated under species trees from the posterior distribution of the *BEAST analysis, with a burn-in of 10%, to the pairwise genetic distances of the H3 dataset, the mismatching specimens being placed according to their COI and 28S sequences. We used the mean clock rate and heredity scalar for H3 from the *BEAST analysis. The results were evaluated using a significance level of $P \geq 0.01$. If the specimens are of hybrid origin we expect significantly shorter distances than those to be expected by ILS alone.

3. Results

COI was successfully sequenced for all specimens, whereas H3 could not be obtained for one specimen, and 28S not for two. After phasing and trimming, the alignments, respectively, consisted of 89 sequences and were 573 bp long for 28S, 134 sequences and 328 bp for H3, and 69 sequences and 588 bp for COI. No stop codons were found in COI or H3, and no non-synonymous substitutions were found in COI. In H3, 11 sequences differed by 1–2 synonymous substitutions compared with the most common amino acid sequence found in the other 123 sequences. However, these substitutions were mostly autapomorphic events among specimens within the groups, and there was no general amino acid separation between the three main groups found in COI (see 3.1).

3.1. Distance analysis and clustering of specimens

The uncorrected p-distances in the COI dataset varied from 0 to 16.6% with a large barcoding gap between 2.0% and 11.4%. Based on the barcoding-gap the sequences were divided into three clusters, one corresponding with *E. andrei*, and two with *E. fetida* 1 and *E. fetida* 2 [sensu 8]. The genetic variation within clusters were low, in *E. andrei* the p-distances varied from 0% to 1.8%, within *E. fetida* 1 from 0 to 2.0%, and in *E. fetida* 2 all sequences were identical. The p-distances between *E. andrei* and *E. fetida* 1 varied from 13.4% to 16.6%, between *E. andrei* and *E. fetida* 2 from 14.5% to 14.8%, and between *E. fetida* 1 and *E. fetida* 2 from 11.4% to 12.6%

3.2. Haplotype networks

In the COI network (Fig. 1A) all lineages form distinct, well separated haplotype groups. However, in both the 28S (Fig. 1B) and H3 networks (Fig. 1C), the sequences of *E. fetida* 1 and *E. fetida* 2 are mixed, and often share haplotypes. In the 28S network there is a clear separation between *E. andrei* and *E. fetida* 1 + 2, but in H3 the division is not as clear, and four sequences of *E. andrei* (two belonging to CE2873,

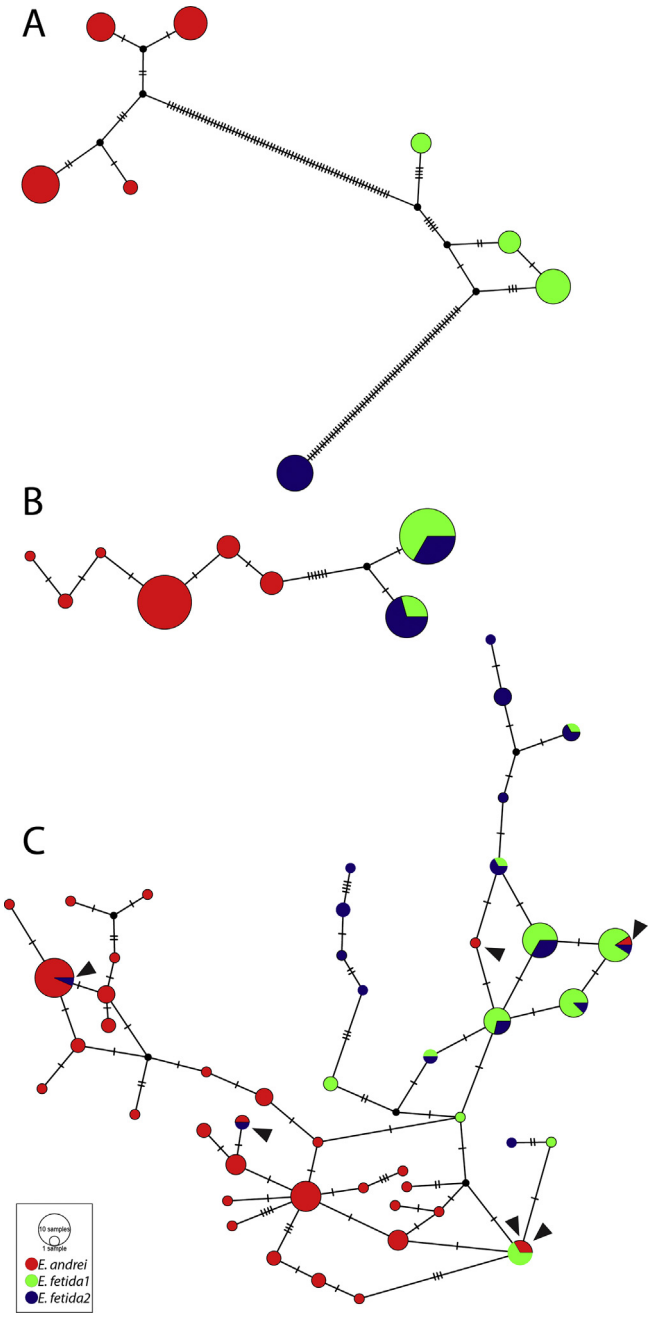

Fig. 1. Statistical parsimony haplotype networks. The size of the circles is relative to the number of sequences sharing that haplotype, the colours correspond to mt lineages, and the hatch marks indicate substitutions. Arrow heads indicate mismatch sequences from specimens of hybrid origin. A. COI network. B. 28S network. C. H3 network. Note that in figures B and C, due to allelic variation, the number of sequences is higher than the number of specimens studied. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and one each from CE6342 and CE6343, respectively) are found within *E. fetida*, and two sequences of *E. fetida* 2 (both belonging to CE4785) are found nested within *E. andrei*.

3.3. Multi-locus species delimitation

All species delimitation analyses support *Eisenia andrei* as a separate species with maximum support, whereas the support for separating *E. fetida* 1 and *E. fetida* 2 varies between analyses. In analysis A, the three species model is preferred with a mean PP of 0.88. In analysis B, the three species model is also preferred, but with a lower mean PP of 0.72. In analysis C, a two species model, combining *E. fetida* 1 and *E. fetida* 2 is preferred with a mean PP of 0.56. Based on the low support for separating *E. fetida* 1 and *E. fetida* 2, the conclusion is that these two groups represent a single species with two divergent mt-lineages.

3.4. Testing for hybridisation

In the JML analysis, 93 pairwise distances were found to be significantly shorter ($P < 0.01$) than what could be explained by ILS alone. All mismatched sequences are found in this set of significantly shorter distances, supporting a hybrid origin of the specimens.

4. Discussion

As mentioned above, hybridisation between *Eisenia fetida* and *E. andrei* under laboratory conditions has been noted before [6,12]. In the present study of non-laboratory animals from Scandinavia, we found evidence for limited hybridisation between the two species too, but only four of our 69 worms bear signs of a hybrid origin. Plytycz et al. [12] only found F1 hybrids with a maternal contribution from *E. andrei*, and only four of seven intra-specific pairs produced offspring at all, and the reproductive success was generally reduced compared to that of intra-specific pairs, indicating the presence of reproductive barriers between the two species. Interestingly, two of the specimens of hybrid origin (CE6342 and CE6343) were found *in copula*. They were found within *E. andrei* in COI and 28S, and have different COI haplotypes, but identical 28S haplotypes, and in H3 both specimens are heterozygous, sharing one haplotype found in *E. fetida*, whereas the other haplotype differs, but is in both cases clustered with *E. andrei*. The identical *E. fetida* H3 haplotype in these two specimens could indicate that the introgression of this haplotype emanates from a single historical hybridisation event.

In our study, there is no discordance between COI and 28S, but instead a few mismatches between H3 and the other two markers. This could be explained by the concerted evolution of the ribosomal genome, resulting in homogenisation, and the removal of introgressed haplotypes from the populations [35]. Although histones are known to be prone to homogenisation too, the lower heterozygosity in 28S compared to H3 (see the haplotype networks, Fig. 1B–C) suggests that, in our case, this process seems to be more severe in 28S.

In Scandinavia, *Eisenia andrei* and *E. fetida* were only found in mixed populations at two localities (see Table 1); an indoor compost in Österåker, Vingåker, Sweden (CE13945-59), and rotting wood in Slattum, Nittedal, Norway (CE16396-99). In both these cases, *E. andrei* seemed to be the most abundant species, but specimens of hybrid origin were not found in these populations.

In other terrestrial clitellates, hybridisation has also been found between species in the *Allobophora chlorotica* complex [36] and the genus *Lumbricus* [37] among the Lumbricidae, as well as in the enchytraeid genera *Hemifridericia* and possibly *Henlea* [29,38].

At one of the Norwegian sites sampled, an outdoor compost in Sudndalen (Hol, Buskerud), at 875 m above sea level, specimens of *E. fetida* 1 and *E. fetida* 2 were found together (see Table 1: CE13211-14, CE19158-60). However, we did not find support for splitting *E. fetida* into two species in any part of our material; haplotypes of both nuclear genes (28S, H3) were mixed in both *fetida* 1 and *fetida* 2 (Fig. 1B–C). Instead it seems that *E. fetida* is just another case of deep intraspecific mt-divergence (between blue and green circles in Fig. 1A), something reported for other earthworm species too [37,39,40]. Pérez-Losada et al. [7], who studied only a limited number of specimens, found that *E. fetida* from Ireland (=*E. fetida* 1) was separated from Spanish populations (=*E. fetida* 2) in both COI and 28S. However, their Spanish 28S haplotype matches one of our haplotypes, and their Irish haplotype is intermediate between the two *E. fetida* haplotypes in our study. Pérez-

Losada et al.'s suggestion of the Irish and Spanish *E. fetida* possibly being two different species was logical, given the data at the time, but the contradictory conclusion of our study highlights the importance of a sufficient sample size in species delimitation analysis, if possible including also different variants from sympatric populations.

Despite the hybridisation between *E. fetida* and *E. andrei*, the two are still well separated species, and no signs of break-down of the species boundaries were noted in the present study. This is in strong contrast with the two mt-lineages of *E. fetida*, which are completely mixed in the nuclear markers, despite that these lineages are almost as well separated in COI as any of them and *E. andrei*.

Acknowledgements

We are grateful to Olle Adolfsson, Eva-Britt Elinsdotter, Egil Boräng, Mårten Klinth, Yingkui Liu, Caroline Rhodén and Endre Willassen, for assisting in the collection of worms; to Anna Ansebo, Per Hjelmstedt, Mårten Klinth, Emilie Lindquist, Maria Lindström, Peter Samson and Marcus Svensson, for molecular lab assistance; and the Swedish Research Council, the Swedish Species Information Centre (ArtDatabanken), the Norwegian Biodiversity Information Centre (Artsdatabanken), the Adlerbert Research Foundation, and the Royal Society of Arts and Sciences in Gothenburg, for financial support.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejsobi.2018.06.003.

References

[1] J.C. Savigny, Analyses de travaux de l'Académie Royale des Sciences pendant l'année 1821, partie physique, Zoologie. Mém Acad Sci Inst France (Hist) 5 (1826) 176–184.
[2] M.B. Bouché, Lombriciens de France: écologie et systématique, I.N.R.A, 1972.
[3] J. Römbke, P. Egeler, Oligochaete worms for ecotoxicological assessment of soils and sediments, in: D.H. Shain (Ed.), Annelids in Modern Biology, Wiley-Blackwell, Hoboken, 2009, pp. 228–241.
[4] D.J. Spurgeon, J.M. Weeks, C.A.M. Van Gestel, A summary of eleven years progress in earthworm ecotoxicology, Pedobiologia 47 (2003) 588–606.
[5] P. Garg, A. Gupta, S. Satya, Vermicomposting of different types of waste using *Eisenia foetida*: a comparative study, Bioresour. Technol. 97 (2006) 391–395.
[6] N. Øien, J. Stenersen, Esterases of earthworms—III. Electrophoresis reveals that *Eisenia fetida* (Savigny) is two species, Comp. Biochem. Physiol. C Comp. Pharmacol. 78 (1984) 277–282.
[7] M. Pérez-Losada, J. Eiroa, S. Mato, J. Domínguez, Phylogenetic species delimitation of the earthworms *Eisenia fetida* (Savigny, 1826) and *Eisenia andrei* Bouché, 1972 (Oligochaeta, Lumbricidae) based on mitochondrial and nuclear DNA sequences, Pedobiologia 49 (2005) 317–324.
[8] J. Römbke, M. Aira, T. Backeljau, K. Breugelmans, J. Dominguez, E. Funke, N. Graf, M. Hajibabaei, M. Perez-Losada, P.G. Porto, R.M. Schmelz, J. Vierna, A. Vizcaino, M. Pfenninger, DNA barcoding of earthworms (*Eisenia fetida/andrei* complex) from 28 ecotoxicological test laboratories, Appl. Soil Ecol. 104 (2016) 3–11.
[9] R. Latif, M. Malek, C. Csuzdi, When morphology and DNA are discordant: integrated taxonomic studies on the *Eisenia fetida/andrei* complex from different parts of Iran (Annelida, Clitellata: Megadrili), Eur. J. Soil Biol. 81 (2017) 55–63.
[10] J. Jaenike, *Eisenia foetida* is two biological species, Megadrilogica 4 (1982) 6–8.
[11] R.W. Sims, B.M. Gerard, Earthworms: Keys and Notes for the Identification and Study of the Species, Brill, London, 1985.
[12] B. Plytycz, J. Bigaj, A. Osikowski, S. Hofman, A. Falniowski, T. Panz, P. Grzmil, F. Vandenbulcke, The existence of fertile hybrids of closely related model earthworm species, *Eisenia andrei* and *E. fetida*, PLoS One 13 (2018) e0191711.
[13] J. Domínguez, A. Velando, A. Ferreiro, Are *Eisenia fetida* (Savigny, 1826) and *Eisenia andrei* (Oligochaeta, Lumbricidae) different biological species? Pedobiologia 49 (2005) 81–87.
[14] S. Jänsch, M.J. Amorim, J. Römbke, Identification of the ecological requirements of important terrestrial ecotoxicological test species, Environ. Rev. 13 (2005) 51–83.
[15] E. Werle, C. Schneider, M. Renner, M. Volker, W. Fiehn, Convenient single-step, one tube purification of PCR products for direct sequencing, Nucleic Acids Res. 22 (1994) 4354–4355.
[16] K. Katoh, K. Misawa, K. Kuma, T. Miyata, MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform, Nucleic Acids Res. 30 (2002) 3059–3066.
[17] M. Stephens, P. Donnelly, A comparison of bayesian methods for haplotype reconstruction from population genotype data, Am. J. Hum. Genet. 73 (2003) 1162–1169.
[18] M. Stephens, N.J. Smith, P. Donnelly, A new statistical method for haplotype reconstruction from population data, Am. J. Hum. Genet. 68 (2001) 978–989.
[19] P. Librado, J. Rozas, DnaSP v5: a software for comprehensive analysis of DNA polymorphism data, Bioinformatics 25 (2009) 1451–1452.
[20] P.D. Hebert, A. Cywinska, S.L. Ball, J.R. deWaard, Biological identifications through DNA barcodes, Proc R Soc Biol Sci Ser B 270 (2003) 313–321.
[21] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6: molecular evolutionary genetics analysis version 6.0, Mol. Biol. Evol. 30 (2013) 2725–2729.
[22] J.W. Leigh, D. Bryant, POPART: full-feature software for haplotype network construction, Methods Ecol Evol 6 (2015) 1110–1116.
[23] M. Clement, Q. Snell, P. Walke, D. Posada, K. Crandall, TCS: estimating gene genealogies, Proc 16th Int Parallel Distrib Process Sym, 2 2002, p. 184.
[24] A.R. Templeton, K.A. Crandall, C.F. Sing, A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation, Genetics 132 (1992) 619–633.
[25] Z. Yang, The BPP program for species tree estimation and species delimitation, Curr Zool 61 (2015) 854–865.
[26] B. Rannala, Z. Yang, Improved reversible jump algorithms for Bayesian species delimitation, Genetics 194 (2013) 245–253.
[27] Z. Yang, B. Rannala, Bayesian species delimitation using multilocus sequence data, Proc. Natl. Acad. Sci. Unit. States Am. 107 (2010) 9264–9269.
[28] Z. Yang, B. Rannala, Unguided species delimitation using DNA sequence data from multiple loci, Mol. Biol. Evol. 31 (2014) 3125–3135.
[29] S. Martinsson, C. Erséus, Cryptic diversity in supposedly species-poor genera of Enchytraeidae (Annelida: Clitellata), Zool. J. Linn. Soc. (2017) zlx084In press https://doi.org/10.1093/zoolinnean/zlx084.
[30] S. Joly, P.A. McLenachan, P.J. Lockhart, A statistical approach for distinguishing hybridization and incomplete lineage sorting, Am. Nat. 174 (2009) E54–E70.
[31] S. Joly, JML: testing hybridization from species trees, Molecular ecology resources 12 (2012) 179–184.
[32] J. Heled, A.J. Drummond, Bayesian inference of species trees from multilocus data, Mol. Biol. Evol. 27 (2010) 570–580.
[33] A.J. Drummond, A. Rambaut, BEAST: bayesian evolutionary analysis by sampling trees, BMC Evol. Biol. 7 (2007) 214.
[34] A.J. Drummond, M.A. Suchard, D. Xie, A. Rambaut, Bayesian phylogenetics with BEAUti and the BEAST 1.7, Mol. Biol. Evol. 29 (2012) 1969–1973.
[35] L.D. Strausbaugh, Concerted evolution, in: S. Brenner, J.H. Miller (Eds.), Encyclopedia of Genetics, Academic Press, 2001, pp. 436–441.
[36] L. Dupont, D. Porco, W.O. Symondson, V. Roy, Hybridization relics complicate barcode-based identification of species in earthworms, Mol Ecol Resour 16 (2016) 883–894.
[37] S. Martinsson, C. Erséus, Cryptic speciation and limited hybridization within *Lumbricus* earthworms (Clitellata: Lumbricidae), Mol. Phylogenet. Evol. 106 (2017) 18–27.
[38] E. Rota, S. Martinsson, C. Erséus, Two new bioluminescent *Henlea* from Siberia and lack of molecular support for *Hepatogaster* (Annelida, Clitellata, Enchytraeidae) (submitted).
[39] I. Giska, P. Sechi, W. Babik, Deeply divergent sympatric mitochondrial lineages of the earthworm *Lumbricus rubellus* are not reproductively isolated, BMC Evol. Biol. 15 (2015) 217.
[40] S. Martinsson, C. Rhodén, C. Erséus, Barcoding gap, but no support for cryptic speciation in the earthworm *Aporrectodea longa* (Clitellata: Lumbricidae), Mitochondrial DNA 28 (2017) 147–155.