Opening Pandora's box: Clitellum in phylogeny and taxonomy of earthworms

(Oligochaeta)

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Abstract. The solution to the current contradictions in earthworm taxonomy and phylogeny is a better understanding of the underlying speciation process. The analysis of size and distribution of clitellar segments and that of tubercula pubertatis in the model homoploid genus *Lumbricus* provides prima facie evidence for the occurrence of the intra-chromosomal autohomoploid hybridization (autohomoploid hybridization = hybridization without changing the ploidy level and taking place between parents from the same panmictic population). The inferred principal mechanism of the autohomoploid hybridization is an unequal crossing recombining paralogous genes organized in a genomic island of divergence (called here "clitellar genomic island of divergence", CGID). Since clitellar segments constitute a prezygotic reproduction barrier and seem to correspond to the underlying genes at CGID on a one-to-one basis, their analysis helps to illuminate the underlying speciation process. The inferred characteristic features of the autohomoploid hybridization in earthworms are: (1) Presence of CGID; (2) Generation of quantitative changes in CGID leading to speciation by means of unequal crossing over (= separation of the process leading to speciation from the rest of genome); (3) Regulated distribution of breaking points; (4) Intra-lineage hybridizations, and (5) Homeotic character of the CGID genes. As far as we know, this is the first case of autohomoploid hybridization described in animals. Probably, it is not exaggerated to conclude that many earthworm evolutionary lineages (species) originated in the process of the described autohomploid hybridization in the CGID or in the process of inter-chromosomal duplications leading to polyploidization of the whole genome. We do not deny the possible existence of allopatric speciation in earthworms caused, for example, by established geographic or behavioural (e.g., assortative mating) barriers to inter-population gene flow. The major consequences of ignoring autohomoploid hybrid speciation in lumbricid earthworms (and presumably in other earthworm families as well) in phylogenetic analyses are: (i) Incorrect inference of phylogenies by applying bifurcating-like phylogenetic analysis instead of reticulate analyses as often seen in the low statistical supports for different clades in constructed bifurcating-like phylogenetic trees and for trees topologies (frequently not even tested). (ii) Misinterpretation of taxonomy and phylogeny by using genetic distance as the sole defining criterion.

Key words. Erthworms, speciation, clitellum, tubercula pubertatis, *Lumbricus*, hybridization.

Introduction

The publication of the tenth volume of "Das Tierreich" (MICHAELSEN 1900) deemed as "Triumph of earthworm taxonomy" (STEPHENSON 1930) provided a conceptual leap in earthworm taxonomy. However, the appearance of contradictions that began possibly with G. E. GATES (1959) culminated in a current "unequal chaos in earthworm taxonomy" (referring to Lumbricidae by BRIONES et al. 2009). To uplift the earthworm taxonomy and phylogeny to higher unity, we attempted to explain the three following ignored or misunderstood phenomena rooted in the speciation process.

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1. Frequently encountered intraspecific variability in number and position of clitellar segments: The change of the positiosn of the clitella, especially if change also includes clitellar segments bearing tubercula, and/or spermathecea, sets up a prezygotic reproduction barrier in biparental (simultaneously hermaphroditic) homoploid or polyploid sexually reproducing earthworms. This happens because a successful exchange of seminal fluid between two earthworm individuals attached one to another in the typical head-to-tail position can only take place if the tubercula position of each individual fits to the location of the spermathecae opening of the partnef (CSUZDI & ZICSI 2003). Here we ask the following question: *Does the occurrence of sympatrically distributed tubercular (e.g., specimens differing in number or position of clitellar segments with expressed tuberculum) and clitellar variants implicate the presence of evolutionary lineage complexes related by common origin?* The alternative way to sexual reproduction in earthworms could be the exchange of spermatophores (BEDDARD 1901, JAMIESON 1988, OMODEO 2000). However, this way of sexual reproduction might have only limited impact since spermatophores are known only in a limited number of earthworm species, and their role in facilitating sexual reproduction in some earthworm species has been disputed (Monroy et al. 2003). In the asexually reproducing earthworm lineages the clitella persist, including segments bearing tubercula pubertatis, probably due to acquisitions of other functions, e.g., facilitating segment regeneration (GATES 1958) and production of cocoons.

2. Bimodal or polymodal intra- and inter-population distribution of body sizes: Existence of bimodal or polymodal inter- and intrapopulation patterns in the total number of body segments was observed and recorded in lumbricids (HOLMSTRUP & SIMONSEN 1996, OMODEO & ROTA 1989, POP 1991). Several lines of evidence indicate that body-pattern evolution (HUGHES & KAUFMAN 2002) and morphological diversification (PICK & HEFFER 2012) are facilitated in Bilatelaria by changes in the expression of Hox genes. The Hox genes determine the identities of body segments along the anteroposterior axis by a spatiotemporal-specific expression (MASTICK et al. 1995). There are no available detailed study of Hox genes in earthworms, but the results obtained from the study of *Perionyx excavatus* indicated the occurrence of 11 paralog groups of which at least four have undergone duplications (CHO et al. 2012). Gene duplications are regarded as the primary mechanism of change among Hox genes (PICK & HEFFER 2012).

3. Lack of statistical robustness for splits on constructed molecular phylogenetic trees: The low statistical support is characteristic for many splits, especially in the basal parts of the constructed bifurcating-like phylogenetic trees (POP et al. 2007, BRIGANDT 2001, JAMES et al**.** 2010, JAMIESON et al. 2002, POP et al. 2003). The low resolution power of phylogenetic analyses is not the result of small sampling sizes only, because repeated analyses on different genes are producing non-consistent results (BRIONES et al. 2009). In addition, growing is the number of cases when two different classically identified species showed very similar or identical sequences of the compared genes. These cases are usually referred as results of wrong identifications of the earthworm specimen from which DNA has been isolated.

Material and methods

Tests of tendencies and trends were done on the regional earthworm assemblage representing the earthworm fauna of Hungary (CSUZDI & ZICSI 2003) (Table 1). As a model taxon for our study of speciation mechanism, we chose the genus *Lumbricus*, the only genus accepted as monophyletic for a long time by earthworm taxonomists (CSUZDI & ZICSI 2003) (see Table 3 for more detailed

Table 1. List of regional earthworm species, represented by earthworm fauna of Hungary, and the sizes and positions of their clitellum and tubercula pubertatis (T) Source of data: CSUZDI & ZICSI (2003). *reported are the most frequent positions and sizes of clitellum and tubercula pubertatis only.

	Beginning of clitellum	No. of clitellar segments*	End of clitellum	Clitellar segments with T*	No. of segments with T
Allolobophora chlorotica	29	9	37	31,33,35	3
A. dacica	29	9	37	29-37	9
A. gestroides	30	11	40	35-40	6
A. hrabei	30	28	57	49-53	5
A. leoni	26	9	34	30, 32	2
A. mehadiensis	36	12	47	42-47	6
A. nematogena	26	8	33	30-32	3
Al. eiseni	25	8	32	0	0
Aporrectodea caliginosa	25	10	34	31-33	3
Ap. georgii	29	6	34	31, 33	2
Ap. handlirschi	27	6	32	28-1/2 32	4
Ap. jassyensis	29	7	35	32-34	3
Ap. longa	28	8	35	32-34	3
Ap. rosea	24	7	32	29-31	3
Ap. sineporis	25	6	30	27-29	3
Ap. dubiosa	37	11	47	44-47	4
Cernosvitovia opisthocystis	25	13	37	25-37	13
Dendrobena auriculata	24	11	34	31-33	3
D. clujensis	28	6	33	30-32	3
D. cognettii	33	4	37	0	0
D. ganglbaueri	24	5	29	25-27	3
D. hortensis	27	6	33	30-1/2 33	3
D. octaedra	29	5	33	31-33	4
D. vejdovskyi	29	5	33	31-32	2
D. veneta	27	6	33	30-31	2
Dendrodrilus rubidus	26	6	31	29-39	2
Eiseniella balatonica	25	6	30	26-29	4
Eisenia fetida	27	4	32	28-30	3
E. lucens	27	6	33	28-31	4
E. spelea	27	6	33	29-31	3
Eiseniella tetraedra	23	4	26	24-25	2
Fitzingeria platyura	25	6	30	26-29	4
F. depressa	25	6	30	26-29	4
F. montana	25	6	30	26-29	4
Helodrilus cernosvitovianus	22	7	29	1/2 26-1/2	1

	Begin-ning of clitellum	No. of clitellar segments*	End of clitellum	Clitellar segments with T*	No. of segments with T
				28	
H. deficiens	26	8	33	30, 31	2
H. mozsaryorum	25	11	35	31-1/2 34	4
Lumbricus baicalensis	28	5	32	29-31	3
L. castaneus	28	6	33	29-32	4
L. polyphemus	39	5	44	40-43	4
L. rubellus	27	6	32	28-31	4
L. terrestris	32	6	37	33-36	4
Octolasion cyaneum	29	6	34	30-33	4
O. lacteum	30	6	35	31-34	4
O. lacteovicinum	29	7	35	1/2 29-1/2 35	5
O. montanum	32	5	36	1/2 32-1/2 36	3
Octodrilus compromisus	29	8	36	29-37	9
Oc. gradinescui	30	9	38	30-38	9
Oc. lissaensioides	29	8	36	29-37	9
Oc. pseudolissaensioides	29	8	36	29-36	8
Oc. transpadanus	30	8	37	30-37	8
Oc. pseudotranspadanus	29	9	37	29-37	9
Octodrioides karawankensis	30	8	37	30-40	11
Proctodrilus antipai	25	9	33	30, 31	2
P. opisthoductus	25	9	33	30-31	2
P. tuberculatus	26	8	33	30/31-31/32	2

information about each species). The validity of obtained speciation model was tested on a case of biclitellate homeosis. As testing case, we chose biclitellate *Lumbricus terrestris* (Gates 1956), the only published case of biclitellate homeosis with enough detailed data in the genus *Lumbricus*. Statistical tests were performed online (WESSA 2012). The hybridization network visualized by means of Dendroscope (HUSON & SCORNAVACCA 2012) (Fig. 2) was constructed by means of the Recomb2007 algorithm (HUSON & KLOEPPER 2007) implemented in SplitsTree (HUSON & BRYANT 2006).

Glossary of terms: Alloploid: A hybrid individual having two sets of chromosomes derived from two different parents. – **Autohomoploid hybrid speciation:** Homoploid speciation within a single parent lineage or species. – **Autopolyploid species:** A species resulting from chromosome doubling within a single parent species. – **Epistasis:** A phenomenon when one or more other genes modify the effects of one gene. – **Haldane's rule**: When in the F1 offspring of two different animal races one sex is absent, rare, or sterile, that sex is the heterozygous (heterogametic) sex. – **Hybrid swarm:** Population of hybrids interconnected by interactions, such as backcrossing and interbreeding, between hybrid individuals. – **Homeotic genes:** "Master control genes" that regulate other sub-ordinate genes to program certain developmental pathways. – **Hybrid dysgenesis:** A syndrome of abnormal traits that is appearing in hybrids after crosses between

certain parental types. – **Homeosis (homoeosis):** Process leading to the transformation of one body part into another body part (Bateson 1894) caused usually by the expression of a mutated homeotic gene. – **Paralogous gene:** Two homologous genes are called paralogous if they diverged after duplication and they are called orthologous if they diverged after speciation (FITCH 1970).

Results

Inter-specific variability in numbers and positions of clitellar segments and of tubercula pubertatis in earthworms

The analysis of the regional earthworm fauna represented by 56 species (Table 1) shows relatively small number of the clitellar segments in the majority of species (Median = 7, Fig. 1). Nevertheless, a relatively large variability in the number of clitellar segments exists (Midrange = 16, absolute range = 24, Fig. 1). The two species with the largest clitella listed in Table 1 are *A. hrabei* with 28 clitellar segments and *C. ophystocistis* with 13 clitellar segments. As we show later, the differences in the number of clitellar segments result from gene duplications. Species with fully functioning duplicated sets of "clitellar genes", e.g., species with duplicated or triplicated basal number of clitellar segments, are easily recognizable. Among the species listed in Table 1, these are for example: *Allolobophora gestroides, A. hrabei*, *A. mehadiensis* and *Cernosvitovia opisthocystis*. The increase in the number of clitellar segments, corresponding to a basal number multiplied by a positive integer, is widely spread in different earthworm families. For example, duplicated or triplicated increases in the basal number of clitellar segments have been observed in Criodrilidae (*Criodrilus ghanyiae* (clitellum with ca. 12 segments), *Biwadrilus bathybates* (clitellum with ca. 19 segments), *Criodrilus lacuum* (clitellum: ca. 31 segments) (BLAKEMORE 2008)). Please, note that part of the clitellar variability recorded in *C. lacuum* and probably in other species of the same genus might be an artifact, caused by increased and decreased numbers of clitellar segments before and after of a very short period of full adult maturity. However, in most hybrid cases, the duplication of the whole set of genes underlying clitellar segments is not accompanied by an increase in number of visible clitellar segments. This happens because out of the both duplicated sets the anterior one has been silenced, as is indicated for example in Table 2. The silenced genes are also excluded from the CGID (see below for explanation).

The average number of clitellar segments, on which tubercula pubertatis is appearing, is smaller than the number of the clitellar segments as expected (median = 3.5). The variability of clitellar segments with tubercula pubertatitis (midrange = 6.5, absolute range = 13) is also smaller in comparison with the variability in all clitellar segments (Fig. 1b,d). Both data sets, e.g., number of clitellar segments and number of tubercula pubertatis, are positively and significantly correlated (N = 56, Pearson correlation, correlation = 0.30, $P = 0.02$ (two-sided test)). However, this significant correlation does not prevent the use of both parameters in the analysis of hereditary pattern because the second parameter, positions of clitellar segments and of tubercula pubertatis, are overlapping but not identical. In fact, in *Lumbricus*, the real prezygotic reproduction barrier is established by a recombinational change in position and number of clitellar segments that usually do not correspond to the full set of clitellar segments (e.g., compare *L. terrestris* (a) and *L. terrestris* (b) in Table 2). Importantly, the distribution of both parameters is unimodal with asymmetric tails (Fig. 1c-d). The bigger tail on the right side (Fig. 1c,d) is expected, partly because earthworm species with none or only one clitellar segment are not known. If one gene would code for a group of clitellar segments

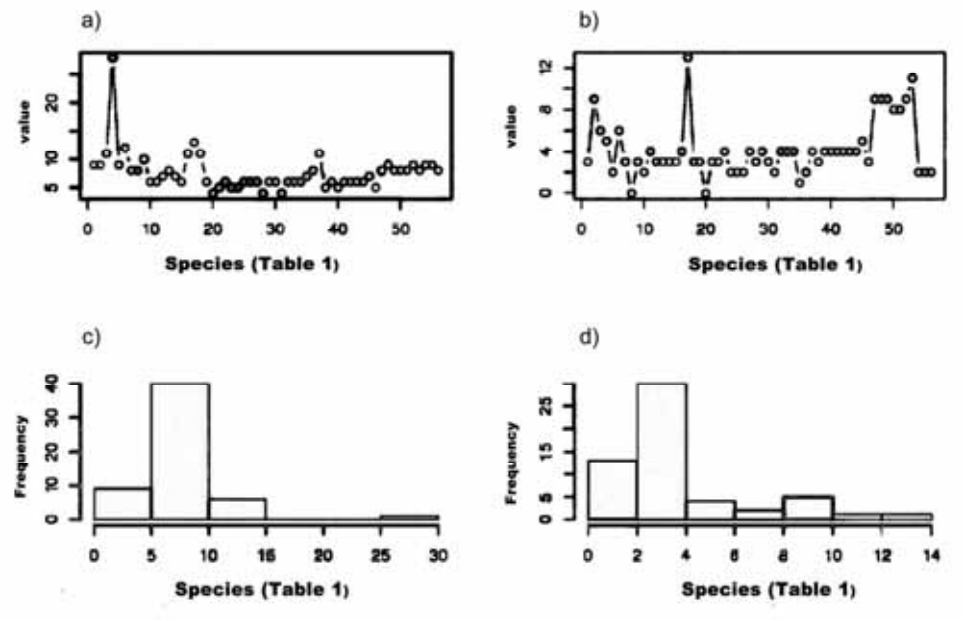

Fig. 1. Plot and histogram of number of clitellar segments (a, c), and number and histogram of segments with tubercula pubertatis (b, d) in 56 species representing regional fauna in Hungary (Table 1).

or segments bearing tubercula pubertatis, e.g., a few segments would be behaving as one block, then one would expect to get polymodal distribution in Fig. 1. The localization of breaking points, around genes corresponding to the first and last clitellar segments, and the preservation of both or at least one of these two segments in hybrids (Table 2), indicate the presence of a clitellar genomic island of divergence (CGID). Again, this points on the homeotic character of the underlying genes because many of them are known to compose compact clusters.

The reconstruction of the recombinational hybridization allows to explain the variability in sizes and positions of clitellar segments and of tubercula pubertatis among the recognized *Lumbricus* lineages (species) (Table 3). By considering the colinearity rule, *L. rubellus*(a) was identified as the most ancestral lineage among the tested lineages (species) because its clitellum begins the most anteriorly, except *L. improvisus* in which clitellum begins at 26 like in *L. rubellus*(a) (Table 2, 3). However, geographically more restricted *L. improvisus* probably originated from the crossing between more widely distributed *L. rubellus x L. rubellus* (Table 2) than vice versa. In addition, a naturally occurring variability in the number and positions of both clitellar and tubercullar segments was recorded in *L. rubellus* but not in *L. improvisus* (Table 1). Table 2 shows that in all *Lumbricus* species, with the exception of *L. rubellus*(a) regarded as the most ancestral lineage, the origin of all species can be tracked directly or indirectly to *L. rubellus*. The mechanism of hybridization is an unequal crossing-over that recombines clitellar segment genes. In our reconstruction (Table 3), all

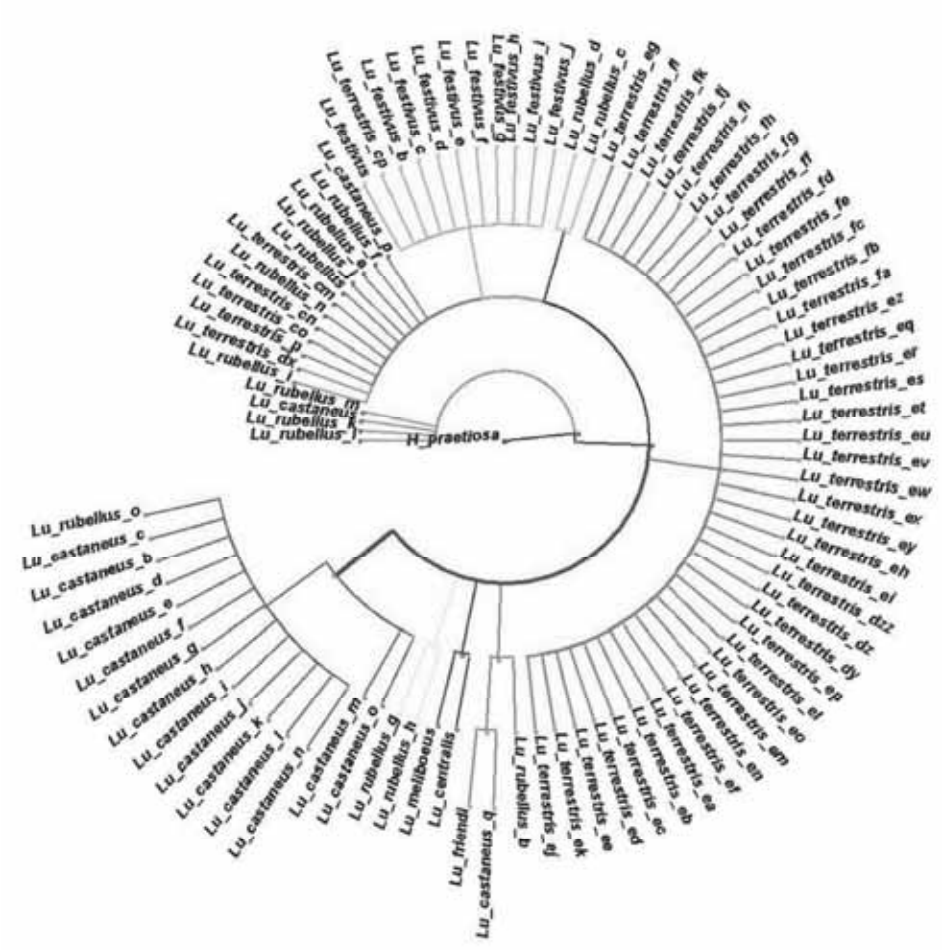

Fig. 2. Rooted circular hybridization network of the aligned (LARKIN et al. 2007, GOUJON et al. 2010) 92 partial (658 bp) COXI sequences (downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank/), details available upon request to authors) of seven *Lumbricus* species and of an outgroup (*Hormogaster praetiosa*). Since support for every edge is 100, the values are not marked in the network.

hybrid lineages (species) corresponding to the current lineages (species) possess the same numbers and positions of clitellar segments and tubercula pubertatis as recorded in reported field observations. Breaking points, where crossing-over operates, appear around the first and the last clitellar segments of the paternal sequences (Table 2). Therefore, the most frequent increase/decrease in the number of clitellar segments in hybrids resulting from autohomploid hybridization would be 2n, 2n-1, 2n+1, and 1n+1, where n = number of clitellar segments in one of the parents.

Table 2. Reconstructed origin of *Lumbricus* lineages (species) by means of recombinational hybridization. Numerals indicate the position of clitellar segment on the earthworm body and the underlying genes associated on the expected one-to-one basis. Visible clitellar segments are marked by grey colour. Positions of the first and last segments in parents and hybrids are underlined. Clitellar segments bearing tubercula pubertatis are marked by bold. Ce: recorded position of clitellum (see also Table 3), Te: recorded position of tubercula pubertatis (see Table 1), ▌: breaking points.

Species	**Parental sequences / Ce, Te**
***L. rubellus* (a)**	(-26-27-28**T**-29**T**-30**T**-31**T**-32-) / Ce: 26-32, Te: 28-31
***L. rubellus* (b)**	(-26-27-28**T**-29**T**-30**T**-31**T**-) / Ce: 26-31, Te: 28-31
***L. rubellus* (c)**	(-27-28**T**-29**T**-30**T**-31**T**-) / Ce: 27-31, Te: 28-31
L. castaneus	(-28-29(**T**)-30(**T**)-31(**T**)-32(**T**)-33-) / Ce: 28-33, Te: 29-32
***L. terrestris* (b)**	(-31-32-33(**T**)-34(**T**)-35(**T**)-36(**T**)-37-) / Ce: 31-37, Te: 33-36
Recombinational hybridization	
***L. rubellus*(a) x *L. rubellus*(a) = *L. badensis* + H2** (nonexistent) (-26-27-**28-29-30-31** ▌-32-) **x** (▌-26-27-**28-29-30-3T**-32-) = (-26-27-28-29-30-31-32(26)-33(27)-**34(28)-35(29)-36(30)-37(31)**-38(32)-) + -32-)	
***L. rubellus*(c) x *L. rubellus*(c) = *L. baicalensis* + *L. rubellus*(d)** (-27 ▌-**28-29-30-31**-) **x** (▌-27-**28-29-30-31**-) = (-27-28(27)-**29(28)-30(29)-31(30)-32(31)**-) + (**-27(28)-28(29)-29(30)-30(31)**-)	
***L. rubellus*(c) x *L. rubellus*(c) = *L. castaneus* + *L. rubellus*(e)** (-27 ▌-**28-29-30-31**-32-) x (▌-27-**28-29-30-31**-32-) = (-27-28(27)-**29(28)-30(29)-31(30)-32(31)**-33(32)-) + (**-27(28)-28(29)-29(30)-30(31)**-31(32)-)	
***L. rubellus*(a) x *L. rubellus*(a) = *L. centralis* + H2** (nonexistent) (-26-27-**28-29-30-31**-32- ▌) x (-26 ▌-27-**28-29-30-31**-32-) = (-26-27-28-29-30-31-32-33(27)-**34(28)-35(29)-36(30)-37(31)**-38(32)-) + (-26-)	
***L. castaneus* x *L. castaneus* = *L. festivus* + H2** (nonexistent) (-28-**29-30-31-32**-33- ▌) x (▌-28-**29-30-31-32**-33)-) = (-28-29-30-31-32-33-34(28)-**35(29)-36(30)-37(31)-38(32)**-39(33)-) + 0 (clitellum absent)	
***L. rubellus*(a) x *L. rubellus*(a) = *L. friendi* + H2 (nonexistent)** (-26-27-**28-29-30-31**-32- ▌) x (-26 ▌-27-**28-29-30-31**-32-) = (-26-27-28-29-30-31-32-33(27)-**34(28)-35(29)-36(30)-37(31)**-38(32)-) + (-26-)..	
***L. rubellus*(a) x *L. rubellus*(a) = *L. improvisus* + H2 (unknown)** (▌-26-27-**28-29-30-31**-32-) x (-26 ▌-27-**28-29-30-31**-32-) = (26(27)-**27(28)-28(29)-29(30)-30(31)**-31(32)) + (26-27(26)-28(27)-**29(28)-30(29)-31(30)-32(31)**-33(32)-))	
I. *L. rubellus*(c) x *L. rubellus*(c) = H1 (unknown) + 0 (no clitellum) **II. H1 x H1 = *L. klarae* + *L. terrestris*(c) (unknown)** I. (-27-**28-29-30-31** ▌-) x (▌-27-**28-29-30-31**-) = (-27-28-29-30-31-32(27)-**33(28)-34(29)-35(30)-36(31)**-) + 0 II. (-32-**33-34-35** ▌-**36**-) x (-32-**33-34-35-36** ▌-) = (-32-**33-34-35**-) + (-32-**33-34-35-36-37**)	

The total body size in terms of the total number of body segments might be related to hybridization. The origin of small species (Table 1) is associated with an increase of the number of clitellar segments by a factor n + 1 (rarely by a factor n-1) where n is the number of clitellar segments in parents (*L. baicalensis*, *L. klarae*, *L. meliobeus, L. castneus*, *L. rubellus* (b, c)). The large species originated by means of gene recombinational duplications of all clitellar segments or almost all segments (*L. badensis*, *L. centralis*, *L. festivus*, *L. friendi*, *L. polyphemus, L. terrestris*). The statistical difference between these two groups is significant (Mann-Whitney test, $P = 0.012$). In addition, the majority of the tested hybrid lineages resulted from hybridizations involving *L. rubellus* (Table 2, Fig. 2). To construct a hybridization network, we chose *Hormogaster speciosa* as the outgroup because of the archaic status of genus *Hormogaster* in comparison with the status of genus *Lumbricus*. It is

Table 3. Species of the genus *Lumbricus* and positions of their clitella, tubercula pubertatis. Please note that the position of spermatecae in all *Lumbricus* species is 9/10, 10/11 cd. Source of data, if not stated otherwise: CSUZDI & ZICSI (2003).

	Position of clitellum	Position of tubercula pubertatis	No of body segments
L. badensis Michaelsen, 1907	31,32-38,39,40 (Brigandt 2001)	34-36, 33-37 (Brigandt 2001)	190-210 (Brigandt 2001)
L. baicalensis Michaelsen, 1900	28-32	29-31	65-105 (Zicsi 1965)
L. castaneus (Savigny, 1826)	28-33	29-32, 29-31 (Zicsi 1965)	55-120 (Zicsi 1965, Lee 1959, Piearce 2007, Edwards & Lofty 1972)
L. centralis Bouché, 1972	1/n 32, 33--38	34--1/2 37	???
L. festivus (Savigny, 1826)	33,34-39 (Edwards & Lofty 1972)	35-38	100-143 (Edwards & Lofty 1972, Michaelsen 1900)
L. friendi Cognetti, 1904	33-1/2 39 (Zicsi 1965)	34-36 (Zicsi 1965)	102-130 (Zicsi 1965, Zicsi & Csuzdi 1999)
L. improvisus Zicsi, 1963	26-31	27-30	???
L. klarae Zicsi & Csuzdi, 1999	32-35 (Zicsi & Csuzdi 1999)	33-35	61-122 (Zicsi & Csuzdi 1999)
L. meliboeus Rosa, 1884	29-33 (Zicsi 1965)	30-32, 33 (Zicsi 1965)	59-124 (Zicsi 1965)
L. polyphemus (Fitzinger, 1833)	37,38,39-43, 44,45,47	37,38,39,40-43, 44,45	90-182 (Zicsi 1965)
L. rubellus Hoffmeister, 1843	26, 27-32	27,28-30,31,32 (Zicsi 1965)	76-145 (Zicsi 1965, Lee 1959, Piearce 2007, Edwards & Lofty 1972)
L. terrestris (Linneaeus, 1758)	31, 32-37	33-36	110-180 (Zicsi 1965, Lee 1959, Piearce 2007, Edwards & Lofty 1972, Sun & Pratt 1931)

clearly seen in Fig. 2 that, as predicted, most of the hybrids are coming from certain lineages of *L. rubellus*. The hybridization network also clearly shows that the same hybrid clusters contain different hybrids originating from the same parental types (named according to the classical species concepts) and that the same nominal earthworm species are composed from hybrids of different origin as regards their parental types and age.

Biclitellate homeosis

In the reported biclitellate *L. terrestris*, the number of body segments and the number of clitellar segments were respectively 196 and 14 (35-38 and 42-51) on the left side of the body and 175 and 11 (32-37 and 40-44) on the right side of the body (GATES 1956). The positions of tubercula pubertatis were 33-36 on the right side of the body and 44-50 on the left side of the body. Similar "monsters" could be produced by irregularities in crossing over during meiosis, especially in the case of polyploid parents (polyploidy is not known in the genus *Lumbricus*), or recombination of non-homologous sequences. In our case, however, only irregular unequal crossing over of the double duplicated clitellar sequence corresponding to *L. terrestris* could explain the observed left/right body side asymmetry in body size (expressed as the total number of body segments) (Table 4). As the comparison of the total number of body segments on both body sides indicates, the meiotic abnormalities were

caused by recombining two sequences of different length (differing by one gene). The proposed scenario is given in Table 4. Please note that the separation of the steps two and three in this table has been made for an illustrative purpose only. We do not expect that the "monstrous" hybrids resulting from the second hybridization existed and were able to reproduce. The steps two and three took place during postmeiotic modifications of DNA in the process called "sister chromatid exchange". Regarding the total number of body segments, the difference between both sides, estimated from the proposed sequences for right and left side, is the same as the difference between the counted numbers of segments (i.e. 21). The parental *L. terrestris* without duplications of the clitellar segments would have probably 168 body segments, which is within the recorded range (Table 3).

Table 4. Reconstructed recombination path from parental sequences of clitellar genes to the observed biclitellate *L. terrestris*.

Step	Recombination
1st duplication - P1 - L. terrestris(a) x P2 - L. terrestris(a) = L. H1 + L. H2	*L. terrestris*(a), -32(27)-33(28T)-34(29T)-35(30T)-36(31T)-37(32)-% x *L. terrestris*(a), %-32(27)-33(28T)-34(29T)-35(30T)-36(31T)-37(32)- = *L.* H1, -32(27)-33(28T)-34(29T)-35(30T)-36(31T)-37(32)-38(32(27))-39(33(28T))-40(34(29T))-41(35(30T))-42(36(31T))-43(37(32))- + *L.* H2, no clitellum.
2nd duplication - P1 - L. H1 x P2 - L. - H1 = L. H3 + L. H4	P1: *L. H1*, -32-33(T)-34(T)-35(T)-36(T)-37-38-39(T)-40(T)-41(T)-42(T)-43-% x *L. H1*, %-32-33(T)-34(T)-35(T)-36(T)-37-38-39(T)-40(T))-41(T))-42(T))-43- = *L.* - H3, -32-33(T)-34(T)-35(T)-36(T)-37-38-39(T)-40(T)-41(T)-42(T)-43-44(32)-45(33T)-46(34T)-47(35T)-48(36T)-49(37)-50(38)-51(39T)-52(40T)-53(41T)-54(42T))-55(43)- + *L.* H4, no clitellum.
2nd duplication - P1 - L. H1 x P2 - L. - H1 = L. H5 + L. H6	*L. H1*, -32-33(T)-34(T)-35(T)-36(T)-37-38-39(T)-40(T)-41(T)-42(T)-43-% x *L. H1*, -32%-33(T)-34(T)-35(T)-36(T)-37-38-39-40(T))-41(T))-42(T))-43- = *L.* - H5, -32-33(T)-34(T)-35(T)-36(T)-37-38-39(T)-40(T)-41(T)-42(T)-43-44(33T)-45(34T)-46(35T)-47(36T)-48(37)-49(38)-50(39T)-51(40T)-52(41T)-53(42T))-54(43)- + *L.* H6, -32- .
Recombination - P1 - L. H3 x P2 - L. H5 = Biclitellate L. terrestris, left side + Biclitellate L. terrestris, right side	%-32-33(T)-34(T)-35(T)%-3(T)-37-38%-39(T)-40(T)-41(T)%-42(T)-43-44(32)-45(33T)-46(34T)-47(35T)-48(36T)-49(37)-50(38)-51(39T)-52(40T)-53(41T)-54(42T))-55(43)- x L. ???(b), %-32-33(T)-34(T)-35(T)-36(T)-37%-38-39(T)-40(T)%-41(T)-42(T)-43-44(33T)%-45(34T)-46(35T)-47(36T)-48(37)-49(38)-50(39T)-51(40T)-52(41T)-53(42T))-54(43)- = Biclitellate *L. terrestris* - left side - H1, -32-33(T)-34(T)-35(T)-36(T)-37-38-39-40-41-42-43-44 + Biclitellate *L. terrestris* + right side - H2, -32-33-34-35-36-37-38-39-40-41-42-43-44(T)-45(T)-46(T)-47(T)-48(T)-49(T)-50(T)-51-52-53-54-55-56-57-58-59-60-61-62-63-64-65-.

Discussion

The growing number of recognized animal hybrid species (DOWLING & SECOR 2012, MALLET 2008, SEEHAUSEN 2004, TWYFORD & ENNOS 2011) invalidates the argument stating that animals are too complex for hybrid speciation to play an important role (MAYR 1963). Though, taxa utilizing the hybridization as an evolutionary orthogonalization strategy (KRÁL 2001) are facing obstacles in the form of hybrid disgenesis (BURTON et al. 2006) and of Haldane's rule (NAISBIT et al. 2002). Haldane's rule is not relevant in earthworms, as well as in many plants, fungi and bacteria, because they do not possess sex chromosomes. Restricting the hybridization process into a genomic island of divergence (NOSIL & FEDER 2012), in which functionally related genes favored by epistatic selection not only tend to occur together but also tend to be inherited together (NEI 2003), limits the detrimental role of hyb-

rid disgenesis on hybrid viability (RAMSEY & SCHEMSKE 2002). Our analysis indicates the presence of such a genomic island of divergence (called "clitellar genomic island of divergence" and abbreviated CGID) in earthworms. The CGID is probably composed of the paralog genes coding for the formation of clitellar segments with or without tubercula pubertatis. Based on the analysis one clitellar segments seems to correspond to one underlying gene. However, the actual number of genes included in CGID might be higher because our analyses indicate that the behaviour of the clitellar segments resemble rather master-control genes than to them subordianted genes. Nevertheless, this allows, by analyzing the changes in numbers and positions of clitellar segments and in tubercula pubertatis, the tracking of the origin of evolutionary lineages (species). Based on our analysis, we inferred the following features of the recombinational autohomoploid hybridization in the genus *Lumbricus*:

1. Presence of CGID. The existence of the clitellar genomic center of divergence is supported by: (i) Unimodal distribution of numbers of clitellar segments and of numbers of the segments with tubercula pubertatis; (ii) The obtained hereditary patterns of clitella and tubercula pubertatis, and (iii) the occurrence of regularities in the distribution of breaking points.

2. Generation of quantitative changes in CGID leading to speciation by means of unequal crossing over. We inferred from the pattern of inheritance of the CGID genes the unequal crossing over as the process generating the observed gene duplications and gene deletions. It should be noted that unequal crossing-over is a common mechanism behind homologous recombinations **(**LADOUKAKIS & ZOUROS 2001, TSAOUSIS et al. 2005**)**, and that the gene duplications are regarded as the primary mechanism of change among Hox genes (PICK & HEFFER 2012).

3. Regulated distribution of breaking points. We observed that in all reconstructed cases of hybrid speciation in genus *Lumbricus*, the breaking points occurred around, i.e., before or after, the CGID genes corresponding to the first anterior and the last posterior clitellar segments. The limitation of the number of potential breaking points might offset the problems with the frequency-dependent minority cytotype disadvantage (HUSBAND 2000) and with the occurrence of meiotic irregularities, and thus increase the chance to successfully establish a new hybrid lineage. However, one should be aware of the fact that evidence pointing to the presence of breaking points inside of the CGID gene sequences is also available, for example in our analysis of the biclitellate *L. terrestris* (Table 4) and the reported hybrid swarm in *L. terrestris.* In the hybrid swarm (GATES 1962), "the clitella in 640 of the worms, identified as *L. terrestris*, were comprised by 4-8 segments and began with xxvii (1 specimen), xxviii (1), xxix (2), xxx (11), xxxi (161), xxxii (409), xxxiii (49), xxxiv (4), xxxvi (1), xxxvii (1)". This means that the clitella began with the expected xxxi and xxxii segments only in 89% of the cases (GATES 1962). Unfortunately, the unknown taxonomic composition of the parental stock and the unknown conditions under which hybridization took place, prevents the drawing of more generalizations. The case of the biclitellate *L. terrestris* rather shows nonviability of the inter-lineage (inter-species) hybrids caused by meiotic irregularities resulting from recombining of two not fully HOMOLOGOUS sequences, in our case differing by one gene. The occurrence of meiotic irregularities also could explain why cases of fully functional duplicated CGID gene sets are rare, and why in the majority of cases part of the duplicated genes is silenced. The appearance of asexual lineages in some earthworm taxa might be an escape route from the harmful effects caused by meiotic abnormalities.

4. Intra-lineage hybridizations. All reconstructed hybridizations in the genus *Lumbricus* took place between two parents representing an identical lineage (Table 2) and in one case

(see *L. terrestris*(b), Table 2) between two lineages representing the same classically determined species. This strategy might lead to saltational changes in hybrid properties (see for example difference in the number of body segments between right and left sides of the biclitellate *L. terrestris* in Table 4) in comparison with parents. This "jump" forces a hybrid to search for a new ecological niche (niche shift) rather than to compete with parents for their niche. Importantly, this mechanism allows for true sympatric speciation in earthworms, e.g., sympatric speciation taking place in a panmictic population (BOLNICK & FITZPATRICK 2007).

5. Homeotic character of the CGID genes. As seen in our analysis, the new set of clitellar genes is positioned posteriorly of the breaking point in the original set of clitellar genes. Only rarely, both duplicated sets are kept to express clitellar segments. This means that visible clitellar segments represent the latest recombined genes, and that information about recombinational history is hidden in the clitellar position along the anteroposterior axis. As shown above, the unequal crossing-over can explain the difference in the number of segments between left and right sides in the biclitellate *L. terrestris*. In Bilatelaria, the identities of body segments along the anteroposterior axis are determined by a spatiotemporal-specific expression of the homeotic genes (MASTICK et al. 1995), and the observed behaviour of the CGID genes might correspond to –, an unusual trait in animals – frequent multiple duplications recorded in different paralog groups of the Hox genes along the anteroposterior axis in the earthworm, *Perionyx excavatus* (CHO et al. 2012).

Consequences of autohomoploid hybridization in earthworms on the inference of phylogeny

In lumbricid earthworms, hybrid speciation seems to be prevalent either in the form of intrachromosomal autohomoploid speciation discussed here or inter-chromosomal allopolyploid speciation discussed elsewhere (VIKTOROV 1997). The extent of autohomoploid hybridization is unknown but, according to similarities in distribution of the variability between size and distribution of clitella in different earthworm taxa, it is widely spread. Also, there are indications of the presence of CGID in both, the alloploids and homoploids. The question is whether allopolyploidy in earthworms results from somatic chromosome doubling of a homoploid hybrid or from polyspermy or through gametic nonreduction (MALLET 2007, RAMSEY & SCHEMSKE 1998).

In hybrid taxa one could expect high rates of speciation resulting from high levels of chromosomal change (BUSH et al. 1977). In earthworms, rapid tempo of speciation – similar to *Helianthus anomalus* in which diploid hybrid genomes are stabilized quickly, after 10-60 generations (UNGERER et al. 1998) – could occur in spite of some contrary opinions (OMODEO 2000). There is a lot of evidence indicating high speed speciation in earthworms: (i) Recorded hybrid swarms; (ii) Shift in position of clitellar segments in earthworms introduced to new regions. For example, the recorded clitellar formula in *L. rubellus* from New Zealand 26, 27-32, 33 (MARTIN 1975), i.e. one position higher posteriorly than the same parameters recorded in other *L. terrestris* in Europe (Table 3). (iii) High lineage diversity, including asexual lineages, present in areas covered by the glaciation shield during the last glacial. (iv) High species diversity and endemism occurring in some hotspots, perhaps, analogically to insects (HADRYS et al. 2012), occurring in taxa with the highest rate of homeobox sequence evolution. The underestimation of the speed of the speciation process might result from the employment of bifurcating-like methods of phylogenetic analysis in the hy-

brid taxon. The speed of the speciation process might not correspond to the observed species richness because the fixation of species also depends on environmental factors, such as the availability of a suitable ecological niche (GROSS & RIESEBERG 2005).

Under the proposed circumstances, the best definition of earthworm species would probably be: distinguishable groups of genotypes that remain distinct in the face of potential or actual hybridization and gene flow (MALLET 1995). Generally, many earthworm species represent such hybrid lineages. Yet, many sexually reproducing lineages that are reproductively separated are probably not recognized as species such as *L. rubellus*(a) and *L. rubellus*(b,c) (Table 2). On the one hand, the analysis of the biclitellate *L. terrestris* indicates that differences in only one gene between otherwise homologous duplicated parental CGID sequences produced a "monster". On the other hand, it might indicate that differences in the number of clitellar segments alone, without differences in the position of tubercula pubertatis, do not establish an enough strong prezygotic reproduction barrier. As a matter of fact, the majority of classical earthworm species includes a variability in number and position of clitellar segments or in spermatecae (difference in position of spermatecae might function as the prezygotic reproduction barrier as well). For example, such variability is recorded in six out of twelve studied species of the genus *Lumbricus* (Table 2). One could ask the question "How many reproductively isolated lineages correspond to the clitellar and tubercular variability 37, 38, 39-43, 44, 45, 47 and 37, 38, 39, 40-43, 44, 45, reported in *L. polyphemus* (Table 3)?". Nevertheless, if reproductively isolated lineages are of recent origin, it might be very difficult to recognize them as established hybrid species because their initial gene frequencies and morphological characters, apart of the CGID genes and morphological characters associated to them, are like the ones of their parents. Alternatively, two lineages after establishment of the secondary contact, might fuse and effectively establish a new species if the reproductive isolation between them is not enough strong.

Conclusion

The presented model of autohomoploid hybrid speciation in earthworms fits to the modern evolutionary synthesis by the formation of the prezygotic reproduction barrier, and to the evo-devo theories by the speed of the speciation process. As a matter of fact, it seems that the rearrangement of a few CGID genes has a strongly expressed phenotypic effect leading to the rapid speciation process that most closely corresponds to the saltational theory of evolution (LASHIN et al. 2012).

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