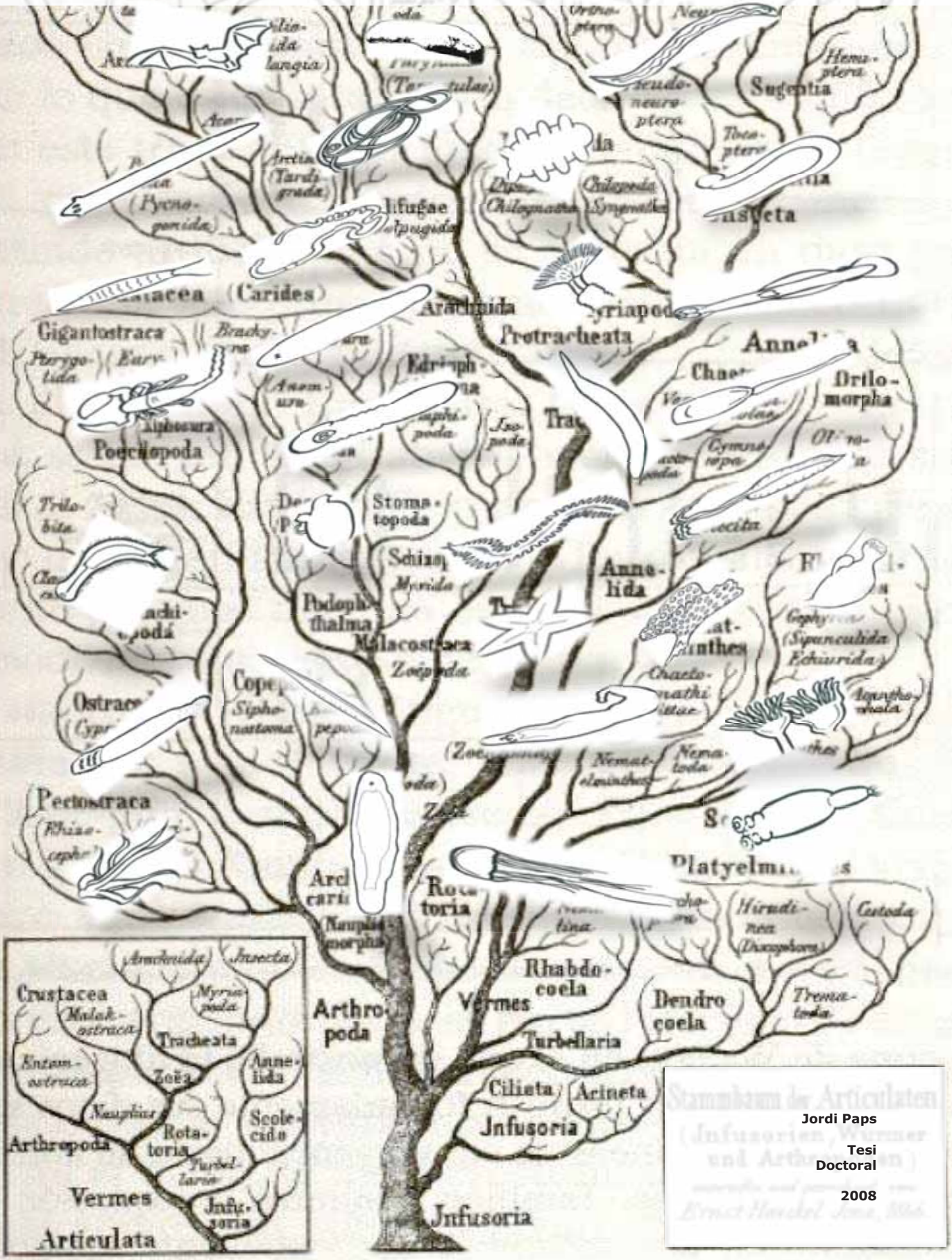
Filogènia Molecular dels Bilaterals: una aproximació multigènica



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Filogènia Molecular dels Bilaterals: una aproximació multigènica

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Apèndix I

Descripció dels fílums bilaterals

Es pretén en aquesta secció donar una pincellada sobre els principals trets que presenten els fílums de bilaterals, per tal de familiaritzar al lector amb les seves característiques. S'ha seguit l'esquema de Hyman per que és senzill i de fàcil organització. Això, però, produeix algunes inconsistències amb els coneixements actuals, com per exemple la situació d'animals acelomats dins de pseudocelomats. Els caràcters que es descriuen són un compendi extret tant de llibres (Valentine 2004; Schmidt-Rhaesa 2007) com d'articles (Zrzavý et al. 1998; Giribet et al. 2000; Schmidt-Rhaesa 2003).

Els antics Acelomats

Els *Platyhelminthes* és el fílum dels **cucs plans** i es consideren els bilaterals amb el pla corporal més simple. Ha estat un grup molt agitat en quant a la seva filogènia, tant a les seves relacions internes com a la seva posició dins dels metazous. Clàssicament s'agrupaven els platihelmints de vida lliure dins de la classe Turbellaria i els paràsits a les classes Trematoda, Monogenea i Cestoda. En canvi, actualment la morfologia reconeix tres grans grups dins dels platihelmints: **catenulats**, **acelomorfs**, i **rabditòfors**.

PLATYHELMINTHES

- 25.000 spp
- Cosmopolites, vida lliure i paràsits
- Acelomats (cos format per parènquima)
- Clivellament espiral (només als *Archoophora*)
- Desenvolupament directe
- Epidermis multiciliada
- No segmentats
- Digestiu cec
- Neoblasts

Els Catenulida són cucs microscòpics aquàtics de vida lliure i en base a la morfologia es disputa la posició com a grup més primitiu de platihelmints amb els acelomorfs (Haszprunar, 1996). Els Acoelomorfa (constituïts per Acoela i Nemertodermatida) també són cucs microscòpics marins i es caracteritzen per les **interconexions entre les arrels dels cilis** de les cèl·lules epidèrmiques; els acelomorfs no tenen **protonefridis**, tenen un sistema nerviós difós i comparteixen un desenvolupament embrionari peculiar anomenat **clivellament espiral en duets**, tot i que l'homologia d'aquest últim tret entre acels i nemertodermàtids no està clara (Jondelius et al, 2004).

Els Rhabditophora comprenen la resta de platihelmints de vida lliure (com les planàries) i tots els paràsits (els Neodermata, a on trobem animals com la tènia i la fasciola). Els catenulats i els rabditòfors més primitius (macrostòmides, haplofaríngides i policlàdides) formen els Archoophora, que presenten **clivellament espiral en quartets**, mentre que a la resta de rabditòfors (Neophora) el desenvolupament està molt modificat degut a la presència de nombroses cèl·lules vitel·lines. Dins dels rabditòfors, alguns membres dels policlàdides passen per **forma larvaria** (larves de Mueller i de Goette), però a la resta el desenvolupament és directe. A diferència dels acelomorfs, catenulats i rabditòfors presenten protonefridis i un sistema nerviós amb cervell. Tots tres grups presenten un tipus cel·lular insòlit, els **neoblasts**, cèl·lules totipotents que els donen grans capacitats regeneratives i que els han fet subjecte d'estudi des de fa molt temps. A molts platihelmints de vida lliure es poden trobar **rabdites**, unes estructures en forma de vara que són secretades per les cèl·lules epidèrmiques amb el mucus. Alguns autors dubten de la monofilia dels platihelmints degut a la feblesa dels caràcters proposats: **protonefridis biciliats**, **epidermis multiciliada** i **manca de mitosis** a les cèl·lules somàtiques (per una discussió, veure Bagunyà & Riutort 2004). La seva simplicitat ha fet que diferents autors els hagin situat com a primera branca de bilaterals, però hi ha d'altres autors que defensen un primer bilateral complex (amb celoma i segmentació) i per tant els platihelmints serien organismes que s'han simplificat secundàriament al reduir el seu tamany (Adoutte *et al.*, 2000; Valentine, 1997).

El fílum *Nemertea* (nom en honor a Nemertes, una nimfa marina de la mitologia grega) també són coneguts com els **cucs cinta**. Malgrat el seu nom vulgar, són generalment cilíndrics, però alguns fan servir la musculatura per aplanar-se a l'hora de nedar. Fan des d'1 mm fins a 30 m, però hi ha cites de nemertins de 60 m, el qual els convertiria en l'animal existent més llarg. Són majoritàriament marins, però n'hi ha algunes espècies d'aigua dolça, terrestres i fins hi tot paràsites.

NEMERTEA

- 1.500 spp
- Majoritariament al bentos marí
- Celoma (Rincocel)
- Clivellament espiral i determinat
- Larva pilidium (només a *Heteronemertea*)
- Probòscide eversible
- No segmentats
- Epidermis multiciliada
- Protonefridis
- Sistema vascular

Presenten una **probòscide** ("abans de menjar") **eversible**; les probòscides, freqüents a molts grups d'invertebrats, són estructures elongades que sorgeixen del cap i participen a l'alimentació de l'animal, generalment fent succió. A altres grups estan connectades al sistema digestiu de l'animal, però aquest no és el cas dels nemertins. Tenen un **sistema nerviós** ben desenvolupat amb **cervell**. La seva **epidermis ciliada**, **parènquima**, **rabdites** i **la suposada manca de celoma** els ha vinculat clàssicament amb els platihelmints; però aquest útim tret va resultar erroni i avui es consideren celomats.

Un calaix de sastre, els antics Aschelminthes

Els *Gnathostomulida* ("boca mandibulada") és un grup petit de cucs marins cilíndrics (0,5 µm-3 mm) que habiten l'espai intersticial de la sorra marina, sobretot en ambients en condicions properes a l'anòxia. Tenen un **cap** i un **tronc** separats per un "**coll**" i a la boca trobem una placa bassal en forma de pinta amb **un parell de mandíbules dentades** definitòries del grup, les quals poden arribar a ser molt complexes. Presenten **clivellament espiral**, són **acelomats** i el **desenvolupament és directe**

Els gnatostomúlids van ser descoberts per Peter Ax l'any 1956, i es van associar al principi als platihelmints (formant els Platihelmintomorpha; Ax, (1956)) degut a la **manca de cavitats corporals**, la **manca de cutícula i anus**, **l'hermafroditisme amb fecundació interna** i **l'epidermis ciliada**, mentre que altres autors els han vinculat amb els gastròtrics (Neotrichozoa; Zrzavý, 1998) degut a que ambdos grups presenten una **epidermis monociliada**.

Els *Rotifera* (els "animalculats" de Leeuwenhoek) són animals vermiformes (menys d'1 mm) que viuen majoritàriament a l'aigua dolça, tot i que alguns viuen a llocs humits (a tolls, al terra, a molses, liquens i bolets). Al cos presenten una mena de "closca" dorsal (**lorica**), **l'epidermis és de tipus sincitial** (amb una capa densa intracel·lular) i una boca amb mandíbules (**trophi**) envoltada per la famosa **corona de cilis**. Presenten **clivellament espiral modificat**, **pseudoceloma** i el **desenvolupament és directe**. Algunes de les seves curiositats són la capacitat de **criptobiosi** (formes de resistència que sobreviuen a condicions ambientals extremes) i la raresa dels mascles (que no es coneixen per a moltes espècies).

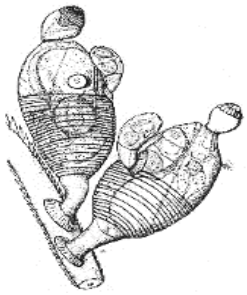
GNATHOSTOMULIDA	***ROTIFERA***	***ACANTHOCEPHALA***
• 100 spp, marins, vida lliure • Acelomats • Mandíbula • Sense circulatori ni anus • Clivellament espiral • Protonefridis • Sense larva • Epidermis monociliada	• 1800 spp, aquàtics, vida lliure • Pseudocelomats • Corona ciliada i Lorica ("closca") • Boca (*trophi*-mandíbula) i anus • Clivellament espiral modificat • Protonefridis • Sense larva • Epidermis sincitial	• 900 spp, paràsits • Pseudocelomats • Probòscide retràctil amb espines i "closca", sense mandíbula • Clivellament espiral • Protonefridis • Larva paràsita acanthor • Epidermis sincitial
		

Alguns autors han proposat que el mecanisme d'alimentació dels rotífers adults podria ser homòleg al de la **larva trocòfora** d'anèl·lids i mol·luscs, però aquesta idea ha rebut moltes crítiques (Haszprunar *et al.*, 1995; Hyman, 1951; Nielsen, 1995).

Els *Acanthocephala* ("cap espinós") són cucs paràsits (2 mm- 80 cm) amb **formes larvàries paràsites** d'artròpodes i adults que viuen a l'intestí de vertebrats. El cos dels adults està composat pel **tronc**, el **coll** i una **probòscide espinosa**. A diferència dels gnatostomúlids i els rotífers, no presenten mandíbules. **L'epidermis és sincitial**, el **clivellament és espiral** i el **pseudoceloma**, molt voluminós, no és un blastocel persistent si no que es genera *de novo* per cavitació del mesoderm.

Els *Micrognathozoa* ("animals petits amb mandíbules") és el fílum descrit més recentment (Kristensen & Funch, 2000). L'única espècie coneguda és *Limnognathia maerski*, descoberta a l'any 1994 a unes fonts de Groenlandia. Són

uns cucs microscòpics (130 µm) amb un **cap** amb dos parts, un **tòrax** i un **abdomen** ovoide. Presenten **tres parells de mandíbules** i l'epidermis dorsal posseeix una "closca" feta de plaques situades a la matriu intracel·lular.

MICROGNATHOZOA	***CYCLIOPHORA***
• 1 spp, aquatics • Acelomats • Mandíbules complexes • "Closca" dorsal • Epidermis cel·lular 	• 3 spp, epibionts marins • Cavitat primària • Digestiu en forma d'U • Multitud de larves • Sense mandíbules • Sense epidermis sincitial 

Els integrants del fílum anomenat *Cycliophora* ("portadors de rodes petites") es van observar per primer cop a l'any 1969, però aleshores es va pensar que eren algun tipus de rotífers modificats. A l'any 1995 es van definir com a nou fílum amb una única espècie, *Symbion pandora*, trobada al Mar del Nord (Funch & Kristensen, 1995); però avui en dia se n'han trobat dues espècies noves, una al Mediterrani i l'altre a les aigües de la costa Est de Nordamèrica. Els cicliòfors tenen un dels cicles vitals més complexes, amb varies larves i un adult amb tres formes diferents: l'asexual, la femella i el mascle. La forma més freqüent és la dels adults asexuals, que viuen enganxats a les parts bucals de crustacis decàpodes, tenen un **cos en forma de sac** (350 µm) i una **boca** similar a un embut amb una **corona de cilis** (que dona el nom al fílum). Presenten una cavitat primària i l'estructura del **sistema digestiu en forma d'U** els va associar amb els entoproctes i ectoproctes.

Els *Priapulida* són uns cucs (500 µm-40 cm) amb forma de cogombre que viuen soterrats a la sorra del fons marí. El seu nom prové de Priapos, un deu grec menor protector dels genitals masculins. Al cos es diferencien dues parts, el **cap** i el **tronc**. Dins del cap trobem l'**introvert**, una probòscide evaginable amb unes "espines" (**escàlids**) de cutícula que porten cèl·lules receptores i serveix per soterrar-se al substrat (Lemburg, 1995). A pesar d'estar agrupat filogenèticament amb alguns "asquelmints" (Hyman, 1951), són **celomats** i a més presenten **clivellament radial**, el qual ha fet que el seu posicionament hagi estat controvertit.

Els *Kinorhyncha* ("trompa amb moviment") es coneixien anteriorment com el fílum *Echinodera*. Es tracta d'un grup de cucs marins microscòpics (menys d'1 mm) que viuen a la sorra (raó per la qual també s'anomenen "dracs del llim") i es troben des de la zona intermareal fins a zones abissals de 5.000 m de profunditat. Com a altres "asquelmints", el cos es divideix en **cap** (l'introvert), **coll** i **tronc**, i està envoltat per una **cutícula quitinosa**. La cutícula del tronc està articulada en **11 segments**, segmentació que afecta al sistema nerviós i el muscular; però aquesta segmentació no es considera homòloga a la d'anèl·lids o artròpodes (Schmidt-Rhaesa, 2006). La disposició de les plaques cuticulars i d'escàlids és força complexa i variada. Presenten una forma juvenil de vida lliure, a vegades anomenada larva, que s'assembla molt a un adult però amb menys segments.

PRIAPULIDA	***KINORHYNCHA***	***LORICIFERA***
• 17 spp, marins • Celomats • Cutícula amb escàlids • Probòscide eversible • Clivellament radial • Protonefridis • Fase larvaria	• 180 spp, marins • Pseudoceloma molt reduït • Cutícula articulada amb escàlids • Probòscide eversible • Clivellament desconegut • Protonefridis • Larva/juvenil amb 6 mudes	• 11 spp, 80 per definir • Pseudocelomats • Cutícula amb escàlids • Probòscide eversible • Clivellament desconegut • Protonefridis • Larva de Higgins
		

El phylum *Loricifera* ("portadors de lorica") van ser descoberts al 1983 pel mateix investigador que va definir els cicliòfors i els micrognatozous, el danès Reinhardt M. Kristensen. Petis cucs (menys de 500 μm) dels sediments marins, els loricífers tenen el cos dividit en cinc parts (**conus bucal**, **cap-introvert**, **coll**, **tòrax** i **abdomen**) i està recobert per una **cutícula quitinosa**. Es l'únic fílum pel qual encara no existeixen dades moleculars.

Els *Nematoda* ("forma de fil") o cucs rodons és el grup més gran dels "asquelmints", amb unes 20.000 spp descrites, tot i que Hyman va estimar que poden existir fins a mig milió. Els nemàtodes es troben a tot arreu: al fons marí, a

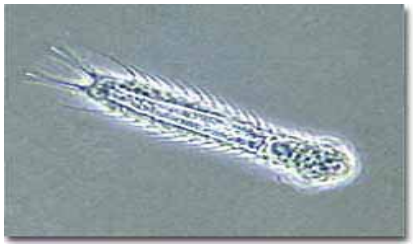
l'aigua dolça, al medi terrestre humit i dins d'altres organismes (són paràsits virtualment de tots els grups de plantes i animals). El seu gran èxit s'explica en base a una estructura simple però eficient que els permet tolerar condicions extremes. Els nematodes tenen el cos cilíndric, recobert de **cutícula reforçada amb col·làgens** i amb els extrems del cos més fins. El **clivellament es bilateral**, diferent del radial o l'espiral. Fan des de micres fins als 7 metres i a la cutícula i l'epidermis trobem **4 solcs** atravessant el cos en sentit anteroposterior: un dorsal, un ventral i un a cada banda del cos. El solc dorsal i el ventral van acompanyats per cordes nervioses, tret compartit amb els nematomorfs.

El nom dels *Nematomorpha* també significa "forma de fil" i es va anomenar aquest grup així per la seva similitud amb els nemàtodes. També anomenats cucs de pèl de cavall, són cucs (1 mm-100 cm) que passen la major part de la seva vida en forma de larva, parasitant insectes, i tenen una forma adulta de curta duració que viu a aigua dolça o al terra (només hi ha 5 espècies marines).

NEMATODA	***NEMATOMORPHA***
• 20.000 spp	• 300 spp
• Sense larva	• Larva paràsita,
• Vida lliure i paràsits	• Adult de vida lliure
• Pseudocelomats	• Pseudoceloma molt reduït
• Cutícula	• Cutícula
• Clivellament bilateral	• Clivellament idiosincràtic
	

Tenen el cos envoltat amb una **cutícula** i els adult entortolliguen el cos fent un nus, fet pel qual també s'anomenen gordiacis en referència al nus gordià. El seu **desenvolupament** sembla ser una variació del patró radial i és catalogat com **idiosincràtic**, el qual vol dir que no s'assembla al de cap altre fílum.

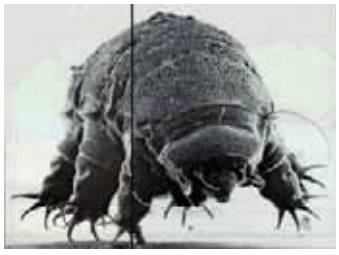
GASTROTRICHA
• 700 spp • Aquatics de vida lliure • Acelomats • Epidermis ventral monociliada • Cutícula que recobreix els cilis • Clivellament bilateral o biradial



Els *Gastrotricha* ("panxes peludes") són uns cucs microscòpics (60 µm-3 mm) que viuen tant a l'aigua marina com a aigua dolça, alimentant-se de bacteris, fongs i protistes. El seu nom fa referència a la presència de **cilis locomotors ventrals** que estan recoberts de **cutícula**, característica que és única al món animal. En els grups més primitius de gastròtrics les **cél·lules són monociliades** com en els gnatostomúlids. A la part posterior del cos tenen **tubs adhesius** en forma de forqueta per enganxar-se al substrat.

Els *Tardigrada* ("caminants lents") també es coneixen com óssos d'aigua i són un dels invertebrats que generen més simpaties per seva aparença d'ossets de peluix. Tenen un cos petit (100 µm-1,5 mm) amb un **cap** indiferenciat i un **tronc** amb 4 parells de **lobopodis** (apèndix sense articulacions) acabats en **ungles** (de 4 a 8 per pota). Viuen en ambients aquàtics i terrestres humits, i mostren capacitats de **criptobiosi** que superen a les dels rotífers. El **clivellament** és únic dins dels metazous, el **desenvolupament** és **directe** i s'han associat tant amb "asquelmints" com amb artròpodes (Nielsen, 2001).

TARDIGRADA
• 600 spp, aquatics • Pseudocelomats • Cutícula amb quitina • Lobopodis amb ungles • Clivellament idiosincràtic



Animals celomats, protostomats, espirals i no segmentats

Els *Mollusca* (del llatí "tou") és un dels fílums d'invertebrats amb més exit. Ocupen tots els hàbitats i presenten una gran variació corporal, des dels cargols i els llimacs fins als musclos, les ostres o les lapes, passant pels *Nautilus*, calamars i escafòpodes. Inclou animals com els pops, considerats els invertebrats més intel·ligents, o els calamars gegants, possiblement els invertebrats més grans (amb el permís dels nemertins anteriorment citats). Hi ha 100.000 espècies actuals descrites (a més de 35.000 fòssils) i es creu que només són la meitat de les espècies existents.

MOLLUSCA

- 100.000 spp
- Cosmopolites
- Celomats
- No segmentats
- Clivellament espiral
- Larva trocòfora

Els mol·luscs són tan diversos que és difícil establir un pla corporal arquetípic; es descriuen com animals amb una **ràdula quitinosa** (un òrgan bucal raspador, absent a bivalvs i alguns aplacòfors), un **peu locomotor ventral** (reduït a aplacòfors) i una **massa visceral dorsal** (a la majoria de grups associada amb una closca) a dins de la qual podem trobar els **ctenidis** (òrgans respiratoris, absents als escafòpodes). Només la massa visceral dorsal és comuna a tots els seus integrants.

Al mol·luscs trobem un veritable **celoma**, **larva trocòfora** (a gasteròpodes, escafòpodes i bivalvs hi ha a més una segona larva, la **veliger**) i **clivellament espiral**. A l'estadi de 64 cèl·lules, als embrions es poden observar un conjunt de cèl·lules que formen una estructura en similar a una creu, i que rep el nom de **creu dels mol·luscs**; els anèl·lids presenten una creu similar. Tots aquests trets els han vinculat amb altres protòstoms espirals com els platihelmints, sipúnculs i annèl·lids (Nielsen, 1995).

Els *Sipuncula* ("petit sifó") també es coneixen com a **cucs cacauet**, degut a la forma del seu cos, o **cucs estrella**, per la corona de tentacles que envolta la seva boca. Són animals vermiformes (2 mm-70 cm) que viuen soterrats al fons marí, per sobre dels esculls coral·lins o dins les closques d'altres animals. El cos no està segmentat i es troba envoltat per una **cutícula** de **col·làgens**. Al **cap** hi ha un **introvert evaginable** envoltat per una **corona de tentacles** i un **tronc** que és bàsicament una bossa celòmica. Els sipúnculs són **celomats** però no presenten sistemes circulatori ni respiratori. L'embrió mostra **clivellament espiral**, amb una agrupació de cèl·lules que recorda a la creu dels mol·luscs, i passen per una **fase larvaria** molt similar a la de la **trocófora**. Per això se'ls ha vinculat tant amb els mol·luscs com amb els anèl·lids.

SIPUNCULA

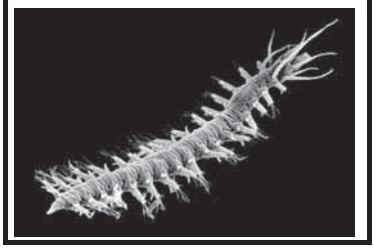
- 320 spp
- Cosmopolites
- Celomats
- Cutícula de col·làgens
- No segmentats
- Clivellament espiral ("creu dels mol·luscs")
- Larva trocòfora

Animals celomats, protostomats i segmentats

Els *Annelida* ("petits anells") són els cucs per excel·lència. Tot i que les llombrius de terra són els més coneguts, els anèl·lids són molt diversos, incloent les sangoneres i multitud de cucs marins, i poden ser microscòpics o arribar fins als 3 m. Tenen el cos envoltat per una **cutícula** de **col·làgens** i dividit en tres parts: el **prostomi** (cap), un **tronc segmentat** i el **pigidi**. La **segmentació del tronc és teloblàstica**, a la qual els segments es generen a la part més posterior del cos (just abans del pigidi) i els nous segments empenyen als antics cap a la part més anterior del cos. Al llarg del cos presenten les **quetes quitinoses**, uns pèls que els ajuden a ancorar-se al substrat.

ANNELIDA

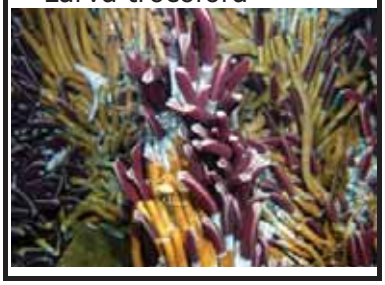
- 20.000 spp
- Cosmopolites
- Celomats
- Cutícula de col·làgens
- Segmentació teloblàstica
- Clivellament espiral ("creu dels anèl·lids")
- Larva trocòfora



Són **celomats** amb sistema circulatori però sense respiratori i mostren **clivellament espiral** (amb una agrupació cel·lular anomenada "creu dels anèl·lids") i una **larva trocòfora** arquetípica. Aquests trets

els han associat amb altres espirals com els mol·luscs, i degut a la segmentació corporal se'ls ha relacionat filogenèticament amb els artròpodes.

POGONOPHORA
• 140 spp
• Sèssils, viuen al fons marí
• Multitud de tentacles
• Celomats
• Espirals o bilaterals?
• Opistosoma segmentat
• Larva trocòfora



Els *Pogonophora* ("portadors de barba") van ser descoberts a l'any 1900 a Indonesia. Són uns cucs molt llargs i prims (amb una longitud de 5-150 cm i 0'1 mm-4 cm de diàmetre) que viuen al fons marí a una profunditats de fins a 10 km. Són organismes sèssils que viuen dins de **tubs de quitina i escleroproteïna** i es caracteritzen per la presència de multitud de **tentacles** (fins a 200.000).

El cos està dividit en quatre parts: el **glòbul cefàlic** o **vestimentum** (d'on surten els tentacles), la **regió glandular** (que secreta el tub), la part més gran és el **tronc** (amb bandes ciliades ventrals i anells on trobem quetes) i a la part posterior hi ha l'**opistosoma** (que es troba fora del tub i presenta fins a **95 segments** i a on també trobem quetes). Cada regió te la seva pròpia cavitat **celòmica**, a l'igual que els tentacles i cadascun dels segments de l'opistosoma. El sistema nerviós consta d'un anell nerviós al glòbul cefàlic i 3 cordes nervioses ventrals longitudinals. Als adults el sistema digestiu està molt reduït o directament absent degut a que aquests animals s'alimenten de bacteris que metabolitzen el sofre i que ells mateixos cultiven dins del tronc, a una estructura anomenada **trophosoma**. Però els juvenils presenten un sistema digestiu complert molt similar al dels anèl·lids.

No està clar si el clivellament és **espiral** (Valentine 2004) o **bilateral** (Barnes 1995), ni tampoc si el celoma apareix per esquizocèlia, tot i que l'enterocèlia sembla estar descartada. Tenen una larva de tipus **trocòfora**. Tots els pogonòfors obtinguts abans de l'any 1964 patien una extracció violenta que els feia malbé i sempre perdien de l'opistosoma. El material incomplert mostrava animals amb tres cavitats celòmiques i es va pensar que la corda nerviosa era dorsal; això els va vincular erròniament als deuterostomats. Però avui en dia el consens és que els pogonòfors estan estretament vinculats amb els anèl·lids.

Els *Echiura* ("cua d'escurçó"), o **cucs cullera**, són organismes marins (1-50 cm) que viuen soterrats a la sorra en tubs en forma de U així com a les roques o als esculls de corall. A la part anterior del cos trobem el **prostomi**, una estructura en forma de cullera molt mòbil i que estesa pot arribar als 2 m, seguit d'un **tronc** posterior. El cos està recobert per una **cutícula** de **col·làgens** i, a l'igual que els anèl·lids, tenen **quetes quitinoses** a la part ventral del tronc. Els equiürs són **celomats**, sense segmentació corporal i amb **clivellament espiral** a on trobem una estructura similar a la a la creu dels anèl·lids i presenten **larva** similar a la **trocófora**. Alguns autors defensen que el tronc mostra **traces de segmentació** (Barnes, 2004). Aquestes característiques els han associat sovint amb els anèl·lids.

ECHIURA

- 140 spp
- Cosmopolites
- Celomats
- Cutícula de col·làgens
- Segmentats?
- Clivellament espiral ("creu dels anèl·lids")
- Larva trocòfora

Els *Onychophora* ("portadors d'ungles") són uns cucs de vida lliure (1,5-15 cm) de longitud, de costums nocturnes i que es troben sobretot a zones tropicals. Els onicòfors caminen d'una forma molt característica, pel qual se'ls anomena vulgarment els **cucs que deambulen**, tot i que també s'anomenen **cucs de vellut** degut a la superfície peluda dels seu cos. Quan van ser descrits per primera vegada al 1826 es va pensar que eren llimacs amb potes. Al **cap** tenen dos ulls, dues antenes i una boca amb un parell de mandíbules. El **tronc és segmentat** amb un nombre variable de parells de **lobopodis** (14 a 43), acabats en **ungles**. Tot i que els onicòfors actuals són terrestres, els representants fòssils són marins i similars als animals d'avui en dia. Durant molt de temps els onicòfors s'han considerat l'intermediari evolutiu entre els anèl·lids i els artròpodes. S'assemblen als anèl·lids en la **cutícula** (molt fina i flexible) i l'estructura de la paret del cos (amb **tres capes musculars**: circular, diagonal i longitudinal. En canvi, les mandíbules són apèndix modificats com als artròpodes i la cutícula és de **quitina**, amb les mateixes capes dels artròpodes; a més fan muda i respiren fent servir tràquees.

ONYCHOPHORA

- 70 spp
- Vida lliure, terrestres
- Celomats
- Cutícula de quitina
- Segmentats
- Clivellament

Els *Arthropoda* ("potes articulades") són els invertebrats amb major èxit, més coneguts i probablement els de major diversitat terrestre. No hi ha cap grup d'invertebrats tan ric en espècies, gran en biomassa total i amb tanta influència a l'ecologia del planeta. Hi ha més d'un milió d'espècies descrites (més de dues terceres parts del total de les espècies animals actuals), ocupant tots els hàbitats i es creu que aquest número és només una petita part del total. D'aquest milió, 300.000 són espècies d'escarbats, fet que va portar a J. B. S. Haldane a dir que "*God has an inordinate fondness for beetles*".

ARTHROPODA

- Més d'un milió d'spp
- Cosmopolites
- Celomats
- Cutícula de quitina
- Segmentats
- Apèndix especialitzats
- Clivellament idiosincràtic

Dins dels artròpodes contemporanis hi ha quatre grups principals: 1) **quelicerats** (aranyes, àcars, escorpins, aranyes marines, etc.), 2) **miriàpodes** (centpeus i milpeus, tots són terrestres), 3) **hexàpodes** (tots els insectes, també són terrestres) i 4) **crustacis** (aquàtics i majoritàriament marins, com ara els crancs, gambes, decàpodes, etc). Malgrat les dècades d'estudis, les relacions internes dels artròpodes encara són tema de debat. Els artròpodes presenten un cos amb regions ben definides i especialitzades (**tagmes**) que varien depenent del grup; per exemple, als hexàpodes trobem 3 regions (cap, tronc i abdomen), mentre que els crustacis presenten dues (cefalotòrax i abdomen) a l'igual que els quelicerats (prosoma i opistosoma). Recobrint tot el cos hi ha una **cutícula quitinosa** molt rica en proteïna (que s'estén fins a l'interior de la boca i l'anus), amb regions més endurides formant plaques (**esclerites**) i de la qual fan mudes a mesura que creixen. El cos està clarament **segmentat** i presenten una enorme varietat d'**apèndix articulats** especialitzats en diferents funcions (antenes, mandíbules, etc). Tot i que clàssicament es considerava que els artròpodes presenten clivellament espiral, avui en dia això està descartat. La gran diversitat dels artròpodes també és reflexa en una gran heterogeneïtat en el seu desenvolupament embrionari i cap grup presenta un clar patró espiral. En quant a la seva posició al regne animal, clàssicament es consideren el grup germà dels anèl·lids (formant el clade *Articulata*) en base a la seva organització **metamèrica**, la **formació teloblàstica dels segments** i la presència de **cavitats celòmiques** i **nefridis** (Schmidt-Rhaesa *et al.*, 1998).

Lophophorata

Els **Lophophorata** ("portadors de lofòfor") és una agrupació d'animals marins que es caracteritzen pel lofòfor, una corona retràctil de tentacles multiciliats, amb forma de ferradura que envolta la boca i que porta el menjar cap aquesta (v. fig 1). La diferència entre aquests tentacles i els d'altres grups és que els dels lofòforats estan celomats (amb l'excepció dels pogonòfors).

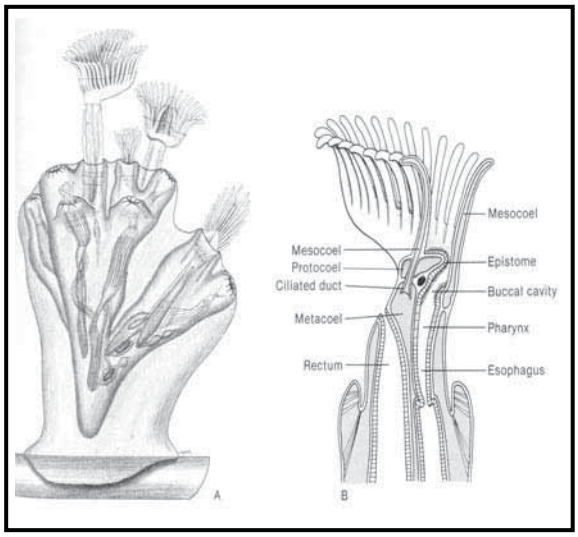
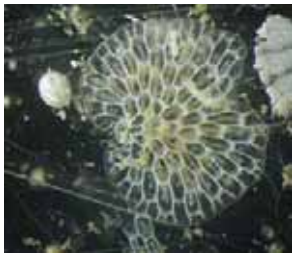


Fig. 1: Un *Lofoforat* i detall del *Lofòfor*; extret de Barnes 1996.

Als lofoforats el tub digestiu te forma d'U i secreten o bé quitina o carbonat càlcic per fer un exoesquelet. És creu que aquests trets són determinats per l'estil de vida sèssil d'aquests animals i tenen un alt grau de convergència. En general, els lofoforats presenten **3 cavitats celòmiques** que es formen tant per **enterocèlia** com **esquizocèlia**, un sistema nerviós **heteroneure** (amb una corda dorsal i una ventral, tret present també als hemicordats i els quetognats), el **clivellament és radial** (amb l'excepció dels entoproctes, que el tenen **espiral**) i en el cas dels braquiòpodes el blastopor dona lloc a l'anus (**deuterostomía**). El lofòfor és similar a estructures presents a equinoderms i hemicordats. Tots aquests trets els han vinculat amb els deuterostomats.

Els *Ectoprocta* ("anus fora") també s'anomenen **briozous** ("animals molsa") o **kamptozous** ("cucs que mouen el cap"), i és el grup més gran de lofoforats amb un registre fòssil molt ric. Tenen formes **solitàries** i **colonials**, amb individus molt petits (0.5 mm). Les colònies són similars als coralls i formen estructures incrustades al substrat o branques que es ramifiquen. Els animals viuen dins d'una mena de closca en forma de caixa amb orificis pels quals poden treure els tentacles. Hi ha colònies on diferents animals s'especialitzen en diferents funcions (defensa, reproducció, fixació al substrat, alimentació, etc.).

ECTOPROCTA	***ENTOPROCTA***
• 5000 spp	• 150 spp
• Marins séssils	• Marins séssils
• Solitaris o colonials	• Colonials
• 3 cavitats celomiques	• Cavitat primària
• Clivellament biradial	• Clivellament espiral
• Larva cyphonauta	• Larva trocòfora
	

Els *Entoprocta* o **endoproctes** ("anus dins") també s'anomenen **cucs calze** i abans formaven un sol fílum amb els ectoproctes, anomenat els ***Bryozoa***. La seva estructura és molt similar a la dels ectoproctes, amb formes séssils que a vegades es troben contigües als ectoproctes. També fan colònies a on tots els integrants provenen d'un mateix individu (0,5-5 mm) que es divideix asexualment. Però hi ha prou diferències per separar-los dels ectoproctes: el endoproctes presenten **l'anus dins de la corona de tentacles**, el clivellament és **espiral**, presenten una cavitat primària i no tenen exoesquelet.

PHORONIDA	***BRACHIOPODA***
• 14 spp	• 150spp
• Marins séssils	• Marins séssils
• 3 cavitats celomiques	• 3 cavitats celomiques
• Clivellament biradial	• Clivellament radial
• Larva actinotroca	• Larves glottidia i tripartita
	

Els *Phoronida* (nom que prové de Isis, la deessa egípcia de la mort) també són coneguts com **cucs ferradura**. És un grup molt petit de 14 espècies, contingudes en dos gèneres que pertanyen a la mateixa família. Viuen dins de **tubs de quitina** al fons marí, enterrats a la sorra o enganxats a roques i closques d'altres animals. Es tracta d'animals molt llargs i prims, amb una mica menys de 20 cm longitud i 3 mm de diàmetre. El lofòfor te altes capacitats regeneratives i s'obre fora del sediment per filtrar partícules nutritives, i a diferència dels altres lofoforats, és **monociliat**. Tot i que presenta les característiques típiques dels lofoforats que els aproximen als deuterostomats, com per exemple el **clivellament radial**, als foronidis el blastopor dona lloc a la boca (**protostomía**) i la larva presenta un parell de protonefridis com a les larves trocòfores dels anèl·lids.

Els *Brachiopoda* ("braç amb peu") són molt diferents dels altres lofoforats pel fet de que tenen el cos contingut dins de dues valves asimètriques (5 mm-8 cm), similars superficialment a les cloïsses. També són coneguts com **closques de làmpara** per que la valva inferior recorda a les làmpares d'oli que feien servir els romans. Les valves estan fetes de carbonat o fosfat càlcic, amb proteïnes o **quitina** i no són homòlogues a les dels mol·luscs, si no que es tracta d'un exemple de convergència. La valva inferior, la més gran, es fixa al substrat i la d'adalt està lliure per moure's. El lofòfor es troba dins de les valves i a vegades treuen les puntes dels tentacles fora de les valves. Existeixen molts fòssils d'aquest grup, un total de 12.000 espècies, i és l'animal celomat més antic trobat al registre fòssil.

Deuterostomats

Els *Echinodermata* ("pell espinosa") és un grup que ha generat molta atenció degut a la seva rellevància ecològica i sobretot per la seva morfologia inusual. I és que els equinoderms adults es diferencien de la resta de bilaterals per presentar **simetria pentaradial**, tot i que les formes larvàries són bilaterals. Dins d'aquest grup trobem animals molt coneguts i variats com les estrelles de mar, els eriçons de mar o els cogombres de mar. Habiten el fons marí i es troben a totes les profunditats possibles, fins hi tot a la fossa de la Marianes (a 11 km de profunditat). Les espècies actuals s'agrupen en 6 classes, però les 13.000 espècies del seu extens registre fòssil s'han assignat a 15 classes addicionals.

Degut a la simetria pentaradial, aquests animals no presenten cap i per tant no es parla d'un eix anterior-posterior sino d'un eix oral-aboral, a on la boca està en contacte amb el substrat. Al marge de la simetria pentaradial, els equinoderms es caracteritzen per un **esquelet calcari** format per diferents plaques (**ossicles**), un **teixit de col·làgens** molt mutable i plàstic que permet canviar la forma i consistència del cos

ECHINODERMATA	***HEMICHORDATA***
• 7000 spp • Marins bentònics • Simetria pentaradial • Celoma enterocèlic • Sistema ambulacral • Clivellament radial • Larva dipleurula	• 100 spp • Marins bentònics • Solitaris i colonials • Celoma enterocèlic • Cos/celoma tripartit • Clivellament radial • Larva tornaria
	

ràpidament, i un **sistema vascular hidràulic** (amb funció de locomoció, reproducció i alimentació) d'origen celòmic que s'estén per tot el cos formant petites potes (**podia** o **ambulacra**).

Tan mateix, com a deuteròstoms que són, el **blastopor** dona lloc a l'anus i el **clivellament** és **radial indeterminat**. El **celoma** es forma per **enterocèlia** i està **tripartit** a les primeres fases del desenvolupament. Presenten larves molt diverses depenent del grup, però totes comparteixen trets que es resumeixen en una larva ideal anomenada **diplèurula**. El sistema nerviós consta d'un anell que envolta la boca i seguint els eixos de simetria sorgeixen 5 branques que es connecten amb una xarxa neuronal subepidèrmica. Alguns fòssils semblen mostrar traces de **fenadures faringies** (veure els vertebrats més endavant). Les seves capacitats de regeneració i un embrió de fàcil accés i manipulació els han perfilat com un dels organismes model clàssics.

El nom dels *Hemichordata* ("mitja corda") prové de la presència d'un tub dorsal buit (**estomocorda**) i degut a la forma particular del cap també són anomenats **cucs gla**. Els hemicordats són animals vermiformes (pocs mil·límetres a 1,5 m) que viuen sobre el fons marí i pràcticament a totes les fondàries. Hi ha dues classes principals, la dels *Enteropneusta* (solitaris) i la classe *Pterobranquia* (que formen colonies similars a les dels briozous).

Els adults tenen un cos clarament tripartit: la **proboscis** (amb un sistema tentacular), el **collar** (amb l'**estomocorda**), i un **tronc** on es troben les **fenadures faringies**. Es creu que tant l'estomocorda com les fanadures són homòlogues a estructures presents als cordats. El sistema digestiu te forma d'U i el nerviós consta d'una corda dorsal principal i una ventral més petita. Com els equinoderms, presenten totes les característiques embrionàries dels deuteròstoms, si bé el celoma es pot formar per **esquizocélia**, **enterocélia** i altres patrons. Presenten una fase larvària anomenada **tornaria** similar a la dipleurula dels equinoderms. Existeix un registre fòssil molt ric a on destaquen els **graptòlits**, utilitzats habitualment per datar els estrats geològics del paleozoic.

Els *Chordata* ("corda") és el fílum més conegut i estudiat, degut a la seva importància ecològica, gran abundància, tamany, gran nombre de representants terrestres i probablement en bona part a que els investigadors mateixos pertanyen a aquest grup. És l'únic fílum que conté organismes invertebrats, els *Urochordata* i *Cephalochordata*, i organismes vertebrats, els *Vertebrata*, el que mostra la gran

diversitat corporal del grup. Dins dels mateixos vertebrats, la varietat és molt gran, incloent animals tan diferents com dofins, rat-penats, els desapareguts dinosaures, taurons, rates, peixos, granotes i un llarg etcètera.

Tot i així, tots els cordats comparteixen una sèrie de trets comuns presents en algun moment del seu desenvolupament, i que poden persistir en l'adult: 1) el **tub neural** és un tub nerviós buit, que corre dorsalment de cap a cua i que comprèn el sistema nerviós central, 2) el **notocordi** és un eix cartilaginós dorsal en forma de vara flexible, que s'estén al llarg de cos entre el tub digestiu i el tub neural i que fa funció d'endoesquelet (en els vertebrats es substitueix per la columna vertebral), 3) les **fenadures faringies** són unes ranures als dos costats de la faringe amb una funció original d'alimentació que ha esdevingut respiratòria i 4) una **cua postanal**, una elongació posterior del cos que va més enllà del final del tub digestiu i conté part del tub neural, el notocordi i la musculatura corporal.

CHORDATA

- 50.000 spp
- Cosmopolites
- Celoma enterocèlic
- Miòmers segmentats
- Fase larvaria
- Clivellament radial

Igual que als equinoderms i els hemicordats, el **blastopor** dona lloc a l'anus, i el **clivellament** és **radial indeterminat**. Algunes diferències dins del grup són que el sistema muscular està segmentat en **miòmers** als cefalocordats i els cordats, trobem **fase larvaria** als urocordats i els cefalocordats i els urocordats presentan cavitat primària.

Incertae sedis, els animals de posició incerta

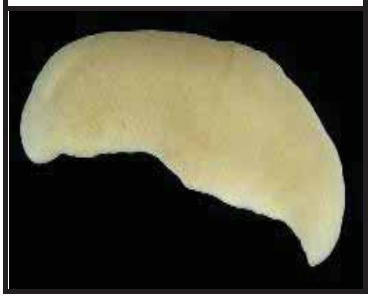
Tot seguit es descriuen breument tres grups de bilaterals amb afiliacions filogenètiques dubtoses. En alguns casos es deu a la manca de coneixements de la biologia dels animals i en altres a la presència de caràcters que són un mosaic de diferents grups. Un d'ells són els *Chaetognatha* són animals marins planctònics amb el cos en forma de fletxa, pel qual també s'anomenen **cucs sageta**. Són cucs petits i transparents, amb cossos allargats i fins, i presenten una mena d'aletes laterals que fan servir per la natació. La boca presenta multitud d'espines amb la qual subjecten la pressa mentre se la mengen; hi ha alguns exemplars de fins a 12 cm que ataquen peixos petits. Es mouen dins de la columna d'aigua, pujant per alimentar-se de nit i estan estretament associats a les corrents marines.

CHAETOGNATHA

Els quetognats són **bilaterals**, **triploblàstics**, amb **desenvolupament directe**. Presenten trets típics tant de protostomats com de deuterostomats, i Darwin ja va expressar que els quetognats "are remarkable for the obscurity of their affinities". Alguns trets típics de deuterostomats són un **blastopor** que dona lloc a l'anus i un **celoma** (tot i que no te peritonéu i per tant s'ha posat en dubte) que clàssicament es creia que es formava per **enterocèlia** (tot i que estudis recents mostren que es genera per un patró idiosincràtic; Kapp 2000); a més el seu **clivellament és radial**, malgrat que el llinatge cel·lular de la cèl·lula 4d es similar al dels espirals. Es considera que tenen el **celoma bipartit**, però alguns autors consideren la cua com una tercera regió (allà es troben els testicles) i per tant consideren que tenen el cos tripartit com els deuteròstoms. El sistema nerviós forma un anell al voltant de la boca com en els equinoderms. En canvi presenten altres caràcter típics de protostomats, com un **sistema nerviós ventral** i la presència de **quitina**. Tots aquests trets han dificultat el seu posicionament filogenètic des d'un punt de vista morfològic.

Xenoturbella és un cuc enigmàtic que va ser descobert al 1949. Fa al voltant d'un centímetre de longitud, viu al fons marí i te un pla corporal molt simple que recorda al dels platihelmints. Originalment es va relacionar amb aquests, concretament amb els acelomorfs, però els trets que comparteix amb ells (**hermafroditisme**, **cilis epidermics**, **estatocists**, la **manca de gònades i anus**) van ser considerats com plesiomòrfics o homoplàsics. Alguns autors van proposar que són deuterostomats neotènics (Reisinger 1960) o bilaterals basals (Ehlers 1997). No se sap res de la seva embriologia, i caldrien noves dades morfològiques per establir la seva posició filogenètica.

XENOTURBELLA



Apèndix II

Altres publicacions

Molecular taxonomy and phylogeny of the Tricladida

Jaume Baguñà, Salvador Carranza, Jordi Paps, Iñaki Ruiz-Trillo and Marta Riutort

Within the free-living Platyhelminthes, the triclads or planarians are the best known group, partly as a result of being suitable for classroom studies but, largely, because they have been the subject of intensive research concerning the cellular bases of regeneration and pattern formation (for general reviews see Baguñà *et al.* 1994, and Baguñà 1998) and, most recently gene expression (Bayascas *et al.* 1997; Orii *et al.* 1999). The Tricladida Lang, 1884, which is best considered a suborder (Ehlers 1985a), forms, together with the suborder Proseriata Meixner, 1938, the Order Seriata. Autapomorphies for the Seriata are their backwards-directed tubiform and plicate pharynx and the division of testes and vitellaria into serially arranged follicles. Proseriata do not have obvious autapomorphies apart from their lack of lamellate rhabdites (Sopott-Ehlers 1985), whereas Tricladida are characterized by its three-branched intestine and its highly modified embryonic development with the presence of a transitory embryonic pharynx. A family of proseriates, the Bothrioplanidae Hofsten, 1907, was proposed by Sopott-Ehlers (1985) as the actual sister group of the Tricladida forming a taxon N.N. (Bothrioplanida + Tricladida) characterized by the presumed lack of epidermal collar-receptors, a tricladoid intestine, and a crossing-over of muscle layers at the root and the tip of the pharynx. A general phylogenetic scheme of Seriata is summarized in Figure 6.1.

The monophyletic status of the Seriata has been questioned both on morphological and molecular grounds. The main morphological argument against the presumed autapomorphies of Seriata is that, without comparative ultrastructural studies, pharynx types are not useful for phylogenetic studies, whereas the serial arrangement of vitellaria is so general that homology is impossible to test (Sluys 1989a). Moreover, data on the ultrastructure of the excretory system indicates a basal location for Proseriata and, therefore, the paraphyly of Seriata (Rohde 1990). This paraphyly was reinforced from molecular data (18S rDNA sequences; Carranza *et al.* 1997). Neighbour-joining (NJ) distance, maximum-likelihood (ML) and maximum parsimony (MP) trees showed Tricladida clustering either with prolecithophorans or rhabdocoels, whereas proseriates appeared as basal neoophorans close to lecithoepitheliates or Neodermata. A more recent study, combining morphological and molecular characters (Littlewood *et al.* 1999a), confirmed the results of Carranza *et al.* (1997) and suggested a clade made by *Urastoma* Dörler, 1900, Fecampiida (*Kronborgia*, Christensen and Kannerworff, 1964) and *Ichthyophaga* Syriamiatnikova, 1949, as the actual sister group of the Tricladida. The morphological synapomorphies shared by the Bothrioplanida and the Tricladida proposed by Sopott-Ehlers (1985a), have been critically examined by Sluys (1989a). Lack of collar-receptor is a secondary absence and, in the lack of further information, has to be considered a weak character. Instead, the tripartite intestinal system and the muscle crossing-over at the pharynx, the latter not uncommon among other platyhelminths (see references in Sluys 1989a), were considered good synapomorphies.

Within the Tricladida, three infraorders usually have been recognized: Maricola (marine planarians), Paludicola (freshwater planarians) and Terricola (land planarians) (reviewed in Sluys 1989a), to which a new one, the Cavernicola was further added (Sluys 1990). Relationships of these infraorders have been the subject of several morphological-based analyses. Sluys' (1989a) analysis is by far the most detailed and valuable. It supported the monophyly of the Tricladida, the Terricola, the Maricola and the Paludicola, and the existence of a new clade, the Terricola-Paludicola clade. Relationships within the infraorders have only been considered in detail within the Paludicola, in which three families are currently recognized: the Dugesiidae Ball, 1974, the Planariidae Stimpson, 1857, and the Dendrocoelidae Hallez, 1892. The Planariidae and Dendrocoelidae are considered derived groups and form the sister-group of the more primitive Dugesiidae. A radically different view of infraorder relationships of Tricladida emerged recently from molecular studies based on sequence data of the 18S ribosomal genes and the presence of an 18S gene duplication shared by the Terricola and the family Dugesiidae of the Paludicola (Carranza *et al.* 1998a,b). The resulting phylogenetic trees strongly indicated that the Paludicola is paraphyletic since the Terricola and one paludicolan family, the Dugesiidae, share a more recent common ancestor than the dugesiids with the other paludicolans (dendrocoelids and planariids). Therefore, it was suggested that the infraorders Terricola and Paludicola are redundant and should be replaced by a new taxon, the Continenticola (Carranza *et al.* 1998a). A comparison between Sluys' (1989a) and Carranza *et al.*'s (1998a) phylogenetic proposals is depicted in Figure 6.2.

We report here partial sequences of the Cytochrome Oxidase I (COI) gene from 21 taxa of the Terricola and the Paludicola, and new complete 18S rDNA sequences from five species of turbellarians. Published 18S rDNA sequences from the large data set of 18S rDNA sequences available from other Platyhelminthes, namely from the Proseriata, the Prolecithophora and the Rhabdocoela, are also included in the phylogenetic analysis. The aims of this paper are to: 1) Reassess the taxonomic status and the phylogenetic position within the Platyhelminthes of the Proseriata, the Bothrioplanida and the Tricladida; 2) Further test the new triclad phylogeny drawn from molecular data (Carranza *et al.* 1998a,b), in particular the paraphyletic status of the Paludicola and the monophyly of Terricola + Dugesiidae; and 3) Test the monophyletic or paraphyletic status of the Terricola and Dugesiidae and its internal phylogeny. Congruence and conflicts between the morphological and molecular phylogenies of the Tricladida are highlighted.

Materials and methods

The current taxonomic classification of the species used in this study is shown in Table 6.1.

Sequencing of the 18S molecule

High molecular weight DNA was purified according to a modification (Garcia-Fernàndez *et al.* 1993) of the guanidine isothiocyanate method initially described for RNA (Chirgwin *et al.* 1979) from live or ethanol-fixed specimens. The entire length of the 18S rDNA molecule was PCR-amplified applying specific primers and conditions described earlier (Carranza *et al.* 1996, 1998a; Littlewood and Smith 1995). Amplification

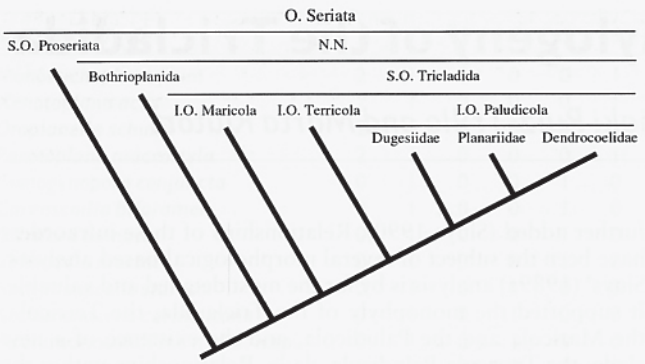


Figure 6.1 Internal phylogenetic relationships of the Seriata after Ehlers (1985), Sopott-Ehlers (1985) and Sluys (1989a), slightly modified. N.N.: unnamed taxon, according to Sopott-Ehlers (1985), formed by the Bothrioplanida and the Tricladida. For the sake of clarity, the Infraorder Cavernicola (Sluys 1990) of the Tricladida is not included.

products were sequenced directly. Sequencing of the clones and the PCR products was performed using an automated sequencer ABI Prism 377, following manufacturer's protocols.

Sequencing of the Cytochrome Oxidase I (COI) molecule

High molecular weight DNA was purified as described for the 18S rDNA gene (see above) from 21 species of Terricola and Paludicola (see Table 6.1). A fragment of approximately 450 nucleotides close to the centre of the cytochrome *c* oxidase subunit I mitochondrial gene was amplified. The primers used (pr-a2 and pr-b2) and the conditions of the PCR reaction were as described in Bessho *et al.* (1992). The PCR products were purified with GenecleanR II kit (BIO 101 Inc.) and directly sequenced using the same primers as for amplification. Cycle sequencing using Dye-labelled terminators (Prism™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit) was performed in a DNA Thermal Cycler Perkin-Elmer 480 according to the manufacturer's instructions and run on an automated sequencer ABI 377.

Sequence alignment

18S rDNA sequence data were aligned by hand with the help of a computer editor (GDE 2.2; Smith S.W. *et al.* 1994). Alignment gaps were inserted to account for putative length differences between sequences. A secondary structure model (Gutell *et al.* 1985) was used to optimize alignment of homologous nucleotide positions. Those positions that could not be unambiguously aligned were subsequently excluded resulting in a total of 1483 positions that could be used in the phylogenetic analyses.

CO I sequences were aligned by eye based on the protein sequences. There is a variable region in the middle of the fragment in which some species have one or two extra amino acids; this region has been excluded from the alignment. Given the variability of the third position of the codons it also was excluded from the phylogenetic analyses, resulting in a total of 232 positions that could be used in the subsequent studies.

The full sequence alignments used in these analyses have been deposited with EMBL under accessions ds41997 (18S) and ds42057 (CO I) and are available via anonymous FTP from FTP.EBI.AC.UK under directory pub/databases/embl/align

Phylogenetic analysis

Data sets were analysed using the programs in PHYLIP package v. 3.52 (Felsenstein 1993). A distance matrix was generated from the aligned sequences using the program DNADIST and corrected with the two-parameter method of Kimura (1980). The distances were then converted to phylogenetic trees using the NJ method of Saitou and Nei (1987) provided by the NEIGHBOR program. Bootstrap resampling (Felsenstein 1985) was accomplished with the use of the programs SEQBOOT (1000 replicates)

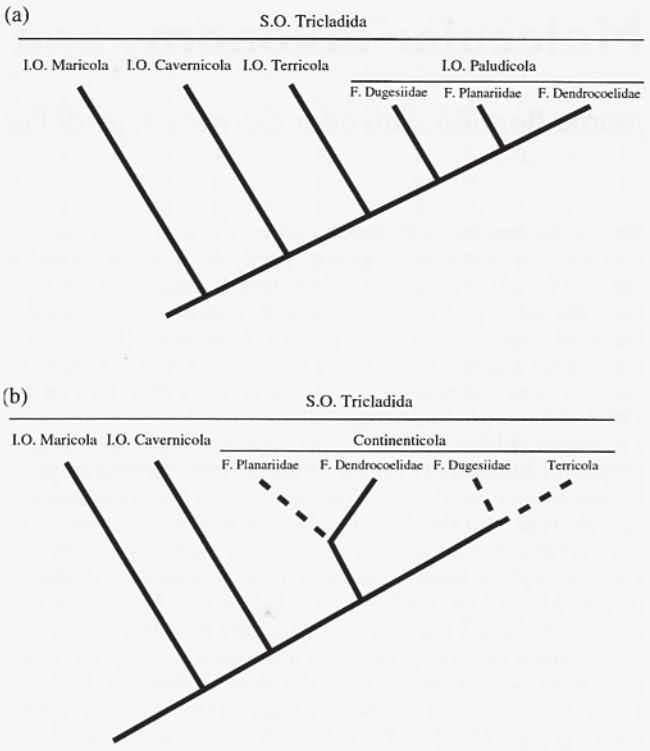


Figure 6.2 a) Phylogenetic relationships of the Tricladida based on morphological characters according to Sluys (1989a); b) Phylogenetic hypothesis for the Tricladida based on molecular characters (18S rDNA sequences and an 18S rDNA duplication event) (Carranza *et al.* 1998a). Dashed lines indicate groups that are not well-supported in the molecular phylogenetic analysis.

and CONSENSE. For the estimation of the maximum-likelihood (ML) trees we used the FastDNAml program v. 1.1.1a (with global rearrangements and reordering of species) (Felsenstein 1981; Olsen *et al.* 1994).

To root the Platyhelminthes internal tree, representatives of lophotrochozoans and ecdysozoans (*sensu* Aguinaldo *et al.* 1997) and from deuterostomates were chosen. For the Terricola + Dugesiidae trees, representatives of families Dendrocoelidae and Planariidae were chosen because they are known to be its sister-group (Carranza *et al.* 1998a).

Results

The phylogenetic position of the Tricladida within the Platyhelminthes

Both NJ and ML methods of phylogenetic reconstruction gave similar results as regards the position of the Tricladida and the Proseriata and the paraphyly of Seriata, only the NJ tree being shown (Figure 6.3). First of all, the Seriata appears as a polyphyletic assemblage, because Proseriata, Bothrioplanida and Tricladida never cluster together. Second, Bothrioplanida never clusters with proseriates and triclads, appearing with poor bootstrap support (NJ tree) as the sister-group of a vast assemblage of neoophorans and in the ML tree as the sister-group of the Neodermata. Third, Proseriata and Tricladida, represented by four or more sequences fall into clear recognizable monophyletic groups, with very high bootstrap support, regardless of the phylogenetic method used. Fourth, although their positions are variable, proseriates fall in both types of trees as a basal clade of neoophorans. And

Table 6.1 Species used in the analysis with classification. Classification of turbellarians following Cannon (1986). 18S sequence (+) and GenBank accession numbers, and COI sequences (*) and GenBank accession numbers (in brackets), are indicated. New 18S rDNA sequences reported in this paper are marked #. Note: The terricolan genus *Artioposthia* is now known as *Arthurdendyus* (Jones and Gerard 1999).

Classification	18S rDNA	COI	Accession number
Phylum Chordata			
Xenopus laevis	+		X04025
Phylum Echinodermata			
Asterias amurensis	+		D14358
Phylum Arthropoda			
Odiellus troguloides	+		X81441
Scolopendra cingulata	+		U29493
Phylum Annelida			
Eisenia foetida	+		X79872
Lanice conchilega	+		X79873
Phylum Mollusca			
Acanthopleura japonica	+		X70210
Nerita albicilla	+		X91971
Phylum Platyhelminthes			
Order Catenulida			
Family Stenostomidae			
Stenostomum leucops	+		U70085
Family Catenulidae			
Suomina sp.	+		L41129
Order Macrostomida			
Family Dolichomacrostomidae			
Paramalostomum fusculum	+		AJ012531
Family Macrostomidae			
Macrostomum tuba	+		U70080
Macrostomum hystricinum #	+		AF051329
Family Microstomidae			
Microstomum lineare	+		U70082
Order Polycladida			
Acotylea			
Family Leptoplanidae			
Notoplana australis	+		AJ228786
Notoplana koreana	+		D85097
Family Planoceridae			
Planocera multitentaculata	+		D17562
Family Discocelidae			
Discocelis tigrina	+		U70078
Cotylea			
Family Pseudocerotidae			
Thysanozoon brochii	+		D85096
Pseudoceros tritriatus	+		AJ228794
Order Haplopharyngida			
Family Haplopharyngidae			
Haplopharynx rostratus	+		AJ012511
Order Lecithoepitheliata			
Family Prorhynchidae			
Geocentrophora sp.	+		U70079
Geocentrophora baltica	+		AF065417
Geocentrophora spyrocephala	+		D85089
Geocentrophora wagini	+		AJ012509
Order Prolecithophora			
Combinata			
Proporata			
Family Pseudostomidae			
Pseudostomum gracilis	+		AF065423
Reisingeria hexaoculata	+		AF065426
Opisthoporata			
Family Cylindrostomidae			
Cylindrostoma fingalianum #	+		AF051330
Cylindrostoma gracilis	+		AF065416
Separata			
Family Plagiostomidae			
Plagiostomum cinctum	+		AF065418
Plagiostomum striatum	+		AF065420
Plagiostomum vittatum #	+		AF051331
Plicastoma cuticulata	+		AF065422
Vorticeros ijimai	+		D85094
Family Ulianiniidae			
Ulianinia mollissima	+		AF065427
Of uncertain status (see text)			
Family Urastomidae			
Urastoma cyprinae	+		U70085
Ichthyophaga sp.	+		AJ012512
Order Rhabdocoela			
Dalyelliida			
Family Dalyelliidae			
Microdalyellia rossi	+		AJ012515
Family Graffillidae			
Graffilla buccinicola	+		AJ012521
Family Pterascolidae			
Pterastericola australis	+		AJ012518
Family Fecampiidae			
Kronborgia isopodicola	+		AJ012513
Temnocephalida			
Family Temnocephalidae			
Temnocephala sp. #	+		AF051332
Temnocephala sp.	+		AJ012520
Typhloplanida			
Family Trigonostomidae			
Mariplanella frisia	+		AJ012514
Family Typhloplanidae			
Bothromesostoma personatum	+		M58347
Mesocastrada sp.	+		U70081
Kalyptorhynchia			
Family Polycystidae			
Gyratrix hermaphroditus	+		AJ012510
Schizorhynchia			
Family Schizorhynchidae			
Diascorhynchus rubrus	+		AJ012508
Family Karkinorhynchidae			
Cheliplana cf. *orthocirra*	+		AJ012507
Order Seriata			
Suborder Proseriata			
Family Bothrioplanidae			
Bothrioplana semperi #	+		AF051333
Family Monocelidae			
Monocelis lineata	+		U45961
Archiloa rivularis	+		U70077
Family Otoplanidae			
Otoplana sp.	+		D85090
Parotoplana renatae	+		AJ012517
Suborder Tricladida			
Infraorder Maricola			
Family Bdellouridae			
Bdelloura candida	+		Z99947
Family Procerodidae			
Ectoplana limuli	+		D85088
Procerodes littoralis	+		Z99950
Family Uteriporidae			
Uteriporus sp.	+		AF013148
Infraorder Terricola			
Family Geoplanidae			
Subfamily Caenoplaninae			
Artioposthia sp.		*	(AF178325)
Artioposthia testacea		*	(AF178305)
Artioposthia triangulata	+		AF033038
Caenoplana caerulea	+		AF033040
Australoplana sanguinea	+		AF033041
Subfamily Geoplaninae			
Geoplana ladislavi		*	(AF178313)
Family Bipaliidae			
Bipalium adventitium		*	(AF178306)
Bipalium kewense	+		AF033039
Bipalium sp.		*	(AF178307)
Family Rhynchodemidae			
Subfamily Microplaninae			
Microplana nana	+	*	AF033042 (AF178317)

Continued overleaf

Table 6.1 Continued.

Classification	18S rDNA	COI	Accession number
Microplana terrestris		*	(AF178318)
Subfamily Rhynchodeminae			
Platydemus manokwari		*	(AF178320)
Infraorder Paludicola			
Family Planariidae			
Polycelis nigra	+		AF013151
Polycelis tenuis	+	*	Z99949 (AF178321)
Crenobia alpina	+		M58345
Phagocata ullala	+		AF013149
Phagocata sp.	+		AF013150
Family Dendrocoelidae			
Dendrocoelum lacteum	+	*	M58346 (AF178312)
Baikalobia guttata	+		Z99946
Family Dugesiidae			
Schmidtea mediterranea	+	*	U31084 (AF178322)
Schmidtea polychroa	+	*	AF013152 (AF178323)
Cura pinguis	+	*	AF033043 (AF178309)
Dugesia subtentaculata	+		M58343
Dugesia etrusca		*	(AF178310)
Dugesia japonica	+	*	AF013153 (D499166)
Dugesia ryukyuensis		*	(AF178311)
Neppia montana		*	(AF178319)
Spathula sp.		*	(AF178324)
Girardia tigrina	+	*	AF013157 (AF178316)
Girardia dorotocephala		*	(AF178314)
Girardia anderlani		*	(AF178315)
Neodermata			
Class 'Monogenea' — incerta sedis			
Udonella caligorum	+		AJ228796
Class Trematoda			
Order Strigeida			
Family Schistosomatidae			
Schistosoma mansoni	+		M62652
Order Echinostomida			
Family Fasciolidae			
Fasciolopsis buski	+		L06668
Order Plagiorchiida			
Family Gyliauchenidae			
Gyliauchen sp.	+		L06669
Class Eucestoda			
Order Cyclophyllidea			
Family Taeniidae			
Echinococcus granulosus	+		U27015

finally, Tricladida forms a monophyletic group with very high bootstrap support (100%) and appears as the sister-group, with a rather weak support (42%) in NJ trees, to the Prolecithophora. Using both phylogenetic methods, the new clade formed by Tricladida + Prolecithophora, shifts the clade formed by *Urastoma*, Fecampiida (*Kronborgia*) and *Ichthyophaga* (Littlewood *et al.* 1999a) to a more external position forming the sister-group of the Tricladida + Prolecithophora and altogether a new clade with a 79% bootstrap support.

Molecular phylogeny of the Tricladida: the monophyly of the Maricola, the paraphyly of the Paludicola, and evidence for a clade Dugesiidae + Terricola

All dugesiids and all the Terricola sampled so far show two types of 18S ribosomal genes homologous to the type I and type II genes described in the dugesiid *Schmidtea mediterranea* Benazzi *et al.* 1975, by Carranza *et al.* (1996). Using this duplication event and the sequences of either type I or type II, it was found that: 1) the Maricola form a monophyletic primitive group; 2) the Terricola and the Dugesiidae cluster together with high support irrespective of the phylogenetic method used (NJ, MP and ML; Carranza *et al.* 1998a,b); and 3) the other paludicolan families, the Planariidae and Dendrocoelidae form the sister-group of the Terricola + Dugesiidae clade. A NJ tree using only type I 18S rDNA sequences is shown in Figure 6.4. The clade Terricola + Dugesiidae has a 100% bootstrap support, and its sister group a support of 99%. ML trees (not shown) had also both sister-groups very well supported.

Although the topology of the tree drawn from COI sequences is different from that derived from 18S rDNA sequences, it also supports with very high bootstrap support (100%) the clustering of Terricola and Dugesiidae and the sister-group character of the Planariidae and Dendrocoelidae (Figure 6.5).

Are the Terricola and the Dugesiidae monophyletic clades?

In both NJ trees drawn from 18S rDNA sequences (Figures 6.3 and 6.4) the monophyly of the Terricola is not supported or very weakly supported. Likewise, trees derived from COI sequences show monophyly of the Terricola, but with very weak support. A similar situation holds for the Dugesiidae, the anomaly in 18S trees being the clustering of the dugesiid *Girardia tigrina* Girard, 1850 (Figure 6.3) within the Terricola, and in COI trees the early branching of *Spathula* sp. Nurse, 1950 (Figure 6.5). Nevertheless, both sets of trees reproduce with high bootstrap support monophyletic assemblages such as the dugesiid genera *Schmidtea* Ball, 1974, and *Dugesia* Girard, 1850. In addition, the COI tree supports the family Bipaliidae, the subfamily Microplaninae and the genus *Girardia* Ball, 1974. It is worth noting the 'anomalous' position in COI trees of the rhynchodemid *Platydemus manokwari* de Beauchamp, 1962, within the Geoplanidae and of the dugesiid species *Girardia anderlani* Kawakatsu and Hauser, 1983, within the genus *Dugesia* and not with the other *Girardia* species.

Discussion

In his detailed analysis on the phylogenetic relationships of the triclads, Sluys (1989a) stated that research in this group 'is in a state of flux'. The advent of molecular studies, largely based on 18S rDNA sequences, has increased the rate of flux. This reflects the apparent instability of the morphological framework due to increasing difficulties of distinguishing homologies from recurrent convergences or homoplasies but, at the same time, also reflects the inconsistencies of most molecular data often based on incomplete sampling and, so far, on a single (namely 18S rDNA) or a few genes.

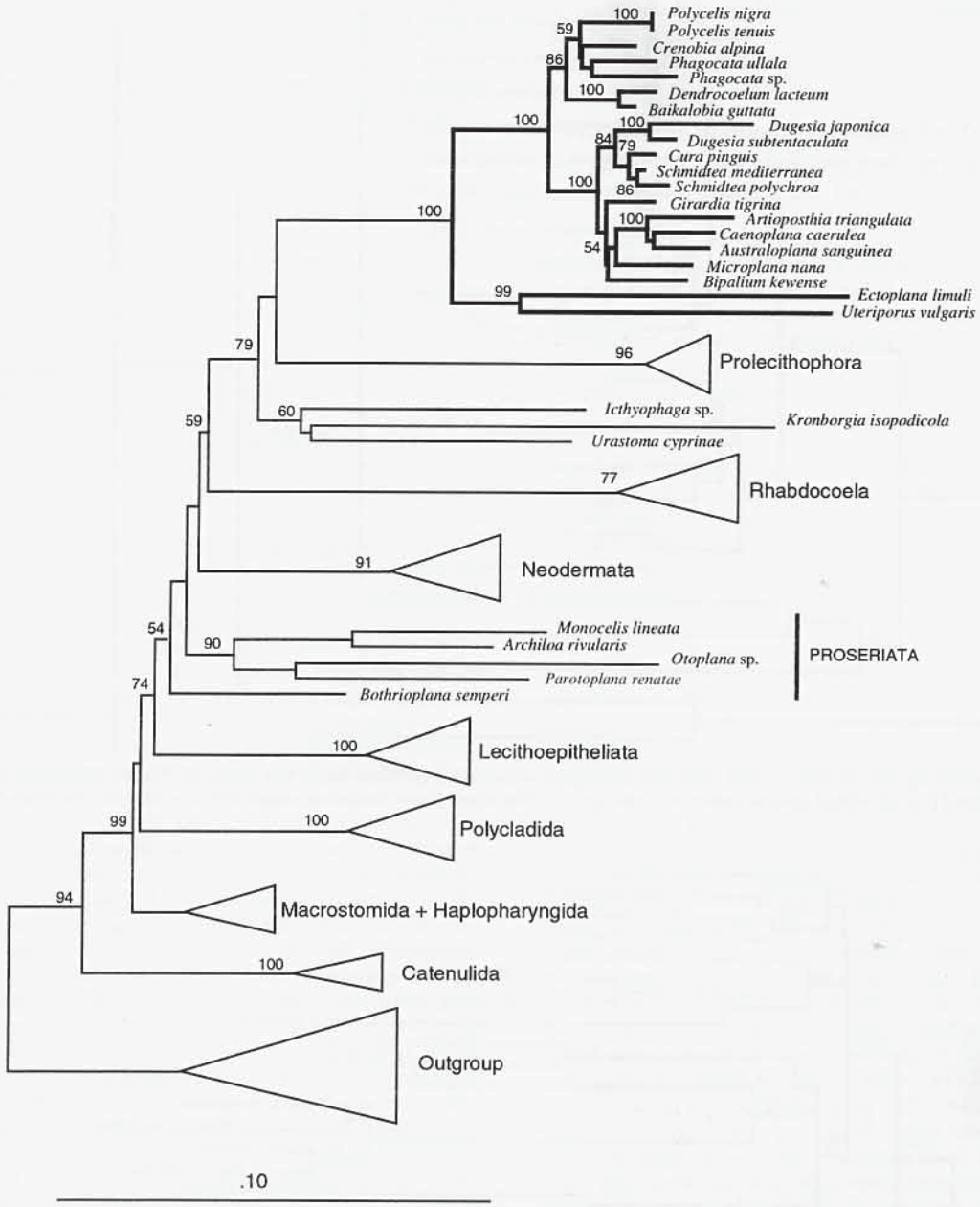


Figure 6.3 Neighbour-joining tree of the Platyhelminthes and other triploblasts (outgroup) based on 18S rDNA data set. Numbers on branches represent bootstrap percentages (n = 1000), only those over 50% being indicated. The tree illustrates the position of the Tricladida (top of the tree, bold lines) and its sister relationship to the Prolecithophora and the paraphyly of the Seriata. For taxa and species names, see Table 6.1. The complete tree with all species names is available on request from the authors. Scale indicates substitutions per position.

The validity of the taxon Seriata and the position of the Tricladida within the Platyhelminthes

The phylogenetic placement of the Tricladida within the Platyhelminthes is an example of these uncertainties. Most taxonomists consider the Tricladida a highly derived group of turbellarians, largely due to the rather complex morphological structure and to the advanced features of embryonic development. The precise phylogenetic position of the Tricladida and its sister-group relationships, however, have not been fully resolved. On the basis of a large set of morphological and embryological characters, Karling (1974) placed the Tricladida close to the Proseriata and not far from Prolecithophora and the Rhabdocoela, a position also supported by Ehlers (1985a) and by Smith *et al.* (1986). Based on protonephridial structure, Rohde (1990) placed them close to the Polycladida whereas the Proseriata fell close to Neodermata. Finally, using a matrix of 65 equally weighted and unordered morphological characters, the 50% majority-rule consensus solution found also suggested that Tricladida and Proseriata do not constitute a monophylum (Littlewood *et al.* 1999a). The Tricladida was found, however, albeit with very poor support, within a clade together with Polycladida, Macrostomida and Haplopharyngida, whereas Proseriata appeared as the sister group of the Fecampiida. Recoding of some characters to take into account proposed synapomorphies for spermiogenesis between the

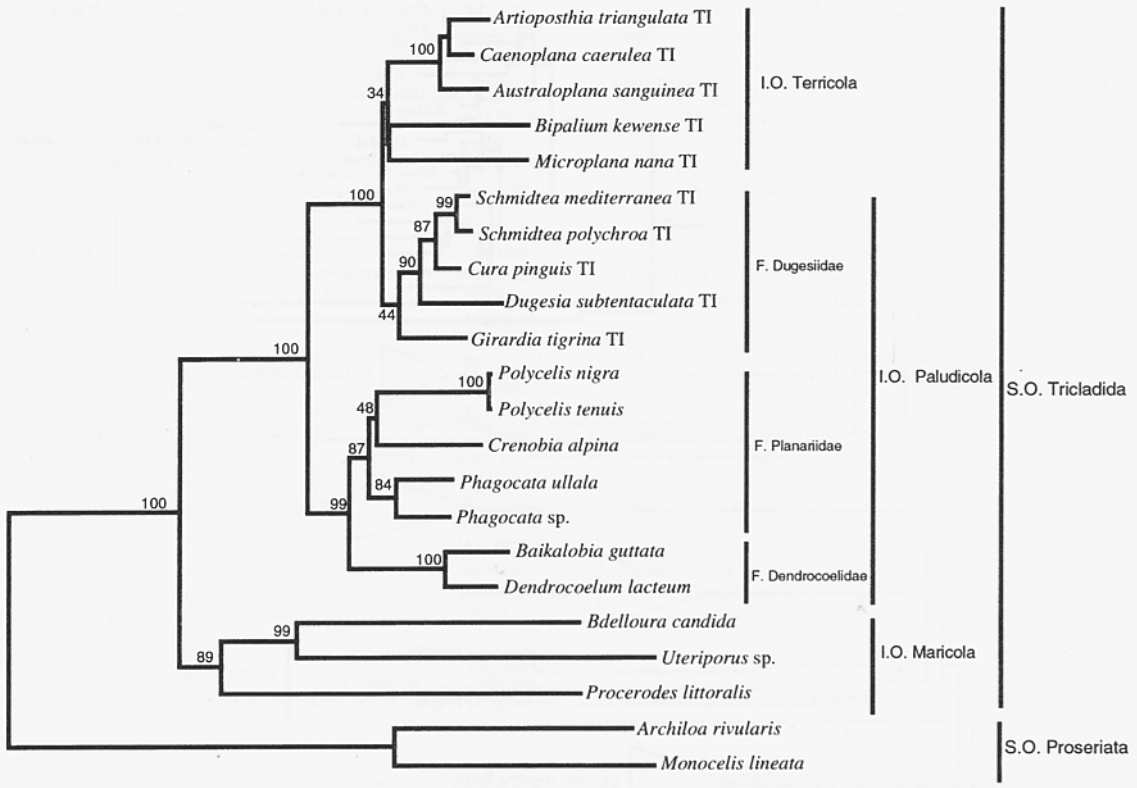


Figure 6.4 Neighbour-joining tree of the Tricladida using 18S rDNA gene sequences with Proseriata as the outgroup. For the Dugesiidae and the Terricola only the type I (TI) 18S rDNA gene sequence (Carranza *et al.* 1998a,b) was used. Bootstrap support (%; n = 1000) indicated above the nodes.

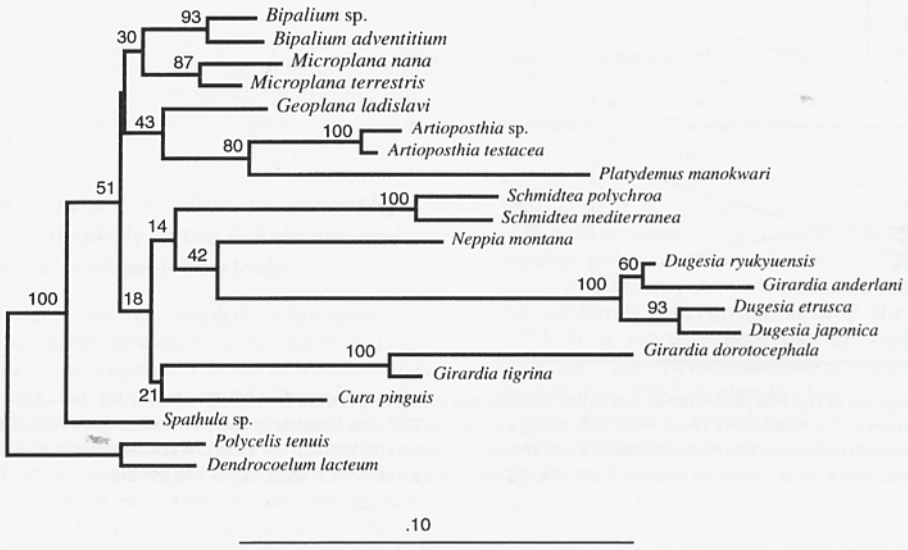


Figure 6.5 Neighbour-joining tree based on partial sequences of the cytochrome oxidase I gene of 21 species of Terricola and Paludicola. Only first and second positions were used for analysis. Numbers on branches represent bootstrap percentages (n = 1000). For taxa and species names, see Table 6.1. Scale indicates substitutions per position.

Fecampiida, *Urastoma* and the Neodermata (Joffe and Kornakova 1998), supported a new tree that, as regards the position of the Tricladida, did not differ substantially to those from previous analyses (Littlewood *et al.* 1999a).

Molecular data based on partial 18S and 28S rDNA sequences have added very little but confusion. Triclads were found either: 1) close to Rhabdocoela (Rohde *et al.* 1995); 2) as a group close to the prolecithophorans (Kuznedelov and Timoshkin 1995); 3) as a basal platyhelminth group branching early, second only to the acoels (Katayama *et al.* 1996); 4) as forming the sister-group to a clade made up of the Acoela and the rhabdocoel fecampiid *Kronborgia* (Jondelius 1998); 5) as the sister-group to the Acoela (Campos *et al.* 1998); and 6) as the sister-group of the Fecampiidae, both forming a sister-group to some rhabdocoels (Litvaitis and Rohde 1999). Studies based on complete 18S rDNA sequences (Carranza *et*

al. 1997; Littlewood *et al.* 1999a; Norén and Jondelius 1999) gave a more consistent position for the Tricladida as they appeared as the sister-group to the Rhabdocoela or to the Prolecithophora (Carranza *et al.* 1997; Norén and Jondelius 1999), or to a clade made by *Urastoma*, Fecampiida (*Kronborgia*) and *Ichthyophaga* (Littlewood *et al.* 1999a). The position of the Proseriata in molecular trees was found to be rather variable. They clustered at the base of the neoophorans (*sensu* Ehlers 1985a) either with the Lecithoepitheliata, the Neodermata or, oddly enough, with the Nemertodermatida. The clustering of Nemertodermatida and Proseriata is, however, unusual and very likely artefactual due to the poor sampling of this group (Carranza *et al.* 1997) and because there are no sound morphological arguments to place them together. When morphological and the 18S rDNA evidence were combined ('total evidence approach'; Littlewood *et al.* 1999a), Tricladida and Proseriata were not monophyletic, i.e., the taxon Seriata is invalid. In addition, Tricladida appeared, as in the molecular trees, as the sister-group to a clade formed by *Urastoma*, Fecampiida (*Kronborgia*) and *Ichthyophaga*.

New sequences of prolecithophorans and rhabdocoels now available from GenBank, added to those available within the large data set of turbellarians, made up 72 full-length sequences of Platyhelminthes (20 from the Tricladida) for analysis. Results show, albeit with a rather weak support (42% bootstrap; Figure 6.3), that prolecithophorans and triclads are monophyletic sister clades. A relationship between both groups had already been advanced by Kuznedelov and Timoshkin (1995) and more recently by Norén and Jondelius (1999). The first study, however, was based on rather short stretches (340–368 bp) of 18S rDNA from a rather restricted sampling of both groups, and lacked suitable outgroups. The second, based on full-length sequences, showed a consistent sister-group relationship between triclads and prolecithophorans and rejected the family Urastomidae as a member of the Prolecithophora. The new position of the prolecithophorans seems to exclude the clade formed by *Urastoma*, Fecampiida (*Kronborgia*) and *Ichthyophaga* as the sister-group of the triclads as suggested by Littlewood *et al.* (1999a). The latter clade and prolecithophorans + triclads form altogether a new clade well supported in NJ (79%) and ML trees, which is worth analysing in the future. The main autapomorphy of prolecithophorans is the aflagellate spermatozoa with no dense bodies and an extensive intracellular membrane system. No synapomorphies are known between prolecithophorans and triclads, though the lack of sclerotized structures, some features of the protonephridial system and the pharynx, and the structure of the female copulatory apparatus are worth exploration. NJ and ML trees also supported the monophyly and the basal position of the Proseriata within the neoophorans, confirming molecular analyses by Carranza *et al.* (1997) and Littlewood *et al.* (1999a). In addition, the Bothrioplanida, here represented by *Bothrioplana semperi*, and considered a proseriate (see Cannon 1986) or the sister-group of the Tricladida (Sopott-Ehlers 1985), was never seen clustering with the Tricladida. Instead it appears as a sister-group, albeit with weak support, to the Neodermata (ML trees), or to a large group of neoophorans (NJ tree; Figure 6.3). Hence, the presumed synapomorphies linking the Bothrioplanida and the Tricladida (a tricladoid intestine and a crossing over of muscle layers at the pharynx; Sopott-Ehlers 1985) may be convergences and should be reassessed. To summarize, available evidence gathered from this as well as from previous molecular analyses, strongly suggests that the Order Seriata is not a valid taxon because the Proseriata and Tricladida are monophyletic unrelated taxa, that the Bothrioplanida is not the sister taxon of the Tricladida, and that the Prolecithophora and Tricladida are likely sister-taxa.

Phylogenetic relationships within the Tricladida: the paraphyly of the Paludicola and the internal phylogeny of the Dugesiidae and the Terricola

The clustering of the Terricola and the paludicolan family Dugesiidae and, therefore, the paraphyly of the Paludicola is mainly based on their sharing of an 18S gene duplication (Carranza *et al.* 1998a,b). That this resulted from a single duplication event in the ancestor of both clades and not from independent duplication events after the split between terricolans and dugesiids was deduced from intra- and interspecific genetic distances found between type I and type II 18S genes (Carranza *et al.* 1998a, 1999). Moreover, using either type I or type II sequences, the Terricola and the Dugesiidae always cluster together with very high bootstrap support in MP and NJ trees (see Figure 6.4 for Type I sequences). In addition to this molecular synapomorphy, a character thought to be an autapomorphy for the dugesiids, the multicellular eye cup, was proposed to be a morphological synapomorphy for terricolans and dugesiids (Carranza *et al.* 1998a).

The phylogenetic tree of paludicolans derived from partial sequences of the cytochrome oxidase c subunit I (COI) gene (Figure 6.5) further reinforces the evidence for a clade Terricola+Dugesiidae, because it shows a 100% bootstrap support for the Planariidae+Dendrocoelidae as the sister-group to the Terricola+Dugesiidae. However, neither 18S gene nor COI gene sequences support the monophyly of either the Terricola and the Dugesiidae rendering them, so far, paraphyletic. In 18S gene trees, the odd placement of *Girardia tigrina* within the Terricola, represents its main drawback. Even so, these trees reproduce the clustering of the genus *Cura* Strand, 1942, and *Schmidtea* Ball, 1974, with the genus *Dugesia* next to them, and *Neppia* Ball, 1974, usually making the external group to the former three. Instead, the genus *Spathula* Nurse, 1950, has, together with *Girardia*, a rather erratic position. The clustering of *Schmidtea* and *Cura* with *Dugesia* contradicts the clustering of *Cura* and *Schmidtea* with *Girardia* (de Vries and Sluys 1991) which was based on a single synapomorphy: the 'angled bursal canal'. This character is only present in *Girardia* (Carranza *et al.* 1998a) and, therefore, has been rejected as a synapomorphy for these three genera (Sluys *et al.* 1998a). In contrast to *Schmidtea* and *Dugesia*, for which more than one species have been sequenced for the 18S gene, only a single species each is so far available for the genera *Girardia* and *Neppia*. This makes the sampling too poor to draw any firm conclusion. Besides, *Neppia* and *Spathula* are poorly defined genera (Sluys 2001, this volume). A similar situation holds for the Terricola, only the clustering of the Geoplanidae (type I gene), represented by three species, being highly supported.

In COI trees, all terricolans and most dugesiids cluster together. However, the very low bootstrap support found does not permit any firm conclusion to be drawn regarding the monophyly of these two clades. Within the Terricola, and despite insufficient sampling, the family Bipaliidae and the subfamily Microplaninae of the Rhynchodemidae appear highly supported. However, the position of the rhynchodemid *Platydemus manokwari* (subfamily Rhynchodeminae) within the Geoplaniidae (also found for type II 18S gene trees; Figure 6.4) suggests that the rhynchodemids are polyphyletic and the geoplaniids are paraphyletic. The family Rhynchodemidae has

always been considered an artificial assemblage, with subfamilies Rynchodeminae and Microplaninae being only loosely related (personal communication, Leigh Winsor 1998). The non-clustering of both subfamilies in both 18S and COI gene trees may reflect this situation, and deserves a better sampling and further studies. As regards the Dugesiidae, the clustering of *Dugesia* and *Schmidtea* and, more loosely, *Neppia* is reproduced as it was for the 18S gene tree, but the single *Cura* species, *Cura pinguis*, does not cluster with them. In addition, the single *Spathula* species falls outside the Dugesiidae.

A special mention must be made of the odd placement in COI trees of *Girardia anderlani* within the genus *Dugesia* (Figure 6.5). Taken at face value it may be either an artefact, a misclassified specimen, or that *Girardia anderlani* does not actually belong to *Girardia* but to *Dugesia*. *Girardia anderlani*, described so far from Brazil (Kawakatsu *et al.* 1983), is externally similar to other species belonging to the genus *Girardia* living in South America. However, it differs internally in the presence of dorsal testes (usually ventral in other *Girardia*, though a few also have dorsal testes) and in their large and very asymmetrical penial bulb. Moreover, its chromosome number of $2x = 18$; $n = 9$, differs to those of most *Girardia* species, usually bearing chromosome numbers of $2x = 8$, 16 or 24; $n = 4$ or 8, the only exception being *Dugesia* (*Girardia*) *cubana* Codreanu and Balcesco, 1973, with $2x = 18$. Chromosome numbers of $2x = 18$ have been described for all species of the genus *Dugesia* belonging to the *Dugesia sicula* group (Baguñá *et al.* 1999). Therefore, given the clustering of *Girardia anderlani* within the *Dugesia* species in COI trees, and its specific karyotype, we suggest it may actually belong to the genus *Dugesia*. Otherwise, and considering that the genus *Dugesia* has not been reported from North or South America (Sluys *et al.* 1998a) some misclassification or cross-contamination may have occurred.

Main conclusions and prospects

To summarize, phylogenetic analyses of 18S ribosomal sequences of 72 Platyhelminthes species, including those of 20 species of triclads, together with the phylogenetic analyses of cytochrome oxidase subunit I partial gene sequences from 21 species of triclads show that: 1) the Tricladida is a monophyletic taxon not related to the Proseriata; therefore, validity of the taxon Seriata is rejected; 2) 18S sequence analyses show for both NJ and ML trees a sister-group relationship between the Tricladida and the Order Prolecithophora; 3) a new clade made by Tricladida + Prolecithophora and a clade formed by *Urastoma*, Fecampiida (*Kronborgia*) and *Ichthyophaga* is well supported in both NJ and ML trees deserving further studies; 4) the Bothrioplanida does not appear in any of the analyses as the sister-group of the Tricladida; 5) 18S rDNA sequence analyses, the duplication event involving the 18S ribosomal gene, and COI sequence analyses, strongly support the paraphyly of the Paludicola and the validity of the Terricola + Dugesiidae clade; and 6) 18S rDNA and COI sequences do not validate so far, probably because of insufficient sampling, the monophyly of the Terricola and the Dugesiidae.

Altogether, the new sister-group relationship between the Tricladida and the Prolecithophora, together with 18S rDNA and COI data reported in Carranza *et al.* (1998a,b) and in this work, support a new phylogenetic hypothesis for the Tricladida which is depicted in Figure 6.6.

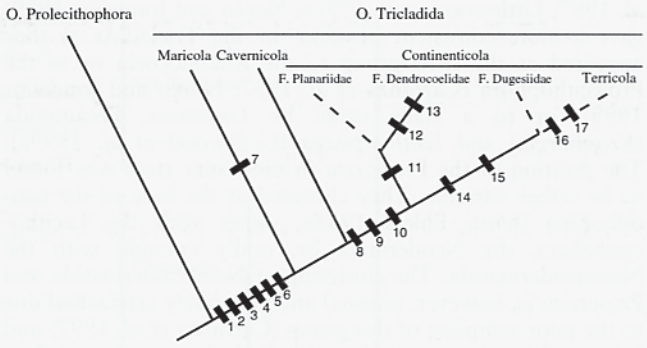


Figure 6.6 A new phylogenetic hypothesis for the Tricladida proposed in the present study. It illustrates the monophyly of the Tricladida and its sister-group relationships to the Order Prolecithophora, the monophyly of the Maricola, the clade formed by the Dugesiidae and the Terricola, and the recently proposed clade, the Continenticola, grouping the present families Dugesiidae, Planariidae, Dendrocoelidae and the Terricola. Selected morphological characters from Sluys (1989a) and the molecular apomorphy (character 15) from Carranza *et al.* (1998a) have been mapped onto the tree, with black prisms referring to derived characters. 1, tricladoid intestine; 2, crossing-over of pharynx muscles; 3, embryology; 4, cerebral position of female gonads; 5, serial arrangement of many nephridiopores; 6, marginal adhesive zone; 7, Haftpapillen in annular zone; 8, loss of Haftpapillen; 9, resorptive vesicles; 10, reduction in number of longitudinal nerve cords; 11, common oviduct opening into atrium; 12, dendrocoelid pharyngeal musculature; 13, anterior adhesive organ; 14, multicellular eye cup with numerous retinal cells; 15, two types of 18S rDNA genes (type I and type II); 16, creeping sole; 17, diploneuran nervous system.

To further support the conclusions here obtained, and to resolve the contradictions posed, further studies will be needed, including a denser sampling of Tricladida taxa for molecular data, complementary sequences from independent genes, and the broad and thorough morphological database already available for the Tricladida (Sluys 1989a, 1990; Sluys *et al.* 1998a), used together in a combined 'total evidence' approach.

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Short Report

The first bilaterian organisms: simple or complex? New molecular evidence

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ABSTRACT The quest for the first bilaterian organisms is the biggest riddle in metazoan evolution and in understanding the evolution of developmental mechanisms. Recent molecular work has regrouped the bilaterian phyla into three superphyletic clades: the Deuterostomia, the Lophotrochozoa and the Ecdysozoa. In these trees, Platyhelminthes, for a long time considered basal bilaterians, have a more derived position among the Spiralia. However, a recent 18S rDNA analysis showed Platyhelminthes to be polyphyletic with one of its orders, the Acoela, as the earliest extant bilaterian. To corroborate such position, we have sequenced new 18S and other nuclear genes, two mitochondrial genes, and examined the number and type of Hox cluster genes in acoels, nemertodermatids and other platyhelminthes and metazoans. Results confirm acoels and nemertodermatids as the earliest extant bilaterians. These results imply that the last common bilaterian ancestor was a small, benthic, direct developer without segments, coelomic cavities, nephridia or a true brain. In addition this argues for an extended pre-Cambrian period within which different simple bilaterian stem lineages emerged from which more complex ones diversified during the Cambrian explosion.

Over 150 years, morphologists and embryologists have proposed different hypothesis on the nature of the first bilaterian organism. Such hypotheses hinge into two basic forms. The first, the planuloid-acoeloid theory, posits a small and structurally simple acoel-like ancestor, similar in its organization grade to today's planulae larvae of cnidarians, from which the rest of bilaterian phyla evolved by stages of increased size and complexity. In this view, today's simple unsegmented acoelomate and pseudocoelomate organisms would be basal bilaterians, whereas coelomate segmented bilaterians should be derived. The alternative hypothesis, suggests instead a rather large and complex organism as the ancestral bilaterian (the so-called 'Urbilateria', Kimmel 1996) bearing a mouth and anus, coelom, segments, a primitive heart and, very likely, some sort of appendages (De Robertis, 1997). Under such conception, acoelomate/pseudocoelomate unsegmented organisms would be structurally simplified organisms derived from coelomate bilaterians. A recently proposed third scenario suggest a small ciliated primary larva with a population of set-aside cells as the ancestral bilaterian (reviewed in Petterson *et al.*, 2000). This hypothesis hinges on the assumption that 'maximal indirect development' is ancestral for bilaterians, direct development being derived. However, several difficulties turn untenable this hypothesis.

In the last 10 years and thanks largely to molecular systematic studies based on 18SrDNA sequencing and Hox cluster synapomorphies, the metazoan phylogenetic tree has been reorganized into three main clades of bilaterian organisms: Deuterostomia, Ecdysozoa and Lophotrochozoa (Aguinaldo *et al.*, 1997). The main casualties of this process have been the acoelomate and pseudocoelomate organisms, once considered intermediate forms between diploblasts and higher bilaterians (coelomates), and now displaced to much higher positions inside the tree (Adoutte *et al.*, 1999). This new scenario backs the 'Urbilateria' hypothesis featuring a large and complex bilaterian ancestor. However, the branching order between these three clades and within each of them is still unresolved leaving, at the very least, the position of acoelomates and pseudocoelomates within them still unsettled. This new status quo was recently upset by an 18S rDNA based sequence work showing that platyhelminth Acoela is the most basal extant bilaterian lineage distinct from the other platyhelmiths (Ruiz-Trillo *et al.*, 1999). These results rendered the Platyhelminthes polyphyletic. More importantly, they implied that the last common bilaterian ancestor was small, benthic, without segments and coelomic cavities, and having direct development. This invalidated the Urbilateria model and resurrected the idea of an extant evolutionary intermediate of 'simple' design as postulated by the planuloid-acoeloid theory.

To further test the position of acoels as basal bilaterians we have undertaken a multigenic approach together with a search for non-homoplasious molecular synapomorphies. In particular we have obtained sequences of 18S rDNA and the mitochondrial genes Cox1 and Cytb from new acoels and from four nemertodermatids, a group of basal platyhelminths close to acoels. In addition, nuclear genes other than 18S (e.g. TPI, triose phosphate isomerase; and MHC, myosin heavy chain), and two mitochondrial genes (Cox1 and Cytb) have also been sequenced from a large set of metazoans. Finally, the order of genes in the mitochondrial genomes of acoels, nemertodermatids and other platyhelminthes as well as the number and type of Hox cluster genes in acoels are at present under study. Data were analysed with parsimony and maximum likelihood methods.

Main results can be summarized as follows: 1) sequences of 18S rDNA from nemertodermatids mapped onto the rDNA general tree of metazoans branch basal to the rest of bilaterians, second only to acoels (Fig. 1); 2) whereas TPI was found to be uninformative, metazoan trees based on the MHC gene se-

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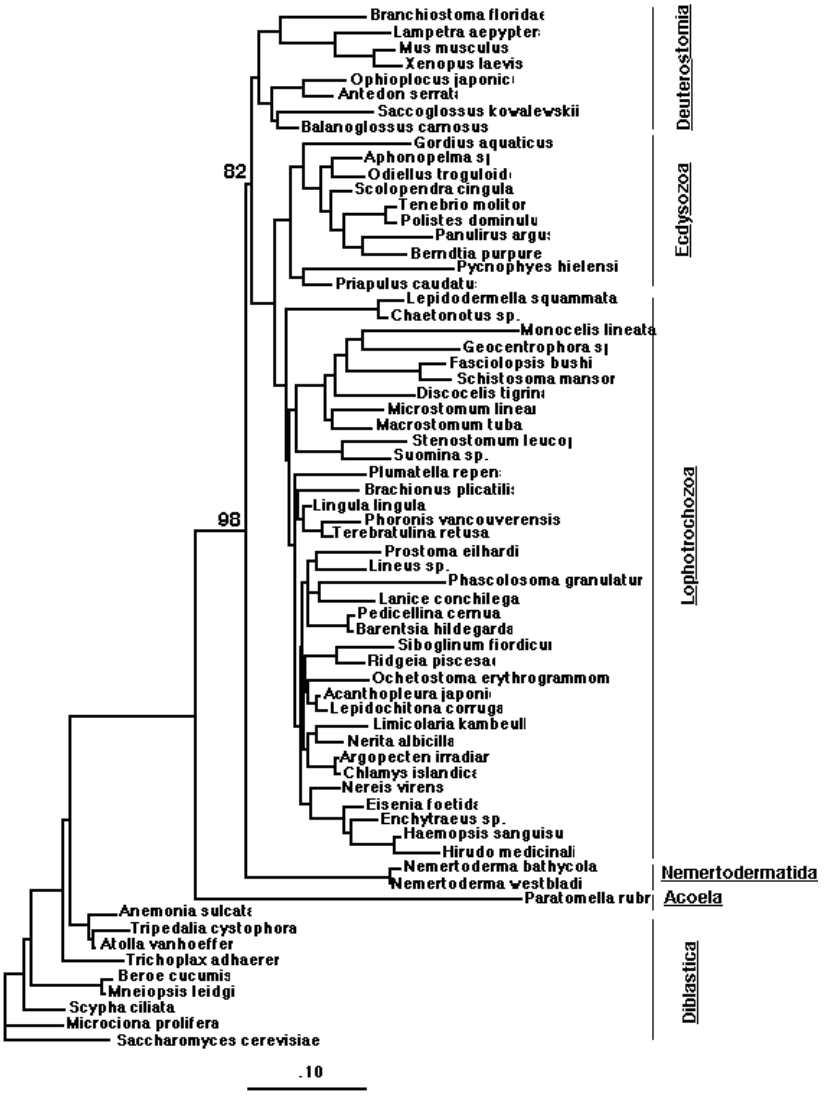

Fig. 1. Maximum Likelihood tree of 18S rDNA sequences from 66 metazoan taxa. *Branch support for the Acoela and Nemertodermatida are indicated at the corresponding nodes. The tree ilustrates the position of both clades as sister groups to the rest of the Bilateria. The rest of the Platyhelminthes branch within the Lophotrochozoa.*

quences resolves into the three bilaterian clades, with acoels branching at the base of the bilaterians; 3) concatenated analyses of 18S rDNA+ two mitochondrial genes show acoels and nemertodermatids to branch separately at the base of the bilaterians; and 4) despite an extensive search carried out in several labs, the number of Hox and ParaHox genes detected in acoels is so far consistently limited to three: a *labial*-like, an *Antennapedia*-like and a *Caudal*-like (Saló *et al.*, 2001). Unfortunately, attempts to detect lophotrochozoan synapomorphies in the *Antp*-like gene of acoels (e.g. the UbdA peptide and the spiralian peptide; Telford, 2000) have so far been unsuccesful. The presently limited number of Hox genes in acoels, if it holds, may also be indicative of its primitiveness.

If acoels and nemertodermatids are basal bilaterians this argues for an extended pre-Cambrian period within which a few different and simple bilaterian lineages emerged from which more complex ones diversified during the Cambrian explosion (Knoll and Carroll, 1999). Moreover, were acoels and nemertodermatids basal bilaterians, the rest of bilaterians should bear some synapomorphies for which acoels and nemertodermatids must have the plesiomorphic condition in common with an outgroup (e.g. cnidarians). Two of these synapomorphies could be the presence of a true brain and protonephridia. Finally, acoels and nemertodermatids will be instrumental to study how the synapomorphies defining all bilaterians (e.g. bilateral symmetry with two orthogonal body axes compared to radial/biradial symmetry and a single body axis in diploblasts, presence of mesoderm or endomesoderm, and clustering of nerve cells into a sort of primitive brain) came about at the diploblast-triploblast transition.

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Original Article

Hox and *ParaHox* genes in Nemertodermatida, a basal bilaterian clade

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ABSTRACT Molecular evidence suggests that Acoelomorpha, a proposed phylum composed of acoel and Nemertodermatida flatworms, are the most basal bilaterian animals. Hox and ParaHox gene complements characterised so far in acoels consist of a small set of genes, comprising representatives of anterior, central and posterior genes, altogether Hox and ParaHox, but no *PG3-Xlox* representatives have been reported. It has been proposed that this might be the ancestral Hox repertoire in basal bilaterians. However, no studies of the other members of the group, the Nemertodermatida, have been done. In order to get a more complete picture of the basal bilaterian Hox and ParaHox complement, we have analysed the Hox/ParaHox complement of the nemertodermatid *Nemertoderma westbladi*. We have found representatives of two central and one posterior Hox genes, as well as an *Xlox* and a *Caudal* ParaHox gene. From our data we conclude that a *PG3-Xlox* gene was present in the ancestor of bilaterians. These findings support the speculation that basal bilaterians already had the beginnings of the extended central Hox set, driving back gene duplications in the central part of the Hox cluster deeper in phylogeny than previously suggested.

KEY WORDS: *Hox, ParaHox, evolution, nemertodermatida, basal bilateria*

Introduction

The Hox gene family encodes for transcriptional regulators of development, which have a characteristic 60 amino-acid DNA binding motif, encoded by the homeobox. Hox genes play an important role in embryonic development, patterning the A/P axis of the majority of metazoans. This role and the colinearity (whereby the gene order in the genome reflects the antero-posterior pattern of expression in the embryo) they frequently exhibit, predate the divergence of protostomes and deuterostomes (Carroll, 1995). The number and type of Hox genes in a particular animal can be indicative of its phylogenetic relationships (de Rosa *et al.,* 1999). Thus, the presence of a particular gene or a particular peptide motif in one of the genes may be a clue to help assign an animal to one or other bilaterian clade. Several examples of these can be found in the literature, such as the presence of an *abd-B* like posterior gene in ecdysozoans whereas *Post 1* and *2* genes are found in Lophotrochozoa (de Rosa *et al.,* 1999), the presence of a particular set of central genes in Lophotrochozoa (*Lox 2, 4* and *5*) in contrast to those found in Ecdysozoa (*Ubx* and *abd-A*) (de Rosa *et al.,* 1999), or the presence of "molecular signatures" in some Hox paralogue groups (e.g., "spiralian peptide" in *Lox5* or a the "UbdA peptide" at 3'of the *Ubx-Lox* genes of protostomes, which is not present in deuterostome genes) (Bayascas *et al.,* 1998; de Rosa *et al.,* 1999; Telford 2000; Galant and Carroll, 2002).

The evolution of Hox genes has been the subject of much discussion over the last decade. A consensus of two sister clusters of genes emerging by duplication from a ProtoHox cluster has imposed over other theories (Brooke *et al.,* 1998). These two clusters, Hox and ParaHox, have undergone different patterns of evolution after their split. Briefly, whereas the Hox cluster expanded by tandem duplications during evolution, the ParaHox cluster did not increase the number of genes. Current hypotheses propose a ProtoHox cluster of either 2, 3 or 4 genes (Garcia-Fernàndez, 2005b). In the 4-gene cluster model, an anterior, a PG3-Xlox, a central and a posterior gene would have given rise to a primordial Hox cluster and a primordial ParaHox cluster of 4 genes. The ParaHox cluster would have then lost the central gene (Brooke *et al.,* 1998); a 3-gene cluster model (with anterior, PG3-Xlox and posterior genes) implies the genesis, by tandem duplication, of the central Hox gene in the Hox cluster, after the

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duplication of the ProtoHox cluster (Finnerty and Martindale, 1999; Ferrier and Holland, 2001); the 2-gene cluster model (one anterior, one posterior) implies the genesis, after the ProtoHox cluster duplication, of the PG3 and central Hox genes and of the *Xlox* ParaHox gene (Garcia-Fernandez, 2005a). The last scenario is based on the absence, so far, of clear Hox PG3 and Central and ParaHox Xlox genes in Cnidarians. Support for the first two hypotheses however comes from branching patterns in phylogenetic trees of the distinct Hox and ParaHox genes, as proposed in Brooke *et al.* (1998) and Kourakis and Martindale (2000); anterior Hox genes group with Gsx genes, PG3 genes group with Xlox genes and Posterior Hox genes group with Cdx genes. The main caveat of the two-gene ProtoHox theory is that, if it were true, it requires sequence convergence of Hox PG3 and Xlox genes, as those two group together. Therefore, based on sequence parsimony, a 3 or 4 genes ProtoHox cluster is more likely. Starting from these models, Hox genes would have undergone several tandem duplications, increasing the complexity of the cluster during evolution, whereas ParaHox genes would not have increased in number (in the 3-gene model) or would have lost the central class gene (in the 4-gene model).

NweCentralI	**YNKYLTRRRRIEIAHALNLTERQ**	
Mye Scr	.....	AB206317.1
Sro Central	F.R.....NL.A.....	AY282610.1
Nvi Dfd	F.R.....C.....	AF151666.2
Mga dfd	.....E.C.S...	AJ534451.1
Dti Dfd	F.....Q..F.....	AJ300661.1
Pfe dfd	F.R.....S.C.A...	AJ272194.2
Pex Scr	F.R..V.....Y...S...	AY439323.1
Pfe scr	F.R.....C.S...	AJ272190.1
Sca Scr	F.R.....C.....	AF393441.1
Mye Antp	..R.....L.G.....	AB206319.1
Pex Antp	..R.....A.....	AY439324.1
Nvi Lox5	..R.....G.....	AF151671.2
Btu Lox5	..R.....T.G.....	AY497425.1
Alo Antp	F.R.....C.....	AF071402.1
NweCentralII	**F.H....K....V..S.C.....**	
Mye abd-A	F.H....K....V..S.C.....	AB206321.1
Doc abd-A	F.H....K.....S.C.....	AB206309.1
Pma lox4	F.H....K....V..S.C.....	AJ876626.1
Nvi Lox2	F.R....K....LS.M.C.....	AF151668.2
Pex Lox4	F.R....K.....C.C.....	AY439327.1
Csa ubx	T.H.....M..S.C.....	AJ007435.1
Zvi AbdA	F.H.....C.....	AY194829
Pca Ubx	F.H.....MSQ..C.....	AF144891.1
Aka Ubx	T.H.....M....C.....	AF011282.1
NweXlox	**FNKYISRPRRIELAAMLNLTERH**	
Npo Xlox	.....	AJ937219.1
Nmi Xlox	.....	AF363234.1
Cva Xlox	.....L.....	U68279.1
Bfl Xlox	.....	AF052464
Cin Xlox	.SR.....	AJ296167
Aty Xlox	.....	AF439973.1
NwePost	**FNVYITRERRSEISRSLNLTDRQ**	
Sro Post	..T.....L..A.....	AY282611.1
Pru PostB	LST....D..L...K..H.S...	AY282607.1
Pru PostA	..N.....G..AKV.G.S...	AY282606.1
Mye Post1	NST..SKS..W.L.QLI..SE..	AB206322.1
Pex Post1	N.G..S.PE.WHL.CQ....E..	AY439328.1
Nvi Post2	G.S....QK.W...CK.H.SE..	AF151673.2
Mye Post2	NSS....QK.W...CK.Q..E..	AB206323.1
Afr AbdB	..A.VSKQK.W.LA.N....E..	X87250.1
Lfo AbdB	..A.VSKQK.W.LA.N....E..	AF362095.1
Aka AbdB	..A.VSKQK.W.LA.N....E..	AF011274.1
Bfl Hox-9	Y.M.L.....Y...QHV...E..	Z35149
NweCad	**YKRYLTLRRRVELACELGLTERQ**	
Nvi Cdx	.S..I.I..KA...QN.N.S...	AY117546.1
Dti Cdx	TQK.VNA..KS.M.RA.Q.....	AJ300663.1
Sro cad	TNQ.I.I..KS...MQV..S...	AY282612.1
Pru cad	TNQ.I.I.KKA...TQV..S...	AY282608.1
Sma cad	.S..I.I..KA...QL...S...	AY562125.1
Gbi cad	.S..I.I..KA...AS...S...	AB191008.1
Aka cad	.S..I.I..KS...QA.N.S...	AF011275.1
Xin cad	TSE.ISTQ.KAY.SRA...S...	CV579460.1
Bfl cad	SNK.I.IK.K.Q..N....S...	L14866.1
Hro cad	FS..I.I..KS...MQ.S.S...	AB031032.1

The Acoelomorpha flatworms have been proposed to be those most basal bilaterian animals and the outgroup of protostomes plus deuterostomes (Ruiz-Trillo *et al.,* 2002, Jondelius *et al.,* 2002, Telford *et al.,* 2003). This clade comprises two groups: the Acoela and the Nemertodermatida. There has been a debate about the monophyly of this group: 18S data suggests paraphyly of the group (Jondelius *et al.,* 2002), but analysis of myosin heavy chain II sequences (Ruiz-Trillo *et al.,* 2002) and the mitochondrial genome (Ruiz-Trillo *et al.,* 2004), plus morphological data strongly support monophyly of the group (Baguñà and Riutort, 2004).

A small set of Hox and ParaHox genes have been found so far in acoel flatworms: one anterior, one central and one posterior Hox gene and only a posterior (*Cdx*) ParaHox gene (Cook *et al.,* 2004). It was proposed, in the light of this data, that the primitive bilaterian had no representative of PG3-Xlox genes (Cook *et al.,* 2004). This conclusion was based only on one of the groups of the Acoelomorpha. Studies in the smaller sister group, the Nemertodermatida, are thus important for clarification of the basal bilaterian condition of the Hox and ParaHox complements.

In this work, we report PCR-generated sequences of Hox and ParaHox genes in the nemertodermatid *Nemertoderma westbladi* which improve our understanding of the complements of these genes in the sister group of the eubilaterians. We demonstrate the presence of a Group3/Xlox ParaHox gene prior to the protostome/deuterostome divergence.

Results

In order to get an accurate view on basal bilaterian Hox and ParaHox content, we performed PCR on the Nemertodermatida *Nemertoderma westbladi* using degenerate primers which coded

Fig. 1. Alignment of the genes of *Nemertoderma westbladi* (black font) isolated, with other bilaterian orthologs (red for Ecdysozoa, green for Lophotrochozoa and yellow for Acoela). *Species names abbreviated as follows.* Afr, Artemia franciscana. Aka, Acanthokara kaputensis. Alo, Archegozetes longisetosus. Aty, Archaster typicus. Bfl, Branchiostoma floridae. Btu, Bugula turrita. Cin, Ciona intestinalis. Csa, Cupiennius salei. Cva, Chaetopterus variopedatus. Doc, Dentalium octangulatum. Dti, Discocelis tigrina. Gbi, Gryllus bimaculatus. Hro, Halocynthia roretzi. Lfo, Lithobius forficatus. Mga, Mytilus galloprovincialis. Mye, Mizuhopecten yessoensis. Nmi, Nephasoma minuta. Npo, Nautilus pompilius. Nvi, Nereis virens. Pca, Priapulus caudatus. Pex, Perionyx excavatus. Pfe, Pachymerium ferrugineum. Pma, Pecten maximus. Pru, Paratomella rubra. Sca, Sacculina carcini. Sma, Strigamia maritime. Sro, Symsagittifera roscofensis. Xin, Xiphinema index. Zvi, Zaprionus vittiger. *Dots denote amino acid identity of a particular gene to the* Nemertoderma westbladi *gene directly above. All* Nemertoderma westbladi *genes shown have unique nucleotide sequences when compared to the NCBI database, ruling out contamination from any known* Hox *or* ParaHox *gene. Orthology has been assigned by blastn best hit.*

```
NewXlox                 FNKYISRPRRIELAAMLNLTERHIKIWFQNRRMKWKKDEAKRRPRPLKSGSSPDSPPSPTMSSLSWISCLKRD*
B.floridae Xlox         ..............................EQ.....L.ESAS.TTPGGN.G.GTAAGGAESTGTSG
C.intestinalis IPF1     .SR.............................Q.ANSKTGKVRDITAEIRDTACEQTAPRDARTQVSD
S.purpuratus Splox      ..............................E....K.LKQDADG.DV.SQ.DIIANDEK.LPDS.TD
H.sapiens Ipf1          ..........V...V...............E.D.K.GGGTAV.GGGVAE.EQDCAVT.GEEL.ALPP
M.musculus IPF1         ..........V...V...............E.D.K.SSGTP..GGGGEE.EQDCAVT.GEEL.AVPP
C.familiaris PDX-1      ..........V...V...............E.D.K.SCGTAP.GVA.AE.EQDCAVS.GEEL.ALPP
```

Fig. 2. Comparison of the partial *NweXlox* sequence that extends 3'of the homeobox (shadowed box is the RACE extended sequence). *Dots denote identity and bars have been placed where no sequence is available. Lack of* Xlox *sequence information outside the homebox in other groups does not allow phylogenetic analysis with this part of the gene. No 100% nucleotide similarity sequence was found in the database, even for the shorter helix1-helix3 sequence. Sequences used are:* B. floridae *AAC39016,* C. intestinalis *AJ296167,* S. purpuratus *NP_999815.2,* H. sapiens *NP_000200.1,* M. musculus *CAA52389.1,* C. familiaris *XP_543155.2.*

for the helix1 and helix3 regions of the homeodomain. The PCR reactions yielded fragments of approximately 115-120 bp, which were subsequently cloned. Sequences of 126 homeobox-containing clones identified a total of five distinct sequences with similarity to particular paralogous Hox and ParaHox groups (PG, genes related by cluster duplication; we will subsequently refer to the gene groups by the deuterostome nomenclature for naming simplification purposes) (Fig. 1). It is plausible that some other Hox or ParaHox genes were missed by our approach, due to sequence divergence or lack of expression in the RNA sample used for the PCR. To minimise the number of genes missed by PCR we used, in addition to general homoebox-degenerated primers, a set of specific primers targeting the different PGs. Nevertheless, in the sister group of Nemertodermatida, the Acoela, a similar number of genes was found (Cook *et al.,* 2004), but noticeably not a representative of the PG3/Xlox gene. We have named the genes with the prefix Nwe (for *Nemertoderma westbladi*) followed by a name that characterises each paralogy group. We have found two clearly distinct central Hox genes (*NweCentral-I* and *NweCentral-II*), one posterior Hox gene (*NwePost*), an Xlox ParaHox gene (*NweXlox*) and a Caudal ParaHox gene (*NweCad*). Further sequence of the *NweXlox* was obtained using a gene specific primer and a 3' SMART adaptor primer as described in the M&M section. Sequences were submitted to GenBank under accession numbers DQ677343-DQ677347.

Two of the genes found are representatives of the central class Hox genes (*NweCentral-I* and *NweCentral-II).* On the basis of sequence similarity, *NweCentral-II* is more closely related to central Hox genes of the PG6-8 group, whereas *NweCentral-I* cannot be assigned to any particular paralogy group. It has been proposed that at the base of bilaterians there was, at the most, one central gene (Garcia-Fernàndez, 2005a). Nemertodermatida have now been found to have two clearly distinct central genes. We propose, in the light of our findings plus the data previously available from Acoela (Cook *et al.,* 2004), that Acoelomorpha have two central genes, one which would group with PG4/5 and a second one that would group with PG6-8. Those genes in the base of the bilaterian clade could have given rise to the set of central Hox genes seen in Eubilateria.

We have also cloned a posterior Hox gene (*NwePost*). Blast hits classify *NwePost* amongst PG10. Posterior lophotrochozoan and ecdysozoan Hox genes differ in sequence and number. Lophotrochozoans possess two posterior Hox genes, whereas ecdysozoans only have one type of posterior gene; they are distinguishable by several diagnostic residues within the helix1-helix3 regions. Deuterostomes have an extended posterior gene complement, also with some diagnostic residues. Therefore, orthology assignment to the posterior genes is usually relatively easy. Nevertheless, as for Acoela (Cook *et al.,* 2004), Nemertodermatida do not share any of the diagnostic residues for posterior Hox genes with any of the three major bilaterian clades, as expected from its position as an outgroup of eubilaterians. Trees built with this short sequence, even though they do not have good bootstrap values, do indeed support the lack of clear relationship to a particular higher bilaterian clade as well (data not shown). Acoela and Nemertodermatida posterior genes are not extremely similar, but share some amino-acid residues that differ from posterior genes of other groups. We suggest that the existence of a single posterior Hox gene in Acoelomorpha is a prototypic feature, even though its sequence has diverged and evolved differently in Acoela and Nemertodermatida after these groups split.

Of the ParaHox genes, a posterior caudal gene was identified (*NweCad*) and, contrary to the model of Cook *et al.* (2004), an *Xlox* representative was found (*NweXlox*). The *NweCad* gene does not possess some residues that have been until now considered typical for Cad proteins, but in BLAST searches, it gives consistently higher homology to the caudal group than to any other gene. *NweXlox* was further elongated by RACE, yielding a longer sequence at the 3' end of the fragment (Fig. 2). The finding of a *NweXlox* gene challenges and changes the proposed ideas for Acoelomorpha Hox and ParaHox complements. Acoelomorpha were thought to lack representatives of the PG3/Xlox genes. Our work has shown they have indeed representatives of all 4 major Hox classes and therefore at the base of bilaterians those classes were present.

Discussion

We have cloned two central Hox genes in Nemertodermatida: *NweCentral-I* and *NweCentral-II*. We have classified *NweCentral-I* as related to PG6-8 whereas *NweCentral-II* cannot be assigned unequivocally to a particular paralogy group. Several hypotheses may account for the presence of these two distinct genes. First, this could be due to an independent duplication in the Nemertodermatida, after the separation from Acoela, from a single ancestral central gene. In this case, Nemertodermatida would have evolved a PG6-8-like gene and acoels evolved a PG4/5-like gene (Cook *et al.,* 2004) from a single, unique ancestral Central (which would be the ancestor of PG4/5 and PG6-8 genes

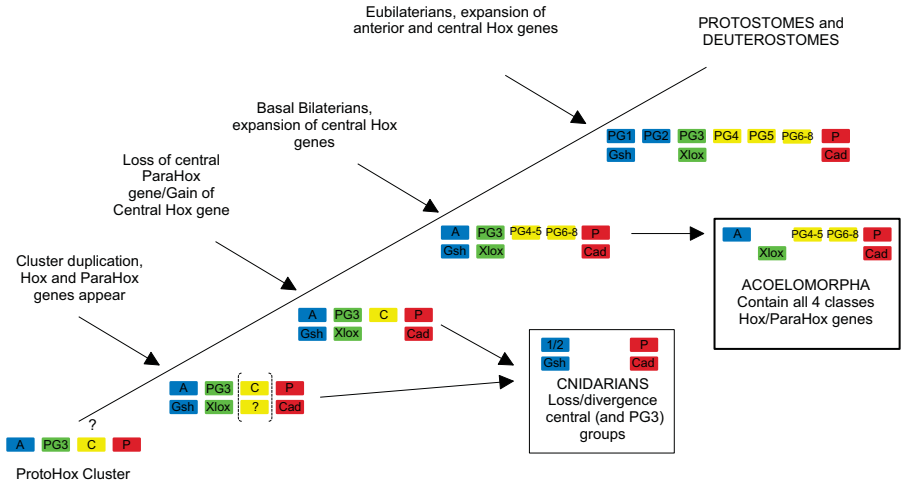

Fig. 3. Proposed evolution of *Hox* and *ParaHox* genes *starting from the 4-gene* ProtoHox *cluster (Brooke* et al.*, 1998) or a 3-gene* ProtoHox *cluster (question mark and dashed parenthesis). Basal bilaterians show a reduced set of genes, but an expansion of the central* Hox *genes, which could have been related to the acquisition of bilaterian features. All four* ProtoHox *gene classes are found in Acoelomorpha.*

of Eubilateria). This would necessarily imply that Nemertodermatida *NewCentral-I/I* and Eubilateria PG6/8 are similar by convergent evolution and that Acoela *SrHox4/5* and Eubilateria PG4/5 are similar by convergent evolution. We find this very unlikely and propose, instead, that Acoelomorpha ancestrally had at least two central genes, one PG4-5 and one PG6-8 and that later evolution led to the loss of the PG6-8 gene in Acoela and of the PG4-5 gene in Nemertodermatida. Subsequently, in Nemertodermatida there was a duplication of the central gene. Another possibility that cannot be ruled out is that Acoelomorpha had ancestrally 3 central genes and two have been kept in Nemertodermatida and one in Acolea (or are yet to be found in each group). Nevertheless, we think *NweCentral-I* is an independent duplication in Nemertodermatida as it has no clear similarity to any other central gene in any phyla.

It has been proposed that the extension of central Hox genes led to the expansion of the higher bilaterians at the time of the Cambrian Explosion (Garcia-Fernandez 2005a, 2005b for latest reviews). From our data, we suggest that the duplication of central Hox genes began earlier in evolution. Hence, the appearance of complex eubilaterians in the Cambrian Explosion would have coincided with less extensive expansion of central Hox genes than previously believed.

We have found a single posterior Hox gene in Nemertodermatida (*NwePost*). This gene is different from the Acoela posterior gene described by Cook *et al.,* even though they share some residues that are not present in the other groups. Nevertheless, neither *NwePost* nor the acoela posterior genes share any diagnostic residues with any of the existing eubilaterian posterior Hox genes. We suggest that the existence of a single posterior Hox gene in Acoleomorpha is a prototypic feature, even though the posterior gene has diverged and evolved in both groups after their split.

We have found two ParaHox genes in Nemertodermatida: *NweCad* (posterior ParaHox gene) and *NweXlox* (*Xlox* representative). Finding the *Xlox* representative changes the vision for the Hox and ParaHox complement in Acoelomorpha. We can now be certain that in Acoelomorpha there is an *Xlox* representative and therefore, given the Acoelomorpha are indeed basal bilaterians, we propose that there were representatives of all 4 classes of Hox genes at the base of the bilaterian lineage (Fig. 3). If that is the case, the model proposed by Ferrier and Holland (2001) (4 distinct classes of Hox genes existed before the divergence of cnidarians and bilaterians) can still be considered, as it is not contradicted by Acoelomorpha data, although still implies losses in Cnidarians, whereas most importantly, the proposed scenario of Cook *et al.* (2004), in which the Xlox/PG3 genes appeared after the origin of the Bilateria must be discarded.

In summary, we speculate, based on the data available and assuming that Acoelomorpha is a monophyletic basal bilaterian group, that the beginning of an extended central Hox set was present at the base of bilaterians. Also, we propose that basal bilaterians had at least all 4 ProtoHox-classes derived genes (and probably five, including two central genes). Our data cannot help in distinguishing which of the 2,3 or 4 gene ProtoHox cluster model is correct (as the ProtoHox cluster duplication occurred before the cnidarian/bilaterian split), but indicates that in the separate branches within the sister group (Acoelomorpha) to the Eubilateria there may have been differential gene loss of certain groups of genes. In Acoelomorpha as a whole we can find at least one gene of each class (Anterior, Group3/Xlox, CentralPG4/5, Central PG6/8 and Posterior) when taking into account both Hox and ParaHox genes, including an *Xlox* representative. We believe that our model for the early evolution of bilaterian Hox/ParaHox clusters, for which caveats derived from PCR screenings and short sequences cannot be discarded, will prompt further research to clarify the long-standing doubt cast on the early function and evolution of the paradigmatic Hox gene family.

Materials and Methods

Adults of *Nemertoderma westbladi* were collected near Kristineberg Marine Research Station (Sweden), immediately immersed in Trizol reagent (Sigma) and kept at 4ºC. To obtain RNA, the protocol for the Trizol Reagent (Sigma) was used according with the manufacturer's specifications. cDNA was obtained by using the SMART cDNA library construction kit (Clontech), after 24 rounds of amplification. This process links specific flanking regions (SMART adaptors) to the DNA, different at the 5' and 3' ends. This cDNA was used as template for PCR amplification using degenerated primers.

A first round of PCR was preformed using primers for the 3' (5'-ATTCTAGAGGCCGAGGCGGCCGACATG-3') or 5' (5'-AAGCAGTGGTATCAACGCAGAGT-3') SMART adaptors combined with degenerate primers of the regions of the helix1 (ELEKEF, QLELE,

YQTLELEK, LELEKE) or the helix3 (WFQNRR, KIWFQN, FQNRR, QVKIWF, QIKIWF) of the homeodomain, respectively. Different sets of primers were used to target classes of different Hox genes. A semi-nested PCR was then performed on the PCR products with the helix1 and helix3 degenerate primers.

The 115-120 bp PCR products obtained were cloned in pSK Bluescript, transformed in *E. coli* JM105 cells and plated on selective medium. Selection of insert containing colonies was done using blue-white screening. DNA minipreparation of single white colonies was preformed using the QIAprep Spin Miniprep kit (Qiagen). Sequence of the clones was performed with M13F primer using a ABI/Prism37100 sequencer. The sequences obtained were compared to the GenBank database using blastn. Anchored PCR of those sequences was performed using specific gene primers and the SMART adaptors primers on all the genes found, although it worked only for the *NweXlox* fragment. Alignments of the sequences were performed using ClustalX.

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Back in time: a new systematic proposal for the Bilateria

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Conventional wisdom suggests that bilateral organisms arose from ancestors that were radially, rather than bilaterally, symmetrical and, therefore, had a single body axis and no mesoderm. The two main hypotheses on how this transformation took place consider either a simple organism akin to the planula larva of extant cnidarians or the acoel Platyhelminthes (planuloid–acoeloid theory), or a rather complex organism bearing several or most features of advanced coelomate bilaterians (archicoelomate theory). We report phylogenetic analyses of bilaterian metazoans using quantitative (ribosomal, nuclear and expressed sequence tag sequences) and qualitative (HOX cluster genes and microRNA sets) markers. The phylogenetic trees obtained corroborate the position of acoel and nemertodermatid flatworms as the earliest branching extant members of the Bilateria. Moreover, some acoelomate and pseudocoelomate clades appear as early branching lophotrochozoans and deuterostomes. These results strengthen the view that stem bilaterians were small, acoelomate/pseudocoelomate, benthic organisms derived from planuloid-like organisms. Because morphological and recent gene expression data suggest that cnidarians are actually bilateral, the origin of the last common bilaterian ancestor has to be put back in time earlier than the cnidarian–bilaterian split in the form of a planuloid animal. A new systematic scheme for the Bilateria that includes the Cnidaria is suggested and its main implications discussed.

Keywords: Bilateria; Cnidaria; Acoela; Nemertodermatida; molecular phylogeny; microRNA

> The time will come I believe, though I shall not live to see it, when we shall have fairly true genealogical trees of each great kingdom of nature.
> (Charles Darwin in a letter to Thomas Huxley, 1857)

1. INTRODUCTION

When in the last third of the twentieth century molecular taxonomists aimed to establish the phylogenetic relationships of all organisms (the Tree of Life), they began with the following two premises: first, the increase in morphological complexity along the phylogenetic tree should run parallel to and be based on an increase in genomic complexity (e.g. number of genes; Cavalier-Smith 1985; reviewed in Hahn & Wray 2002) and second, genes and proteins should have a constant, clock-like rate of change over time (Zuckerkandl & Pauling 1965; Kimura & Ohta 1973). Therefore, under a perfect molecular clock, protein and DNA sequences would result in a complete Tree of Life delineating its main cladogenetic events. Morphological characters and new genes appearing at each node could then be used to guide an understanding of the evolution of morphological characters.

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One contribution of 17 to a Discussion Meeting Issue 'Evolution of the animals: a Linnean tercentenary celebration'.

Some clues emerging from molecular biology and developmental genetics in the early 1990s proved both premises to be flawed. First, lower and higher organisms encode very similar families of transcription factors and signal transduction molecules. In other words, variation in morphological complexity in metazoan evolution is probably correlated with or caused by the variation in the amount of interactions of a more or less similar set of genes. Second, the rates of change in genes and proteins proved to be anything but clock-like and vary according to the gene, protein, lineage, site and period studied (Easteal 1985; Nei & Kumar 2000).

One main consequence of these changes is the long-lasting difficulties in resolving the so-called ancient radiations, that is, cladogenetic events which occurred a long time ago and for which morphology, fossils and molecules have, so far, not provided satisfactory answers. Here, we address arguably the most important conundrum: the origin and radiation of bilateral animals (the Bilateria).

2. THE BILATERIA: BASIC FEATURES AND TWO QUESTIONS

Bilaterians include all Metazoa with bilateral symmetry either in the adult stage or, in those cases where bilateral symmetry turned to radial symmetry (e.g. echinoderms), in the larval stage. All bilaterians are triploblastic, which means the presence of a third middle layer or mesoderm, from which most organs

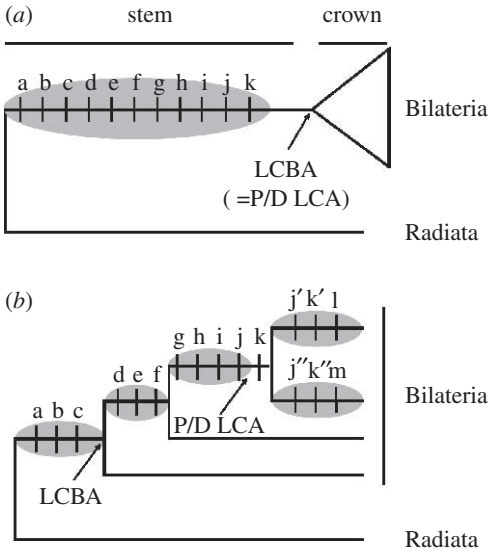

Figure 1. Coincident changes in the branch leading to the LCBA node under (*a*) the complex Urbilateria hypothesis (archicoelomate theory; Remane 1963; Kimmell 1996; Adoutte *et al.* 2000) and (*b*) the simple Urbilateria hypothesis (planuloid–acoeloid theory; Hyman 1951; Salvini-Plawen 1978). Note that in the complex Urbilateria scenario, characters (a–k) clump at the LCBA node that by definition corresponds to the last common ancestor of protostomes and deuterostomes (P/D LCA). Under the simple Urbilateria hypothesis, new clades intercalate and separate the LCBA from the P/D LCA helping to distribute character changes (a–m) across a series of stem branches and to polarize them. Under this scenario, the LCBA is morphologically simpler than the P/D LCA. Note that characters j and k could be either monophyletic (j,k) or di- or polyphyletic (j′, j″, k′, k″). l and m represent protostome- and deuterostome-specific characters. Grey ovals indicate the stem branches where key innovations (new characters) appeared.

form; so, true organs arise only in the triploblasts. Finally, many bilateral animals show a concentration of sensory structures and nerve cells at the anterior end of the body (e.g. cephalization). These features are widely considered basic apomorphies for the Bilateria. However, two questions remain. First, did the first bilaterian bear just this basic set of characters or, as some theories and hypotheses suggest (see below), did they also feature other characters (e.g. true brain, through gut, excretory system, body cavities (coelom), segments and even appendages and simple hearts and eyes) making them rather complex organisms? Second, do some non-bilaterian clades, traditionally considered radially or biradially symmetric (e.g. cnidarians), exhibit bilateral features and, hence, should they be considered true bilaterians?

The scores of theories advanced since Haeckel's gastraea theory on the nature of the first bilaterian (for recent updates see Willmer (1990) and Holland (2003)) could be separated into two main groups. The archicoelomate theories contemplate basic bilaterian traits such as bilaterality (hence a D–V axis) and mesoderm appearing concurrently with advanced characters such as coelom and segments. Therefore, the first bilaterians were segmented and coelomate and derived from radially symmetric, non-segmented, acoelomate cnidarians, either under larval or adult appearance (Remane 1963; Holland 2003). Under this hypothesis, the last common bilaterian ancestor (LCBA) appears as a rather complex organism (dubbed complex Urbilateria; De Robertis & Sasai 1996; Kimmell 1996; Carroll *et al.* 2001) and is defined as the last common ancestor of Protostomia and Deuterostomia (hence, the P/D LCA; for a clarifying terminology, see Valentine (2006)). The alternative group of theories feature a more gradual scenario starting from sexually reproducing pelagic organisms (protoplanula or archiplanula), akin to present-day cnidarian planula larvae, already exhibiting some bilateral symmetry (see Salvini-Plawen (1978) for a thorough review). Under this scenario, the LCBA was a morphologically simple organism and the P/D LCA would be relegated to an internal node within the Bilateria. From this simple LCBA originated the cnidarian polyps, which settled on the substratum, as well as a stock of acoelomate, non-segmented, early bilaterians vaguely similar to present-day acoel and nemertodermatid flatworms (planuloid–acoeloid theory). From the latter stock, other acoelomates as well as pseudocoelomate and coelomate, segmented and non-segmented protostomes and deuterostomes gradually evolved.

In terms of character changes necessary between ancestors and descendants, the phylogenetic consequences of these conflicting scenarios are very different. Under the archicoelomate scenario, the number of coincident characters present at the LCBA (=P/D LCA) node is large (figure 1*a*). This makes it difficult to place them into any temporal order along the stem leading to the LCBA. It also implies either a large number of extinctions of intermediary taxa (stem ancestors) or a wholesale correlated transformation from one life form (radial) to another (bilateral). In contrast, the planuloid–acoeloid scenario posits a reduced number of characters at the stem leading to the LCBA and features fewer and simpler stem ancestors, a simple LCBA and a later origin for the P/D LCA (figure 1*b*). Hopefully, and under both scenarios, phylogenetic advances may uncover fossil or extant clades that break coincident character changes at the stem. The intercalation of these new clades will distribute inferred character changes across a series of branches instead of having them solely at the LCBA node (Donoghue 2005; Valentine 2006).

As regards whether clades outside the Bilateria do exhibit bilateral symmetry, recent genomic and gene expression analyses have shown that besides genes involved in A–P polarity, gastrulation, endodermal and germ cell specifications, cnidarian anthozoans have numerous orthologues of bilaterian gene families previously thought to be absent in 'radial' organisms (for specific references see Finnerty *et al.* 2004; Martindale *et al.* 2004; Martindale 2005; Matus *et al.* 2006*a*; Rentzsch *et al.* 2006). Importantly, the presence and expression in cnidarians of many of the genes involved in D–V patterning in bilaterians match ideas (going back to Stephenson (1926) and held by Hyman (1951) and Salvini-Plawen (1978)) of a second or directive axis in cnidarians (specifically in anthozoans), perpendicular to the oral–aboral (O–AB) axis (Finnerty *et al.* 2004). Were it so, cnidarians and

bilaterians might have evolved from an already bilateral ancestor, putting the origin of the bilaterians even further back in time.

3. CURRENT APPROACHES TO UNRAVEL THE ORIGIN AND EVOLUTION OF BILATERIANS

To establish whether the first bilaterians had the basic or an expanded set of embryological/morphological key characters or novelties (see above), two main approaches are currently used: (i) molecular phylogenies to sample taxa as close as possible on either side of the origin of evolutionary novelties and (ii) comparing the expression patterns of homologous genes related to these novelties among bilaterians and non-bilaterians as a criterion of homology of the corresponding anatomical structures.

Building molecular phylogenetic trees under rigorous phylogenetic inference methods aims to identify potential earliest branching bilaterians bearing novelties (e.g. symmetry, mesoderm, through gut, nephridia, coelom, segments, etc.), derived from ancestors that did not possess such features. Extant 'non-bilaterian' or 'pre-bilaterian' metazoan groups must also be searched for to be used as appropriate outgroups. As Raff (2000) states, phylogeny provides three important kinds of information: (i) it can determine the direction in which developmental features evolve, (ii) it allows evolutionary rates to be inferred, and (iii) it allows homology statements to be formulated or, conversely, tested. Information of type (i) and (iii) is particularly important as it helps to determine the 'true' groups before and after a morphological novelty and so avoid mistaken comparisons of gene expression patterns in non-homologous features (see below).

The rationale behind comparing expression patterns of developmental control genes between closely or distantly related taxa is that if in two different species orthologous genes are expressed in a similar position, these areas or regions are considered homologous, even across phyla, and should have been present in their last common ancestor. However, attempting to infer structural homology from molecular expression is fraught with difficulties (Abouheif 1997). Some of the genes tested (namely the HOX and some D–V genes; Arendt & Nübler-Jung 1994; De Robertis & Sasai 1996) are good examples of homologous genes used across phyla in homologous patterning mechanisms that result in rather different structures, i.e. HOX genes patterning the A–P axis in arthropods and chordates. Other sets of genes whose expression in embryos was used to deduce homology of structures across phyla (i.e. *DLL*/*distal-less* for appendages and *PAX6*/*eyeless* for eye development; Panganiban *et al.* 1996; Gehring & Ikeo 1999) most likely represent homologous genes patterning non-homologous structures. Finally, because most of these genes are already expressed in cnidarians, greater care needs to be exercised when homologizing morphological structures on the basis of gene expression alone (Abouheif 1997; Wagner 2007).

Such difficulties make the use of molecular characters for reconstructing a backbone tree of the Metazoa and the Bilateria an attractive option. Thereafter, morphological and gene expression characters can be mapped onto the tree to decorate specific nodes and branches.

4. MOLECULAR PHYLOGENY OF THE BILATERIA: NEW DATA

In the last 10 years, molecular data have greatly changed perspectives on the relationships of the Bilateria. The so-called 'new animal phylogeny' (Adoutte *et al.* 2000), initially based on 18S rDNA sequence data alone, split the Bilateria into three superphyla: Deuterostomia, Ecdysozoa and Lophotrochozoa, widely accepted today. A major result was to shift all acoelomate and pseudocoelomate groups, traditionally considered at the base of the Bilateria, into the Ecdysozoa and Lophotrochozoa (Adoutte *et al.* 2000). Further nuclear and mitochondrial markers and combined morphological–molecular studies also support these findings (Peterson & Eernisse 2001; Halanych 2004).

In all schemes concerning the early history of bilaterians, the flatworms (phylum Platyhelminthes) had a central role—their simple morphology (acoelomate, non-segmented with a blind gut) coupled with a gradualistic view of evolution made them the perfect transitional taxon from cnidarian diploblasts to bilaterian triploblasts. Their monophyly, however, has always been in dispute (Smith *et al.* 1986). The first comprehensive molecular trees of the Platyhelminthes and other bilaterian and non-bilaterian phyla using 18S rDNA sequences (Carranza *et al.* 1997; Ruiz-Trillo *et al.* 1999; Jondelius *et al.* 2002) ran contrary to morphological analysis: Platyhelminthes was polyphyletic with two of its orders, the Acoela and the Nemertodermatida, branching as early bilaterian clades while the rest of the Platyhelminthes (Catenulida + Rhabditophora) fell within the Lophotrochozoa (reviewed in Baguñà & Riutort 2004*a*,*b*). Importantly, the early branching position of acoels and nemertodermatids also contradicted one of the tenets of the new animal phylogeny: the non-basal position of acoelomate organisms. Sequences of other nuclear genes (including HOX cluster genes) and mitochondrial genes (Ruiz-Trillo *et al.* 2002, 2004; Cook *et al.* 2004) corroborated this early branching position. It is important to point out that based on perceived morphological synapomorphies, Acoela and Nemertodermatida are classified as sister groups forming the taxon Acoelomorpha (Ehlers 1985). However, because in most molecular trees they branch paraphyletically (Jondelius *et al.* 2002; Ruiz-Trillo *et al.* 2004; Wallberg *et al.* 2007), the monophyletic status of the Acoelomorpha is here left open and from now on dubbed 'Acoelomorpha'.

Notwithstanding these advances, the cladogenetic events at the base of the Bilateria and among most phyla belonging to the three big superphyla remain poorly resolved. This lack of resolution, also reproduced using large datasets of genes and taxa, was thought to result from the high levels of stochastic changes along presumably closely spaced cladogenetic events such as the origin and radiation of bilaterians (Rokas & Carroll 2006). However, besides stochastic errors and short time spans, incongruent or unresolved phylogenies stem from systematic errors. These errors are due to inaccuracies of the methods used in tree

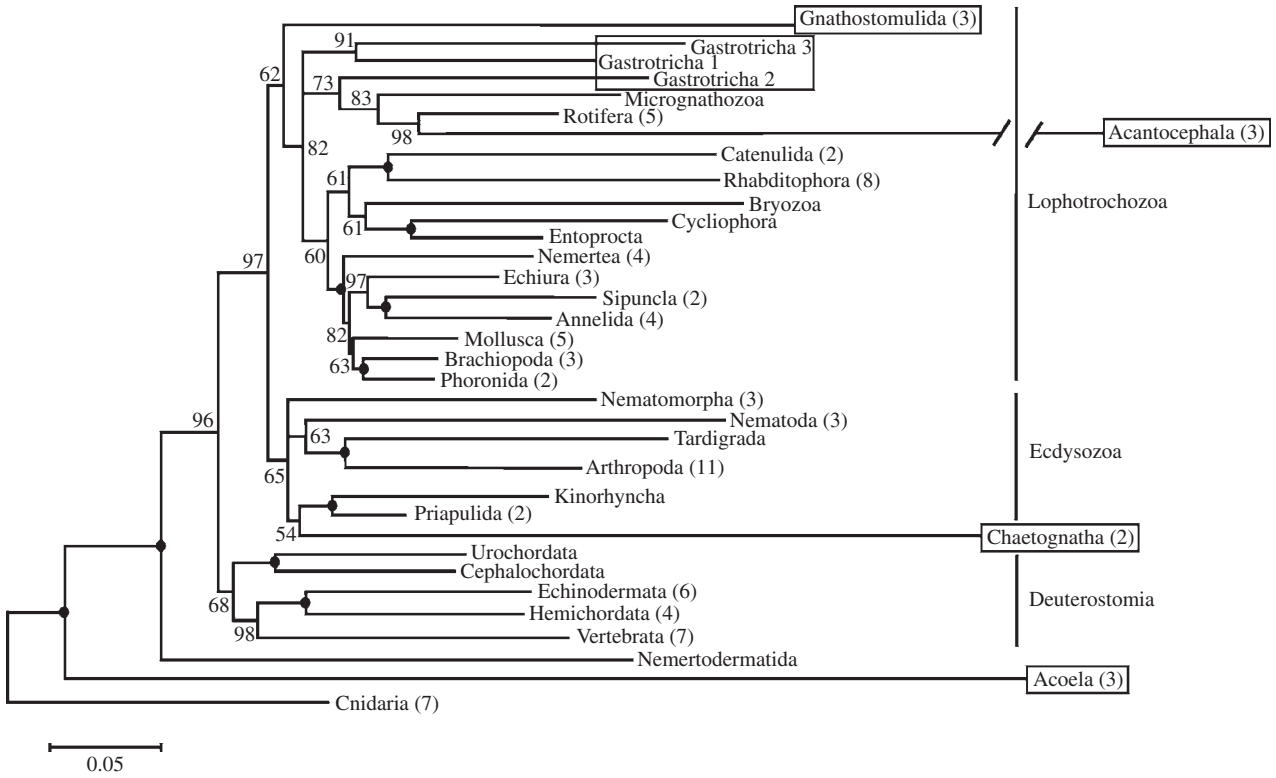

Figure 2. Bayesian analysis of 18S + 28S sequences (3696 nts) from 106 metazoan representatives with Cnidaria as the outgroup. Posterior probabilities are indicated when less than 100%, otherwise a bullet is present on the node. Phyla are collapsed and numbers in parentheses indicate the number of taxa sampled if more than one. The monophyly of each phylum has maximum support (except for gastrotrichs). Boxed groups correspond to long-branched or problematic groups for which specific analyses were carried out (see electronic supplementary material). The scale bar indicates the number of changes per site.

reconstruction directly related to model misspecifications (Felsenstein 2004). To avoid them and to improve tree reconstruction, several approaches are currently available (Philippe & Telford 2006): allowing for the numerous observed heterogeneities of the evolutionary process in models of sequence evolution (e.g. CAT model of Lartillot & Philippe (2004); Lartillot *et al.* 2007); increasing the number of taxa; removing the fastest evolving positions from the dataset; and excluding fast-evolving taxa to avoid long-branch attraction effects (LBA; Felsenstein 2004). Furthermore, the so-called rare genomic changes (RGCs; Rokas & Holland 2000; Rokas & Carroll 2006) are considered more reliable characters than conventional linear, homoplasy-sensitive sequences to avoid these problems and resolve these cladogenetic events.

In what follows, we observe these guidelines for ribosomal and nuclear gene sequences and introduce unconventional RGCs, such as microRNAs, to bilaterian phylogeny. Our main aims were to test again the position of the 'acoelomorph' flatworms as early branching bilaterians (Ruiz-Trillo *et al.* 1999, 2002) and to single out early branching phyla at the base of the three superphyla. Finally, and based on the growing consensus of cnidarians as true bilaterians (Finnerty *et al.* 2004; Martindale 2005), a new systematic proposal for the Bilateria is suggested.

(a) *Linear (quantitative) markers*

(i) *Ribosomal genes*

To minimize mutational saturation and homoplasies from ribosomal gene sequences and to avoid LBA effects, we used methods less sensitive to LBA (maximum likelihood and Bayesian inference), model modifications such as rate heterogeneity across sites and the slowest evolving taxa available. From 564 18S and 142 28S rDNA sequences, a combined 18 + 28S rDNA dataset of over 3700 bps was obtained with 104 taxa for 28 bilaterian phyla and the outgroup. A basic dataset was obtained avoiding six long-branch (LB) phyla (Acoela, Gnathostomulida, Gastrotricha, Acanthocephala, Bryozoa and Chaetognatha) and a tree was built which reproduced the backbone of the new animal phylogeny with very high support (for further details, see electronic supplementary material). When LB phyla were introduced into this basic tree, either individually or in combination, the topology remained unchanged and statistically highly supported (figure 2; Paps *et al.*, unpublished data). Contrary to expectations, LB phyla did not cluster together at the base but fell at specific places within each superphylum: acanthocephalans with rotifers; gnathostomulids and gastrotrichs as early branching lophotrochozoans; bryozoans as sister group to a clade of cycliophorans and entoprocts; chaetognaths, albeit with very low support (bootstrap BP = 0.54), as sister group to Scalidophora; and, most importantly, a paraphyletic 'Acoelomorpha' comprising Acoela and Nemertodermatida that were, with maximum support, the earliest branching bilaterian clades.

(ii) *Other nuclear genes*

A second tree was obtained from a dataset of 13 genes (18 + 28S genes and 11 nuclear genes) from 71 species

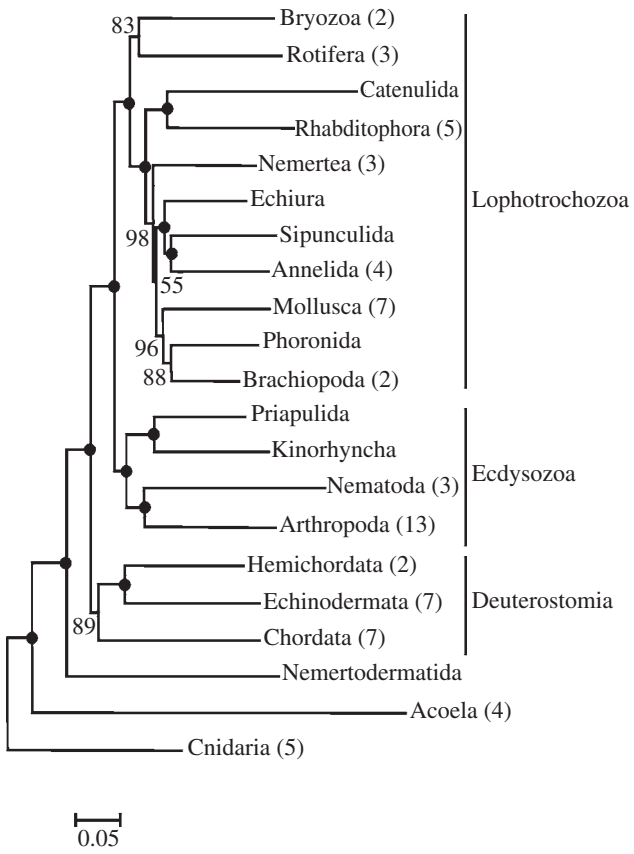

Figure 3. Bayesian analysis of concatenated sequences for 18S, 28S and 11 nuclear protein genes (9290 nts) from 74 metazoan representatives with Cnidaria as the outgroup. Posterior probabilities are indicated when less than 100%, otherwise a bullet is present on the node. Phyla are collapsed and numbers in parentheses indicate the number of taxa sampled if more than one. The monophyly of each phylum has maximum support. The scale bar indicates the number of changes per site. For further details, see electronic supplementary material.

of 21 bilaterian phyla and the outgroup. Figure 3 summarizes the tree drawn from the concatenated dataset (J. Paps, J. Baguñà & M. Riutort 2007, unpublished data; see electronic supplementary material). Of the six long-branch phyla included in the 18+28S tree, only two (Acoela and Bryozoa) could be analysed and included here. The three superphyla appeared again with maximal support, except for a 0.89 BP value for deuterostomes. Within each superphylum, phyla grouped similarly, albeit more robustly, than in the ribosomal tree. Two differences are noteworthy: first the clustering, with low support, of Rotifera with Bryozoa (BP=0.83) and second, the low support (BP=0.55) of annelids and relatives with molluscs, phoronids and brachiopods forming, together with nemerteans, a highly supported Eutrochozoa group (BP=1.00) sister to the rhabditophoran platyhelminths. Finally, and most importantly, 'Acoelomorpha' branched paraphyletically and with maximal support at the base of the Bilateria.

(iii) *Phylogenomics using expressed sequence tags*

Because trees reconstructed from sequences of few genes, even from many taxa, are prone to stochastic errors while those with few taxa and many genes may generate systematic errors, gathering many sequences from many taxa might counter both sources of errors and produce a well-resolved animal phylogeny (Philippe & Telford 2006). From several species of each phylum, 5000 expressed sequence tags (ESTs; drawn from cDNA libraries) is considered a reasonable number from which to select a set of suitable genes that, under appropriate evolutionary models, will result in better and more robust phylogenetic trees than those drawn from gene- or species-poor trees.

We produced an EST collection from the acoel *Convoluta pulchra* from which 68 different protein-coding genes were unequivocally assigned to a dataset of conserved single-copy genes from 51 species belonging to 10 different bilaterian phyla and 4 outgroup phyla. An alignment of 11 959 amino acids was established and trees inferred by maximum likelihood under the standard WAG model (Whelan & Goldman 2001) and by PhyloBayes analyses with the CAT mixture model that overcomes LBA artefacts when other models fail (Lartillot *et al.* 2007). Whereas the standard WAG model groups together the two long branches of Platyhelminthes and the acoel, the resulting tree under the CAT model, with and without the outgroup, strongly rejects this grouping. Platyhelminthes (with the exception of the acoel) fell within the lophotrochozoans. The acoel studied instead clustered either with all deuterostomes, to Xenoturbellida, or to Ambulacraria. Importantly, when the outgroup was removed, the acoel remained in a basal position, sister group to the Xenoturbellida (Philippe *et al.* 2007).

This is the first time using a large set of data that acoels were shown not to belong to the classical Platyhelminthes, making the latter polyphyletic. The deuterostome affinities of the acoels, however puzzling, seem to contradict the early emergence of acoels at the base of the Bilateria (Ruiz-Trillo *et al.* 1999). Nonetheless, the very fast evolutionary rates shown by the acoel *C. pulchra* make it not the best acoel species to study even using the CAT method. This calls for new data from slowly evolving acoels (and from its putative sister group, the Nemertodermatida) to solve this challenging phylogenetic problem.

(b) *Qualitative markers*

(i) *HOX cluster genes*

HOX and ParaHOX genes from five species of acoels and a single nemertodermatid have recently been isolated and analysed (Cook *et al.* 2004; Jiménez-Guri *et al.* 2006; Baguñà *et al.* 2008; M. Q. Martindale 2004, personal communication). Overall, the maximal set deduced for both taxa would consist of an anterior, one/two central and one posterior HOX genes, and one representative each of the *Xlox* and *Cdx* ParaHOX genes. This putative simple HOX gene cluster in 'acoelomorphs' has been considered (Baguñà & Riutort 2004*a*) intermediate between the expanded set (at least seven out of eight paralogy groups) of most bilaterians and the simpler set of HOX/ParaHOX genes in cnidarians (only anterior and posterior HOX and ParaHOX genes reported with no representatives of central genes; Chourrout *et al.* 2006; Ryan *et al.* 2007). Preliminary analyses of anterior, central and posterior HOX genes in the acoel *Symsagitiffera roscoffensis* using BAC libraries and

fluorescent *in situ* hybridization (FISH) show them located on different chromosomes (E. Moreno, J. Baguñà & P. Martínez 2007, unpublished data); therefore, the putative HOX cluster is dispersed. Preliminary results on HOX gene expression in the acoel *Convolutriloba longifissura* show a coarse collinear expression along the A–P axis (Hejnol & Martindale in press; M. Q. Martindale 2004, personal communication). However, until whole genome sequences of acoels or nemertodermatids are made available, the presence of new, undetected, HOX and ParaHOX genes in these taxa could not be ruled out and, therefore, the precise number and type of HOX cluster genes will remain unsettled.

(ii) *microRNA sets*

The recently discovered microRNAs (miRNAs) represent new and powerful molecular markers to examine unique genetic and/or biochemical apomorphies relatively immune from homoplasy. The main phylogenetic asset is the rough correlation between the number of different miRNAs with both the hierarchy of metazoan relationships and the number of differentiated cell types as a measure of morphological complexity (Sempere *et al.* 2006). When a large set of non-paralogous miRNAs were traced along a wide range of taxa using northern blots, 21 miRNAs were found common to protostomes and deuterostomes of which none is present in sponges and two in cnidarians (Sempere *et al.* 2006). Protostomes had 12 additional specific miRNAs and deuterostomes had 7. Platyhelminthes, represented by a marine polyclad, had almost all protostome miRNAs excluding the two ecdysozoan-specific miRNAs so far detected, confirming that they are lophotrochozoan protostomes. Interestingly, the sole acoel included, *Childia* sp., had only a subset (six miRNAs) of the miRNAs shared by protostomes and deuterostomes.

Recently, we examined the miRNA complement of a second acoel taxon, *Symsagitiffera roscoffensis*, and three additional rhabditophoran platyhelminth taxa, one polyclad and two triclads. *Symsagitiffera roscoffensis* possesses an identical subset of miRNAs to *Childia* sp. found across protostomes and deuterostomes, and none of the miRNAs unique to protostomes or planarians (Sempere *et al.* 2007). This supports again the polyphyly of the Platyhelminthes and that the Acoela are early branching bilaterians. Were acoels members of the Platyhelminthes and simply had a reduced number of miRNAs due to secondary loss, then one would expect acoels to bear a mosaic or 'salt-and-pepper' pattern of miRNAs such that some primitive (triploblast specific) and some, but not all, derived (nephrozoan- and protostome-specific) miRNAs would be detected (Sempere *et al.* 2007).

(c) *Summing up*

This report and previous studies (reviewed in Baguñà & Riutort 2004*a*) are consistent with the view that acoels and nemertodermatids are early branching bilaterian lineages (figures 2 and 3). However, two features of these figures need clarification. First, the conflict between the topology of these trees (acoels and nemertodermatids as early branching bilaterians) and trees recovered from EST analyses (acoels as deuterostomes; Philippe *et al.* 2007). Second, the taxonomic status of acoels and nemertodermatids, either branching paraphyletically at the base (Jondelius *et al.* 2002; Ruiz-Trillo *et al.* 2004) dismissing the 'Acoelomorpha' as a valid taxon (Wallberg *et al.* 2007) or, as morphologists claim (Smith *et al.* 1986), sister groups forming a monophyletic Acoelomorpha.

Our EST analysis incorporated a large number of characters, though few phyla were included and acoels were represented by a single species that unfortunately had very fast clock behaviour. In contrast, ribosomal and nuclear gene analyses included fewer characters, but they had better phyla and within-phyla sampling, and acoelomorphs were represented by four out of five species (which included a nemertodermatid), some short branched. Such differences translate into striking differences in bootstrap support values as regards acoelomorph position: maximal for nuclear genes and very weak in the EST analysis (see fig. 1 in Philippe *et al.* 2007). Waiting for new data from slowly evolving acoels and nemertodermatids, current evidence and HOX cluster gene and miRNA datasets favour the topology represented in figure 4 as regards the phylogenetic position of the acoelomorphs and of the acoels in particular.

Support for a monophyletic 'Acoelomorpha' in molecular trees relies solely on myosin heavy chain II sequences (Ruiz-Trillo *et al.* 2002). All remaining trees and those reported here (figures 2 and 3) recover the Acoela and the Nemertodermatida as the first two separate branches within the Bilateria with high support. This dismisses the 'Acoelomorpha' as a valid taxon (Wallberg *et al.* 2007). How does this molecular evidence fit with claimed morphological synapomorphies linking acoels with nemertodermatids (i.e. the special structure of the basal body-rootlet system complex and the ciliary tips and the fine structure of the frontal organ; Smith *et al.* 1986)? Most of these structures occur in other metazoan groups including the Xenoturbellida, recently proposed to be a deuterostome (Bourlat *et al.* 2006). Moreover, these structures are only superficially similar and probably originated by convergence. Together with differences in sperm morphology, neurotransmitter patterns and embryonic cleavage patterns between acoels and nemertodermatids, most evidence is consistent with Acoela and Nemertodermatida as separate early branching clades and with the 'Acoelomorpha' as a non-monophyletic clade (Wallberg *et al.* 2007).

A second important outcome from figure 2 is the presence, albeit with moderate to low support, of acoelomate (Gnathostomulida and Gastrotricha) and pseudocoelomate (Rotifera) groups as early branching lophotrochozoans. In addition, the new Platyhelminthes (Platyhelminthes *sensu lato* excluding the acoelomorphs) either alone or with other phyla appears as sister group to the spiralian Eutrochozoa (annelids, molluscs and relatives) and not buried within the Lophotrochozoa. Together with the recent placement of the acoelomate Xenoturbellida as sister group to all deuterostomes (Perseke *et al.* 2007) or to the Ambulacraria (Bourlat *et al.* 2006), these data suggest the need to re-evaluate in depth the so-called new animal phylogeny (Adoutte *et al.* 2000).

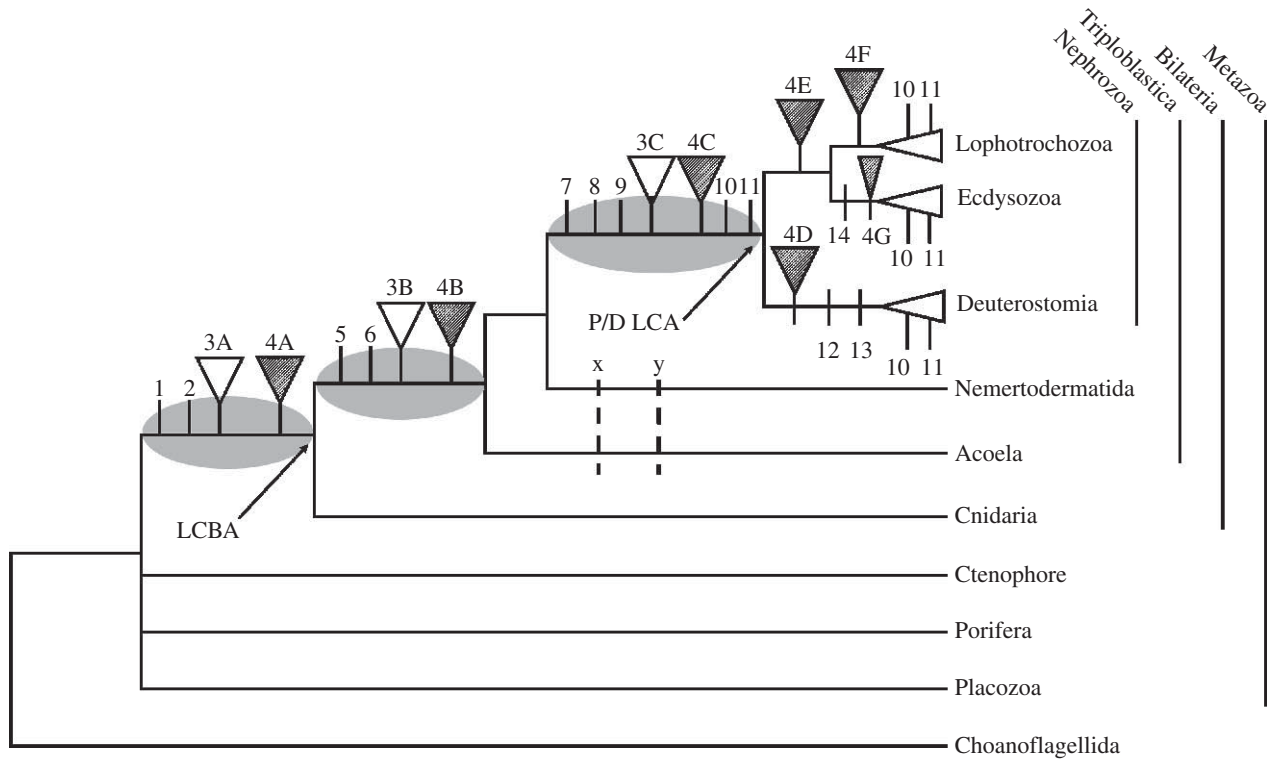

Figure 4. A new systematic proposal for the Bilateria. Morphological and molecular characters (HOX cluster genes and microRNA sets) have been mapped onto a backbone tree drawn from 18S + 28S rDNA and 11 nuclear genes. The new Bilateria includes the Cnidaria and the former Bilateria, now dubbed as Triploblastica, the latter split into a paraphyletic 'Acoelomorpha' (Acoela and Nemertodermatida) and the rest of the bilaterians or Nephrozoa. Note that the LCBA for cnidarians + Triploblastica is less complex than the ancestor for Triploblastica. Bilaterian autapomorphies (vertical solid lines and empty and hatched inverted triangles) are as follows: 1, D–V axis; 2, bilateral symmetry; 3, HOX/ParaHOX clusters (3A: 2 HOX/2 ParaHOX); 4, microRNA sets (4A: basic bilaterian set). The Triploblastica have some autapomorphies that exclude cnidarians: 3B (4 Hox/3 ParaHox); 4B, a miRNA set of five out of six genes; 5, mesoderm; 6, clustered nerve cells at the anterior end. Finally, the Nephrozoa (= Protostomes + Deuterostomes at the P/D LCA node) will have some autapomorphies that exclude acoelomorphs: 3C, an expanded HOX cluster gene of seven to eight genes; 4C, a nephrozoan miRNA set of 20 or more genes; 7, small anterior brain ganglia and ventral nerve cords; 8, through gut (mouth + anus); 9, excretory system (= protonephridia). As suggested by some authors, other autapomorphies of Nephrozoa would be: 10, coelomic cavities; 11, body segmentation, though they may have a monophyletic or a polyphyletic origin. Some autapomorphies for the Deuterostomia, Lophotrochozoa and Ecdysozoa are indicated: 4D–4G, specific miRNA sets; 12, post-anal tail; 13, gill slits; 14, ecdysis. x, y (broken lines), postulated synapomorphies for a monophyletic Acoelomorpha (Smith *et al.* 1986). x, special structure of the basal body-rootlet system complex and the ciliary tips; y, fine structure of the frontal organ. Grey ovals indicate the stem branches where key innovations appeared. See text for further details and main references.

5. THE BILATERIA: A NEW SYSTEMATIC PROPOSAL

In all zoological textbooks, cnidarians (anthozoans + medusozoans) are classified as organisms with radial symmetry. Although in most anthozoan cnidarians bilaterally symmetric features (e.g. slit-shaped mouth, internal mesenteries and asymmetric siphonoglyphs in the polyp form) were noted in the past (Stephenson 1926; Hyman 1951; Salvini-Plawen 1978; Willmer 1990), they were not taken as evidence for bilaterality because anthozoans were considered derived cnidarians and hence their internal bilateral features as secondarily evolved. Recent molecular phylogenies, however, have shown that Cnidaria and Bilateria are sister groups (Medina *et al.* 2001; Wallberg *et al.* 2004) and, importantly, that the Anthozoa is not a derived cnidarian clade but a basal group rendering its bilaterally symmetric features as possible plesiomorphies for the cnidarians (Collins 2002). Hence, cnidarians could originally be truly bilaterian (Finnerty *et al.* 2004; Martindale 2005) albeit secondarily modified to radiality (externally) owing to their predominantly sessile life style.

The homology between the O–AB axis of cnidarians and the A–P axis of bilaterians is now widely accepted, though the precise equivalences between oral (O) and aboral (AB) ends of cnidarians to anterior (A) and posterior (P) ends of bilaterians are disputed (O = P and AB = A: Salvini-Plawen 1978; Meinhardt 2002; Rentzsch *et al.* 2007; Baguñà *et al.* 2008; O = A and AB = P: Finnerty *et al.* 2004; Martindale 2005; Matus *et al.* 2006*b*). Moreover, the presumed homology of the 'directive axis' of cnidarians to the bilaterian D–V axis, initially based on the transient asymmetric expression of the cnidarian orthologues of *BMP2/4/dpp* and other D–V genes, has been amply corroborated by the asymmetric expressions of scores of 'endodermal', 'mesodermal' and 'neural' genes (reviewed in Martindale 2005; Matus *et al.* 2006*b*).

The increasing evidence of cnidarians as bilateral in origin, new molecular data (figures 2 and 3 and microRNA datasets) backing the acoelomorph

flatworms as the earliest extant branching bilaterian and the presence of acoelomate/pseudocoelomate groups at the base of the lophotrochozoans and deuterostomes prompt us to suggest a new systematic proposal for the origin and evolution of the Bilateria (figure 4). Under this proposal, Cnidaria are considered true bilaterians and the sister group to a less-inclusive bilaterian clade, here named Triploblastica, which comprises present-day bilaterians with a true mesoderm. Within the Triploblastica, molecular evidence (figures 2 and 3) favours the early branching of a paraphyletic 'Acoelomorpha' (acoels first and nemertodermatids second) sister group to the traditional protostome+deuterostome clade, or Nephrozoa (*sensu* Jondelius *et al.* 2002). Apomorphies of the new Bilateria would be the establishment and consolidation of a new D–V axis and the ensuing bilateral symmetry, and the appearance of a basic HOX cluster (2 HOX/2 ParaHOX genes) and a minimal set (two out of three genes) of miRNAs. Plesiomorphies of the new Bilateria, shared with the ctenophores, would be an A–P axis (O–AB in cnidarians and ctenophores), diploblasty (ectoderm+endoderm), the presence of muscle cells not forming a true mesoderm (Burton 2008) and the presence of a nerve net. Another key apomorphy of Triploblastica is the clustering of nerve cells at the anterior end from which longitudinal bundles of nerve fibres spring. Such characters probably run parallel to the first expansion of HOX/ParaHOX clusters (group 3 and central HOX genes; character 3B) and new miRNAs (character 4B). After acoelomorphs and other extant (Xenoturbellida) and extinct acoelomate/pseudocoelomate groups split, the radiation of Nephrozoa resulted in protonephridia, a through gut, and the progressive development of a more concentrated nervous system (layers of nerve cells surrounding a neuropile and defined as ventral nerve chords). Because the last scenario led to the appearance of true organs and more elaborate A–P and D–V axial patterns, full sets of HOX cluster genes (character 3C) and new sets of miRNAs (characters 4C–4G) were also required.

The name Planulozoa was recently proposed by Wallberg *et al.* (2004) to define a clade comprising Cnidaria and Bilateria. As such, Planulozoa is formally equivalent to the name Bilateria, here proposed for the more inclusive clade (Cnidaria+Triploblastica). Suggested synapomorphies for the Planulozoa are the presence of endodermal myoepithelial musculature, septate junctions in epithelial cells, symmetrically arranged spermatozoon heads with a mid-piece and a set of several clustered HOX genes (Wallberg *et al.* 2004). Such features, however, are plesiomorphic or have not been tested in other phyla, and, when referring to HOX clustering in cnidarians, seem at odds with most recent genomic data (Chourrout *et al.* 2006; Ryan *et al.* 2007). Moreover, the name Planulozoa stems from the presumed similarities between the planula larva and acoel worms, both being vermiform and having an apparent polar development of the nervous system (for specific references, see Wallberg *et al.* 2004). Again such characters are weakly defined, have to be tested in other phyla and refer to a hypothetical process contemplated in the planuloid–acoeloid theory. Instead, the new Bilateria here proposed is defined by specific descriptive characters (D–V axis, bilateral symmetry and microRNA sets).

The first asset of the proposal set forth over that contemplated in the new animal phylogeny (see figure 1 for comparison) is that key changes or innovations in bilaterian evolution are spread along several stem branches allowing character states to be polarized. In particular, it unlinks D–V axis formation from mesoderm formation: the first appearing in the last common ancestor (equal to LCBA) of Cnidarians and Triploblastica and the second originated in the LCA of Triploblastica. It has been claimed that some members of the cnidarian Medusozoa possess a mesodermal derivative, the entocodon (Seipel & Schmid 2005), and that members of both Cnidaria and Ctenophora possess striated muscle, a mesodermal derivative (Seipel & Schmid 2006). This would imply that the last common ancestor of Ctenophores and Cnidaria+Bilateria had already been a triploblast bearing striated muscle (Martindale *et al.* 2004; Seipel & Schmid 2005; Boero *et al.* 2007). However, striated muscle in cnidarians, namely in anthozoans, is epitheliomuscular; the entocodon and the mesoderm have very different developmental origins (the first from ectoderm and the second from the endoderm); and striated muscles in ctenophores, while truly muscular, non-epithelial and derived from the endoderm, are very distinct from triploblastic striated muscles (reviewed in Burton 2008). Therefore, the more parsimonious scenario is that the LCBA in figure 4 was a diploblast and that triploblastic mesoderm, cnidarian entocodon and striated musculature in Cnidaria, Ctenophora and Triploblastica had independent origins. Under this scenario, it could be predicted that genes involved in mesodermal patterning and differentiation in triploblasts (i.e. *snail*, *twist*, *forkhead*, *brachyury*, *mef2* and *GATA*) are primarily linked with the endoderm in diploblasts, and that patterning genes involved in muscle development within each lineage and in the formation of the hydrozoan entocodon (i.e. *mef2*, *Id*, *msx*) bear little or no similarity in expression. Both predictions are borne out from recent molecular data (Finnerty *et al.* 2004; Martindale *et al.* 2004; Burton 2008). This strengthens the view of separate, independent origins for muscle cells in the three clades, and for the origin of mesoderm in triploblasts from the bipotential endoderm (equal to mesoendoderm) of the LCBA. Therefore, the presence of a D–V axis in the LCBA unlinks character 2 (D–V axis) from character 5 (mesoderm), pointing to cnidarians as the group of choice to analyse the origins of the D–V axis and bilateral symmetry, and to acoels and nemertodermatids to explore the origins of mesoderm and of a more centralized nervous system.

The second asset of the phylogenetic tree in figure 4 is the closer similarities between the new LCBA and the ancestor envisaged in the planuloid–acoeloid theory than between the former and the complex Urbilateria postulated in the archicoelomate theory.

Unless acoels and nemertodermatids are shown to be ancestral but simplified or just derived bilaterians, the new LCBA leads to a smooth morphological and developmental transition from a bilateral, diploblastic planuloid to a bilateral, triploblast acoeloid, and from the latter to more complex higher bilaterians. Alternatively, bilateral symmetry may have evolved under selective pressure for improved internal circulation in a cnidarian–bilaterian ancestor, inferred to be a sessile, bilaterally symmetrical animal (Finnerty 2005). Additional internal manifestations of bilateral symmetry evolved subsequently in bilaterians. As for most proposals on the origin of bilateral organisms with directive locomotion based on the enterocoel–archicoelomate hypotheses, the main stumbling blocks for its acceptance are the undefined developmental mechanisms and the uncertain functional continuity of intermediates between a sessile ancestor and a benthic crawling descendent. This makes it more plausible that, as stated in the planuloid–acoeloid theory, bilaterality first originated in small, bottom-dwelling organisms.

6. SUMMARY AND PROSPECTS

Phylogenetic analysis using molecular markers, under strict conditions to avoid stochastic and systematic errors, have corroborated the position of acoel and nemertodermatid flatworms as the earliest extant branching members of the Bilateria. This reinforces the planuloid–acoeloid theories that see stem bilaterians as stocks of small, benthic, simple organisms probably derived from planuloid-like organisms and from which present-day cnidarians also probably arose. In addition, new molecular data are helping to overcome the simple subdivision of Bilateria into the three large and poorly internally resolved superclades introducing 'minor' phyla (e.g. Gnathostomulida, Gastrotricha, Chaetognatha, Xenoturbellida) into new positions that will force a re-evaluation of the new animal phylogeny (Adoutte *et al.* 2000). Finally, and most importantly, the growing consensus to consider the Cnidaria bilaterally symmetric in origin (Finnerty *et al.* 2004; Martindale 2005) leads us to suggest a new systematics for the Bilateria, which considers the Cnidaria as bilaterians and sister group to the rest of Bilateria, now dubbed as Triploblastica to indicate the appearance of mesoderm as one of the most important events in animal evolution.

In the upcoming years, refinements in data acquisition, evolutionary models, fossil record, molecular phylogenies, gene expression data (see expression of developmental genes in embryos of acoels, Hejnol & Martindale in press) and functional evo–devo studies will be instrumental to test the soundness of the new proposal as well as to sort out the sequential evolution of clades at the base of the Deuterostomia, the Ecdysozoa and the Lophotrochozoa.

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Agraïments

Quan ets petit, hi ha vivències que creus que mai et passaran a tu, i que només els hi passen als que són més grans que tu. Des de coses petites, com ser prou gran com per que et donin les claus de casa, tenir rellotge propi o anar sol al cole, a coses més importants com tenir fills, casar-se o acabar la carrera. Una de les vivències que sempre m'ha quedat molt lluny era la d'escriure els agraïments de la tesi, i ja veus, aquí estem. Tantes vegades que he fullejat els agraïments de les tesis dels altres, i ara em toca fer els meus. Ara ve un tros de rotllo, així que si algú només vol llegir la seva dedicatòria com jo faig sempre, podeu buscar els vostres noms en negreta. Ah! I no us piqueu pels motes, exerciu el sentit de l'humor sisplau jejeje.

Han estat molts anys al departament, o al "depar" com diem els joves (ejem!), plens d'experiències i relacions amb molta gent. Totes elles han estat enriquidores en un sentit o en un altre, i per mi sempre hi haurà un nucli de gent sense la qual el departament no seria el depar. Evidentment, dir quines persones formen part d'aquest nucli és totalment subjectiu i depèn de com cadascú "sent" la forma de ser del departament; i no són només els més "amiguets", si no aquella gent que defineix el caràcter i personalitat del depar. I dels "amiguets", haig de dir que l'ambient al departament és totalment favorable a les amistats i a passar-ho bé... i jo m'ho he passat molt bé, i he rigut molt com podeu atestiguar tots, jejeje.

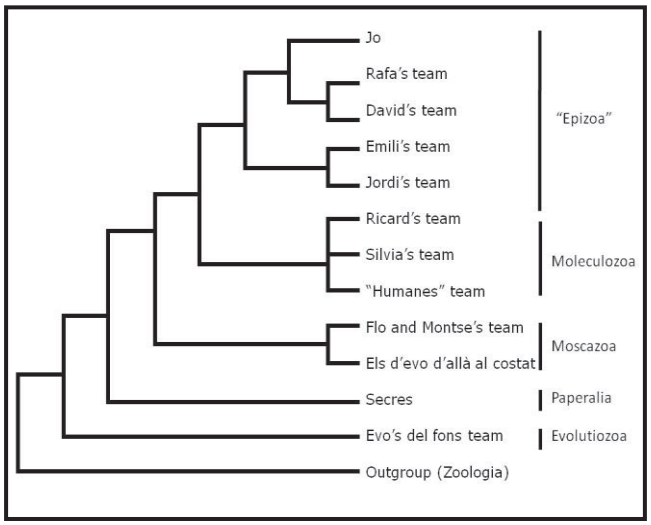

Fig 0. Esquema de les **meves** relacions evolutives amb la gent del departament. A diferència dels arbres filogenètics, els grups més propers indiquen relacions més antigues i de proximitat geogràfica, no busqueu segones intencions ni dobles sentits. I al contrari que els arbres de la meva tesi, en general les relacions més antigues son les més robustes. Noms entre cometes indiquen grups no monofilètics.

I bé, deixo de liar-me, per que haig d'anar a enquadernar això d'aquí poc. Aquests agraïments són més "gràcies per fer-m'ho passar bé" que no un "gràcies per deixar-me el marcador de pes molecular aquell dia"; evidentment estic agraït a tota la gent que m'ha deixat el seu marcador, i de tots els favors científics en general. I dins dels agraïments personals, espero no deixar-me a ningú. I si em deixo algú sapigueu que no és premeditat, si no fruit de la presa i de la memòria, que ja fa molt que rondo per aquí. Com començo? Aix, no se... porto tat de temps escrivint els articles i la tesi, que crec que faré servir el mateix esquema aquí.

La **Figura 0** mostra com s'han anant desenvolupant les meves relacions dins del departament des de que hi vaig entrar. També cal dir que hi ha un abans i un després de l'event "anar a l'efici nou"; aquest event, d'una importància similar a l'explosió càmbrica, va fer canviar les relacions entre els grups, tot i que no entre individus específics. Les descripcions són més o menys cronològiques. De veritat, espero no deixar-me a ningú!

El clade més proper és el format per la gent de **Rafa** "batallitas" **Romero** i **David** "fenotip nòrdic" **Bueno**. Sempre recordaré aquests dos éssers, heretges de la filogènia (jajjaa us la devia, pesats!), així com a la resta de gent d'aquell lab, amb molt de carinyo. Els primers amb els que em vaig relacionar i amb els que encara tinc lligams molt forts. La primera que em ve al cap és evidentment **Juani** "belleza ibérica" **Fernández**, gran amiga, gran persona (no només per que és molt alta), grans converses i sempiterna parella de ball a les "noches locas" del depar. **Jordi** "Yo soy tu padre" **Solana**, el meu Chewbacca de les pràctiques de genètica, el meu Han Solo de las festes i possiblement l'home del departament amb el que m'he fet més petons a la boca (a ver como queda tu reputación despues de esto). I com no, el gran **Victor** "pollito" **Hamburguer and Hamburguer**... ah no, era **Hernández Hernández**, company de goigs i penes, poeta del carrer i el terror de les nenes... sempre recordaré el curs de Banyuls (com vam emprenyar al Rafa per poder anar), les xerrades, les festes, les glories i les desglories... quina sort que tens Anna (això ho pots interpretar com vulguis, jejeje). **Miquel**

"Capullotplanariocalvoide" **Vila**, que està com una cabra (que us haig d'explicar), que malgrat el seu escepticisme filo-molecular, sempre ha mostrat un autèntic interès i carinyo per mi i els meus resultats. **Susana** "Decathlon" **Reigada**, un altre gran persona, amb la que vaig compartir protocols desastrosos i molts riures. També m'enrecordo molt de **Vane** "Tierra Media" **Sancho**, grans converses frikis, fan de l'Ally McBeal i una de les estudiants més intel·ligents que ha passat pel depar. **Albertito** "no m'equivoco mai" **Cardona**, un tio que sent genuïna passió per la ciència i amb el que vaig compartit moltes discussions, totes de bon rotllo... no se si el vaig arribar a convèncer de que no ens inventem els arbres, jejeje. **Francesc** "mio-culé" **Cebrià**, un tio molt centrat, molt intel·ligent i sempre disposat a donar una ma. A l'època post-edifici nou, **Carolina** y **Maryam**, grans companyes de lab, sempre amb un somriure càndid a la cara i amb les que he tingut una convivència genial... espero que sent tan tranquil·les, el meu riure no us així molestat massa, jejeje. **Joan Anton** "trilobit" **Vela**, sempre el recordaré pels puros, els trilobits, les separatas (crec sincerament que ets un dels que publica més al depar) i les partides d'escacs amb pique contra el Víctor... i per la fatídica frase "jo vaig trigar més d'un any a acabar la tesi" (que cabrón). I a les "últimes" incorporacions com el **Cisco** "distalless" o el **Xema** "tinc un Mac de nena", molta sort i paciència amb el Rafa, jajaja!

El següent clade és el de l'**Emili** "veus com l'esport no es sa" **Saló** i **Jordi** "Homeòtic" **Garcia**. L'Emili, com diria la Ona, és un dels grans tipets que ronda pel depar, que sempre s'ha preocupat genuïnament per mi i m'ha donat molts bons consells fins el punt de que m'he sentit membre honorari del seu grup, amb molt d'orgull!!! Ah! També recordaré totes les vegades que em vas fer passar-te el correu electrònic d'un ordinador a un altre, jajaja, maldito Outlook, menys mal que vas agafar al Kike. I el bon gust amb les Erasmus daneses. Del Jordi sempre m'ha agradat la seva forma de veure l'evolució i les seves "palles mentals" sobre l'evolució dels Hox. Bé, qui hi ha dins d'aquest grup? Evidentment el **Javi** "hechamos un cigarrito" **González**, tot i que em deu més favors ell a mi que jo a ell, jejeje; però no m'importa. No hace falta que diga mucho más, verdad? Un beso. **Kike** "vamos a fumar, no?" **Taboada**,

un altre dels meus grans companys que va succeir al Javi a la Gran Cadena dels Grans Fumadors, amb el qual he compartit tantes coses, bones i dolentes, i amb el que comparteixo filosofia de vida... siempre recordaré aquel verano que pasamos juntos en el Servei d'Esports de la UB. **Mete** "Jordi malo malo" **Handberg Thorsager** (seguro que lo he escrito mal), per la seva innocència però eficiència nòrdica, gran sentit de l'humor, realment un dels angelets que corre pel lab. També m'enrecordo de l'inimitable **Eva** "no me toques las narices" **Jiménez**, la qual s'ha guanyat totes les coses bones que li han passat i totes les que li passaran; a veure si tornem a la Fira de Nadal noieta! La **Cris** "transgènica" **González**, també una gran amiga i una de les més divertides, aquelles xerrades sobre les relacions de parella, bons consells íntims dels que malauradament mai vaig fer cas i els piques a les partides del Risk... molta sort allà on aneu tu i el Fèlix. **Maria** "angelet" **Marsal**, companya de carrera i un altre dels angelets del depar. La **Loli** "no me gusta Camela" per ser tan divertida, soportar al Javi González, i per aquella tornada del congrés d'Innsbruck repassant totes i cadascuna de les xerradas del meeting. I tanta altre gent que va passar pel lab (les daneses!), i les noves incorporacions, la **Marta** "fiestera" **Iglesias**, l'alemanyot rar aquell (**Kay**, que grande escribir el último párrafo de la tesis mientras Alemania pierde la eurocopa) o el **Josep** "integer value" **Abril**. I a la **Ula**, tots ens enrecordem de tu. De la gent del Jordi també tinc molts bons records, l'**Anna** "rastas" **Rosannas**, el **Josep**, la **Senda** "ojazos", el **Salvatore** "mafiosi" i tota la nova fornada d'evodevitos que tenen un gran background evolutiu: **Nacho**, **Manuel** & **Champi**. Tot i que no està a l'arbre (difícil de situar), una abraçada ben forta pel **Pere** "a on cauen els Acels aquesta setmana?" **Martínez** i la seva gent.

Dels *Moleculozoa* sempre recordaré amb molt de carinyo els dinars amb el **Ricard** "quan acabes la tesi?" **Albalat**, amb un gran sentit de l'humor i capacitat d'encaixar les "puyes". Otro tanto per la **Neus** "impermeable" **Cols**, una de les cares més amables del departament amb un sentit de l'humor infinit. La **Roser** "molecular" **González** i la **Gemma** "rinxols d'or" **Marfany** per que van ser unes profes genials que em van inspirar molt. La **Silvia** "Mac" **Atrian** per ser capaç de comprendre el gran enigma que són els ordinadors de

la poma (els mata la simplicitat!). El **Jon** "petò de puça" **Permanyer**, un altre gran tipet, membre del club dels fumadors, etern jubilat observant als "guasos" en acció i que sempre m'ha ajudat molt i donat grans consells... el Jon és tan útil que és una de les tres coses que em portaria a una illa deserta. Menció especial per les xafardaries, i converses amb **Laura** "maruja" **Tio** (un beso enorme pa ti y toda tu familia!!!! Y parad de reproduciros ya, por Dios!!!), que va treure los rulos que hay en mi. I molts petons per la **Roser** "viva windows 2000" **Urreizti**, i el seu marit **Guillem** "Vampiro" **Plasencia** (ya os habeis casado, no? En el limbo del depósito no me he enterado). A l'**Anna** "no saps on t'has ficat" **Diaz** (que Deu t'ajudi), la **Laura** "yo no he visto ninguna ranita" **Godoy** i el **Jordi** "alter" **Doménech**, amb els quals he rigut molt. A l'**Ester** "sempre agobiada" **Pomares** per les xerrades als capvespres, tot i que vaig perdre almenys mig any de tesi, jajaja! L'**Anna** "no soc cridanera" **Bosch** que sempre ha fet les coses més interessants. El **Miquel** "pèl blau" **Tusón** (sí tio, no amaguis, quan et vaig coneixer portaves el pèl blau... o lila?), que et vagi molt bé per NY. La **Mónica** "hazme un masajito" **Cózar** per totes les vegades que m'he aprofitat de la seva debilitat per demanar-li productes i kits i altres coses. La **Noe** "rompo el suelo de la discoteca cuando bailo" per ser tan maca i carinyosa, el **Raul** "no me cogieron en Factor X" **Santamaria** i tota la gent "de l'edifici nou", tant els jefes (**Lluisa, Susanna, Dani** i **Bru**) com les nenes: **Lidia** "la dolça", **Laura** "melenas", el gran **Isaac** "melón" (si tio, acabo de dir que ets una nena i un melón), **Oriel** "mirada penetrante", **Gessamí** "Soprano", **Susanna** "la rubia"... i segur que em deixo algú. I aquí també ficaré a la **Mari**, omnipresent tant a l'edifici antic com al nou, per la seva infinita paciència, bon humor i obrirme les portes de casa seva. No cambies Mari!

Dins dels *Moscozoa*, a l'edifici "antic" hi havia dos grups: els de desenvolupament i els d'evolució. Dels primers estic agraït als seus jefes, **Montse & Flo**, per diversos motius, però sobretot... per tenir la major concentració de doctorandes guapes de tot el departament! Oi quin goig fa sempre entrar en aquell lab! Que no s'ofenguin les noies dels altres grups, eh? Els nois també són molt guapos, però això ja no és cosa meva. D'aquí

recordaré a totes les nenes, la **Montse** "Big Brother" **Amorós**, la **Cristina** "estoy confundida" **Pallarés**, la **Marta** "me va la marcha" **Sesé**, la **Mireia** "pràctiques de genètica" **Angulo**, la **Isabel** "no bailo jotas... cuando estoy sobria" **Almudí**, la **Cora** "viva Madrid" **Bergantiños** i d'altres noies amb les que no he interactuat tant però que també són molt guapes. I dels nens també me'n recordo, of course: el **Manel** "no hace falta tener pelos para tener una novia que te cagas" **Bosch**, el **Sergi** "terror de las nenas" **Bertran**, l'**Adrià** "mirada enigmàtica**" Punset** i el **Xavier** "mireu noies, aquí teniu un exemple de doctorand guapo" **Vilana**. Vosaltres també sou molt guapos! L'altre grup dels *Moscozoa* d'aquell lab són els d'evolució, el **Lluís** "mathematics rules!" **Serra**, el **Frances** "FORÇA BARÇA!!!" **Mestres**, la **Marta** "ojazos" **Pascual** i el **Joan** "politènic" **Balanyà**. Amb tots sempre he tingut xerrades amenes i interessants, sempre fent gala d'un bon sentit de l'humor. Tot i que no perdonaré mai al Francesc que m'espantés a les estudiants (Frances dixit: "Charlotte, be careful, Jordi is a vulpture"). Molts de records per tots els seus estudiants, com l'**Elisabet**, el **Pep**, el **Ferran**, la **Cinta**, el **Pedro** i d'altres que em dec oblidar.

Si des del lab dels *Moscozoa* anaves cap a fora del depar, et trobaves la secretaria del departament. Crec que és un dels clades amb el que estic més endeutat i al que dec més favors. Aix, quins temps aquells amb la **Nuria** i la **Irene**, aquelles xerrades ben d'hora al matí quan arribava al lab. I a l'edifici nou, la **Roser**, la **Rosa Maria** i l'**Àfrica**, que a més de ser super-eficaces i saber-ho tot (molt important per mi, que soc un desastre amb la paperassa), sempre s'han interessat de veritat per com m'anava tot i per ajudar-me de la millor forma possible. Moltes gràcies a totes i molts petons.

I l'últim grup del depar son "els d'evolució", que es trobaven a l'"altre banda". Tot i que no els hi he dit mai, tant ells com algun profe de Zoo són responsables de la meva situació actual... que suposo que és d'agraïr, jejeje. L'**Elvira** "amo a Benzer" **Juan** per que amb la seva força va fer que m'interessés encara més per la Genètica, i **Julio** "has probado a hacerlo con el DnaSP?" **Rozas** per que va ser la primera persona que em va explicar quelcom

d'evolució a la meva vida, a les mítiques classes d'OVE, de forma clara i cristal·lina... i bé, l'efecte va ser que aquí estic ara! La **Montse Aguadé** i la **Carme Segarra** (a les dues els hi podria aplicar el mote "aquest cop et presentes a l'examen?") van cimentar els meus coneixements d'evolució molecular a partir dels quals em vaig ficar a la filogènia molecular; amb totes dues m'ho he passat molt bé (encara recordo les partides de cartes al tren de camí a Gandia) i sempre m'han ajudat molt. La **Montse** "coordinadora de pràctiques" **Papaceit** per ser tan afectuosa però ferma a la vegada, a l'igual que la **Dorcas Orengo**, ja que ambdues m'han hagut d'aguantar les meves tonteries. I TOTS els estudiants del grup, amb els quals m'ho he passat molt bé tant al depar com arreu de la geografia del país... em sap greu per que és un grup tant gran que segur que em deixo a algú. **Alex** "otro terror de las nenas" **Farrerons** per ser l'únic no fumador del grup dels fumadors, la **Úrsula** "terremoto" **Ramírez** (se te hecha de menos!), **Carlos** "doctorado en paralelo" **Arboleda**, la **Sara & Alex**, el **Michael**, l'**Inés**, l'**Eva**, el **Filipe**, els **Davids**, l'**Anna**, l'**Albert** i l'inimitable **Sebas**.

Ja arribem a l'outgroup, els zoòlegs. A molts d'ells els hi dec la major part de les meves mostres, i als que estic molt agraït per que sempre m'han tractat com un mes del seu departament. Aquí hi ha un altre culpable de la meva situació actual, el **Xavier** "Tunicado" **Turon**, tant per les classes de Zoologia general com per les de sistemàtica d'OVE; allà va ser on vaig tenir contacte per primer cop amb la part "organísmica" de l'evolució, que és la que trobo més màgica, i amb els invertebrats que per mi són infinitament més interessants que els vertebrats. Encara recordo una classe (no em facis dir l'any que em fa vergonya) a on explicaves que alguns moleculars COMENÇAVEN a proposar que els anèl·lids i els artròpodes no eren grups germans... imaginat. La **Creu** "nematoda" **Palacín** pel seu interés en el que faig (amb tota la feina que te ara, encara para per preguntar-me com em va) i per que som dels pocs frikis interessats en la meiofauna, encara recordo aquelles pràctiques on pensàvem que havien trobat un gnatostomulat, jejejej! El **Manel** "opitobranquio" **Ballesteros** per la seva amabilitat quan vam anar a mostrejar a Roses i el seu interès. I als "joves" que van arribar més tard i van

renovar la sang dels filogenètics: **Salvi** "lagarto" **Carranza** i **Miquel Àngel** "Parsimony forever!" **Arnedo**. Ambdós exsuden entusiasme per aquest món i només diré que de gran vull ser com ells (però amb menys fills que el Salvi, i menys canes que el Kele). També he passat bons moments amb els i les estudiants de Zoo, sobretot la gent del Xavi, el Salvi i el Kele. Tot i que ja fa temps que va marxar de Zoo, es mereix una menció especial el **Gonzalo Giribet**, que no només em va fer sentir com a casa quan vaig anar a Boston, si no que a més d'obrir-me les portes del seu lab també em va obrir les portes de casa seva (Big Lewosky FTW!); aquella va ser una de les experiències més importants d'aquesta tesi, tot i que jo soc un sapastre i no li he agraït prou. And I can't forget **Matthias Obst** and **Martin Sorensen,** though it can look like I do. A big hug for you guys, good old times back there in Boston and in Ravenna, you were very special for me.

I ja se'ns ha acabat l'arbre... però encara queda gent. Les meves dues famílies, la filogenètica i la de veritat. Al principio estava... el **Carles** "me encanta que los planes salgan bien" **Ribera**, que al marge de molts bitxos, també li dec molts riures; malgrat que és un caos en potes, sempre ha estat molt afectuós (d'una forma totalment heterosexual) i sempre m'ha mostrat un aspecte molt pràctic de feina. I sobretot, em va ensenyar a jugar amb Nitrogen líquid, Déu meu, mira que hi vam ficar de coses allà dins! La meva "amant secreta", la **Gemma** "madre de mi hijo secreto" **Blasco**, li agraeixo el seu gran humor i la seva força, que fes totes les PCRs que a mi no em sortien (moltíssimes!!!) i que no es deprimís en l'intent... tot i que estic a punt de posar-te a la carpeta d'spam de l'e-mail, jejeje. Un beso muy gordo mi niña. La meva germaneta "bessona", la **Mercè** "no puc obrir els ulls a les fotos" **Loukota**, que em va acompanyar a les primeres passes del laboratori durant molt de temps (recordes les "abductions"?; vam compartir moltes coses, des d'històries fins a material de laboratori, tot això uneix molt. I sobretot em va ensenyar la tenacitat a l'hora de perseguir el que vols. Molta sort Merche!

El meu germà gran, l'**Inyaki** "filiprim" **Ruiz-Trillo**, per la seva paciència i que em va ensenyar pràcticament tot de la feina al lab. I sobretot, la seva

filosofia de la vida respecte la ciència, tant a nivell pràctic com filosòfic. I per mostrar un sentit de l'humor dels més aguts que hi ha pel departament, i per la seva humilitat. Per ser l'únic home amb el que he compartit llit i que m'ha respectat, i ha tolerat que arribés a les tantes de la nit (a Gandia). De gran també vull ser com tu, però amb uns kilos més. I les dues germanes "petites" (no us ho prengueu malament!!!), que ja s'han fet grans i fan la seva vida, l'**Ona** "cul inquiet" **Àlvarez** i l'**Eva** "friendly colleja" **Lázaro**. A vosaltres també us agraeixo moltes coses, començant pel bon humor i acabant per hores i hores de feina i de festa. Quan vam anar a l'edifici nou estava preocupat pel canvi d'"ambient": la Mercè i l'Inyaki ja no hi eren, els d'epi i mol estàven a un altre pis. Però em veu ensenyar que estava equivocat i em veu renovar amb la vostra efervescència per la feina. Molta sort en tot el que feu! I que jo ho vegi!

Jaume "organismic" **Bagunyà**, al qual li agraeixo moltes coses: la passió per l'evolució, una visió integradora de la mateixa i l'entusiasme per la xarrera, jejeje. Tot i que jo ja estava encaminat cap a l'evolució, les seves inoblidables classes d'embriologia em van fer adonar de la importància del desenvolupament en l'evolució, per fi lligant l'evolució de les molècules amb la dels organismes. Més enllà dels agraïments cientifico-acadèmics (que són moltíssims), li dec molts bons consells, molts cafès, moltes "palles mentals" sobre l'evolució dels bitxos i també molts riures. Per que malgrat no ser doctorand seu, m'ha tractat com si ho fos, i hi ha invertit molt de temps en mi. De gran també vull ser com tu, però amb un PC Windows.

A més de la meva mare biològica, tinc la sort de tenir una mare a Biologia, la **Marta**. Bé, ja vam dir que arribaríem algun dia (ejem!) i que segur que riuríem molt pel camí. I així ha estat! Moltes gràcies per la teva paciència infinita, per tots els ànims, pels cops a l'esquena i els cops al cul (figurats, eh?!?!), per suportar les meves tonteries, i per ser una amiga de veritat a més d'una directora ideal. M'has ensenyat els fonaments d'aquesta ciència, una que no és precisament senzilla, la rigurositat científica per fer-la i la minuciositat per les coses ben fetes. Sobretot, sempre m'has tractat com un igual: has respectat les meves decisions, m'has escoltat i quan m'he equivocat m'has

redirigit de la millor forma possible; i no només parlo de la feina. Moltes gràcies Marta, et dec moltes coses. De gran també vull ser com tu, però sense pits... tot i que això em penso que no ho podré evitar.

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