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Alternative oxidase in animals: unique characteristics and taxonomic distribution

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SUMMARY

Alternative oxidase (AOX), a ubiquinol oxidase, introduces a branch point into the respiratory electron transport chain, bypassing complexes III and IV and resulting in cyanide-resistant respiration. Previously, AOX was thought to be limited to plants and some fungi and protists but recent work has demonstrated the presence of AOX in most kingdoms of life, including animals. In the present study we identified AOX in 28 animal species representing nine phyla. This expands the known taxonomic distribution of AOX in animals by 10 species and two phyla. Using bioinformatics we found AOX gene sequences in members of the animal phyla Porifera, Placozoa, Cnidaria, Mollusca, Annelida, Nematoda, Echinodermata, Hemichordata and Chordata. Using reverse-transcriptase polymerase chain reaction (RT-PCR) with degenerate primers designed to recognize conserved regions of animal AOX, we demonstrated that AOX genes are transcribed in several animals from different phyla. An analysis of full-length AOX sequences revealed an amino acid motif in the C-terminal region of the protein that is unique to animal AOXs. Animal AOX also lacks an N-terminal cysteine residue that is known to be important for AOX enzyme regulation in plants. We conclude that the presence of AOX is the ancestral state in animals and hypothesize that its absence in some lineages, including vertebrates, is due to gene loss events.

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Key words: mitochondria, cyanide-resistant respiration, animal evolution, sulfide metabolism.

INTRODUCTION

Mitochondria exist in eukaryotic cells and are important for, among other vital functions, producing ATP by oxidative phosphorylation. In contrast to the respiratory electron transport chain (ETC) of vertebrate mitochondria, the ETCs of many eukaryotes are branched due to the presence of alternative NAD(P)H dehydrogenases and/or additional terminal oxidases such as alternative oxidase (AOX) (McDonald, 2008; Rasmussen et al., 2008). Electrons enter the ETC *via* complexes I and II and are passed through ubiquinol to complexes III and IV (Bendall and Bonner, 1971) (Fig. 1). Proton pumping by complexes I, III and IV develops the mitochondrial proton motive force, which is used by F_1F_O ATPase to synthesize ATP. By contrast, AOX accepts electrons directly from ubiquinol and reduces O_2 to H_2O. Because AOX bypasses complexes III and IV, no protons are pumped by these complexes and ATP production capacity is lowered (Moore and Siedow, 1991) (Fig. 1). Whereas complex IV is inhibited by cyanide (CN), AOX is not, and therefore a characteristic of AOX function is O_2 consumption that persists in the presence of CN (Bendall and Bonner, 1971). AOX is inhibited by salicylhydroxamic acid (SHAM) and *n*-propyl gallate (*n*PG) (Lambowitz and Slayman, 1971; Siedow and Girvin, 1980).

At one time, AOX was thought to be limited to plants and some fungi and protists but bioinformatics studies have found AOX sequences in organisms from all kingdoms (except Archaebacteria), including animals (McDonald et al., 2003; McDonald and Vanlerberghe, 2005; McDonald and Vanlerberghe, 2006). Moreover, reports of CN-resistant O_2 consumption in animal mitochondria date to as early as 1971 (Hall et al., 1971). Such CN-resistant respiration has been seen in the annelid worms *Arenicola marina*, *Nereis pelagica* and *Marenzelleria viridis* (Völkel and Grieshaber, 1996; Hahlbeck et al., 2000; Tschischka et al., 2000), the sipunculid worm *Sipunculus nudus* (Buchner et al., 2001), the molluscs *Arctica islandica* and *Geukensia demissa* (Tschischka et al., 2000; Parrino et al., 2000) and the arthropod millipedes *Euryurus leachii* and *Pleuroloma flavipes butleri* (Hall et al., 1971). Several of these studies suggest that this CN-resistant respiration might be due to a non-heme oxidase related to the AOX of plants but molecular sequences or other conclusive data for AOX were lacking. Recently, AOX DNA sequences were found for the first time in four animals belonging to the phyla Mollusca, Nematoda and Chordata (McDonald and Vanlerberghe, 2004). Subsequently, an AOX cDNA from the chordate *Ciona intestinalis* Linnaeus (a sea squirt) was expressed in cultured human kidney cells and localized to the mitochondria (Hakkaart et al., 2005). This allotopically-expressed AOX conferred CN-resistant, *n*PG-sensitive respiration to the cells, indicating that it was catalytically active (Hakkaart et al., 2005). The presence of AOX in animals is a recent discovery so the taxonomic distribution of AOX in this kingdom has not been addressed thoroughly. Moreover, there is little information about how animal AOX sequences compare with those from other kingdoms and what implications this may have for enzyme regulation.

In the present study, we used bioinformatics and molecular biology to demonstrate that the taxonomic distribution of AOX in the animal kingdom is broad. AOX coding sequences allowed us to identify several characteristics, including a unique C terminus that can be used to distinguish animal AOXs from those of other kingdoms. Our experimental data and an *in silico* analysis of data from public molecular databases indicate that animal AOX genes are expressed in a variety of species, tissue types, developmental stages and under

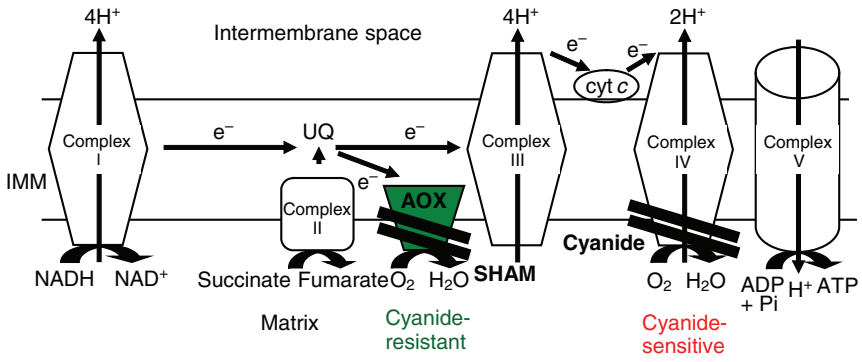

Fig. 1. The respiratory electron transport chain of mitochondria showing the position of alternative oxidase (AOX), which introduces a branch-point at the level of ubiquinol. Complexes I, III and IV move protons across the inner mitochondrial membrane to the intermembrane space, producing a proton gradient which is used by complex V to synthesize ATP. Complex IV is inhibited by cyanide whereas AOX is cyanide-resistant. The legend is as follows: cyt *c*, cytochrome *c*; e⁻, electrons; IMM, inner mitochondrial membrane; UQ, ubiquinol pool; SHAM, salicylhydroxamic acid.

environmental conditions where AOX might provide protection from oxidative damage or ETC inhibitors. These findings challenge the linear ETC model that is often presented for animals.

MATERIALS AND METHODS

In silico analyses – recovery of novel AOX sequences

Sequence similarity searches used the tBLASTn program at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) to search the non-redundant database and sequenced genomes using the AOX sequences previously recovered from *C. intestinalis* (TC94316), *Meloidogyne hapla* Goeldi (BM901810) and *Crassostrea gigas* Thunberg (BQ426710) (McDonald and Vanlerberghe, 2004). These sequences were used to search the NCBI trace archive, the Department of Energy Joint Genome Institute (www.jgi.doe.gov/), the J. Craig Venter Institute (www.jcvi.org/), the Sea Urchin Genome Project at the Human Genome Sequencing Center at the Baylor College of Medicine (www.hgsc.bcm.tmc.edu/projects/seaurchin/), the *Ciona savignyi* Herdman project at the Broad Institute (www.genome.wi.mit.edu/annotation/ciona/background.html) and the gene indices at the Dana-Farber Cancer Institute and Harvard School of Public Health (http://compbio.dfci.harvard.edu/tgi/tgipage.html). Novel AOX sequences identified were then used to search for additional AOX sequences in the above databases.

In silico analyses – identification and verification of animal AOX sequences

Putative AOX protein sequences had their identity verified using multiple sequence alignments with other AOX sequences and the presence of one or more iron-binding sites was used as positive identification (McDonald et al., 2003). Multiple sequence alignments of AOX proteins from a variety of species from several kingdoms identified characteristics that differed in animal AOXs compared with those of other organisms. These characteristics were then used to identify *bona fide* animal AOX sequences. Multiple sequence alignments of AOX proteins were generated using the Clustal X program (Thompson et al., 1997).

RNA isolation

Tissues were stored in RNAlater (Ambion, Austin, TX, USA) or frozen in liquid nitrogen and stored at –80°C until use. Total RNA was extracted from each tissue using TRIzol reagent according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). All RNA isolations exhibited an $A_{260/280}$ ratio >1.5.

Amplification and sequencing of animal AOX cDNAs

Two sets of degenerate primer pairs were designed using Omiga 2.0 (Genetics Computer Group, Madison, WI, USA) and a subset of the animal AOX DNA sequences. The primers were designed to bind around the first and third iron-binding sites (Set #1) or the second and fourth iron-binding sites (Set #2) of the AOX sequence, respectively (Fig. 2). Animal Degenerate Set #1 primers were: Forward 5′-GGNGTNCCHGGHATG-3′ and Reverse 5′-CBAGRTANCCNACRAAHC-3′. Animal Degenerate Set #2 primers were: Forward 5′-GRGAYYAYGGNTGGATHCAYAC-3′ and Reverse: 5′-TGRTGWGCYTCRTCNGCHC-3′. DNA was eliminated from RNA samples using amplification grade DNase I (Invitrogen Life Technologies). One–2 μg of total RNA was used as template in reverse-transcriptase polymerase chain reactions (RT-PCR) using the Access RT-PCR System (Promega, Madison, WI, USA). All RT-PCR experiments were run with a positive control supplied with the kit and a negative control lacking reverse transcriptase. The RT-PCR program for the degenerate animal AOX reactions was one cycle at 48°C for 45 min, one cycle at 94°C for 2 min, 30 cycles of 94°C for 30 s, 57°C for 1 min, 68°C for 1 min and one cycle at 68°C for 7 min. RT-PCR products were run on 1% or 1.5% agarose gels and cDNAs were purified with the QIAquick Gel Extraction Kit (Qiagen, Mississauga, Ontario, Canada). cDNAs were ligated into the pGEM-T Easy vector (Promega) and used to transform XL-1 Blue cells. White colonies were picked from LB amp^{100}-selective agar plates, containing 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) and isopropyl β-D-1 thiogalactopyranoside (IPTG), and 5 ml of overnight LB amp^{50} culture was used to isolate plasmids using the QIAprep Spin Miniprep Kit (Qiagen). Plasmids were digested with *Eco*RI and those containing inserts of the expected size were sent for DNA sequencing (Cortec DNA Service Laboratories, Kingston, Ontario, Canada or the DNA Sequencing Facility at Robarts Research, London, Ontario, Canada). DNA sequences were converted into amino acid sequences using the ExPasy translate tool (http://ca.expasy.org/tools/dna.html) and were confirmed to be AOX sequences using the criteria outlined in the section above (*In silico* analyses – identification and verification of animal AOX sequences).

RESULTS

Recovery of novel animal AOX sequences using bioinformatics

The bioinformatic identification of AOX (see Materials and methods) resulted in 25 animal AOX sequences, including 18 sequences that have been reported previously (McDonald and Vanlerberghe, 2004; McDonald and Vanlerberghe, 2006) (Table 1). AOX protein sequences were inferred from AOX genes in the genomes of *Branchiostoma floridae* Hubbs, *C. intestinalis*, *Capitella* sp. I Fabricius 1780, *Nematostella vectensis* Stephenson and from a full-length AOX sequenced from *C. gigas* by rapid amplification

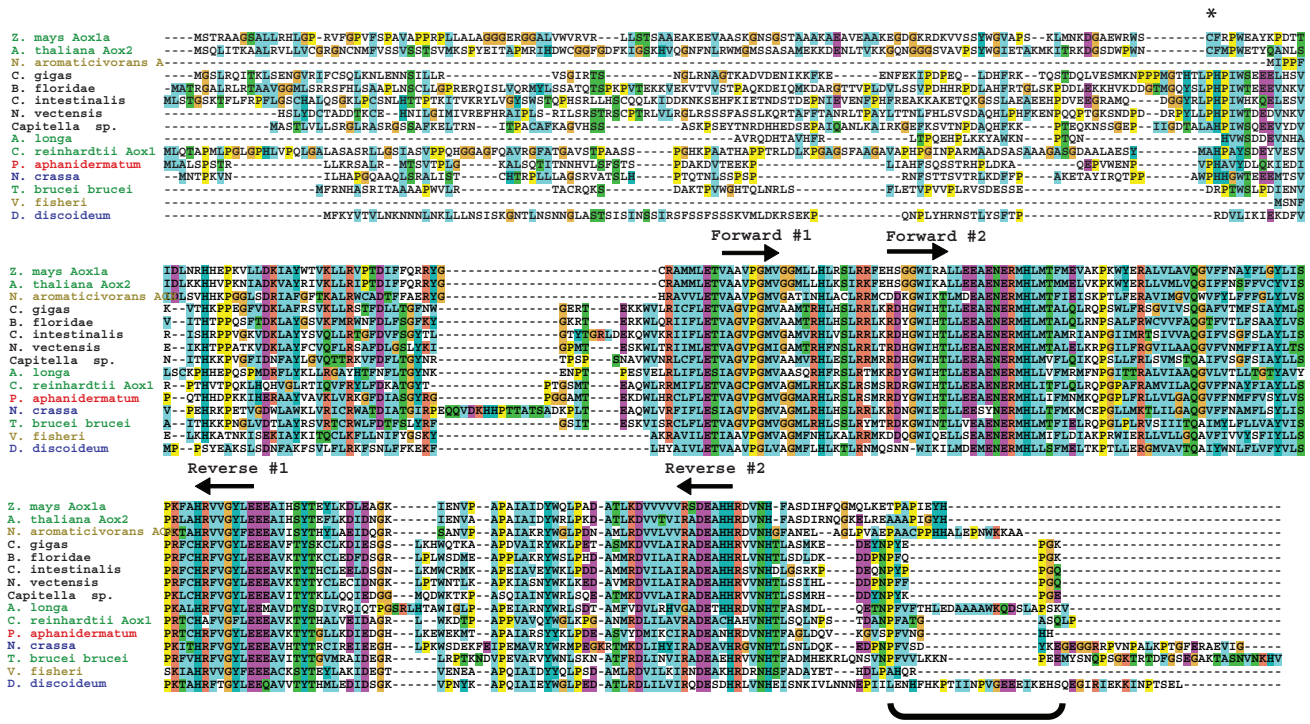

Fig. 2. A multiple sequence alignment of full-length alternative oxidase (AOX) proteins from a variety of species and kingdoms. The binding positions of the designed degenerate animal AOX primers are indicated by the arrows above the alignment. The conserved regulatory cysteine of angiosperm AOX proteins is denoted by a star. A C-terminal region of the AOX protein that is diagnostic of animal AOXs is denoted by the bracket. Animal species are listed in black, red lineage organisms in red, green lineage organisms in green, fungi and slime mould in blue and bacteria in tan.

of cDNA ends (RACE) (G.C.V., S. Amirsadeghi, A.E.M., D. Y. Zhao and R. E. Harrison, unpublished). A multiple sequence alignment of these proteins showed that they shared several features (Fig. 2) that distinguish animal AOXs from those of members of the green lineage (*Chlamydomonas reinhardtii* Dangeard, *Astasia longa* Pringsheim, *Trypanosoma brucei brucei* Gruby), the red

Table 1. A summary of animal alternative oxidase (AOX) sequences recovered using bioinformatics

Phylum	Species	Database and identifier	Relevant citation or webpage
Placozoa	*Trichoplax adhaerens*	NCBI; NZ_ABGP01000201 350281-351960	Srivastava et al., 2008
Porifera	*Oscarella carmela*	NCBI; EC370323	Nichols et al., 2006
	Reniera sp.	NCBI; 858481109	www.jgi.doe.gov/sequencing/why/3161.html
Cnidaria	*Acropora millepora*	NCBI; DY587694	Technau et al., 2005
	Clytia hemisphaerica	NCBI; CU430547	Not applicable
	Hydra magnipapillata	NCBI; 668978988	Not applicable
	*Nematostella vectensis**	NCBI; XM_001635879	Putnam et al., 2007
Nematoda	*Meloidogyne hapla*	NCBI; BM901810	Martin et al., 2009
	Pratylenchus vulnus	NCBI; CV200442	Martin et al., 2009
Annelida	*Capitella* sp.*	JGI genome	http://genome.jgi-psf.org/Capca1/Capca1.home.html
	Alvinella pompejana	NCBI; GO114366	www.jgi.doe.gov/sequencing/why/3135.html
Mollusca	*Aplysia californica*	NCBI; EB344940	Moroz et al., 2006
	*Crassostrea gigas**	NCBI; FJ607013	Gueguen et al., 2003
	Crassostrea virginica	NCBI; CD649081	Not applicable
	Ilyanassa obsoleta	NCBI; FK717789	Not applicable
	Lottia gigantea	JGI genome	http://genome.jgi-psf.org/Lotgi1/Lotgi1.home.html
	Lymnaea stagnalis	NCBI; ES576122	Not applicable
	Mytilus californianus	NCBI; ES402065	Not applicable
	Mytilus galloprovincialis	NCBI; FL489835	Venier et al., 2009
Echinodermata	*Strongylocentrotus purpuratus**	NCBI; XM_001192213	Sodergren et al., 2006
Hemichordata	*Saccoglossus kowalevskii*	NCBI; 1698837910	Not applicable
Chordata	*Branchiostoma floridae**	JGI genome	Putnam et al., 2008
	*Ciona intestinalis**	TIGR genome	Dehal et al., 2002
	Ciona savignyi	TIGR genome	www.genome.wi.mit.edu/annotation/ciona/background.html
	Molgula tectiformis	NCBI; CJ360866	Gyoja et al., 2007

Sequences marked with an asterisk are complete protein coding sequences, all others are partial sequences.

Table 2. A summary of animal alternative oxidase (AOX) sequences recovered by RT-PCR with animal degenerate primers designed to amplify a 400 bp product

Phylum	Species	NCBI identifier	Reference
Porifera	*Ephydatia muelleri*	FJ607015	Present study
Mollusca	*Anadara ovalis*	FJ607014	Present study
	Crassostrea gigas	FJ177509	McDonald and Vanlerberghe, 2004; G.C.V., S. Amirsadeghi, A.E.M., D. Y. Zhao and R. E. Harrison, unpublished; present study
	Crassostrea virginica	FJ607013	Present study
	Mercenaria mercenaria	FJ607016	Present study

lineage (*Pythium aphanidermatum* Edson 1923), a free living protist (*Dictyostelium discoideum* Raper), a fungus (*Neurospora crassa* Shear and Dodge), two bacteria (*Novosphingobium aromaticivorans* Balkwill 1997, *Vibrio fischeri* Beijerinck 1889) and two plants (*Zea mays* Linnaeus, *Arabidopsis thaliana* Linnaeus Heynh.) (Fig. 2). In particular, the animal AOXs lack an N-terminal cysteine residue present in the angiosperm enzymes (Fig. 2). Animal AOXs have a characteristic C-terminal motif that is unlike other AOX proteins (Fig. 2, details below). Using this diagnostic tool, it was determined that the AOXs identified from *Trichoplax adhaerens* Schulze, *Oscarella carmela* Vosmaer, *Aplysia californica* Cooper, *Molgula tectiformis* Nishikawa and *C. savignyi* are *bona fide* animal AOXs. Other putative animal AOXs were identified from partial sequences (Table 1) but the C-terminal regions are missing and therefore more data must be collected before their origin can be determined definitively.

We determined that the AOX sequence from the animal *Hydra magnipapillata* Ito is not of animal origin but appears to be from a higher plant source. This may represent a horizontal gene transfer event from a symbiont to the *Hydra* as was recently proposed for a plant-like peroxidase gene (Habetha and Bosch, 2005). It may also be the result of contamination of the animal sample with external material during collection or processing and illustrates the need for caution when interpreting data collected from public databases.

Recovery of novel animal AOX sequences using RT-PCR

Degenerate animal AOX RT-PCR primers were tested on *C. gigas*, which is known to contain an AOX transcript (McDonald and Vanlerberghe, 2004). Primer Set #1 (see Materials and methods) did not amplify a product whereas Primer Set #2 amplified a ~400 bp cDNA, which was confirmed to be AOX by sequencing (see Fig. S1 in supplementary material). To ascertain if the primer pair would work in other species, we used RNA from the Eastern oyster *Crassostrea virginica* Gmelin 1791 and a freshwater sponge *Ephydatia muelleri* Lieberkuhn 1855. Primer Set #2 amplified an AOX sequence from both species (see Fig. S1 in supplementary material). The *C. virginica* AOX sequence matched a partial sequence identified by bioinformatics (Tables 1 and 2). The AOX from *E. muelleri* was novel and most similar to other sponge sequences recovered by bioinformatics (Tables 1 and 2). The primers were also able to amplify AOX from two bivalve molluscs: the northern quahog *Mercenaria mercenaria* Linnaeus and the cockle *Anadara ovalis* Bruguiere (Table 2). Therefore, RT-PCR using the degenerate animal AOX primers identified three novel animal AOX sequences that were not present in public molecular databases and therefore could not be recovered using bioinformatics (Table 2).

Characteristics of animal AOX protein sequences

MitoProt II software (Claros and Vincens, 1996) predicts that the AOX of *C. intestinalis* has a 0.8855 probability of mitochondrial import. This prediction is supported by recent work that shows that this AOX localizes to mitochondria when expressed in human kidney cells (Hakkaart et al., 2005). This software calculates a probability of 0.8691 that *C. gigas* AOX will also localize to mitochondria. We therefore predict that all animal AOXs will be mitochondrial proteins.

A multiple sequence alignment of animal AOX proteins from several phyla shows that all of the glutamate and histidine iron-binding residues (Berthold and Stenmark, 2003) are absolutely conserved (see Fig. S2 in supplementary material). As noted above, animal AOXs possess a C-terminal N-P-[YF]-X-P-G-[KQE] motif that is not present in AOX proteins from other kingdoms (see Fig. S2 in supplementary material). This region therefore represents a diagnostic tool for the identification of animal AOX proteins.

One region of interest is the epitope recognized by the widely used AOX antibody (AOA) (Elthon et al., 1989; Finnegan et al., 1999). An alanine in position 2 of this sequence must be present for the antibody to recognize an AOX protein (Finnegan et al., 1999). All animal AOX proteins for which data exist have an alanine in this position (see Fig. S2 in supplementary material). Several of the animal AOX proteins differ from plant AOXs at position 9 of this sequence but such alterations are also present in algal, fungal and protistan AOXs that cross-react with the AOA antibody (Finnegan et al., 1999); therefore, we predict that AOA will recognize most animal AOX proteins.

Taxonomic distribution and expression of animal AOX sequences

Combining bioinformatics and RT-PCR yielded 28 putative animal AOX sequences (Tables 1 and 2). AOX was found in the genome of *T. adhaerens*, one of the few identified members of the Placozoa (Table 1). AOX is in three species belonging to Porifera (Table 1). Bioinformatics found AOX sequences in the marine demosponges *O. carmela* and *Reniera* sp. Schmidt. These represent mRNAs isolated from whole tissue containing embryos and from different developmental stages (Nichols et al., 2006) (see Table S1 in supplementary material). RT-PCR using degenerate animal AOX primers recovered an AOX sequence from the freshwater demosponge *E. muelleri* (see Fig. S1 in supplementary material; Table 2). AOX in the Cnidaria includes two anthozoans, the coral *Acropora millepora* Ehrenberg 1834 and the starlet sea anemone *N. vectensis*, and two hydrozoans *Clytia hemisphaerica* Linnaeus 1767 and *H. magnipapillata* (Table 1). As noted above we suspect the *H. magnipapillata* sequence (Table 1) is not animal in origin. In *N. vectensis,* expressed sequence tags (ESTs) indicate that AOX is expressed throughout development, including the larval stage (Putnam et al., 2007) (see Table S1 in supplementary material). Our degenerate primers did not recover cDNAs in *Xenia* sp. Lamarck (coral), the anemones *Metridium senile* Linnaeus 1761 and *Tealia feline* Linnaeus 1761, the jellyfish *Eutonina indicans* Romanes 1876 or the hydrozoan *Obelia* sp. Peron and Lesueur.

Table 3. Complete animal genomes searched for alternative oxidase (AOX) where no sequence was detected

Phylum	Species	Common name and description
Nematoda	*Brugia malayi*	Parasitic filarial worm causes lymphatic filariasis and elephantiasis in humans
	Caenorhabditis briggsae	Nematode worm model system
	Caenorhabditis elegans	Nematode worm model system
	Heterodera glycines	Soybean cyst nematode
	Pristionchus pacificus	Free-living nematode
	Trichinella spiralis	Nematode parasite causing trichnellosis (or trichinosis) in humans
Arthropoda	*Acyrthosiphon pisum*	Pea aphid
	Aedes aegypti	Mosquito vector for dengue and yellow fever
	Anopheles gambiae	African malaria mosquito
	Apis mellifera	Honeybee
	Bombyx mori	Domestic silkworm
	Culex quinquefasciatus	Southern house mosquito vector for Western Nile Virus
	Drosophila melanogaster	Fruit fly
	Ixodes scapularis	Black-legged tick
	Nasonia vitripennis	Parasitoid wasp
	Pediculus humanus corporis	Human body louse
	Tribolium castaneum	Red flour beetle
Chordata	*Bos taurus*	Cow
	Canis lupus familiaris	Dog
	Danio rerio	Zebrafish
	Equus caballus	Horse
	Felis catus	Cat
	Gallus gallus	Chicken
	Homo sapiens	Human
	Macaca mulatta	Rhesus macaque
	Monodelphis domestica	Opossum
	Mus musculus	Common mouse
	Ornithorhynchus anatinus	Duck-billed platypus
	Ovis aries	Sheep
	Pan troglodytes	Chimpanzee
	Rattus norvegicus	Rat
	Sus scrofa	Pig
	Taeniopygia guttata	Zebra finch
	Takifugu rubripes	Japanese pufferfish
	Xenopus tropicalis	Western clawed frog

In bilateral animals, members of both the Protostomia and Deuterostomia contain AOX. In the Lophotrochozoa, AOX is found in several molluscs (Table 1). AOX is present in the gastropods *A. californica*, *Ilyanassa obsoleta* Say 1822, *Lottia gigantea* Sowerby and *Lymnaea stagnalis* Linneaus (Table 1). AOX is also present in several bivalves including *C. gigas* (Pacific oyster), *C. virginica* (Eastern oyster), the northern quahog *M. mercenaria*, the California mussel *Mytilus californianus* Conrad, the Mediterranean mussel *Mytilus galloprovincialis* Lamarck and the cockle *A. ovalis* (Tables 1 and 2). Prior work in *C. gigas* detected AOX transcripts in the gill, heart, adductor muscle, hemolymph and mantle tissues (McDonald and Vanlerberghe, 2004). EST data indicate that AOX is detected in *C. gigas* exposed to sewage or bacterial challenge (Medeiros et al., 2008; Roberts et al., 2009) (see Table S1 in supplementary material). RT-PCR using degenerate animal AOX primers detected AOX transcript in *C. virginica* adductor muscle (see Fig. S1 in supplementary material), and EST data show that AOX is also expressed in gill tissue (see Table S1 in supplementary material). RT-PCR data shows that AOX is expressed in the gill of *M. mercenaria* (Table 2). EST data indicates that AOX is expressed in the central nervous system of *A. californica*, including the cerebral ganglion, metacerebral neuron and pedal-pleural ganglia (Moroz et al., 2006) (see Table S1 in supplementary material). EST data indicate that AOX is expressed in the adductor muscle of *M. californianus*, several tissues of *M. galloprovincialis* and the brain of *L. stagnalis* (see Table S1 in supplementary material). Our degenerate primers did not yield AOX cDNAs in the bivalves *Placopecten magellanicus* Gmelin 1791 (scallop), *Modiolus modiolus* Linneaus 1758 (northern horsemussel), the gastropods *Buccinum undatum* Linnaeus (waved whelk), *Littorina littorea* Linnaeus 1758 (periwinkle), *Nucella lapillus* Linneaus 1758 (dog whelk) or the cephalopod squid *Loligo pealeii* Lesueur. In the Annelida, AOX was present in the polychaete worm *Capitella* sp. and the Pompeii worm *Alvinella pompejana* Desbruyeres and Laubier (Table 1). In *Capitella* sp. AOX is expressed during various stages of development and in *A. pompejana* it has been detected in the posterior tissues of adults (see Table S1 in supplementary material). Our degenerate primers did not amplify a product in *Lumbricus terrestris* Linnaeus (common earthworm).

In the Ecdysozoa, AOX is present in two plant parasitic nematodes, *M. hapla* and *Pratylenchus vulnus* Allen and Jensen (Table 1). AOX transcript is present in parasitic adult females and several other developmental stages (Martin et al., 2009) (see Table S1 in supplementary material). Our degenerate primers did not recover a product in *M. hapla* eggs. We did not find AOX orthologs in the genomes of several other species of nematodes (Table 3). We did not find AOX in any arthropod, including those for which genomes are available (Table 3). Moreover, our degenerate primers did not yield an AOX for the cricket *Acheta domesticus* Linnaeus, the spider *Latrodectus hasselti* Thorell, the mealworm *Tenebrio molitor* Linnaeus and the crustaceans *Hemigrapsus nudus* Dana 1851 (purple crab) and *Carcinus maenas* Linnaeus 1758 (green crab). Within the Deuterostomia, bioinformatics found AOX in

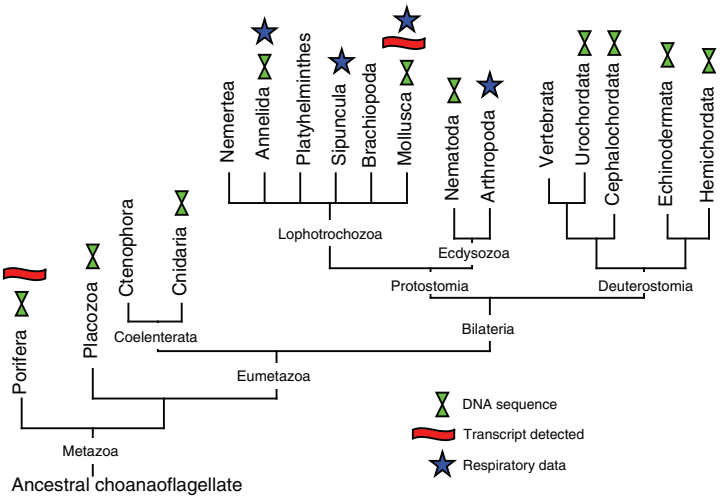

Fig. 3. A schematic representing the relationships of various phyla and subphyla of the animal kingdom showing the known distribution of alternative oxidase (AOX), based on different lines of evidence. For a particular group, a green helix symbol indicates bioinformatic evidence for AOX, a red stripe indicates that reverse-transcriptase polymerase chain reaction (RT-PCR) using degenerate animal AOX primers provides evidence for AOX expression and a blue star indicates that cyanide-resistant respiration has been measured, suggesting the presence of AOX. The relationships depicted are based on the work of Philippe et al. (Philippe et al., 2009).

members of Echinodermata (*Strongylocentrotus purpuratus* Stimpson 1857), Hemichordata (*Saccoglossus kowalevskii* Agassiz 1873) and Chordata (Table 1). However, our degenerate primers did not amplify AOX cDNA from several echinoderms, including the red sea cucumber *Cucumaria miniata* Brandt 1835, the ochreous starfish *Pisaster ochraceus* Brandt 1835 and the urchins *Lytechinus pictus* Verrill 1867 and *Strongylocentrotus droebachiensis* Muller 1776.

Within the chordates, bioinformatics results show AOX in the subphylum Cephalochordata in the Florida lancelet *B. floridae* (amphioxus, Table 1) with the transcript detected in larvae and adults (Yu et al., 2007) (see Table S1 in supplementary material). AOX is present in the subphylum Urochordata in tunicate ascidians, including *C. intestinalis* (sea squirt), *C. savignyi* and *M. tectiformis* (Table 1), where AOX is detected in the cleaving embryo and just prior to hatching (Gyoja et al., 2007) (see Table S1 in supplementary material). AOX is expressed in *C. intestinalis* during many stages of development and in blood cells (see Table S1 in supplementary material). Despite the availability of several complete genomes (Table 3), we found no bioinformatic evidence of AOX in members of the subphylum Vertebrata. Our primers did not recover AOX cDNAs from the basal vertebrates lamprey (*Petromyzon marinus* Linnaeus) and hagfish (*Eptatretus stoutii* Lockington 1878).

DISCUSSION

The origin of AOX in animals

We identified AOX sequences in 28 animal species representing nine phyla. Our data indicate that the presence of the AOX gene in animals predates the radial/bilaterial symmetry divide. Representatives from both protostomes and deuterostomes have AOX (Fig. 3). AOX is also found in members of the Lophotrochozoa and the Ecdysozoa (Fig. 3).

AOX is present in several species of extant fungi, in the ichthyosporeans *Capsaspora owczarzaki* and *Sphaeroforma arctica*, and in the choanoflagellates *Monosiga brevicollis* and *Monosiga ovata* (McDonald, 2008). This is significant as choanoflagellates are thought to represent the closest living relatives of animals (King et al., 2008). The identification of AOXs in ichthyosporeans, choanoflagellates and basal animal phyla, such as Placozoa and Porifera, suggests that the presence of AOX is the ancestral state in the animal kingdom and that it has spread by vertical inheritance. This implies that the lack of AOX in vertebrates and arthropods results from gene loss events.

Future directions

The presence of AOX in the respiratory ETC may allow animals to acclimate to stressful conditions, particularly those that inhibit the cytochrome pathway. For example, we found AOX in two plant-parasitic nematodes, *M. hapla* and *P. vulnus* and hypothesize that AOX plays a role in pathogenesis *via* metabolic flexibility in this system. If the host plant generates cyanogenic compounds, nitric oxide or other toxic metabolites that could inhibit ETC complex IV (Cooper and Brown, 2008), AOX would permit continued respiration and ATP synthesis, albeit at a lower rate. The presence of a branched ETC may allow the lugworm *A. marina* to use hydrogen sulfide as a respiratory substrate or to involve AOX in a pathway of rapid sulfide detoxification (Hildebrandt and Grieshaber, 2008). Molecular evidence for AOX in this organism is lacking (unfortunately we did not have access to *A. marina* tissue, and limited bioinformatics data are available). AOX was found in the Pompeii worm *A. pompejana*, a thermophilic annelid that inhabits the sides of deep-sea hydrothermal vents (Shin et al., 2009). We hypothesize that the metabolic flexibility afforded by the presence of AOX may help the animal to survive the low pH, high temperature and high metal ions found in its environment (Shin et al., 2009). Living in this habitat would require the ability to detoxify the large amounts of sulfide vented by hydrothermal chimneys and it has been hypothesized that symbiotic bacteria contribute to the survival of *A. pompejana* in this way (Campbell et al., 2003). However, the AOX EST recovered from *A. pompejana* is not bacterial in origin, as it shares a high degree of sequence similarity with the AOX recovered from another annelid *Capitella* sp. and has an extended N-terminal region typical of eukaryotic, but not prokaryotic, AOXs. Therefore, in a manner similar to that postulated for *A. marina*, AOX may allow *A. pompejana* to detoxify the sulfide in its environment without the aid of symbionts; these symbionts may instead be an important source of fixed carbon for *A. pompejana* (Campbell et al., 2003). AOX may serve to ameliorate the generation of reactive oxygen species by preventing over-reduction of the respiratory ETC under conditions that inhibit complex IV, similar to the situation seen in tobacco cell cultures (Maxwell et al., 1999). In fact, recent work shows that expression of the AOX from *C. intestinalis* in human cells exhibiting cytochrome *c* oxidase deficiency can decrease the sensitivity of these cells to oxidative stress (Dassa et al., 2009). AOX may be capable of influencing developmental pathways and/or patterning due the possible role that it might have in apoptosis as exhibited by tobacco (Robson and Vanlerberghe,

2002) and cellular differentiation processes examined in the slime mould *Dictyostelium discoideum* (Jarmuszkiewicz et al., 2002).

AOX is present in several animals that are model systems or are emerging as such. For example, the ascidian chordate *C. intestinalis* is used as a model system for studying the central nervous system and for studies of animal gene evolution (Meinertzhagen and Okamura, 2001; Kamesh et al., 2008). The starlet sea anemone, *N. vectensis*, has rapidly emerged as a model system for the investigation of gene families and developmental patterning in animals (Matus et al., 2008). The sea hare, *A. californica*, is used to study the central nervous system and memory formation (Geiger and Magoski, 2008; Hawkins et al., 2006). *Branchiostoma floridae* (amphioxus) has been used in gene evolution studies, especially those examining homeobox genes involved in embryonic development (Takatori et al., 2008). These animals represent excellent model systems for exploring questions about the physiological role of AOX due to their experimental tractability, the availability of genomic resources and the broad community studying diverse aspects of the biology of these species.

AOX is not present in several other animals that serve as research models (Table 3). Species such as *Drosophila melanogaster* Meigen, *Caenorhabditis elegans* Maupas 1900, *Rattus norvegicus* Berkenhout 1769 and *Homo sapiens* Linnaeus all lack AOX and therefore could be used as heterologous expression systems. The AOXs of different organisms have been expressed in the bacterium *Escherichia coli* Migula 1895 and the yeasts *Schizosaccharomyces pombe* Lindner and *Saccharomyces cerevisiae* Hansen (none of which contain a native AOX protein) and have revealed a great deal about AOX function and post-translational regulation (Nihei et al., 2003; Stenmark and Nordlund, 2003; Suzuki et al., 2004; Crichton et al., 2005; Mathy et al., 2006; Magnani et al., 2007). The expression of the *C. intestinalis* AOX in human kidney cells (Hakkaart et al., 2005) and *D. melanogaster* (Fernandez-Ayala et al., 2009) indicates that this approach is feasible in animal systems.

Our experimental approach identified unique characteristics of animal AOX that can be used to further define the taxonomic distribution of this enzyme. For example, within the molluscs the cDNAs of *C. gigas* and *C. virginica* detected by RT-PCR matched AOX sequences previously recovered *via* bioinformatics but the cDNAs from *A. ovalis*, *M. mercenaria* and *E. muelleri* were novel, and demonstrated the utility of our degenerate primers as a means of identifying AOX in organisms where little or no sequence data are currently available (Table 2). Our primers, based on 10 sequences from animals in six phyla, exhibited a success rate of ~20% (five out of 26 species screened). While this low success rate might indicate that relatively few animal species express AOX, we predict that the use of primers designed to target the AOXs of a particular phylum will probably achieve a higher success rate. Future work should target phyla where data on AOX are lacking (e.g. Ctenophora and several Lophotrochozoan phyla). A more robust search for AOX in arthropods [especially millipedes (Hall et al., 1971)] and vertebrates would be valuable. Once better kingdom sampling has been achieved, an AOX protein phylogeny could test our hypothesis that AOX arose early in the animal lineage.

We predict that animal AOX is targeted to mitochondria. To our knowledge there has been no research on a native AOX protein *in vitro* or *in vivo* in an animal. Our analysis indicates that this will be possible because the AOA is expected to recognize its epitope in animal AOX proteins (see Fig. S2 in supplementary material). Indeed, the AOA has recently been demonstrated to recognize the AOX of *C. intestinalis* expressed in human cell lines (Dassa et al., 2009). In particular, determining how animal AOX is regulated at the post-translational level will be of great interest. The activity of AOX in angiosperms is regulated *via* the redox status of an intersubunit disulfide bond (Umbach and Siedow, 1993). This mode of regulation in plants requires the presence of a key regulatory cysteine residue in the N-terminal region of the protein, which is absent in all animal AOXs examined to date (Fig. 2 and Fig. S2 in supplementary material). It remains to be determined whether animal AOXs are monomeric or dimeric enzymes (McDonald, 2008).

This work demonstrates that AOX genes and AOX mRNA are present in several animal phyla. Future work will need to investigate whether the presence of AOX genes and mRNA translate into a functional AOX protein and to examine the possibilities of AOX gene loss, pseudo genes or untranslated mRNAs in animals. The confirmation of the presence of AOX in animals indicates that some animals probably possess a branched ETC. This also has direct implications for theoretical models of mitochondrial bioenergetics, which assume a linear ETC (Nazaret et al., 2008). Future models and experiments should be designed with this in mind.

LIST OF ABBREVIATIONS

AOA	AOX antibody
AOX	alternative oxidase
cDNA	complementary DNA
CN	cyanide
EST	expressed sequence tag
ETC	electron transport chain
*n*PG	*n*-propyl gallate
RT-PCR	reverse-transcriptase polymerase chain reaction

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