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## **Characterization of Cu/Zn-SODs in sympatric species: a comparison of Zebra and Quagga mussels**

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## Abstract

Zebra mussels (*Dreissena polymorpha*) are commonly used as sentinel organisms in freshwater biomonitoring. but the displacement of this species by quagga mussels (*Dreissena rostriformis bugensis*) has raised the issue of their discrimination after field sampling. Even if those two species exhibit specific morphological characteristics, confusion can occur. However, behind the visible phenotypes lie important physiological and metabolic differences that may affect the interpretation of biomarkers in risk assessment. In this study, we present a characterization of Cu/Zn-SODs (superoxide dismutases) in *Dreissena spp.* with a comparison of activity profiles from individuals originating from North America and Europe. A phylogenetic tree was constructed and dreissenid SOD sequences appear to be closely related to clam species sequences. A specific isoform at an isoelectric point value of 4.95 was observed only for quagga mussels independently of their origin while zebra mussels exhibited several polymorphic profiles even for a single sampling site. A comparison of transcript sequences was conducted and the observed differences were highlighted. Alignment of amino acid sequences shows a high level of identity between the two species associated with minor changes in the predicted secondary structures.

## Keywords

Biomonitoring; Oxidative stress; Sentinel; Invasive species; *Dreissena*

## Introduction

Bivalves such as *Dreissena polymorpha* are widely used as sentinel organisms in freshwater biomonitoring programs (Hanana *et al.*, 2017; Binelli *et al.*, 2015; Faria *et al.*, 2014; Pain-Devin *et al.*, 2014; Navarro *et al.*, 2011). They have a wide geographical distribution, a filtering and fixed adult lifestyle, significant bioaccumulation capacity, high abundance and are resistant to complex stresses. Originating from the Ponto-Caspian basin, *Dreissena polymorpha* is a well-known invasive species of freshwater rivers in Western Europe and North America (Ginn *et al.*, 2018; Marescaux *et al.*, 2015). A rapid expansion of a related species, *Dreissena rostriformis bugensis*, quagga mussel, has been observed in Western Europe where *D. rostriformis bugensis* may displace *D. polymorpha*, a shift previously observed in North America's Great Lakes (Sturtevant *et al.*, 2019; Kerambrun *et al.*, 2018; Potet *et al.*, 2018; Matthew *et al.*, 2015). In Western Europe, first observations of quagga mussels were made in the Hollands Diep in 2006 (Matthews *et al.*, 2015; Molloy *et al.*, 2007) and in the Meuse River in 2008 (Marescaux *et al.*, 2012; Vaate and Beisel, 2011). In France, *D. rostriformis bugensis* expansion has since been reported in the Rhône drainage and the Oise River (Kerambrun *et al.*, 2018).

In this context, efficient discrimination of the two species became crucial. Zebra and quagga mussels indeed present morphological differences, but shell morphotypes can vary and lead to misidentification. Indeed, morphological indices based on length, width and height, have been proposed (Teubner *et al.*, 2016; Beggel *et al.*, 2015), but the risk of erroneous classification has already been underlined (Kerambrun *et al.*, 2018; Beggel *et al.*, 2015). For instance, in relation with their habitats, quagga mussel exhibit different shell morphotypes through a developmental plasticity (Peyer *et al.*, 2010). In a biomonitoring context, it appears essential to clearly and correctly identify the species in order to properly analyze ecotoxicological responses (Madeira *et al.*, 2019; Vernon and Jha, 2019; Farkas *et al.*, 2017). Genetic methods based on DNA markers, therefore requiring nucleic acid extractions, have already been developed to differentiate *Dreissena* species (Marescaux and Van Doninck, 2013; Voroshilova *et al.*, 2010). Hence in general, RFLP (Restriction Fragment Length Polymorphism) analysis of the fragment of the mitochondrial gene COI allows the discrimination of the two species (Voroshilova *et al.*, 2010).

Superoxide dismutases (SODs) are classic biomarkers in ecotoxicological studies (Manduzio *et al.*, 2004). These enzymes exhibit an oxido-reductase activity that catalyzes dismutation of superoxide anion into molecular oxygen and hydrogen peroxide which is in turn used by the catalase enzyme. Superoxide dismutases therefore represent first line antioxidant defenses. Depending on their metal catalytic cofactors, SODs are classified into distinct groups, (for details see revue by Abreu and Cabelli, 2010) and only copper/zinc SODs and manganese SOD are expressed in animals, the last form being exclusively mitochondrial. Cu/Zn-SODs include extra- and intracellular SODs, each one encoded by a specific gene. The extracellular form contains a signal peptide with a cleavage site for its secretion. The intracellular form, a major SOD, is often referred to as cytosolic Cu/Zn-SOD, although it has been localized not only in the cytosol but also in all cellular compartments, including the nucleus and the internal mitochondrial membrane, excluding the mitochondrial matrix (Zelco *et al.*, 2002). SOD activity is often measured on tissue homogenates, but it can also be measured by gel zymography, as developed by Beauchamp and Fridovich (1971), a method that highlights isoforms. Indeed, SODs present distinct properties including electrophoretic mobility and differential susceptibility to inhibitors such as cyanide, making possible a separate study of the different allozymes. Thus, after isoelectrofocusing in native conditions, intracellular Cu/Zn-SOD (SOD1) activity is revealed at pH between 4 and 5 while the other SODs are observed at more basic pH (Manduzio *et al.*, 2003).

In the present work, we chose to focus on the intracellular Cu/Zn-SOD and investigate its characteristics in the two *Dreissena* mussels in order to assess whether the sister species

could be used interchangeably in environmental surveys. Geographically distant sites were selected for sampling and an in-depth study of intracellular Cu / Zn-SOD was carried out. Enzymatic profiles were analyzed by zymography, and we demonstrated that Cu/Zn-SOD exhibits distinct patterns in the two *Dreissena* species. In order to understand such differences, data mining of published sequences was performed, allowing us to build a phylogenetic tree that places the sister species side by side with clams as their closest neighbors. Finally, structure prediction for both Cu/Zn SODs was conducted and revealed very similar shapes.

## Methods

All chemicals were of analytical grade and were used as received. Water for preparation of the solutions was purified using a Milli-Q purification system. Nitrobluetetrazolium (NBT), riboflavin, aprotinin and purified Cu/Zn-SOD from bovine erythrocytes and isoelectric focusing markers were purchased from Sigma.

Mussels were collected in one location in the St. Lawrence River (Quebec, Canada) and in several locations in the French territory. Sampling maps were previously published in Evariste *et al.*, (2018) for the Canadian part and in Pain-Devin *et al.*, (2014) for the French side. The Canadian site is located upstream of Montreal city at Pointe-des-Cascades (45°19'49.9"N; 73°58'11.8"W). This site is receiving water masses from the Laurentian Great Lakes and was previously shown to be a low-polluted area (Evariste *et al.*, 2018; Kwan *et al.*, 2003). In Europe, the sampling sites are located in the rivers of north France, namely the Meuse (E5°32'04; N48°53'14), Moselle (E6°04'43; N49°03'58), Vilaine (W2°18'30; N47°31'22) and Oise (E2°30'36; N49°16'50). These sites have already been the subject of environmental monitoring under the research program IPOC (Interactions between pollution and climate changes: development of an improved monitoring strategy, ANR-12-ISV7-0004-01, 2013–2015), and the presence of *Dreissena* is confirmed, either as monospecific assemblages or as sympatric populations of both species (Kerambrun *et al.*, 2018, 2016). Hemolymph collections were withdrawn from the posterior adductor muscle sinus using a 0.5 mL syringe with a 0.33 mm x 12 mm needle and stored at –80°C. Mantles were preserved in 70% ethanol. After dissection, gills and digestive glands were immediately frozen in liquid nitrogen and stored at –80°C until further analysis.

Genetic species identification was carried by Restriction Fragment Length Polymorphism (RFLP) analysis performed on the mitochondrial COI gene using RSA I enzyme according to a method adapted from Voroshilova *et al.* (2010). For details, total genomic DNA was extracted from mussel mantle using phenol/chloroform/isoamyl alcohol (25:24:1). The fragment of COI mitochondrial DNA containing the RSA I restriction site was amplified using

COI universal primers (5'-GGTCAACAAATCATAAAGATATTGG-3' and 5'-TAAACTTCAGGGTGACCAAAAAATCA-3') from Black *et al.* (1994). PCR amplifications were performed in a volume of 25  $\mu$ L containing 1 $\times$ Taq polymerase buffer, 2mM MgCl<sub>2</sub>, 80  $\mu$ M deoxynucleotides (dNTPs), 10 pmol of each primer, 0.5 units of TAQ Pol DNA polymerase (Euromedex, France) and about 10 ng of total genomic DNA. The following steps were applied for the PCR: denaturation at 94°C for 4 min, then 35 cycles at 94°C for 50 s, 56°C for 50 s, 72°C for 1 min, and a final step at 72°C for 10 min. Resulting fragments were hydrolyzed using RSA I restriction endonuclease (Euromedex, France), following the manufacturer's instructions. Restriction fragments were visualized under UV light after electrophoresis on a 2% agarose gel in Tris Borate EDTA 0.5% buffer, and staining with ethidium bromide.

Gills and digestive glands were homogenized by Precellys grinding in an ice-cold solution of 100 mM Tris, 60 mM saccharose, 60 mM mannitol, 3 mM EDTA, 1.5  $\mu$ M aprotinin, pH 7.5. The homogenates were centrifuged at 10,000 g, at 4 °C for 15 min. After centrifugation, supernatants containing the cytosolic fraction were collected. If analyses were done the same day, samples were kept at 4°C otherwise they were stored at -80°C. Protein concentration of each extract was measured with bovine serum albumin as a standard according to the Bradford method (Bradford, 1976).

Isoelectric focusing (IEF) was carried out in a Multiphor II equipment (Amersham Pharmacia Biotech, France) on gels with pH range 4.0-5.0 for a better resolution of Cu/Zn-SOD isoforms. The isoelectric point values (pI) were determined in regard with an isoelectric focusing calibration kit. Electrophoreses were performed at 60 mA at 4°C for 2 hours. Current was applied on electrode strips soaked either into an anode solution (1 M H<sub>3</sub>PO<sub>4</sub>) or into a cathode solution (1 M glycine). Polyacrylamide gel electrophoresis (PAGE) was performed with a 10% polyacrylamide running gel at 150 mV at 4°C for 2 hours. In both electrophoreses, purified bovine Cu/Zn-SOD was loaded as control.

Revelation of SOD activity was performed on gel immediately after the achievement of electrophoresis using the nitrobluetetrazolium (NBT) photochemical method of Beauchamp and Fridovitch (1971). The gels were soaked for 30 min in 2.5 mM NBT, in darkness, under gentle agitation and then incubated in a solution of 1 M phosphate buffer (pH 7.8) containing 0.05 mM riboflavin and 0.4% TEMED (v/v) for 30 min, once again in the dark and under constant agitation. Finally, the gels were exposed to light for 15 min in a 10 mM phosphate buffer (pH 7.8). Achromatic bands in the gels indicated SOD activity. The gels were scanned using GS-800 densitometer, BIO-RAD and subsequently analyzed by Image Lab (version 4.1, BIO-RAD) software.

Transcripts encoding Cu/Zn-SOD sequences were searched in annotated transcriptome databases. For details, databases were constructed using either gills or digestive gland of

quagga and zebra mussels found in the French rivers. Following translation using the SIB Bioinformatics Resource Portal (tool, Translate, <https://web.expasy.org/translate/>), the protein's local structural features were predicted from the primary sequence using NetSurfP-2.0 (<http://www.cbs.dtu.dk/cgi-bin/webface2.fcgi>, Klausen *et al.* 2019). For both species Cu/Zn SOD sequences, variant annotations such as Single Nuclear Polymorphisms (SNPs) and INsertion/DEletions events (INDELs) were issued from previously published transcriptomes (Péden *et al.*, 2019). As mentioned in the study, variant callings were realized with GATK following the best practices found on the GATK website (McKenna *et al.*, 2010). Phylogenetic trees were generated with the maximum composite likelihood (ML) algorithm in Mega v6 software (Tamura *et al.*, 2013). The Akaike Information Criterion was used to determine the best evolution model and JTT was retained for SOD. Statistical support for the nodes was estimated used by a bootstrap with 1,000 pseudoreplicates (Felsenstein, 1985). Genetic sequences of SOD proteins of other species used in these phylogenetic analyses were obtained from the GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>).

## Results and Discussion

### *Characterization of Cu/Zn SODs*

Zymograms of Cu/Zn-SOD activity show specific contrasted patterns that clearly discriminate quagga and zebra individuals (Figure 1A). Indeed, the quagga mussel is characterized by a major band at pI 4.95. The purified bovine erythrocyte Cu/Zn-SOD loaded as a control exhibited the same pI. By contrast, the zebra mussel shows multiband profiles at more acidic isoelectric points. In detail, two characteristic patterns were obtained, one with a major band at pI 4.6, the other with 3 major bands at pI 5.0, 4.8 and 4.6, suggesting higher genetic variability in the zebra than in the quagga mussel population (Figure 1B). Species identity of individuals sampled in France was confirmed morphologically and genetically (Figure 1C).

### *Comparison of Cu/Zn SOD profiles of geographically distant mussels*

Zymogram profiles of animals collected in France were compared to those of mussels from Canada (Figure 2). All quagga mussels, at any site, exhibit the same characteristic profile with a main Cu/Zn-SOD band at pI 4.95. For the zebra mussels, the two main profiles described above were found. Thus, it appears that differences in electrophoretic mobility of Cu/Zn-SOD isoforms are clearly species-related since they are conserved in geographically remote areas.

### *Cu/Zn-SOD profiles as a tool for species discrimination*

Because zymographic profiles are different between the two species, the question of using this characteristic to recognize the different species has arisen. Hemolymph constitutes a good candidate for this application because this tissue is minimally invasive and relatively easy to collect. The tests showed that 20  $\mu$ L of hemolymph was sufficient to visualize SOD and discriminate between species after IEF (data not shown). We also checked that samples prepared for other enzymatic tests with 10mM Tris buffer pH7 or 100 mM phosphate buffer pH7.5 allow the construction of zymographic profiles. Hence samples stored at minus 20°C could be used later for species identification by SOD zymogram analyses. Such a result is consistent with the very high stability of this enzyme. Finally an element that can limit the use of such a test is the availability of hardware allowing the IEF. Therefore, SOD profiles were tested on classic non denaturing gel electrophoresis PAGE. As shown in Figure 3, zebra and quagga mussels are clearly discriminated with a separate main band for each species.

#### *Phylogenetic analysis of Cu/Zn-SODs*

A phylogenetic tree was constructed based on the intracellular Cu/Zn-SOD amino acid sequence from the two dressenid species and other invertebrates and vertebrates (Figure 4). The tree shaped two main clades (even branch nodes show a low bootstrap value). Interestingly, the dressenid SODs are not clustered in the marine mussels and oyster group but are closely related to sequences of SOD from clam species (*Mactra quadrangularis* and *Meretrix meretrix*). Moreover, the percentages of identities between all those SODs sequences are close to 70% showing a high level of conservation as it has already been described in other studies relating to the sequencing of SOD in *Dreissena sp.* or in other bivalves (Wu *et al*, 2017; Anju *et al*, 2013; Gao *et al*, 2012; Ni *et al.*, 2007; Geret *et al*, 2004).

#### *Comparison of secondary structures*

Alignment of amino acid sequences were performed with the two cytosolic Cu/Zn-SOD sequences from *D. polymorpha* and *D. bugensis*. The sequences exhibited two conserved cysteine residues (Cys-58 and 146) needed for disulfide bond formation and highly conserved residues involved in copper and zinc fixation (Figure 5).

Amino acid comparison shows only 11 variations among the total of 153 amino acids signifying 93% of identity between the 2 sequences. Of the 11 variations, 5 amino acids share similar properties. For instance, the switch from glutamine to glutamic acid and that of cysteine to serine don't lead to major property changes. In such a way, it raised to 96% the similarity between the cytosolic Cu/Zn-SODs of the two species. At this point, both proteins were identical at a very high level, with only single amino acid modification points. However, these differences lead to minor changes in secondary sequence with the appearance of an  $\alpha$ -helix (position 75), a longer  $\beta$ -sheet (position 101) and longer  $\alpha$ -helix between position 130 and 135 in *Dreissena polymorpha* comparing *Dreissena rostriformis bugensis* (Figure 6).



Additionally, 40 SNPs and INDELS events were found on both Cu/Zn-SOD transcript sequences (data not shown) with respectively 10 and 14 events within *Dreissena polymorpha* and *Dreissena rostriformis bugensis* coding sequences (Figure 7). Among those events, 6 were silent for *Dreissena polymorpha* and 10 for *Dreissena rostriformis bugensis*. Remarkably, only one SNP impacts the secondary protein's sequence, and it is present in the *Dreissena polymorpha* sequence. Moreover, these slight differences could be accompanied by changes in the quaternary structure and may explain the differences in isoelectric point observed between the two species.

## **Conclusions**

The characterization of Cu/Zn-SODs has allowed us to identify significant differences between the isoforms of zebra and quagga mussels and propose their use as species enzymatic markers. At the nucleotide sequence level, the transcripts are also different, which allows the use of specific primer sets to measure Cu/Zn-SOD gene expression in these two species (Kerambrun *et al.*, 2018). Hence cytosolic Cu/Zn-SOD can be used as a discrimination marker for quagga and zebra mussels at the functional, structural and transcriptomic levels. Moreover, SOD is a stable enough enzyme to permit *Dreissena* species verification based on a posteriori analyses of homogenates prepared for other enzymatic assays. The study of Cu/Zn-SOD in *Dreissena* sp. revealed the existence of isoforms specific to zebra and quagga mussels and, incidentally, provided a tool for species discrimination using a simple enzymatic test. Finally, as superoxide dismutases are among the first defenses against ROS damages, differences in Cu/Zn-SOD isoforms in the two dreissenid species could contribute to a possible contrasting response in sensitivity to oxidative stress and metal pollutants as already observed (Louis *et al.*, 2019). Further studies should shed light on this issue.

## **Declaration of interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## **Data availability**

Data are available on request to the corresponding author (beatrice.rocher@univ-lehavre.fr).

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## Bibliography

Abreu, I.A., and Cabelli, D.E., 2010. Superoxide dismutases - a review of the metal-associated mechanistic variations. *Biochimica et Biophysica Acta. Proteins and Proteomics*. 1804, 263–274. <https://doi.org/10.1016/j.bbapap.2009.11.005>.

Anju, A., Jeswin, J., Thomas, P.C., Paulton, M.P., and Vijayan K.K., 2013. Molecular cloning, characterization and expression analysis of cytoplasmic Cu/Zn-superoxide dismutase (SOD) from pearl oyster *Pinctada fucata*. *Fish & Shellfish Immunology*. 34, 946-950. <http://dx.doi.org/10.1016/j.fsi.2012.12.024>.

Beauchamp, C., and Fridovich, I., 1971. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry*. 44, 276–287. [https://doi.org/10.1016/0003-2697\(71\)90370-8](https://doi.org/10.1016/0003-2697(71)90370-8).

Beggel, S., Cerwenka, A., Brandner, J., and Geist, J., 2015. Shell morphological versus genetic identification of quagga mussel (*Dreissena bugensis*) and zebra mussel (*Dreissena polymorpha*). *Aquatic Invasions*. 10, 93–99. <https://doi.org/10.3391/ai.2015.10.1.09>.

Binelli, A., Della Torre, C., Magni, S., and Parolini, M., 2015. Does zebra mussel (*Dreissena polymorpha*) represent the freshwater counterpart of *Mytilus* in ecotoxicological studies? A critical review. *Environmental Pollution*. 196, 386–403. <https://doi.org/10.1016/j.envpol.2014.10.023>.

Black, M., Folmer, O., Hoeh, W., Lutz, R., and Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*. 3, 294–299.

Bradford, M.M., 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry*. 72, 258–254.

Evariste, L., David, E., Cloutier, P.-L., Brousseau, P., Auffret, M., Desrosiers, M., Groleau, P., E., Fournier, M., Betoulle, S., 2018. Field biomonitoring using the zebra mussel *Dreissena polymorpha* and the quagga mussel *Dreissena bugensis* following immunotoxic responses. *Is*

there a need to separate the two species? *Environmental Pollution*. 238, 706-716.  
<https://doi.org/10.1016/j.envpol.20187.03.098>.

Faria, M., Ochoa, V., Blázquez, M., Juan, M.F.S., Lazzara, R., Lacorte, S., Soares, A.M.V.M., and Barata, C., 2014. Separating natural from anthropogenic causes of impairment in Zebra mussel (*Dreissena polymorpha*) populations living across a pollution gradient. *Aquatic Toxicology*. 152, 82–95. <https://doi.org/10.1016/j.aquatox.2014.03.020>.

Farkas, A., Ács, A., Vehovszky, Á., Falfusynska, H., Stoliar, O., Specziár, A., and Győri, J., 2017. Interspecies comparison of selected pollution biomarkers in dreissenid spp. inhabiting pristine and moderately polluted sites. *Science of The Total Environment*. 599–600, 760–770. <https://doi.org/10.1016/j.scitotenv.2017.05.033>.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*. 39, 783–791. <https://doi.org/10.1111/j.1558-5646.1985.tb00420.x>.

Gao X., He C., Liu H., Li H., Zhu D., Cai S., Xia Y., Wang Y., Yu Z., 2012. Intracellular Cu/Zn superoxide dismutase (Cu/Zn-SOD) from hard clam *Meretrix meretrix*: its cDNA cloning, mRNA expression and enzyme activity. *Molecular Biology Reports*. 39, 10713-10722.  
<https://doi:10.1007/s11033-012-1962-8>.

Geret, F., Manduzio, H., Company, R., Leboulenger, F., Bebianno, M.J., and Danger, J.M., 2004. Molecular cloning of superoxide dismutase (Cu/Zn-SOD) from aquatic molluscs. *Marine Environmental Research*. 58, 619–623.  
<https://doi.org/10.1016/j.marenvres.2004.03.052>.

Ginn, B.K., Bolton, R., Coulombe, D., Fleischaker, T., and Yerec, G., 2018. Quantifying a shift in benthic dominance from zebra (*Dreissena polymorpha*) to quagga (*Dreissena rostriformis bugensis*) mussels in a large, inland lake. *Journal of Great Lakes Research*. 44, 271–282. <https://doi.org/10.1016/j.jglr.2017.12.003>.

Hanana, H., Turcotte, P., André, C., Gagnon, C., and Gagné, F., 2017. Comparative study of the effects of gadolinium chloride and gadolinium – based magnetic resonance imaging contrast agent on freshwater mussel, *Dreissena polymorpha*. *Chemosphere*. 181, 197–207.  
<https://doi.org/10.1016/j.chemosphere.2017.04.073>.

Kerambrun, E., Rioult, D., Delahaut, L., Evariste, L., Pain-Devin, S., Auffret, M., Geffard, A., and David, E., 2016. Variations in gene expression levels in four European zebra mussel, *Dreissena polymorpha*, populations in relation to metal bioaccumulation: A field study. *Ecotoxicology and Environmental Safety*. 134, 53–63.  
<https://doi.org/10.1016/j.ecoenv.2018.02.051>.

Kerambrun, E., Delahaut, L., Geffard, A., and David, E., 2018. Differentiation of sympatric zebra and quagga mussels in ecotoxicological studies: A comparison of morphometric data, gene expression, and body metal concentrations. *Ecotoxicology and Environmental Safety*. 154, 321–328. <https://doi.org/10.1016/j.ecoenv.2016.08.018>.

Klausen, M.S., Jespersen, M.C., Nielsen, H., Jensen, K.K., Jurtz, V.I., Sønderby, C.K., Sommer, M.O.A., Winther, O., Nielsen, M., Petersen, B., *et al.*, 2019. NetSurfP-2.0: Improved prediction of protein structural features by integrated deep learning. *Proteins: Structure, Function, and Bioinformatics*. 87, 520–527. <https://doi.org/10.1002/prot.25674>.

Kwan, K.H.M., Chan, H.M., De Lafontaine, Y., 2003. Metal contamination in zebra mussels (*Dreissena Polymorpha*) along the St. Lawrence River. *Environmental Monitoring Assessment*. 88, 193–219. <https://doi.org/10.1023/A:1025517007605>.

Louis, F., Devin, S., Giambérini, L., Potet, M., David, E., and Pain-Devin, S., 2019. Energy allocation in two dreissenid species under metal stress. *Environmental Pollution*. 245, 889–897. <https://doi.org/10.1016/j.envpol.2018.11.079>.

Madeira, D., Mendonça, V., Madeira, C., Gaitero, C., Vinagre, C., and Diniz, M.S., 2019. Molecular assessment of wild populations in the marine realm: Importance of taxonomic, seasonal and habitat patterns in environmental monitoring. *Science of The Total Environment*. 654, 250–263. <https://doi.org/10.1016/j.scitotenv.2018.11.064>.

Manduzio, H., Monsinjon, T., Rocher, B., Leboulenger, F., and Galap, C., 2003. Characterization of an inducible isoform of the Cu/Zn superoxide dismutase in the blue mussel *Mytilus edulis*. *Aquatic Toxicology*. 64, 73–83. [https://doi.org/10.1016/S0166-445X\(03\)00026-2](https://doi.org/10.1016/S0166-445X(03)00026-2).

Marescaux, J., Molloy, D.P., Giamberini, L., Albrecht, C., and Van Doninck, K., 2012 First records of the quagga mussels, *Dreissena rostriformis bugensis* (Andrusov, 1897), in the Meuse River within France. *BioInvasions Records*. 1, 273–276. <https://dx.doi.org/10.3391/bir.2012.1.4.05>.

Marescaux, J., and Van Doninck, K., 2013. Using DNA barcoding to differentiate invasive *Dreissena* species (Mollusca, Bivalvia). *ZooKeys* 365, 235–244. <https://doi.org/10.3897/zookeys.365.5905>.

Marescaux, J., Boets, P., Lorquet, J., Sablon, R., Van Doninck, K., and Beisel, J.-N., 2015. Sympatric *Dreissena* species in the Meuse River: towards a dominance shift from zebra to quagga mussels. *Aquatic Invasions*. 10, 287–298. <https://doi.org/10.3391/ai.2015.10.3.04>.

Matthews, J., Schipper, A.M., Hendriks, A.J., Yen Le, T.T., bij de Vaate, A., van der Velde, G., and Leuven, R.S.E.W., 2015. A dominance shift from the zebra mussel to the invasive quagga mussel may alter the trophic transfer of metals. *Environmental Pollution*. 203, 183–190. <https://doi.org/10.1016/j.envpol.2015.03.032>.

McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., Garimella, K., Altshuler, D., Gabriel, S., Daly, M., *et al.*, 2010. The Genome Analysis Toolkit: A MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Research*. 20, 1297–1303. <https://doi.org/10.1101/gr.107524.110>.

Molloy, D.P., bij de Vaate, A., Wilke, T., and Giamberini, L., 2007. Discovery of *Dreissena rostriformis bugensis* (Andrusov 1897) in Western Europe. *Biol Invasions*. 9, 871–874.

Navarro, A., Faria, M., Barata, C., and Piña, B., 2011. Transcriptional response of stress genes to metal exposure in zebra mussel larvae and adults. *Environmental Pollution*. 159, 100–107. <https://doi.org/10.1007/s10530-006-9078-5>.

Ni, D., Song, L., Gao, Q., Wu, L., Yu, Y., Zhao, J., Qiu, L., Zhang, H., and Shi, F., 2007. The cDNA cloning and mRNA expression of cytoplasmic Cu, Zn superoxide dismutase (SOD) gene in scallop *Chlamys farreri*. *Fish & Shellfish Immunology*. 23, 1032–1042. <https://doi.org/10.1016/j.fsi.2007.04.008>.

Pain-Devin, S., Cossu-Leguille, C., Geffard, A., Giambérini, L., Jouenne, T., Minguez, L., Naudin, B., Parant, M., Rodius, F., Rousselle, P., *et al.*, 2014. Towards a better understanding of biomarker response in field survey: A case study in eight populations of zebra mussels. *Aquatic Toxicology*. 155, 52–61. <https://doi.org/10.1016/j.aquatox.2014.06.008>.

Péden, R., Poupin, P., Sohm, B., Flayac, J., Giambérini, L., Klopp, C., Louis, F., Pain-Devin, S., Potet, M., Serre, R.-F., *et al.*, 2019. Environmental transcriptomes of invasive *Dreissena*, a model species in ecotoxicology and invasion biology. *Sci Data*. 6, 234. <https://doi.org/10.1038/s41597-019-0252-x>.

Peyer, S., Hermanson, J.C., Lee, C.E., 2010. Developmental plasticity of shell morphology of quagga mussels from shallow and deep-water habitats of the Great Lakes. *J Exp. Biol.* 213, 2602–2609. <https://doi:10.1242/jeb.042549>.

Potet, M., Giambérini, L., Pain-Devin, S., Louis, F., Bertrand, C., and Devin, S., 2018. Differential tolerance to nickel between *Dreissena polymorpha* and *Dreissena rostriformis bugensis* populations. *Sci Rep.* 8, 700. <https://doi.org/10.1038/s41597-019-0252-x>.

Sturtevant, R.A., Mason, D.M., Rutherford, E.S., Elgin, A., Lower, E., and Martinez, F., 2019. Recent history of nonindigenous species in the Laurentian Great Lakes; An update to Mills et al., 1993 (25 years later). *Journal of Great Lakes Research*. 45, 1011–1035.

<https://doi.org/10.1016/j.jglr.2019.09.002>

Tamura, K., Stecher, G., Peterson, D., Filipinski, A., and Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution*. 30, 2725–2729. <https://doi.org/10.1093/molbev/mst197>.

Teubner, D., Wesslein, A.-K., Rønne, P.B., Veith, M., Frings, C., and Paulus, M. 2016. Is a visuo-haptic differentiation of zebra mussel and quagga mussel based on a single external morphometric shell character possible? *Aquatic Invasions*. 11, 145–154.

<https://doi.org/10.3391/ai.2016.11.2.04>.

Vaate, A., and Beisel, J.-N. 2011. Range expansion of the quagga mussel *Dreissena rostriformis bugensis* (Andrusov, 1897) in Western Europe: first observation from France. *Aquatic Invasions*. 6, S71–S74. <https://doi.org/10.3391/ai.2011.6.S1.016>.

Vernon, E.L., and Jha, A.N. 2019. Assessing relative sensitivity of marine and freshwater bivalves following exposure to copper: Application of classical and novel genotoxicological biomarkers. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 842, 60–71. <https://doi.org/10.1016/j.mrgentox.2019.01.008>.

Voroshilova, I.S., Artamonova, V.S., Makhrov, A.A., and Slyn'ko, Yu.V. 2010. Natural hybridization of two mussel species *Dreissena polymorpha* (Pallas, 1771) and *Dreissena bugensis* (Andrusov, 1897). *Biol Bull Russ Acad Sci*. 37, 542–547.

<https://doi.org/10.1134/S1062359010050158>.

Wu, J., Bao, M., Ge, D., Huo, L., Lv, Z., Chi, C., Liao, Z., and Liu, H. 2017. The expression of superoxide dismutase in *Mytilus coruscus* under various stressors. *Fish & Shellfish Immunology*. 70, 361–371. <https://doi.org/10.1016/j.fsi.2017.08.018>.

Zelko, I.N., Mariani, T.J., and Folz, R.J. 2002. Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. *Free Radical Biology and Medicine*. 33, 337–349.

[https://doi.org/10.1016/S0891-5849\(02\)00905-X](https://doi.org/10.1016/S0891-5849(02)00905-X).

## Legends

**Figure 1:** Characterization of Cu/Zn-SOD profiles by zymography.

A- Representative zymogram demonstrating distinct specific profiles of Cu/Zn-SOD activity between *Dreissena polymorpha* and *Dreissena rostriformis bugensis*. Achromatic bands were obtained after isoelectrofocusing and revelation of SOD activity by the nitrobluetetrazolium photochemical method of Beauchamp and Fridovich, 1971.

B- Profiles of Cu/Zn-SOD activity in *Dreissena* samples collected from different tissues. Zymograms show an identical pattern in gills and digestive gland for one individual. L1 to L6, animals correspond to zebra mussels, except L4, a quagga mussel. Cu/Zn-SOD isoforms were separated according to their pI using non-denaturing IEF.

C- Species identification by Restriction Fragment Length Polymorphism (RFLP) analysis. RFLP profiles of Rsa I- digested PCR products of cytochrome c oxidase subunit I gene amplified from *Dreissena polymorpha* and from *Dreissena rostriformis bugensis*.

**Figure 2:** Distribution of Cu/Zn-SOD profiles in *Dreissena* samples collected from different areas. Animals were sampled in France (4 rivers) and in Canada (Saint-Laurent River). Cu/Zn-SOD isoforms were separated according to their pI using non-denaturing IEF. Three patterns were obtained after NBT staining. For details, in the Moselle River a unique band corresponding to quagga mussels is observed. In the Vilaine and Meuse, two distinct patterns of Cu/Zn-SOD activity were observed corresponding only to zebra mussels. In the sites where the two species of *Dreissena* coexist (Oise and Saint-Laurent Rivers) the three patterns of SOD activity are found, similar to those obtained for the two species in the other sampled sites.

**Figure 3:** Zymogram of Cu/Zn-SOD allozymes after PAGE and NBT revelation from *Dreissena rostriformis bugensis* and from *Dreissena polymorpha*. Homogenates are separated in a 10% PAGE. Images of NBT staining are presented in inversed grey colors for a better visualization of the bands. Arrows indicated Cu/Zn-Sod activity.

**Figure 4:** Phylogenetic analysis of Cu/Zn-SODs. Phylogenetic trees were generated with the maximum composite likelihood algorithm in Mega v6 software (Tamura et al., 2013). Statistical support for the nodes was estimated used by a bootstrap with 1,000 pseudoreplicates (Felsenstein, 1985).

**Figure 5:** Amino acid sequence alignment of Cu/Zn-SODs. In line 1, Drpol, the primary sequence of *Dreissena polymorpha*; in line 2, Drbug, the primary sequence of *Dreissena rostriformis bugensis*. Amino acid differences were shaded; the residues required for copper

(His-47, -49, -64 and -120) and zinc (His-64, -72, -81 and Asp-84) fixation are in bold. The two cysteines predicted to form a disulfide bond were underlined.

**Figure 6:** Secondary structure representation of Cu/Zn-SODs. Models of protein's local structural features were predicted from the primary sequences of *Dreissena polymorpha* (up) and *Dreissena rostriformis bugensis* (down) using NetSurfP-2.0.

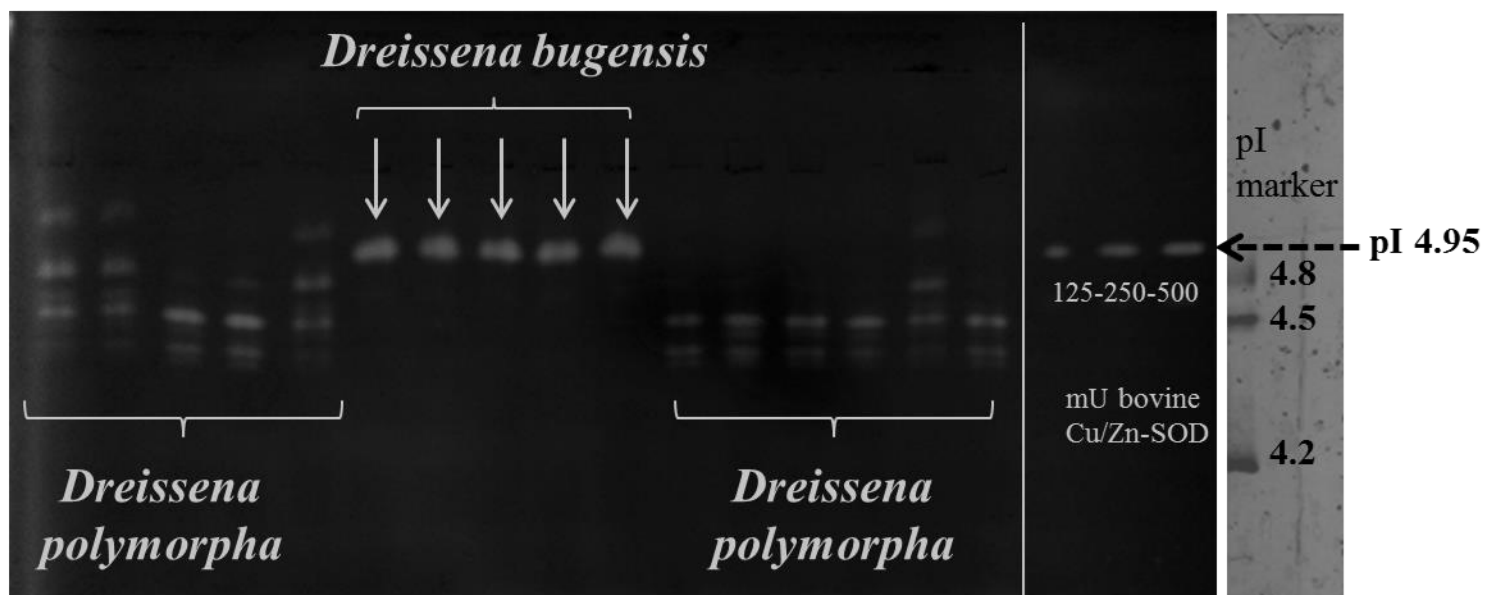
Three secondary sequence differences (black rectangles) were observed between both species, with Q → E in position 75 creating a small  $\alpha$ -helix in polymorpha, C → S in position 101 leading to a smaller  $\beta$ -sheet in quagga, and a smaller  $\alpha$ -helix in quagga between positions 130 and 135 with no sequence difference.

**Figure 7:** SNP positions in Cu/Zn-SOD nucleotide sequence for *Dreissena polymorpha* (Dp SODC) and *Dreissena rostriformis bugensis* (Db SODC). SNPs are highlighted by shaded residues. In the squares, SNPs that changes the amino acid sequence without changes at the secondary structure level. The double square corresponds to the only SNP that impacts the secondary sequence of the protein.

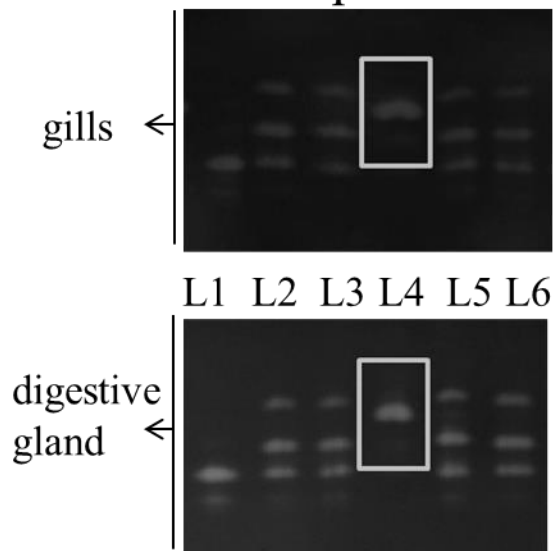


Fig. 1

A. Representative Zymogram



B. Tissue comparison



C. RFLP analysis

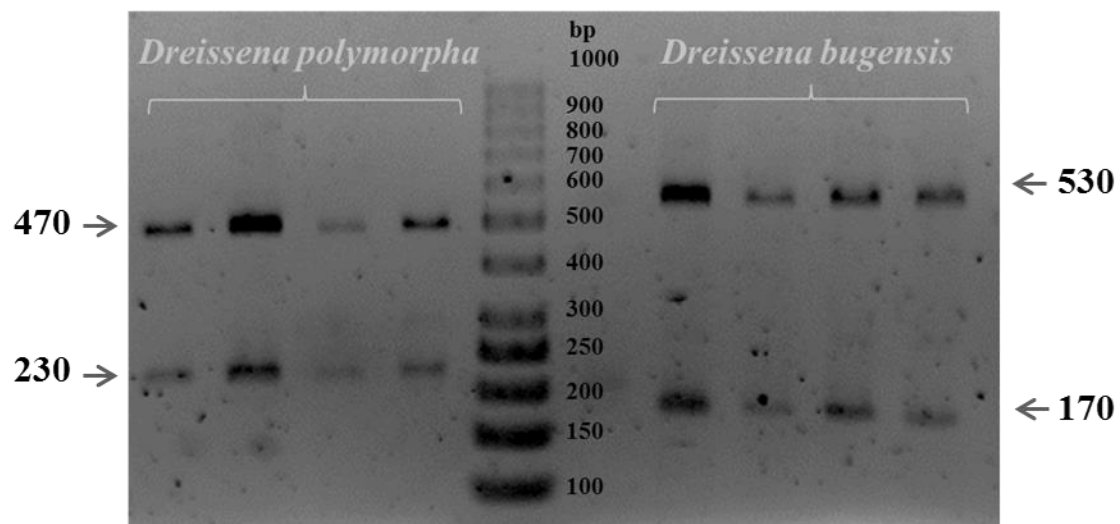


Fig. 2

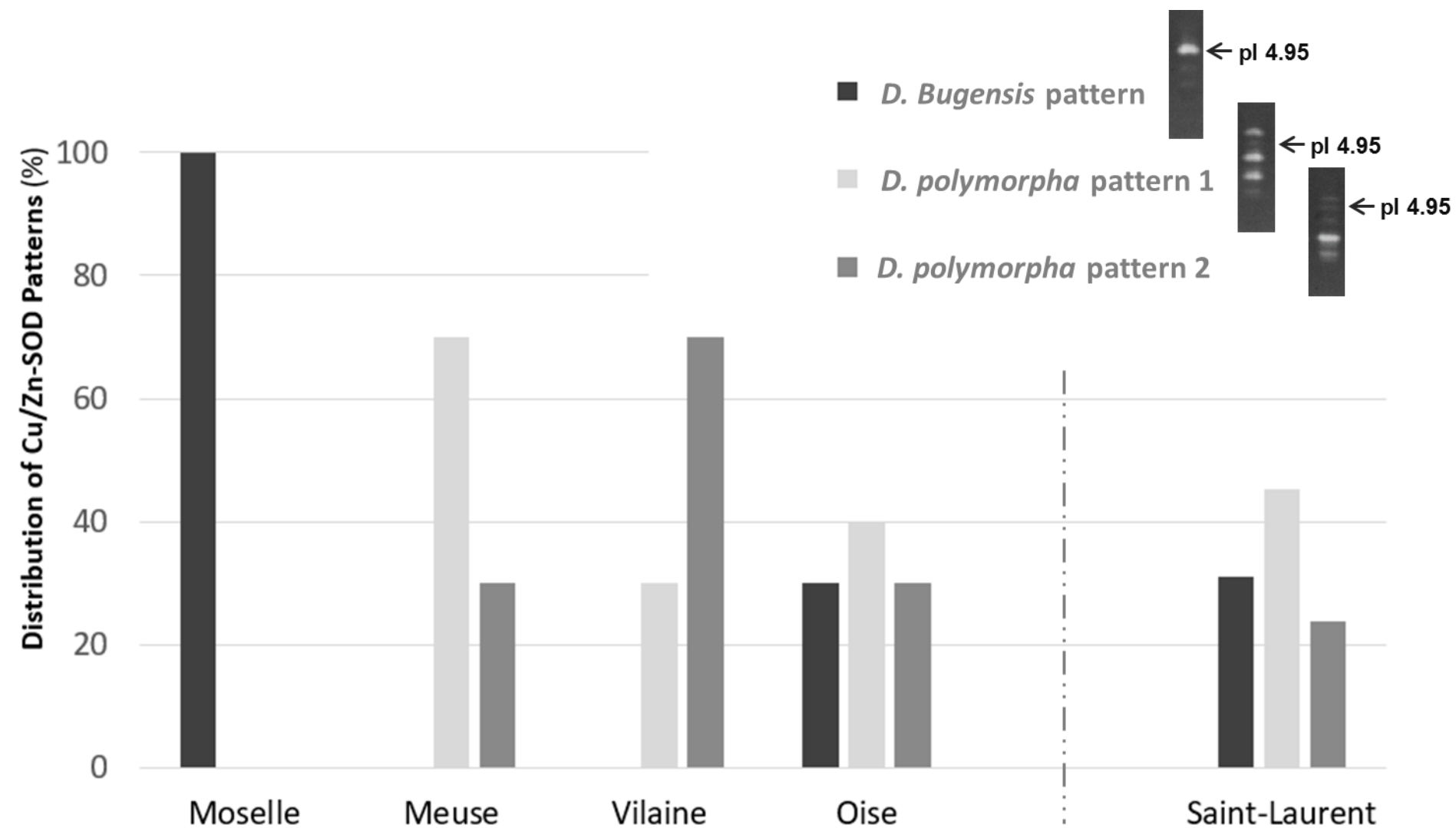


Fig. 3

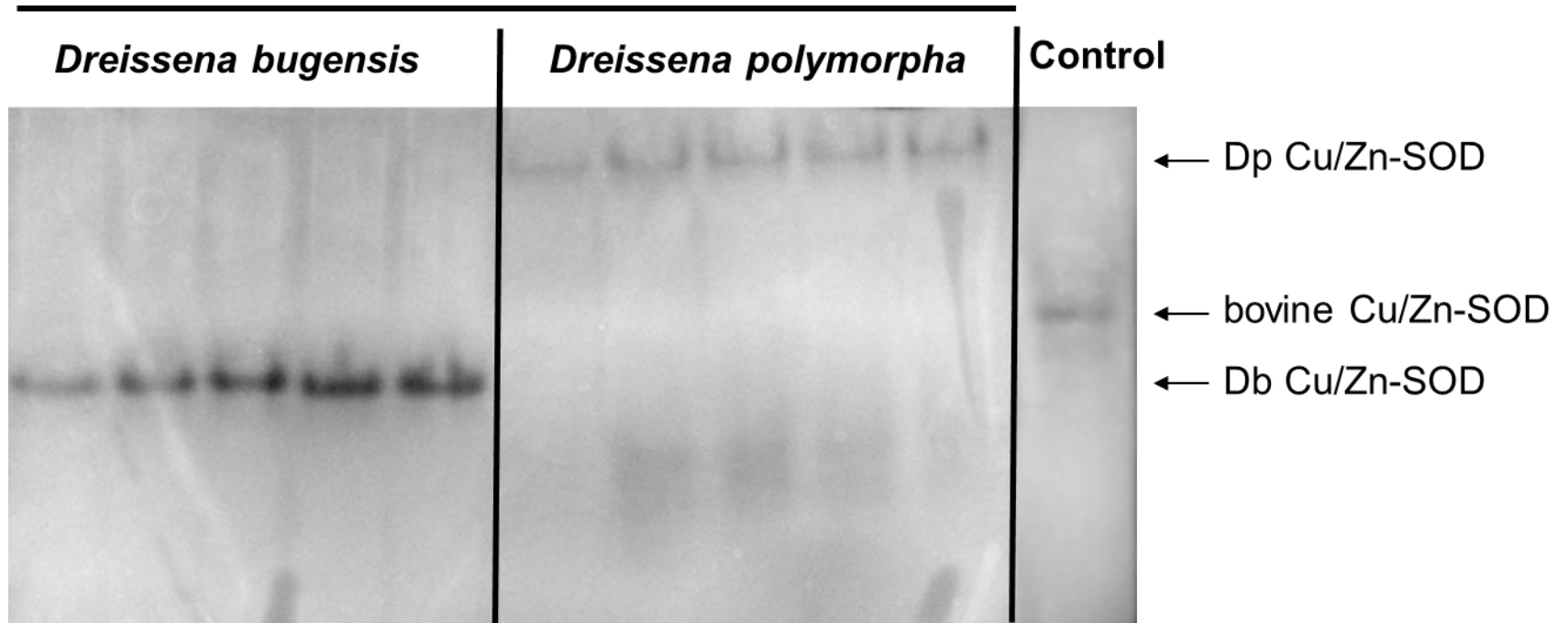


Fig. 4

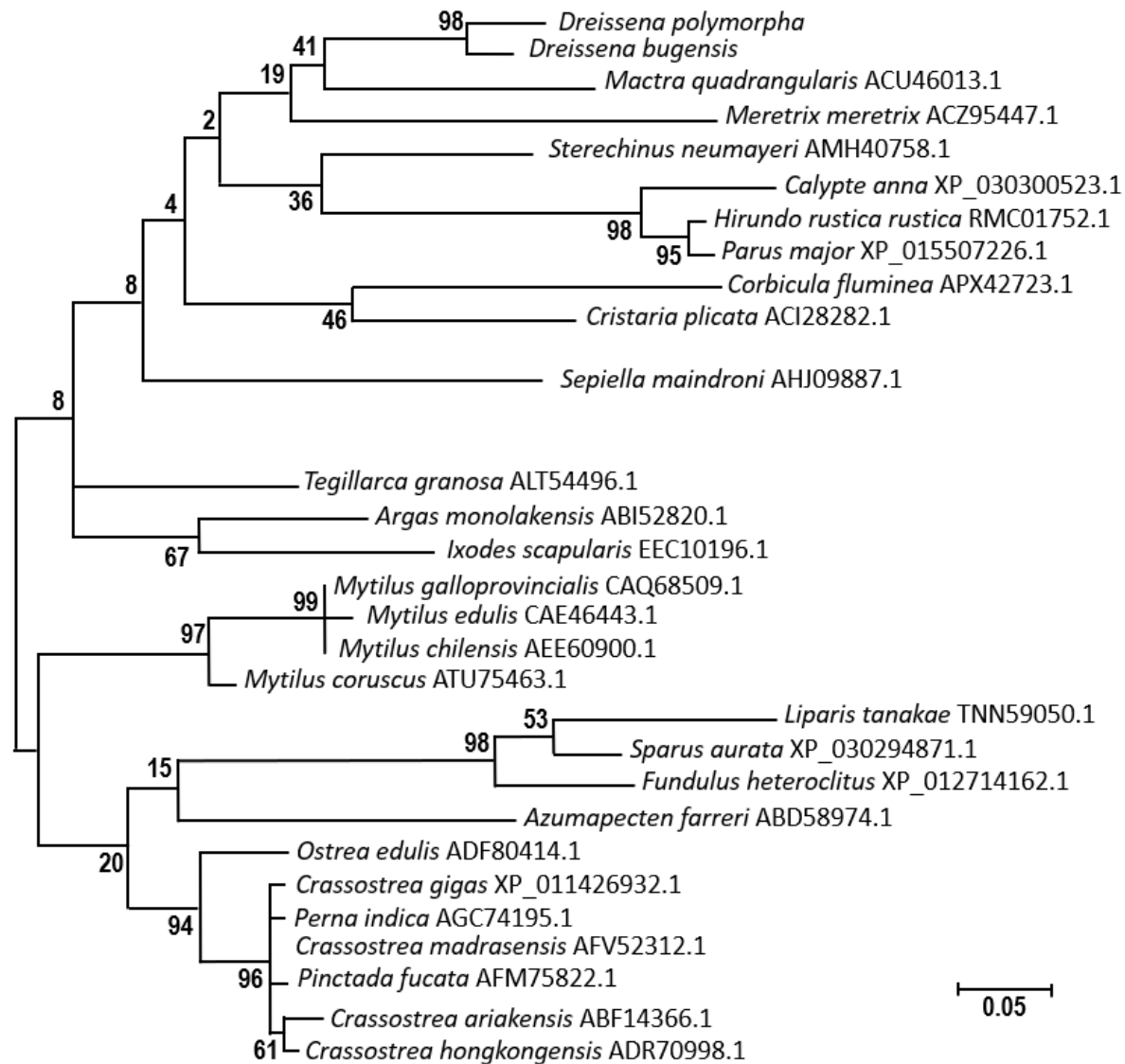


Fig. 5

Drpol> MPINACCVLK GDGVVTGVIH FTQEGPGAPV KLSGKITGLA EGQHGFHVHE<sup>Cu Cu</sup>

Drbug> MPINACCVLR GDGVVTGVIN FTQEAPGAPV KLTGKISGLA EGQHGFHVHE

1 10 20 30 40 50

Drpol> FGDNSNGCVS AGPHFNPSGK THGAPQDEER HAGDLGNVTA TAETCDVNIT

Drbug> FGDNSNGCVS AGPHFNPHGK THGAPEDEER HAGDLGNVKA TPETCDVNIT

60 70 80 90 100

Drpol> DCKITLTGPE SIIGRTMVIH<sup>Cu</sup> ADVDDLKGG HELSKTTGNA GARLACGVIG IAK

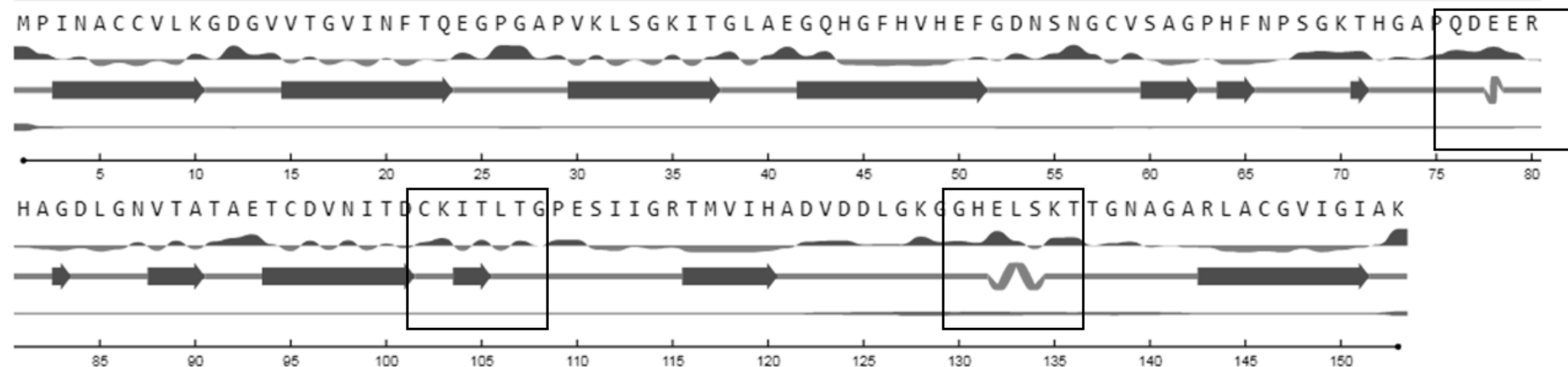
Drbug> DSKISLTGPE SIIGRTMVIH ADVDDLKGG HELSKTTGNA GARLACGVIG IAK

110 120 130 140 150

Fig. 6

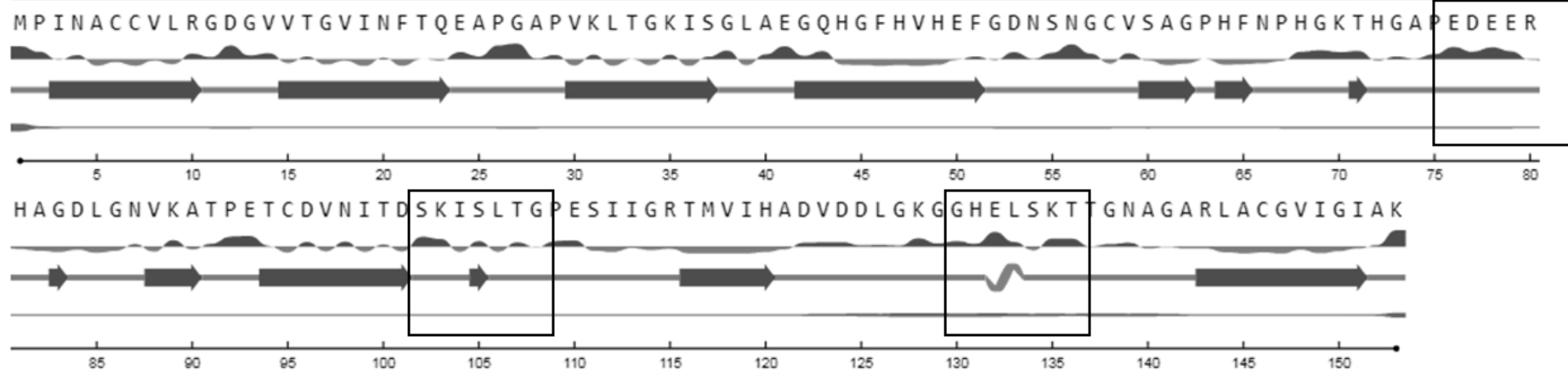
CuZnSOD ZM

Export CuZnSOD



CuZnSOD QM

Export CuZnSOD



<http://www.cbs.dtu.dk/cgi-bin/webface2.fcgi>: Relative surface access Red, accessible zone  
 Blue, buried sequence ; Secondary structures: Helix Sheet coil Disorder :   
 Thickness represents residue disorder probability.

Fig. 7

>Dp\_SODC

```
atg cca att aac gca tgc tgt gtt ctt aaa ggc gat ggt gtc gtt acg ggt gtt att/a a/cat  
M P I N A C C V L K G D G V V T G V I/I N/H  
ttt aca caa gag ggt cca ggt gc/aa cca/t gta aaa ttg agt ggc aaa att aca ggc tta gca  
F T Q E G P G A/E P/P V K L S G K I T G L A  
gag gga cag cat ggc ttc cat gtg cat gag ttt gga gac aac agc aat ggg tgt gta agt  
E G Q H G F H V H E F G D N S N G C V S  
gct gga ccc cac ttc aat cca agt g/tgc aaa act cac ggg gct cct cag/t gat gag gaa a/cga  
A G P H F N P S G/C K T H G A P Q/H D E E R/R  
cat gcc gga gat ctg gga aac gtg aca gca acg gca gag acg tgc/t gat gtg aac atc aca  
H A G D L G N V T A T A E T C/C D V N I T  
gac tgc aag att act ctg act ggc cca gaa tct atc att ggc cgt acc atg gtg att cat  
D C K I T L T G P E S I I G R T M V I H  
gct gat gta gac/t gac ctt ggg aag gga ggt cat gaa ctc tcc aag acg aca ggc aat gct  
A D V D/D D L G K G G H E L S K T T G N A  
gga gct cgt ctg gca tgt gga gta att ggc atc/t gcc aag tag  
G A R L A C G V I G I/I A K -
```

>Db\_SODC

```
atg cca att/a aac gca tgc tgt gtg ctt ag/aa ggc gat ggt gtc gtt acg ggt gtt att aat  
M P I/I N A C C V L R/K G D G V V T G V I N  
tt/g t aca caa gag gct cca ggt gca c/tca gta/c aag ttg act ggc aaa att agt ggc tta gca  
F/C T Q E A P G A P/S V/V K L T G K I S G L A  
gag gga cag cat ggc ttc cat gta cat gag ttt gga gac aac agc aat gga tgc gta agc  
E G Q H G F H V H E F G D N S N G C V S  
gct ggg ccc cac ttc aat cca/g c/aac ggc aaa acc cat ggc gct cct gaa gat gag gaa aga  
A G P H F N P/P H/N G K T H G A P E D E E R  
cat gcc gga gat ttg gga aat gtg/c aag gcg acc cca gag acg tgt gac gtg aac atc aca/t  
H A G D L G N V/V K A T P E T C D V N I T/T  
gac agc aag atc tcc ctc act ggc cca gaa tct atc att ggc cgt/a acc atg gtg att cat  
D S K I S L T G P E S I I G R/R T M V I H  
gct gac gta gac/t gac ctt ggg aag gga ggt cat gaa ctc tcc aag aca acc ggc aat gcc/t,g  
A D V D/D D L G K G G H E L S K T T G N A/A,A  
gga gct cgt ctg/t gca tgt gga gtc atc ggc atc/t gcc aag tag  
G A R L/L A C G V I G I/I A K -
```