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PIONEER PROJECTS

LATTECO - LATERAL GENE TRANSFER AS A RADICALLY NOVEL MECHANISM FOR ECOLOGICAL ADAPTATIONS

CONTRACT - BR/314/PI/LATTECO

FINAL REPORT

19/12/2019

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Published in 2019 by the Belgian Science Policy Office
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Schön, I. Lateral gene transfer as a radically novel mechanism for ecological adaptations. Final Report. Brussels : Belgian Science Policy Office 2019 – 36 p. (BRAIN-be - (Belgian Research Action through Interdisciplinary Networks))

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SUMMARY

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While adaptation in multicellular organisms usually requires recombination and genetic exchange as part of sexual reproduction, bacteria can exchange genes laterally among each other without sex. This can, for example, cause fast antibiotic resistance. Novel studies using whole genome data have shown that successful lateral or horizontal gene transfer (HGT) is not limited to the bacterial world, but also occurs in metazoans, higher organisms and can be quite frequent. For example, the genomes of certain plant parasitic nematodes contain up to 3% of foreign genes, the genomes of bdelloid rotifers up to 8 %. The ostracod *Darwinula stevensoni* has several traits that make it a good candidate for frequent HGT. We hypothesize that HGT has enabled this species to survive in very different aquatic environments in spite of the fact that sex is absent or very rare. Life history experiments have shown that *D. stevensoni* can survive much wider ranges of temperature and salinity than other non-marine ostracods. During this pioneer project, we have investigated HGT with high throughput sequencing at three different levels: RNA, genomic DNA and the ostracods' bacterial communities, which could have acted as donors for HGT.

Objectives

1. To conduct metagenomic studies on *D. stevensoni* with different life histories to compare the diversity of associated bacterial faunas in these ostracods, because these bacteria could have acted as potential donors for HGT.
2. To culture these ostracods for assessing the ecological effects of their bacteria.
3. To obtain large scale transcriptome (RNA) data from *D. stevensoni* and identify candidate genes for HGT within these transcriptomes, using automated bioinformatic pipelines.
4. To localize foreign genes in an existing genomic cosmid library of *D. stevensoni* and sequence those in their genomic DNA background (thus eliminating contaminations).
5. To apply bioinformatics analyses on the obtained sequence data from objectives 3 & 4 to confirm candidate cases of HGT and to predict the putative functions of these genes.

Conclusions

LATTECO successfully characterised the microbiome of the ostracod species *D. stevensoni*. We found that ostracods harbour a specific bacterial fauna that is clearly different to bacteria from their aquatic environment and that ostracod microbiomes vary between different geographic populations of *D. stevensoni*. Proteobacteria dominated the microbiome but hundreds of different operational taxonomic units of bacteria (resembling species) were found, which could have acted as potential donors for HGT. When ostracods were cultured with antibiotic, they only survived at moderate antibiotic concentrations. This result indicates that certain components of the microbiome are essential for the survival of *D. stevensoni*. We also found that the endosymbiotic bacteria *Cardinium* is present in all three superfamilies of non-marine ostracods including the Darwinulidae and are evolutionary different from all other *Cardinium* bacteria known to date (Schön et al. 2018). There is a significant link between asexuality and the presence of *Cardinium* in non-marine ostracods (Schön & Martens, in press), which deserves to be further studied to verify if these bacteria cause asexuality in ostracods as they do in other terrestrial arthropods (Hunter et al. 2003; Ros & Breeuwer 2009; Duron et al. 2008 a & b).
By applying three different approaches, we identified about 200 genes in the transcriptome of *D. stevensoni* that could potentially have originated by HGT. This number is in the same order of magnitude as the 253 genes with HGT origin in a collembola genome (Faddeeva-Vakhrusheva et al. 2017). One third of the potential HGT genes of *D. stevensoni* had a bacterial origin, one third came from eukaryotes and the evolutionary history of the remaining third was too complex to be derived. Most genes with potential HGT origin of *D. stevensoni* coded for molecular

functions and could thus be of high relevance for the ecology and adaptations of this ostracod, especially in the absence of sex and meiosis. A final confirmation of HGT with additional methods has become essential while the LATTECO project was running because of a flawed study on tardigrades. More than 30% of HGT in a tardigrade genome were initially reported by Boothby et al. (2015); as a second study Koutsovoulos et al. (2016) showed later that the majority of these genes were due to bacterial contamination and the correct frequency of HGT in this tardigrade was only around 2%. Verifying potential HGT genes from the *D. stevensoni* transcriptome by sequencing the DNA of the flanking regions of these genes in DNA has thus become essential to be able to publish our results. Unfortunately, such verification has turned out to be very difficult for two reasons: The only draft genome from Illumina sequencing that is currently available for *D. stevensoni* is highly fragmented. Most genes with potential HGT origin could not be located in this genome but this does not necessarily mean that they are absent as such; they might only be absent in the fragmented genome. Sequencing parts of a low coverage genomic DNA library that is available for *D. stevensoni* and that was foreseen as alternative for a high-quality genome revealed that the library contains an unexpectedly large amount of repetitive DNA and transposons. This allowed us to study the transposon landscape of this ostracod in more detail (Schön et al. submitted). However, these features make it very difficult to localise genes with potential HGT in individual clones of the library and even more difficult to sequence these genes and their flanking regions. These problems could also not be solved with repeated screening or by using PacBio long read techniques. The project could thus provide an extensive list of potential HGT genes, but their final validation will need to wait until high quality genome data of *D. stevensoni* will become available in the future.

Keywords

Adaptation, changing environments, gene transfer, bacteria, sex

SAMENVATTING

Context

Terwijl adaptaties in multicellulaire organismen meestal recombinatie en genetische uitwisseling als onderdeel van de seksuele voortplanting vereisen, kunnen bacteriën zijdelings genen met elkaar uitwisselen zonder seks. Dit kan bijvoorbeeld leiden tot snelle antibioticaresistentie. Nieuwe studies die gebruik maken van volledige genoomgegevens hebben aangetoond dat succesvolle laterale of horizontale genoverdracht (HGT) zich niet beperkt tot de bacteriële wereld, maar ook voorkomt in metazoans (hogere organismen) en vrij frequent kan zijn. Zo bevatten de genomen van bepaalde plantenparasitaire nematoden tot 3% vreemde genen, de genomen van bdelloïde rotiferen tot 8%. Het mosselkreeftje Darwinula stevensoni heeft verschillende eigenschappen die het een goede kandidaat maken voor frequente HGT. We veronderstellen dat HGT deze soort in staat heeft gesteld om te overleven in zeer verschillende aquatische milieus, ondanks het feit dat seks afwezig of zeer zeldzaam is. Experimenten hebben aangetoond dat D. stevensoni veel grotere temperatuur- en zoutbereiken kan overleven dan andere niet-mariene ostracoden. Tijdens dit pioniersproject hebben we HGT onderzocht met nieuwe sequentietechnieken op drie verschillende niveaus van de ostracoden: RNA, genomische DNA en de bacteriële gemeenschappen (mogelijke donor voor HGT).

Doelstellingen

1. Het uitvoeren van metagenomische studies van D. stevensoni van verschillende populaties om de diversiteit van de geassocieerde bacteriële faunas in deze ostracoden te vergelijken. Deze bacteriën zouden als potentiële donoren van HGT kunnen hebben gefungeerd.
2. De ostracoden kweken voor de beoordeling van de ecologische effecten van hun bacteriën.
3. Het verkrijgen van grootschalige transcriptoomgegevens (RNA) van D. stevensoni en het identificeren van kandidaatgenen voor HGT binnen deze transcriptoomen, gebruikmakend van geautomatiseerde bio-informaticapijpleidingen.
4. Vreemde genen lokaliseren in een bestaande genomische cosmid bibliotheek van D. stevensoni en het bepalen van de flankerende regio's van deze genen in hun genomische DNA-achtergrond (om contaminaties als oorzaak van valse HGT te elimieren).
5. Het toepassen van bio-informatica-analyses op de verkregen sequentiegegevens van doelstellingen 3 & 4 om kandidaat-gevallen van HGT te bevestigen en de vermeende functies van deze genen te voorspellen.

Besluiten

LATTECO heeft met succes het microbioom van de ostracode D. stevensoni gekarakteriseerd. We hebben vastgesteld dat ostracoden een specifieke bacteriële fauna herbergen die duidelijk verschilt van bacteriën uit hun aquatisch milieu en dat de microbomen van ostracoden variëren tussen de verschillende geografische populaties van D. stevensoni. Alfa proteobacteriën domineerden het microbioom, maar er werden honderden verschillende operationele taxonomische eenheden van bacteriën (die lijken op soorten) gevonden, die als potentiële donoren voor HGT hadden kunnen fungeren. Toen ostracoden werden gekweekt met antibiotica, overleefden ze alleen bij gematigde antibioticaconcentraties. Dit resultaat geeft aan dat bepaalde componenten van het microbioom essentieel zijn voor het overleven van D. stevensoni. We vonden ook dat de endosymbiotische bacterie Cardinium aanwezig is in alle drie de superfamilies van niet-mariene ostracoden, waaronder de Darwinulidae, en dat ze een andere oorsprong hebben dan alle andere bekende Cardinium bacteriën (Schön et al. 2018). Er is een significant verband tussen aseksualiteit en de aanwezigheid van Cardinium in niet-mariene ostracoden (Schön & Martens, in press), dat verder bestudeerd moet worden om na te

gaan of deze bacteriën aseksualiteit veroorzaken in ostracoden zoals ze dat ook doen in andere terrestrische arthropoden (Hunter et al. 2003; Ros & Breeuwer 2009; Duron et al. 2008 a & b). Door drie verschillende benaderingen toe te passen, identificeerden we ongeveer 200 genen in het transcriptoom van D. stevensoni die mogelijk door HGT zouden kunnen zijn ontstaan. Dit aantal is in dezelfde orde van grootte als de 253 genen met HGT oorsprong in een collembola genoom (Faddeeva-Vakhrusheva et al. 2017). Één derde van de potentiële HGT genen van D. stevensoni had een bacteriële oorsprong, één derde kwam van eukaryoten en de evolutionaire geschiedenis van het resterende derde had een te complexe evolutionaire geschiedenis. De meeste genen met potentiële HGT oorsprong van D. stevensoni coderen voor moleculaire functies en zouden dus van groot belang kunnen zijn voor de ecologie en aanpassingen van deze ostracode, vooral bij gebrek aan seks en meiose. Een laatste bevestiging van HGT met extra methoden werd noodzakelijk gedurende het LATTECO-project, toen de ware resultaten van een gebrekkige studie naar tardigrases aan het licht kwamen. Meer dan 30% van de HGT in een tardigrade genoom werd aanvankelijk gerapporteerd door Boothby et al. (2015); een tweede studie (Koutsovoulos et al. 2016) toonde later aan dat het merendeel van deze genen te wijten was aan bacteriële contaminatie en dat de correcte frequentie van HGT in deze tardigrade slechts ongeveer 2% bedroeg. Het verifiëren van potentiële HGT genen van het D. stevensoni transcriptoom door de flankerende regio's van deze genen te sequencen in het DNA is dus essentieel om onze resultaten te kunnen publiceren. Helaas is een dergelijke verificatie om twee redenen zeer moeilijk gebleken: Ten eerste is het enige draftgenoom van Illumina dat momenteel beschikbaar is voor D. stevensoni zeer gefragmenteerd. Ten tweede konden de meeste genen met potentiële HGT-oorsprong niet in dit genoom worden gelokaliseerd. Dit betekent niet per se dat ze afwezig zijn; ze zijn misschien alleen maar afwezig in het gefragmenteerde genoom. Het sequeneren van delen van een low coverage genomische DNA-bibliotheek die beschikbaar is voor D. stevensoni en die was voorzien als alternatief voor een genoom van hoge kwaliteit, toonden aan dat de bibliotheek onverwacht veel herhalende DNA en transposons bevat. Dit stelde ons in staat om het transposonlandschap van deze ostracode nader te bestuderen (Schön et al. ingediend). Deze kenmerken maken het echter zeer moeilijk om genen met potentiële HGT te lokaliseren in individuele klonen van de bibliotheek en nog moeilijker om deze genen en hun flankerende regio's te sequeneren. Deze problemen konden ook niet worden opgelost met herhaalde screening of met behulp van PacBio technieken. Het project had dus een uitgebreide lijst van potentiële HGT genen kunnen opleveren, maar de uiteindelijke validatie ervan zal moeten wachten tot er in de toekomst high quality genoomgegevens van D. stevensoni beschikbaar zullen zijn.

Trefwoorden

Adaptatie, veranderende omgevingen, genenoverdracht, bacteriën, seks

RESUME

Contexte

Alors que l'adaptation chez les organismes multicellulaires nécessite habituellement une recombinaison et un échange génétique dans le cadre de la reproduction sexuée, les bactéries peuvent échanger des gènes latéralement entre elles sans reproduction sexuée. Cela peut, par exemple, provoquer une résistance rapide aux antibiotiques. De nouvelles études utilisant des données génomiques complètes ont montré que le transfert latéral ou horizontal de gènes (HGT) n'est pas limité au monde bactérien, mais se produit aussi chez les métazoaires, les organismes supérieurs et peut être assez fréquent. Par exemple, les génomes de certains nématodes parasites des plantes contiennent jusqu'à 3% de gènes étrangers, les génomes de rotifères bdelloïdes jusqu'à 8%. L'ostracode Darwinula stevensoni présente plusieurs caractéristiques qui en font un bon candidat pour le HGT fréquent. Nous émettons l'hypothèse que le HGT a permis à cette espèce de survivre dans des milieux aquatiques très différents malgré l'absence ou la rareté de reproduction sexuée. Des expériences sur le cycle biologique ont montré que D. stevensoni peut survivre dans des plages de température et de salinité beaucoup plus larges que d'autres ostracodes non marins. Au cours de ce projet pionnier, nous avons étudié le HGT avec un séquençage à haut débit à trois niveaux différents : L'ARN, l'ADN génomique et les communautés bactériennes des ostracodes, qui auraient pu servir de donneurs de HGT.

Objectifs

1. Mener des études métagénomiques sur D. stevensoni avec différents cycles biologiques pour comparer la diversité des faunes bactériennes associées chez ces ostracodes, car ces bactéries pourraient avoir été des donneurs potentiels de HGT.
2. Elever ces ostracodes pour évaluer les effets écologiques de leurs bactéries.
3. Obtenir des données de transcriptome (ARN) de D. stevensoni et identifier des gènes candidats pour le HGT dans ces transcriptomes, en utilisant des pipelines bioinformatiques automatisés.
4. Localiser les gènes étrangers dans une banque de cosmides génomiques existants de D. stevensoni et séquencer leur ADN génomique de base (éliminer la possibilité que le HGT soit effectivement dû à une contamination bactérienne).
5. Appliquer des analyses bioinformatiques sur les données de séquences obtenues à partir des objectifs 3 et 4 pour confirmer des cas poteniels de HGT et prédire les fonctions putatives de ces gènes.

Conclusions

LATTECO a caractérisé avec succès le microbiome de l'espèce d'ostracodes D. stevensoni. Nous avons constaté que les ostracodes abritent une faune bactérienne spécifique qui est clairement différente des bactéries de leur milieu aquatique et que les microbiomes des ostracodes varient entre les différentes populations géographiques de D. stevensoni. Les protéobactéries dominaient le microbiome, mais des centaines d'unités taxonomiques opérationnelles différentes de bactéries (ressemblant à des espèces) ont été trouvées, qui auraient pu servir de donneurs potentiels de HGT. Lorsque les ostracodes ont été élevés avec des antibiotiques, ils n'ont survécu qu'à des concentrations modérées d'antibiotiques. Ce résultat indique que certains composants du microbiome sont essentiels à la survie de D. stevensoni. Nous avons également constaté que la bactérie endosymbiotique Cardinium est présente dans les trois superfamilles d'ostracodes non marins, y compris les Darwinulidae, et qu'elle évolue différemment de toutes les autres bactéries Cardinium connues à ce jour (Schön et al. 2019). Nous avons également trouvé un lien significatif entre l'asexualité et la présence de Cardinium dans les ostracodes non marins (Schön & Martens), qui mérite d'être étudié davantage pour

vérifier si ces bactéries causent l'asexualité des ostracodes comme elles le font dans d'autres arthropodes terrestres (Hunter et al. 2003; Ros & Breeuwer 2009; Duron et al. 2008 a & b).
En appliquant trois approches différentes, nous avons identifié environ 200 gènes dans le transcriptome de D. stevensoni qui pourraient avoir pour origine un HGT. Ce nombre est du même ordre de grandeur que les 253 gènes d'un génome de collemboles ayant pour d'origine un HGT (Faddeeva-Vakhrusheva et al. 2017). Un tiers des gènes vHGT provenant potentiellement d'un HGT étaient d'origine bactérienne, un tiers provenait d'eucaryotes et l'histoire évolutive du tiers restant était trop complexe pour être dérivée. La plupart des gènes de D. stevensoni ayant potentiellement pour origine un HGT codaient pour des fonctions moléculaires et pourraient donc être d'une grande importance pour l'écologie et les adaptations de cet ostracode, surtout en l'absence de reproduction sexuée et de méiose. Une confirmation finale de HGT avec des méthodes supplémentaires est devenue essentielle alors que le projet LATTECO était en cours en raison d'une étude imparfaite sur les tardigrades. Plus de 30% de HGT dans un génome tardigrade ont été initialement signalés par Boothby et al. (2015); comme une deuxième étude a montré plus tard que la majorité de ces gènes était du à une contamination bactérienne et la fréquence correcte de HGT dans ce tardigrade était seulement environ 2% (Koutsovoulos et al. 2016). La vérification des gènes HGT potentiels du transcriptome de D. stevensoni par séquençage de l'ADN des régions flanquantes de ces gènes est donc devenue essentielle pour pouvoir publier nos résultats. Malheureusement, cette vérification s'est avérée très difficile pour deux raisons : Le seul génome issu du séquençage Illumina actuellement disponible pour D. stevensoni est très fragmenté. La plupart des gènes ayant une origine potentielle de HGT n'ont pas pu être localisés dans ce génome, mais cela ne signifie pas nécessairement qu'ils sont absents en tant que tels ; ils pourraient seulement être absents dans le génome fragmenté. Le séquençage de parties d'une banque d'ADN génomique à faible couverture disponible pour D. stevensoni, prévue comme alternative pour un génome de haute qualité, a révélé que la banque contient une quantité étonnamment importante d'ADN répétitif et de transposons. Cela nous a permis d'étudier plus en détail le paysage de transposons de cet ostracode (Schön et al. soumis). Cependant, ces caractéristiques rendent très difficile la localisation de gènes présentant un potentiel HGT dans les clones individuels de la banque et encore plus difficile le séquençage de ces gènes et de leurs régions adjacentes. Ces problèmes ne pouvaient pas non plus être résolus par un dépistage répété ou par l'utilisation des techniques de séquence longue PacBio. Le projet pourrait donc fournir une longue liste de gènes HGT potentiels, mais leur validation finale devra attendre que des données de haute qualité sur le génome de *D. stevensoni* soient disponibles dans l'avenir.

Mots-clés

Adaptation, environnements changeants, transfert de gènes, bactéries, reproduction sexuée

1. INTRODUCTION

Novel gene combinations provide the evolutionary arena on which natural selection will act. In most multicellular animals (metazoans), this is achieved by meiotic recombination as part of sexual reproduction. Bacteria do not have germ lines and thus do not have sex in a strict sense. Nevertheless, they can exchange genetic material horizontally through lateral or horizontal gene transfer (HGT), even between distantly related species. This is an efficient mechanism as is illustrated by the fast adaptation of bacterial pathogens which can quickly become resistant against antibiotics or can acquire foreign virulent factors, and thus of high relevance for society. Lerminiaux & Cameron (2019) reviewed all known cases of HGT in clinical environments and showed that 11 out of 12 priority antibiotica-resistant pathogens are known or suspected to be transformable and subjected to HGT. Besides clinical applications, HGT might also be highly relevant for microbiomes and their contribution the health of the host. A new study on *E. coli* in the gut microbiome of mice recently showed that HGT is more important than mutations to acquire genetic variability. HGT turned out to be especially important for metabolic functioning of these bacteria and provided important fitness advantages (Frazão et al. 2019).

Evolutionary patterns in the tree of life have long been regarded to be fundamentally different between bacteria and metazoans, with HGT being essentially limited to the prokaryotic world. The only widely accepted cases of HGT between bacteria and (ancestral) metazoans occurred during the evolution of the eukaryotic cell when bacterial endosymbionts turned into cell organelles and this was accompanied by massive gene transfers from bacterial to nuclear host genomes (Timmis et al. 2004).

With the onset of sequencing studies, bacterial genes had been found in the genomes of metazoans; but these were not confirmed by later studies (Kidwell 1993; Syvanen 1994; Stanhope et al. 2001). Contamination was the most likely explanation for these puzzling results. Even authentic examples for HGT dealt only with one or two bacterial genes without obvious advantage for the hosts (Woolfit et al. 2008; Klasson et al. 2009) or could not be ultimately proven (Kiko 2010). Consequently, HGT was considered to be insignificant for eukaryotic evolution in general.

The novel, high-throughput techniques for generating DNA sequences have meanwhile provided whole-genome data from various metazoans organisms. In evolutionary biology, few topics have received more attention, more than 11,000 papers have been published on this topic since 1985 (Ku & Martin 2016). In the last years, HGT between kingdoms has been demonstrated in more than 20 groups of multi-cellular eukaryotic species, including algae, insects, and few other invertebrates like nematodes, rotifers, sea quirts (Schönknecht et al. 2013a), beetles (McKenna et al. 2019), a collembola (Faddeeva-Vakhrusheva et al. 2017) and one shrimp species (Yuan et al. 2013). With the exception of one study on tardigrada (Boothby et al. 2015; Koutsovoulos et al. 2016), contamination is an unlikely explanation for these examples as foreign genes contain spliceosomal introns (unknown in real bacteria), are effectively expressed in the eukaryotic hosts (Flot et al. 2013) and their genomic position is flanked by "true" host genes (Danchin et al. 2010). Schönknecht et al. (2013a) consider these examples as "the tip of the iceberg" and conclude that this kind of horizontal or lateral gene exchange is not only far more common than previously thought, but also of very high relevance for eukaryotic evolution, including

animals although the question if HGT is continuous, remains controversial (Ku & Martin 2016; Daninch 2016). Besides antibiotica resistance, HGT has also other, high relevance for medicine and human health as has been proposed by e. g. Robinson et al. (2013) and Riley et al. (2013). They showed that the rate of HGT with bacterial origin was higher in human cancer samples compared to controls.

Case studies show that bacterial genes of HGT have provided novel adaptations to extreme environments, such as ice-binding proteins in diatoms and algae living in sea ice (Raymond & Kim 2012) or thermophily in a red algae (Schönknecht et al. 2013b). Other examples of foreign genes provide more general evolutionary advantages. The pea aphid *Acyrthosiphon pisum* has acquired a gene for the biosynthesis of red carotinoids, important to determine its colour, most likely from fungi (Moran & Jarvik 2010). Even if more preyed upon, bright red aphids have lower rates of parasites (Losey et al. 1997). In the silkworm, newly and horizontally acquired genes enhance disease-resistance and toxin degradation (Gilbert & Cordaux 2013) while the Pacific white shrimp, *Litopenaeus vannamei*, has acquired 14 genes via HGT being related to energy metabolism and defence (Yuan et al. 2013), and HGT of plant cell wall-degrading enzymes from fungi and bacteria has been a key factor promoting evolution of herbivorous beetles. However, the frequency of foreign genes in most host genomes ranges between 0.001 and 0.01%, which is very low (Schönknecht et al. 2013a). In two noticeable exceptions, foreign genes comprise several % of host genomes and originate from bacteria, fungi or plants. The root knot nematode *Meloidogyne incognita* has a global distribution and parasitizes a wide range of plant hosts, including many agricultural important crops. Its genome contains around 3% of foreign genes (Paganini et al. 2012), being involved in the establishment of a feeding structure, metabolism of plant compounds and plant defense suppression, as well as in general parasitic features such as degradation of carbohydrates and proteins of plant cell walls. It has even been postulated that this nematode and related species could only develop a parasitic lifestyle because of the horizontal acquisition of these genes (Danchin et al. 2010). Two species of bdelloid rotifers, *Adineta ricciae* (Boschetti et al. 2012) and *A. vaga* (Gladyshev et al. 2008; Flot et al. 2013), contain up to 8-9% of foreign genes, which are involved in, for example, toxin degradation or the generation of antioxidants and key metabolites, and DNA repair (Hecox-Lea & Mark Welch 2018). HGT in bdelloid rotifers is ancient and more frequent in habitats with desiccation (Eyres et al. 2015). Interestingly, the nematode *M. incognita* as well as these two bdelloids reproduce asexually, thus having lost the possibility to acquire novel gene combinations through sexual recombination. According to Dunning Hotopp (2011), evolutionary theory could perfectly well explain why asexuals or species with endosymbionts and thus close proximity with bacteria should show more frequent HGT. This has important implications for society, especially for pest management, and agriculture as many agriculturally used crops or aquacultures consist of monocultures and/or clonal lineages with very little or no genetic variation and high risk for insufficient adaptive potentials. But the potential of HGT for these organisms is not known at all.

The non-marine ostracod species *Darwinula stevensoni* has these four features making it a likely candidate for frequent HGT. (1) Schönknecht et al. (2013a) mentioned old age (hundreds of millions of years) as potentially important for the high proportion of HGT in red

algae and nematodes. Ostracods have the best fossil record of all recent arthropods, allowing for the absolute dating of evolutionary events. They exist as a group for at least 485 myr (Williams et al. 2008), and darwinulid ostracods for at least 360 myr (Martens 1998). The species *D. stevensoni* has been around for at least 20 myr (Straub 1952).

(2) Ecological experiments have shown that *D. stevensoni* has an exceptionally large tolerance to different temperature, salinity (Van Doninck et al. 2002) and oxygen (Rossi et al. 2002) conditions. It is also ubiquitous and cosmopolitan as it occurs in a wide range of different aquatic habitats (Rossetti & Martens 1998) around the globe (Schön et al. 2012). In this respect, it resembles the ecology (wide host spectrum) and distribution (worldwide in tropical and sub-tropical regions) of the root knot nematode *M. incognita*, a species in which at least 3% of the genome has originated via LTG (Danchin et al. 2010).

(3) *Darwinula stevenson* is also one of the few examples of putative ancient asexuals and has either no sex at all or a very rare or cryptic form of it (Smith et al. 2006; Martens & Schön 2008; Schön et al. 2009). Thus, HGT might constitute an important alternative mechanism for genome plasticity. In this way, *D. stevensoni* also resembles *M. incognita* and the bdelloid rotifers described above, which have the highest frequencies of confirmed HGT so far.

(4) Molecular techniques have already revealed that *D. stevensoni* hosts a wide variety of bacteria, including *Cardinium* (Schön et al. 2018). *Cardinium* is a known endosymbiont in terrestrial arthropods (Hunter et al. 2003; Ros & Breeuwer 2009; Duron et al. 2008 a & b) where it can distort sex ratios. Studies of other endosymbionts, with *Wolbachia* as the best-known example, have shown that the physical closeness of eukaryotic hosts and their endosymbionts occurring in the germ line greatly facilitates LGT (Dunning Hottop 2011).
For these four reasons, we chose this ostracod as model species to study HGT in non-marine ostracods. As described in more detail below, we aimed at investigating HGT at the RNA and DNA level of the genome of *D. stevensoni*. We also characterized the bacterial fauna of this ostracod to identify potential bacterial donors for HGT. To assess the importance of HGT for the persistence of *D. stevensoni* in long evolutionary time frames of many millions of years, we also estimated the frequency of HGT and identified the function of genes with HGT.

2. METHODOLOGY AND RESULTS

2.1 Characterising the bacterial fauna of *D. stevensoni*

2.1.1 Endosymbiotic bacteria

314 ostracod specimens from 22 morphospecies and 35 genetic species were analysed for the presence of endosymbiotic bacteria, including *D. stevensoni*. The publication of these results was the first report on endosymbionts in non-marine ostracods (Schön et al. 2019, epublished 2018). We extracted DNA from individual ostracods with routinely used methods (see for example Schön et al. 2012) mainly using a classic, PCR-based approach with primers for four bacteria known to manipulate reproduction in other arthropods: *Wolbachia*, *Rickettsia*, *Spiroplasma* and *Cardinium* (Schönet al. 2018). We used published primers that are specific for each bacterial group (Jeaprakash & Hoy 2000; Gotoh et al. 2007; Davis et al. 1998; Majerus et al. 1999) and PCR cycle conditions as described in Schön et al. (2018). We also

designed our own primer combinations from Gotoh et al. (2007) & Duron et al. (2008b) to obtain the full 16S of *Cardinium* in different ostracod lineages (Schön et al. 2018). PCR products were sequenced automatically and directly with Sanger sequencing using the PCR primers in-house on an ABI3130X with the BigDye kit. Sequence data have been submitted to GenBank (see Schön et al. (2018) for more details). Additionally, we used high throughput sequencing methods to analyse the bacterial fauna of nine ostracod samples in detail, including *D. stevensoni* to verify the results on absence of presence of *Cardinium* with classic PCR-based techniques. The high throughput 16S data were analysed with bioinformatic pipelines on the Nephele platform to identify bacteria and test their abundances statistically. Our study (Schön et al. 2018; Figure 1) was the first one ever analysing and describing the microbiome of non- ostracods. All sequence data were also used for phylogenetic analyses to reveal the evolutionary position of bacteria from non-marine ostracods and their relationship to similar bacteria from other metazoans.

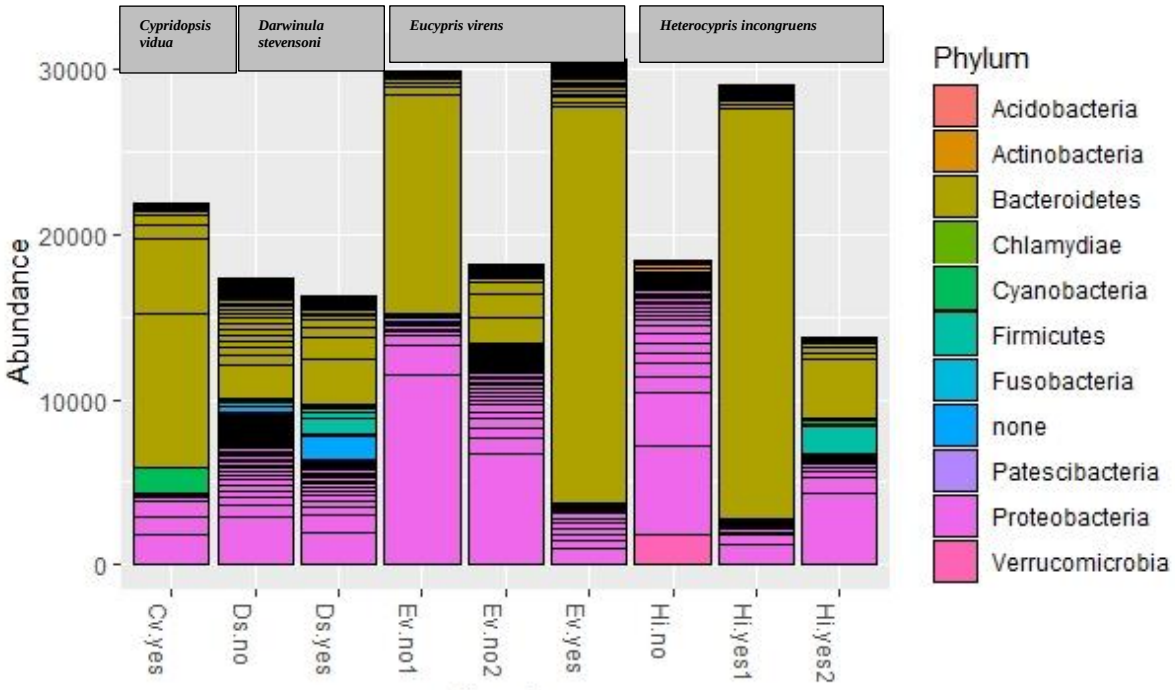

Figure 1: First published non-marine ostracod microbiomes. Based on high-throughput 16S amplicon sequencing, analysed with DADA2 (Callahan et al., 2016) and plotted with phyloseq (McMurdie & Holmes, 2013) and ggplot (Wickham, 2016). *Cardinium* belongs to the phylum Bacterioidetes. Modified from the supplementary material of Schön et al. (2018).

We showed that only *Cardinium* bacteria were present in non-marine ostracods with an overall prevalence of 38% (Schön et al. 2018). Most species of the Cyprididae with mixed reproduction were infected while the distribution of *Cardinium* was patchy in the Darwinulidae and did not occur in all populations of *D. stevensoni*. These results cannot be explained by technical problems as the results of the classic and high throughput approaches fully matched (Schön et al. 2018).

Phylogenetic analyses revealed that *Cardinium* of non-marine ostracods are different from any *Cardinium* strains known to date from other arthropods and also vary between the three superfamilies of non-marine ostracods (Schön et al. 2018). Additional statistical analyses of the presence or absence of *Cardinium* in non-marine ostracods also using data of the studies by Mioduchowska et al. (2018) and Çelen et al. (2019) showed a significant link of *Cardinium* infections with asexuality (Schön & Martens, in press). Whether these bacteria indeed cause asexuality, needs to be further investigated in future studies.

2.1.2 Characterising the entire microbiome of *D. stevensoni*

We used general primers for the V1-V2 and V3-V4 regions of bacterial 16S and 16S from endosymbionts (Claesson et al. 2010) that had been successfully used in other studies (Dethlefsen et al. 2008; De Tender et al. 2015; Freese & Schink 2011) for a high throughput sequencing approach at the Genomics Core of the KU Leuven. Our aim was to characterize and compare the bacterial fauna of *D. stevensoni*, its habitat water and sediment in Belgium, Spain and the UK with at least three replicates each. Sediment DNA was extracted with the commercial Powersoil DNA kit, DNA from filtered water with the Gentra Puregene kit from Qiagen and DNA from ostracods with the Blood and Tissue kit as routinely used in our lab. The first PCR protocol used 25μl of the HotStarTaq Master Mix (1.5 mM $MgCl_2$ and 200 μM each dNTP), 10pmol of each primer and 1-2 μl DNA (comprising 20-50 ng) in a Biometra Thermal Cycler (Westburg), starting with 15 min @95°C and 26 (soil) to 30 or 42 (ostracods) cycles of: 50 sec @ 95°C, 50 sec @ various annealing temperatures and 1 min @ 72°C, followed by a final elongation step of 10 min at 72°C. Annealing temperatures varied from 54°C (for endosymbiont V3-V4), 56°-60°C (universal V3-V4) and 60°C (V1-V2 for water samples) to 62°C (for sediment samples). To verify PCR success, electrophoresis of the amplicons was performed on 1.5% agarose gels, followed by staining with GELRED and photographing under UV fluorescence, and purified with the Agencout AMPure beads kit. The second PCR of all amplicons included adding DNA barcodes for multiplexing and Illumina adaptors followed again by purification with the Agencout AMPure beads kit and their concentrations measured with the qbit before amplicons were pooled for multiplexing. High throughput sequencing was successful for all ostracod samples using the endosymbiont primers (see attached manuscript) and in 99 of the 103 samples from *D. stevensoni* and its aquatic environment. High throughput sequencing yielded reads between 4 and more than 90 million basepairs per sample with a quality score of at least Q30 with an average of 19.63 million reads. A quality score of Q30 is equivalent to an error rate 0.001. We used DADA 2.0 (Callahan et al. 2016) on the Nephele platform (Office of Cyber Infrastructure and Computational Biology (OCICB), National Institute of Allergy and Infectious Diseases (NIAID); Nephele; http://nephele.niaid.nih.gov, 2016) for assembling reads, quality filtering, removal of chimers, reduction to unique reads and identification of bacterial operational taxonomic units (OTUs) by 99% comparisons to the SILVA database release 128. Bacterial compositions of the different samples were statistically compared per replicates, geographic location, habitat (open water, sediment, ostracods) and 16SrRNA region (V1-V2 and V3-V4) and graphically displayed. Here, the most relevant examples from the large number of figures and statistical

comparisons that were successfully generated, are described and discussed. These results are currently also prepared for publication in *Microbiome* for early 2020.

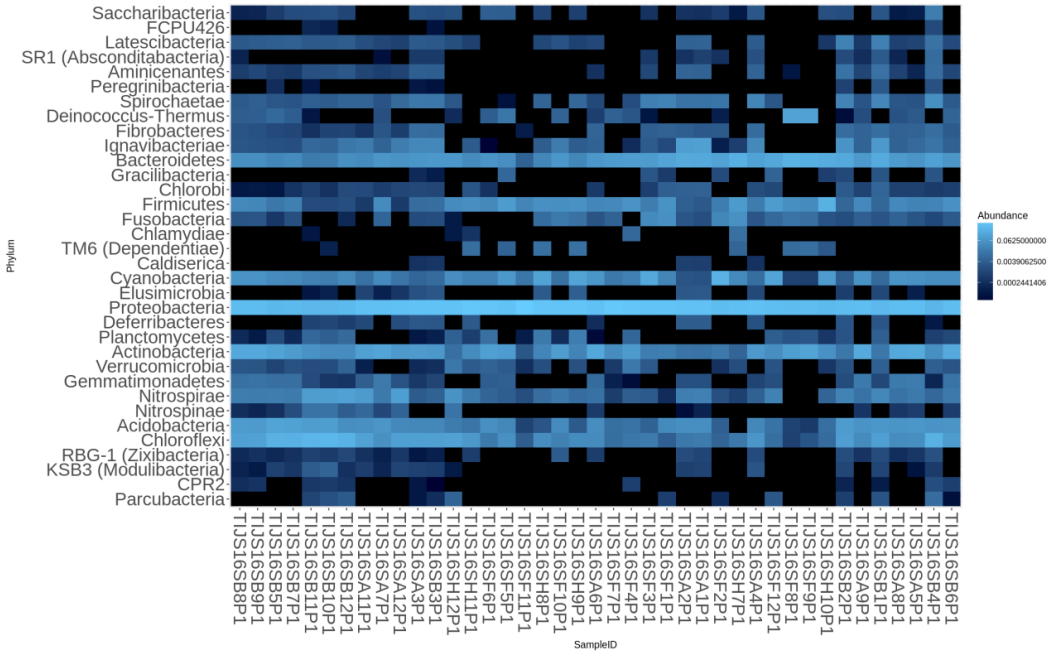

Figure 2: Heatmap of abundance of different bacteria phyla, analysed with V3_V4 primers.

Proteobacteria are most abundant in all samples as illustrated by the light blue in Figure 2, followed by Bacteriodetes. A dominance of Proteobacteria was also found in the bacterial community of a temperate freshwater lake in the North US (Oh et al. 2011), Lake Zürich and its *Daphnia* cladocerans (Eckert & Pernthaler 2014) and in guts of *Daphnia* from Lake Konstanz (Freese & Schink 2011).

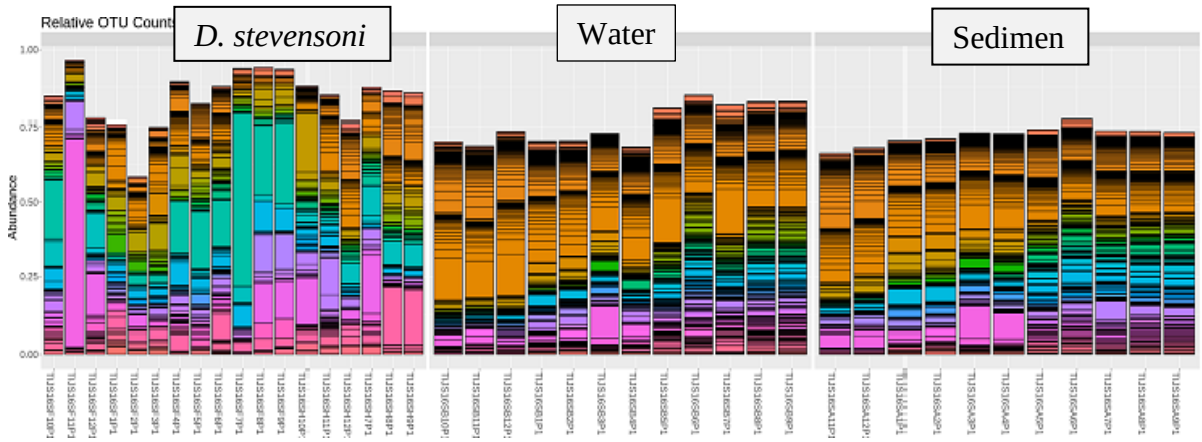

Figure 3: Relative counts of bacteria genera with high throughput sequencing of 16SrRNA amplicons with V3_4 primers.

Concerning variation among treatment groups, the ostracod *D. stevensoni* clearly harbours other bacteria than its environments (see Figure 3), illustrating that the observed microbiome of ostracods is genuine, including endosymbiotic bacteria. In contrast, the bacterial diversity of open water and sediment samples seems to be more similar to each other. In the ostracods

(Figure 3), *Cardinium* (in pink) is one of the most abundant bacteria genera, further confirming the results of Schön et al. (2018).

2.2 **Ecological relationships between bacteria & ostracods**

We first sampled living specimens of three non-marine ostracod species from Belgium: *D. stevensoni*, *Herpetocypris chevreuxi* and *Heterocypris incongruens*. We thus extended the original plan to only study the effects of *D. stevensoni* because of the exceptionally long generation time of this species of about a year (Van Doninck et al. 2003a), while the other two species have generation times of about 4-5 weeks (Meisch 2000), depending on the culturing conditions. All three species reproduce asexually in Belgium (Meisch 2000).

Specimens from the three species were cultured under controlled light and temperature conditions for acclimatization to the laboratory conditions and for allowing to optimize culturing conditions and food. A food selection experiment testing eight different food sources (*Calanus*, *Daphnia*, *Cyclops*, krill, black mosquito larvae, Chironomidae larvae, spinach and rotten wood) revealed that all three ostracod species preferred spinach as food supply. The optimized culture conditions further included 16 hours of light at 20°C in 40 ml of sterilized EPA water (standardized water containing $NaHCO_3$, $CaSO_4$, $MgSO_4$ & KCl with a pH of 7.4-7.8) without oxygen supply. We tested the effects of the antibiotic penicillin which is known to especially affect the endosymbiotic bacterium *Cardinium* (Vanthournout et al. 2011). As described above, this endosymbiont has been found in many non-marine ostracod species (Schön et al. 2018). In an experiment running for 28 weeks (and thus comprising more than three generations of *Heterocypris incongruens*, several generations of *Herpetocypris chevreuxi* and one generation of *D. stevensoni*), we tested the effects of 0, 0.001%, 0.01% and 0.1% of penicillin at 20°C. For each treatment, the life history of two replicates of 10 individual ostracods each were studied per species. In a second experiment, we monitored life history of the three species at 24°C for ten weeks with all other conditions being equal to also test for effects of higher temperatures. All data were analyzed statistically to test for significant differences between species and treatments.

Our experiment was the first of its kind to study the effects of antibiotic treatment on non-marine ostracods. We found significant differences in mortality between the three asexual ostracod species with and without antibiotic treatment ($p@20°C = 0.0378$; $p@24°C = 0.005829$). *Herpetocypris chevreuxi* was the most sensitive and died at the highest and second highest penicillin concentrations within a single generation (or one month), regardless of the temperature regime ($p = 0.7199$). We found the same results repeatedly with new sets of individuals (but only one experiment is shown here). In contrast, *Heterocypris incongruens* survived best at the highest antibiotic concentrations but stopped with its reproduction after having been exposed to penicillin for three generations at 20°C ($p = 0.7557$). Penicillin most likely also influences life history and reproduction of *D. stevensoni* as specimens died when being exposed to the highest antibiotic concentrations. To confirm these preliminary promising results, we plan to conduct additional experiments with *D. stevensoni* in the future, which are necessary because of its long generation time, and also plan to expose ostracods to temperatures above 30°C, which could also have a lethal effect on

endosymbionts. Since the three investigated ostracod species are asexual, our cultures consisted only of females. It has been shown for endosymbiotic *Wolbachia* bacteria feminizing males that exposure to penicillin and thus curing the infection changed phenotypic female mites back to males (Weeks et al. 2001) or stop reproduction in terrestrial arthropods (Pike & Kingcomb 2009). Similarly, in spiders with *Cardinium* infections, antibiotic treatment changed the sex ratios of populations and made them less female-biased (Vanthournout et al. 2011). None of these results were observed in our experiments indicating that *Cardinium* has no feminizing effects in non-marine ostracods. We plan to confirm our results with additional exposure experiments to penicillin, which will need to run for several years because of the long generation time of *D. stevensoni* (Van Doninck et al. 2003a).

2.4 Identify candidates for HGT from transcriptomes (RNA)

RNA extractions with the Qiagen RNA extraction kit using living *D. stevensoni* and conducted in collaboration with the University of Lausanne were very successful and produced the first high quality *de novo* transcriptome of an ostracod, which was used to identify candidates for HGT. Analysing the transcriptome of *D. stevensoni* with BUSCO v. 3.0 (Simao et al. 2015) showed that this transcriptome contains 97% of known eukaryotic genes and 97.1% of known arthropod genes at the DNA level and 95% and 95.1%, respectively, at the protein level. These numbers thus indicate that the transcriptome is of very high quality. In collaboration with the French project partner Prof Etienne Danchin, we used his alien index approach (Rancurel et al. 2017) to identify genes potentially being the result of HGT. We found 191 transcripts with an alien index >30 (forming 125 functional groups) that were also present in the three replicates of the *D. stevensoni* transcriptome. Their possible horizontal origin was further confirmed by the novel, yet unpublished phylogenetic FastTree algorithm developed by the group of Prof Danchin. We identified another 1250 other transcripts that are possibly the result of HGT but did not occur in all three replicates of the transcriptome. These were not further analysed here.

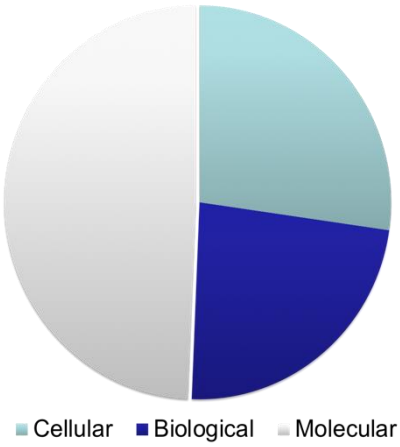

Figure 4A: Functions of genes with likely HGT origin according to Gene Ontology.

The majority of the 191 genes potentially originating from HGT codes for molecular functions according to their Gene Ontology (Figure 4A) and could thus have important functions for the

ecology and adaptations of the ostracod *D. stevensoni*. One third of these genes has a potential bacterial origin, and one third a eukaryotic origin (Figure 4B).

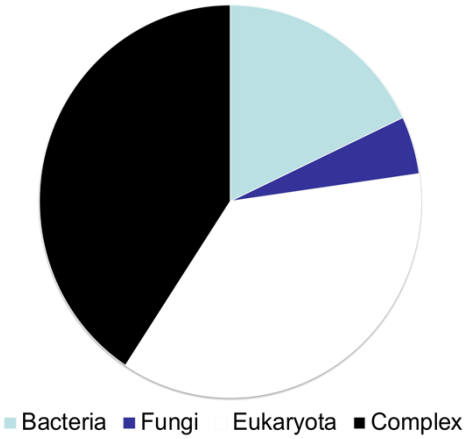

Figure 4B: Origin of genes with likely HGT origin.

The flawed study of Boothby et al. (2015) on tardigrades illustrates the importance of confirming potential cases of horizontal gene transfer with additional methods. Their study claimed to have identified more than 30% of horizontally transferred genes in a tardigrade genome, the highest frequency ever reported. The vast majority of these were in fact the result of bacterial contamination as additional analyses by Koutsovoulos et al. (2016) showed and the frequency of HGT in that genome was only 2.6%.

In the LATTECO project, we were going to sequence the flanking regions of potential HGT genes: only if the flanking regions of genes with HGT were part of the ostracod genome could we be sure that these genes were indeed an authentic case of HGT. If the flanking region originated from bacteria, contamination was the most likely explanation (Figure 5). This kind of analyses could not be conducted from the RNA sequence data of the transcriptome but needed to be carried out at the DNA level because transcriptome data only contain expressed genes not the transgenic or other non-coding regions.

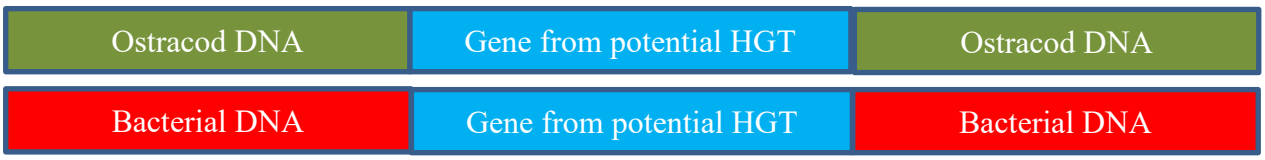

Figure 5: Confirming HGT by sequencing the flanking regions of genes from potential HGT events at the DNA level.

We first used the draft genome of *D. stevensoni* that was assembled in close collaboration with Prof Tanja Schwander of the University of Lausanne. This draft genome is with more than 50,000 contigs still rather fragmented and not of sufficient high quality to ensure that it includes all genomic regions of *D. stevensoni*, which are difficult to assemble from short Illumina sequence data. Of the 191 genes with potential HGT that we had identified in the transcriptome, only 14 were also present in the draft genome of *D. stevensoni;* another three genes were identified in

the genome of the marine shrimp *Litopenaeus vannamei* (Zhang et al. 2019). These results illustrate that most genes with potential HGT cannot be detected in the draft genome of *D. stevensoni*, and that this draft genome is therefore unsuitable to confirm HGT by checking sequences of the flanking regions. Also, the shrimp genome was not really suitable for this purpose, which can be explained by the large evolutionary distance between decapods and ostracods.

Because no other genomic resources for ostracods are yet available, we also screened an existing genomic library of *D. stevensoni* with low genomic coverage for 33 genes with potential HGT and the most interesting functions for ecological adaptations or DNA repair. This part of the work was conducted in collaboration with the INRA lab in Toulouse and the results are summarized in Table 1. The first step of this verification procedure was to design primers of the genes with potential HGT for generating labelled hybridisation probes. The match of these probes with genomic DNA of *D. stevensoni* was tested by PCR (see Table 1 below, "Probe validation"). We could validate 18 probes for the 33 genes, which were subsequently used for hybridization of the entire library (containing thousands of cosmids). When hybridized to the library, probes for 15 genes gave positive signals with the library indicating that these genes are present in the DNA library and most likely authentic results of HGT. We identified 107 positive cosmids that could potentially contain these genes. To ensure that the 107 cosmids indeed contained the target genes, a second round of PCR amplification was used for validation. Only 46 cosmids containing 10 genes of our interest could be validated, whose ends were first test sequenced and, if successful, subsequently sequenced with long read Pac Bio technology. The latter sequencing was only successful for 11 cosmids. We can confirm the presence of seven genes with the cosmid sequence data by mapping the gene sequence data from the transcriptome to DNA sequence data from each cosmid in Geneious v19.3; some genes were present in multiple cosmids (Table 2). Unfortunately, the validation procedure failed for the majority of the cosmids potentially containing genes with HGT. To identify the reason for the low success rate, we repeated validation by PCR amplification of the target genes inside cosmids multiple times. We found varying lengths of the target genes, which are most likely the result of special structures where target genes are arranged in tandem or multiple copies behind each other. Some of the genes for which we were screening, occurred in isoforms and multiple genes in the draft genome (Table 1), making such genomic structures more likely. It is also very probable that genes clustered among transposable elements; estimating the frequency of these mobile genetic elements with repeatmasker (Smit et al. 2013-2015) using a custom transposon library of *D. stevensoni* (Schön et al., submitted) revealed that about 10% of the sequences of the cosmids that could successfully be sequenced, were indeed transposons. The cosmids that could not be sequenced, most likely contain a much higher frequency. Both features, tandem repeats and transposons, make it technically very difficult to sequence cosmids. Because of the low success rate of sequencing cosmids that contained genes with HGT, we stopped validating additional genes of potential HGT origin with the genomic library approach.

As the final validation step could not be successfully completed, we cannot confirm at this stage that all 191 potential genes in the transcriptome of *D. stevensoni* with high probability are indeed genuine examples for HGT. This will require further confirmation from high quality

genome data (see below). These results can also not be published without additional confirmation. Interestingly, 191 genes is in the same order of magnitude as the 253 genes that were identified in another arthropod (Collembola) genome as HGT (Faddeeva-Vakhrusheva et al. 2017), increasing the probability that most of these genes come indeed from HGT. The remaining 1150 genes of *D. stevensoni* with potential HGT origin did not match all of our criteria (presence in three replicates of the transcriptome, alien index > 30 and phylogenetic support). The likelihood that they indeed come from HGT events is thus lower and they will require even more solid confirmation and validation with high quality genome data of *D. stevensoni*.

Another study that was not part of the LATTECO project and had sequenced 95 other cosmids of the library found a high frequency of transposable elements in these cosmids, reaching 35% of the sequenced 3 million basepairs (Schön et al. submitted). In that data set, coding regions only contributed to 8% of the sequence data and 98% of all BUSCO genes were missing. The majority of transposable elements furthermore showed signs of recent activity. The results on sequencing the cosmid library of *D. stevensoni* from the LATTECO project described above and of Schön et al. (submitted) both indicate that a considerable part of this library contains non-coding regions such as repetitive DNA and transposons and is thus not well suited to screen for coding genes including those of HGT.

Interestingly, it is now established that also transposons can be transferred horizontally between different organisms including crustaceans (Dupeyron et al. 2014) and that this other kind of HGT is supposed to be especially common in aquatic environments (Metzger et al. 2018). Due to incorrect cut and paste mechanisms, transposons can also move other parts of the host genome with them, including genes (Bourque et al. 2018). The fact that the genomic surroundings of most HGT genes consists of transposons (as the sequences contained at least 10% of transposons) emphasizes the importance of the potential link between HGT and transposons in *D. stevensoni* that we found. Further studying the transposon landscape of this ostracod species could thus investigate an alternative, evolutionary mechanism also resulting in frequent HGT. It is planned to continue this line of research in 2020 in further collaboration with Dr Irina Arkhipova from the Marine Biological Laboratory (MBL) in Woods Hole, USA.

The LATTECO project provided very promising results by identifying 191 genes with potential HGT origin in *D. stevensoni*. As shown above, the majority of these genes came from bacteria, a likely scenario given the specific bacterial fauna of this ostracod. Most genes with potential HGT coded for molecular functions, which would be of very high importance for the adaptation of *D. stevensoni* to a wide range of temperatures and salinities REF and possibly also for the high UV resistance of this species REF. The 191 genes require to be further validated for their HGT origin before final conclusions can be drawn and published. Obtaining a high quality of *D. stevensoni* genome assembly that would be suitable for this task is currently ongoing through a new collaboration with Dr Irina Arkhipova at the MBL (see below “4. Perspectives” for more details), and the continuation of this collaboration for 2020 is currently planned.

Table 1: Results for confirming potential HGT genes with DNA sequencing of the genomic library of *D. stevensoni*. # = number. Only validated probes were used for hybridization. Only positive and validated cosmids were sequenced. Genes with multiple isoforms are underlined.

Probe #	Gene name	Probe validation	Hybridization	# positive cosmids identified	# positive cosmids validated/sequenced
11	Dst.1070	Yes	Yes	2	2/2
12	Dst.13367	No			
13	Dst.16693	No			
14	Dst.21526	No			
15	Dst.50695	Yes	Yes	1	0/0
16	Dst.53049	Yes	Yes	2	2/2
17	Dst.63063	No			
18	Dst.65566	Yes	Yes	3	3/3
19	Dst.70264	No			
20	Dst.118459	Yes	Yes	1	1/1
23	Dst.121837	Yes	Yes	3	3/3
24	Dst.136996	Yes	Yes	1	1/1
25	Dst.149997	Yes	Yes	1	1/1
26	Dst.176337	Yes	Yes	1	0/0
27	Dst.177453	Yes	Yes	1	1/1
28	Dst.181992	No			
29	Dst.183243	No			
30	Dst.194417	Yes	No	0	
31	Dst.114965	No			
32	Dst.126622	Yes	Yes	14	6/0
33	Dst.183353	No			
34	Dst.187801	Yes	Yes	25	0/0
35	Dst.48052	No			
36	Dst.53055	No			
37	Dst.68007	No			
38	Dst.71431	Yes	Yes	0	
39	Dst.77703	Yes	Yes	25	0/0
40	Dst.87408	Yes	Yes	27	21/0
41-44	Dst.1526	No			
45	Dst.1526	Yes	Yes	0	
46	Dst.63063	Yes	Yes	6	0/0
47-52	Dst.102002	No			
53-58	Dst.143576	No			
59-64	Dst.162058	No			

For those seven genes that were successfully sequenced within cosmids of the library, we also estimated the GC content with the online tool http://www.endmemo.com/bio/gc.php. With the exception of gene 121837 in cosmid 15I07 (Table 2), in all other cases, the GC content was lower in the entire cosmid than in the gene of potential HGT origin. Our estimated GC content of individual cosmid sequence data matched values of other invertebrate genomes lying between 27.4% and 45.22% (https://web.archive.org/web/20110809040326/http://esper.lab.nig.ac.jp/study/genome/?page=genome_composition_database_species_list); also, estimating the GC content for all 13

cosmids of the LATTECO project or another 95 cosmids of the study by Schön et al. (submitted) provided similar estimates around the 43-44% (Table 2). The GC content of prokaryotic genomes is usually higher (31.9% to 67.9%; http://insilico.ehu.es/oligoweb/index2.php?m=all). We can observe from the available data on GC content that the genes with potential HGT have markedly higher GC content than the DNA sequences from the genome of *D. stevensoni* present in the library (Table 2). The best explanation for this pattern is that these genes were only recently transferred from bacteria with one exception, gene 121837. Because of the limited number of genes for which we could estimate the GC content, no statistical tests were conducted, and the conclusions remain preliminary.

Table 2: Estimates of GC content from successfully sequenced cosmids containing genes with potential HGT.

Cosmid name	GC content of whole cosmid in %	Length of cosmid in basepairs	Target gene#	GC content of target gene in %
>Dst-F-Holl-02K10	42.540684	44059	65566	47.96748
>Dst-F-Holl-06H22	41.332051	29113	65566	47.96748
>Dst-F-Holl-11H10	42.662278	42227	65566	47.96748
>Dst-F-Holl-07K22	46.158017	38730	177453	60.626398
>Dst-F-Holl-08N24	46.099686	39484	53049	50.612245
>Dst-F-Holl-17P18	46.953023	37529	53049	50.612245
>Dst-F-Holl-22A16	41.111111	40320	1070	47.070707
>Dst-F-Holl-35G10	40.525952	36125	1070	47.070707
>Dst-F-Holl-22C15	49.234378	29845	121837	46.566164
>Dst-F-Holl-14O05	49.941296	25552	121837	46.566164
>Dst-F-Holl-15I07	47.285874	18809	121837	46.566164
>Dst-F-Holl-42F18	42.117715	48609	139669	45.247148
>Dst-F-Holl-42P10	42.698993	38029	118459	50.486431
All 13 cosmids	44.1400	468431		
95 other cosmids from Schön et al. (submitted)	43.8800			

2.3 Additional project results

2.3.1 Provided training of staff members involved in the project

Early Career Researcher Tijs Van Den Berghe received hands-on training in:

- Ostracod sampling and sorting
- Culturing of non-marine ostracods
- Monitoring life histories of non-marine ostracods
- RNA extractions from non-marine ostracods
- Developing and applying DNA extraction methods to non-marine ostracods and environmental (sediment & water) samples

- Amplification of 16S regions using various universal primers, purification of amplicons & library preparation for high throughput sequencing techniques including multiplexing of samples
- Application of high throughput sequencing methods for estimating bacterial diversities with 16S data
- Bioinformatic analyses of high throughput 16S sequence data including quality filtering, demultiplexing and identification of OTU's
- Presentation of scientific results

Early Career Researcher Marie Cours received hands-on training in :

- sampling and preparing living ostracods for whole genome sequencing

2.3.2 Received external training in novel techniques and skills relevant to the LATTECO project

Formal training of Isa Schön:

- Jan-Feb 2019 Phylogenomics Workshop 2019, 22.01-04.02.2018, Cesky Krumlov, Cz Republic. (funded by RBINS)
- Jan-Feb 2018 Population genomics Workshop 2018, 21.01-03.02.2018, Cesky Krumlov, Cz Republic. (funded by RBINS)
- June 2017 Workshop "VSC Users day 2017", Academy of Science, Brussels, Belgium
- January 2017 Genomics Workshop 2017, 09.-21.01.2017, Cesky Krumlov, Cz Republic. (funded by RBINS)
- November 2015 Gent, Belgium: BITS (Bioinformatics Training and Service Facility) training: Linux for bioinformatics
- March 2015 Leuven, Belgium: BITS training: RNA-Seq analysis for differential expression

Hands-on training of Isa Schön:

- Aug-Sept 2019 Applying Oxford Nanopore long read sequencing technologies, analyses of transposable elements, genome annotations. 29.07.-12.09.2019, Marine Biological Laboratory, Woods Hole, USA.
- Oct 2015-Nov 2016 Analysing transcriptome data; identifying HGT from genome data with different approaches; training provided by partner Prof Danchin and his research group.
- Oct 2015-Nov 2016 Conducting RNA extractions; analyzing transcriptome sequence data statistically for differences in gene expression;

training provided by Genomics Core staff members at the KU Leuven.

2.3.3 Founding and expanding of scientific networks

The LATTECO project has significicantly expanded the scientific network of the co-ordinator Isa Schön. This is partly thanks to the involvement of the French partner Prof Etienne Danchin and his research group, which advanced the project significantly regarding all methods for HGT. The scientific network now for example also includes postdoc Georgios Koutsolvoulos who has been correcting the flawed tardigrade study. In collaboration with Prof Danchin and Prof Richard Cordaux from the University of Poitiers, we plan to organize a Jacques-Monod Conference on HGT in 2021. The application for this conference will be submitted in January 2020. Using genomic techniques in non-marine ostracods received a lot of interest from the evolutionary research community and opened several new possibilities for collaborations. During and after the LATTECO project, novel collaborations have been initiated with Prof Tanja Schwander from the University of Lausanne, which has resulted in draft genomes of three non-marine ostracod species including *D. stevensoni*, the target species of LATTECO. Likewise, the collaboration with Dr Irina Arkhipova from the Marine Biological Laboratory in Woods Hole, USA, has been re-initiated. As a result, I. Schön received a prestigious Whitman fellowship in summer 2019, which allowed her to start developing long read sequence techniques to *D. stevensoni*, which will eventually produce the required high-quality genome of *D. stevensoni* to test for HGT (see also 4. Perspectives). The project coordinator was asked to give a presentation at the Evolution conference in Marseille, France, on ancient asexual ostracods, was invited to a workshop on asexuality at the University of Namur in 2018 to give an oral presentation, and will also participate and give a talk on asexual ostracods in the session on asexuality at the EVENET conference in January 2020 in Kortrijk. The RBINS was invited to act as host for a Marie-Curie postdoc grant of Joao Martins on ostracod genomics; we are still awaiting the outcome of the application. Characterizing the microbiome of *D. stevensoni* has reestablished a former collaboration with Prof Francesc Mezquita from the University of Valencia in Spain. The techniques used to study the bacterial fauna are currently also applied in the GEN-EX pioneer BRAIN project for Tsunami research; this new involvement in geology has significantly expanded the scientific network of the LATTECO project partners. A new and close collaboration with the Genomics Core at the KU Leuven initiated studies on differences in gene expression in non-marine ostracods for the first time ever, which can be continued in new projects in the future.

3. DISSEMINATION AND VALORISATION

3.1 Scientific reviews

Schön, I. & Martens, K. 2016. Ostracod (Ostracoda, Crustacea) genomics – promises and challenges. *Marine Genomics* 29, 19-25. http://dx.doi.org/10.1016/j.margen.2016.03.008

3.2 Presentations at scientific conferences

3.2.1 Oral presentations

1. **Schön I.** & Martens K. 2016. Are darwinulid ostracods ancient asexuals? Oral presentation at the 20th Evolutionary Biology Meeting, 20.-23.09.16, Marseille, France.

2. **Schön I.** 2016. Ancient asexual scandals – 200 million years without sex? Invited talk to the departmental seminar at Institut Cavanilles de Biodiversitat i Biologia Evolutiva, University of Valencia, 27.10.16.

3. **Schön I**. & Martens K. 2017. Phylogenetic and genomic studies of ancient asexual darwinulid ostracods. Invited talk at the workshop "Genome evolution in asexual organisms", University of Namur (Belgium) March 28-30, 2017.

4. **Schön I.**, Van Den Berghe T. & Martens K. 2017. Genomics in Ostracoda (Crustacea) – novel tools to answer long-standing evolutionary questions. Oral presentation at the session "Crustacean Genomics", The Crustacean Society Meeting, Barcelona June 2017.

5. **Schön I.** & Martens K. 2016. Are darwinulid ostracods ancient asexuals? Oral presentation at the 20th Evolutionary Biology Meeting, 20.-23.09.16, Marseille, France.

6. **Schön I**. 2019. Developing ostracods as emerging models for research on evolution and biodiversity". Invited talk, brown bag lunch, 25.08.2019, Woods Hole, USA.

7. **Schön I**., Martens K., Brandon M.L. & Arkhipova I. 2019. The transposable landscape of the putative ancient asexual *Darwinula stevensoni* (Crustacea, Ostracoda). Oral presentation at the Mobile Genetic Elements 2019 conference, 29-31.8.2019, Woods Hole, USA.

3.2.2 Poster presentations

8. **Van den Berghe T**., Martens K., Mezquita F. & **Schön I.** 2016. Metagenomics of the non-marine ostracod *Darwinula stevensoni*. Flanders Annual Meeting of Ecology 2016, 19.12.16, Gent, Belgium.

9. **Van Den Berghe T**., Martens K. & **Schön I**. 2017. Metagenomics of the non-marine ostracod *Darwinula stevensoni* (Crustacea, Ostracoda). Zoology 2017, Nov. 23.-24.11.2017, Wageningen.

10. **Van Den Berghe T.**, Martens K. & **Schön I.** 2017. Metagenomics of the non-marine ostracod *Darwinula stevensoni* (Crustacea, Ostracoda). 18th International Symposium on Ostracodology. Santa Barbara, USA, August 2017.

11. **Schön I**., **Van den Berghe T**., Mesquita-Jones F. & Martens K. 2018. First "omic" results of the putative ancient asexual *Darwinula stevensoni* – evidence for horizontal gene transfer and a

high load of transposable elements. Jaques-Monod-Conference on "Evolution of reproductive systems", Roscoff, France, April 2018.

12. **Schön I**., **Koutsolvoulos G**., **Danchin E**., Mezquita-Jones F. & Martens K. 2019. Horizontal gene transfer in the putative ancient asexual ostracod *Darwinula stevensoni*? 9th European Ostracodologist's Meeting, Gdansk, Poland, 18-22 July, 2019.

3.2.3 Organisation of workshops, symposia etc.

Schön, I. 2016. Co-convener of the Ecological genomics symposium at the Joint Symposium Eco-Evolutionary Dynamics and Flanders Annual Meeting of Ecology, Gent, Belgium, 19.-21.12.16.

Schön, I. 2016. Member of the scientific organization committee of the Zoology 2016 conference, Antwerp, Belgium, 15.-16.12.16.

Schön, I. 2017. Member of the scientific organization committee of the Zoology 2017 conference. Wageningen, The Netherlands, 23.-24.11.17

Schön, I. 2018. Member of the scientific organization committee of the Zoology 2018 conference, Antwerp, Belgium, 15.-16.12.18.

3.2.4 Other means of dissemination and valorisation

Microbiome analyses and HGT have become an essential part of the lessons in Molecular Ecology at the University of Hasselt where these research topics are taught every year to students of the 3rd Bachelor in biology.

4. PERSPECTIVES

As had been anticipated in the original project application, the framework of the LATTECO project has indeed been able to provide a solid basis to further develop genomic techniques at the host institute (RBINS) and the OD Nature. The training that the project coordinator received during the project in various genomic and transcriptomic techniques has significantly broadened her expertise that now includes all cutting-edge methods of genetics and genomics. There are at least five research areas which could be significantly strengthened during the LATTECO project and which have a very high potential to be further developed into future collaborations and projects: (1) The use of high throughput sequencing methods for environmental studies as they were used in LATTECO to characterize the bacterial fauna of *D. stevensoni* and its environments is also applicable to other research questions. Similar methods are currently being used in the GEN-EX BRAIN pioneer project and are also an essential part of a project application on phytoplankton monitoring of the North Sea in collaboration with UGent that is currently under evaluation. It is also planned by the OD Nature at the RBINS to explore the possibilities of these techniques further for other monitoring programs of the North Sea. For obtaining samples from Spain, we have successfully started collaborating again with Prof Mezquita from the University of Valencia. The results of the microbiome of D. stevensoni will be jointly published in 2020 and it is planned to continue this collaboration in the future for similar research questions. (2) We will further study *Cardinium* endosymbionts in non-

marine ostracods and their influence on their hosts' biology and evolution. We applied for funding to the Moore Foundation in August 2019. Although the application was not selected, it provides a suitable draft to seek funding elsewhere with the same project partners, namely Prof Tanja Schwander (University of Lausanne, Switzerland), Prof Maurine Neiman (University of Iowa, USA), Prof Anne Willems (University of Gent), and Dr D. Mark Welch (MBL, Woods Hole, USA). Through a novel collaboration at the MBL with Dr J. Mark Welch, we have started visualizing bacteria inside ostracods and their tissues with bacterial probes and Fluorescent-In-situ-Hybridisation (FISH). This method will be complementary to genetic techniques as in Schön et al. (2018) to test if *Cardinium* is indeed an endosymbiont of non-marine ostracods. The first pictures successfully using this technique have been produced in summer 2019 and one of these pictures just won the annual MBL photo contest. It is planned to continue this collaboration with Dr J. Mark Welch in 2020 and beyond. (3) The collaboration with Prof Tanja Schwander (University of Lausanne, Switzerland) on comparative studies of asexual genomes is still ongoing; as described above, draft genomes and transcriptomes of three species of non-marine ostracods have been assembled, including *D. stevensoni*. Publication of these genomes is foreseen for 2020, and these draft genomes will provide the required Illumina data to assemble high quality genomes with a hybrid approach using novel sequence data from long read technology. (4) A collaboration with Dr Irina Arkhipova from the MBL on ostracod transposons was reinitiated which dates back 10 years ago. During summer 2019 and being funded by MBL through a prestigious Whitman fellowship to Isa Schön, the transposon landscape of *D. stevensoni* was analysed using DNA sequence data of the cosmid library. Contrary to patterns in other ancient asexuals (Rodriguez & Arkhipova 2016 ; Bast et al. 2016), we found a high diversity of transposons in this ostracod species, and most transposons had been recently active. These data will be published in a special issue in Gene in early 2020, and it is planned to continue this collaboration for the next years. For this purpose, protocols for extraction high molecular weight DNA and applying Oxford Nanopore Technology to ostracods were optimised at the MBL in summer 2019. The aim is to obtain a high quality genome of *D. stevensoni* with long DNA reads that can be analysed for transposons and for genes of potential HGT origin. The first reads from 2019 will be complemented with additional sequencing data in 2020 to complete genome assemblies. It is planned to apply the same techniques to other non-marine ostracods in the next years. High quality genomes are becoming a new standard for evolutionary and ecological research; their availability from non-marine ostracods will be an essential prerequisite to acquire novel project funding. (5) In collaboration with Dr Gregory Maes and Dr Alvaro Cortes Calabuig from the Genomics Core at the KU Leuven, we successfully sequenced transcriptomes of *D. stevensoni* from four geographic populations with the Quantseq technique differing in latitude, salinity and temperature. We found significant differences in gene expression between these populations, which are currently analysed in more detail. It is planned to further study genes with expression differences with qPCRs. This could provide important insights into the very genes that are responsbile for ecological adaptations of *D. stevensoni*, for example for surviving in a wide range of temperatures and salinities.

5. PUBLICATIONS

5.1 Peer-reviewed

1. Smith A.J., Horne D.J., Martens K. & **Schön I**. 2015. Class Ostracoda. In "Thorpe & Covich's Freshwater Invertebrates", Elsevier, pp 757-780.

2. **Schön, I.** & Martens, K. 2016. Ostracod (Ostracoda, Crustacea) genomics – promises and challenges. *Marine Genomics* 29, 19-25. http://dx.doi.org/10.1016/j.margen.2016.03.008

3. **Schön I**. & Martens K. 2018. "Paradox of Sex." In Oxford Bibliographies in Evolutionary Biology. Ed. Jonathan Losos. New York: Oxford University Press (updated version).

4. **Schön I**. & Martens K. 2018. Sexual, unisexual and asexual reproduction in animals. Encyclopedia of Reproduction, 2nd edition, 2nd edition, Volume 6, pp. 3-9.

5. **Schön I**., Kamiya T., **Van den Berghe T**., Van den Broecke L. & Martens K. 2019. The evolutionary history of novel *Cardinium* strains and their non-marine ostracod (Crustacea) hosts. *Molecular Phylogeny and Evolution* 130, 406-415. 10.1016/j.ympev.2018.09.008. Epub 2018 Sep 21.

6. **Schön I**. & Martens, K. 2019. Does *Cardinium* cause asexuality in non-marine ostracods? *Hydrobiologia*, in press. 10.1007/s10750-019-04110-2

7. **Schön I**., Martens K., Brandon M.L. & Arkhipova I. (submitted). The transposable landscape of the putative ancient asexual *Darwinula stevensoni* (Crustacea, Ostracoda). Submitted to *Gene*.

5.2 Not peer-reviewed (abstracts)

8. **Schön I.** & Martens K. 2016. Are darwinulid ostracods ancient asexuals? Abstract book of the 20th Evolutionary Biology Meeting, 20.-23.09.16, Marseille, France.

9. **Van den Berghe T**., Martens K., Mezquita F. & **Schön I.** 2016. Metagenomics of the non-marine ostracod *Darwinula stevensoni*. Abstracts of the Flanders Annual Meeting of Ecology 2016, 19.12.16, Gent, Belgium.

10. **Schön I**. & Martens K. 2017. Phylogenetic and genomic studies of ancient asexual darwinulid ostracods. Abstract of the workshop "Genome evolution in asexual organisms", University of Namur (Belgium) March 28-30, 2017.

11. **Schön I.**, **Van Den Berghe T.** & Martens K. 2017. Genomics in Ostracoda (Crustacea) – novel tools to answer long-standing evolutionary questions. Abstract for session

"Crustacean Genomics", The Crustacean Society Meeting, Barcelona June 2017.

12. **Schön I., Van Den Berghe T.,** Mesquita-Joanes F. & Martens K. 2017. The application of "omics" to Darwinula stevensoni (Crustacea, Ostracoda). Abstracts of the 18th International Symposium on Ostracodology. Santa Barbara, USA, August 2017.

13. **Van Den Berghe T.,** Martens K. & **Schön I.** 2017. Metagenomics of the non-marine ostracod Darwinula stevensoni (Crustacea, Ostracoda). Abstracts of the 18th International Symposium on Ostracodology. Santa Barbara, USA, August 2017.

14. **Van Den Berghe T.,** Martens K. & **Schön I.** 2017. Metagenomics of the non-marine ostracod Darwinula stevensoni (Crustacea, Ostracoda). Abstracts of "Zoology 2017", Nov. 23.-24.11.2017, Wageningen.

15. **Schön I**., **Van den Berghe T**., Mesquita-Jones F. & Martens K. 2018. First "omic" results of the putative ancient asexual *Darwinula stevensoni* – evidence for horizontal gene transfer and a high load of transposable elements. Abstracts of the Jaques-Monod-Conference on "Evolution of reproductive systems", Roscoff, France, April 2018.

16. **Schön I**., **Koutsolvoulos G**., **Danchin E**., Mezquita-Jones F. & Martens K. 2019. Horizontal gene transfer in the putative ancient asexual ostracod *Darwinula stevensoni*? Abstracts of the 9th European Ostracodologist's Meeting, Gdansk, Poland, 18-22 July, 2019: 94.

17. **Schön I**., Martens K., Brandon M.L. & Arkhipova I. 2019. The transposable landscape of the putative ancient asexual *Darwinula stevensoni* (Crustacea, Ostracoda). Abstract book of the Mobile Genetic Elements 2019 conference, 29-31.8.2019, Woods Hole, USA

5.3 Publications planned for 2020

The microbiome of the putative ancient asexual ostracod *Darwinula stevensoni*– foreseen for *Microbiome*

Evidence for loss of functional meiotic genes in the putative ancient asexual *Darwinula stevensoni* – foreseen for *Heredity*

UV repair in the putative ancient asexual *Darwinula stevensoni* – foreseen for *PLoS Biol*ogy

Comparative genomics of non-marine ostracods with sexual and asexual reproduction (in collaboration with Prof Schwander, University of Lausanne) – foreseen for *Science*

5. ACKNOWLEDGEMENTS

Andy Vierstraten (University of Gent), Franz M. Heindler & Bart Hellemans (KU Leuven), Zohra Elouaazizi (RBINS) are thanked for technical assistance with the molecular work. Koen Martens, Marie Cours & Jeroen Venderickx (RBINS) are acknowledged for their valuable help with sampling, identifying and culturing non-marine ostracods. Tanja Schwander & Patrick Tran Van (University of Lausanne, Switzerland) are thanked for providing the draft genome data of *D. stevensoni*. The MBL at Woods Hole, USA, is thanked for funding a Whitman fellowship for Isa Schön.

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ANNEXES - LINKS TO PUBLICATIONS

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