

The maximum length of a proposal is 12 pages, including a maximum of 4 pages for the description of the proposed research

1a. Details of proposal

Title: **Sexual selection and the evolution of coiling direction in snails (Mollusca: Gastropoda)**

Area: **Geo and Biosphere**

1b. Field(s) of research

main field of research	
code:	description:
22.90.00	biologie, overig
If applicable: other fields of research (in order of relevance):	
code:	description:
22.20.00	biogeografie, taxonomie
22.60.00	dierkunde

1c. Details of applicant

Name: **Menno Schilthuisen**

Gender: **Male**

Date of birth: **22 April 1965**

Promotion date: **15 November 1994**

Institution: **Naturalis Biodiversity Center and IBL, Leiden University**

Position: **Professor**

Permanent position: **Yes**

E-mail: **menno.schilthuisen@naturalis.nl**

Research School: **Production Ecology and Resource Conservation**

Name and address of the responsible person at your institution (e.g. scientific director of the institute or dean of the faculty): **Prof. Dr. Erik Smets and Prof. Dr. K. Biesmeijer (scientific directors)**

1d. Alternative contact

Name: **Dr. Joris Koene**

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1e. Renewed application?

Yes

File number of the previous application: **ALW1PJ/13052**

Main changes: Our previous application was deemed "excellent" by both of the reviewers. The ALW committee expressed certain reservations, particularly that the general applicability was not sufficiently demonstrated. In the current revised application, we have paid particular attention to this matter by making it abundantly clear that our project is meant to be applicable to coiling direction in all Gastropoda, not just the genus *Amphidromus*; *Amphidromus* and the family to which it belongs (Camaenidae) just happen to be an ideal group with which to test some key predictions about the evolution of chirality. In addition, we also emphasize more clearly how the developmental and evolutionary processes involved in chirality in this taxon may be similar to those in other animal asymmetries, such as visceral asymmetry in vertebrates, including humans.

1f. Applying for: PhD student

2a. Composition of the research group

List all staff members involved in the proposed research: provide name, initials, titles and type of involvement, e.g. daily guidance, technician, thesis supervisor, advisor.

Name and title	Specialization	Institution	Involvement
Prof. Dr. M. Schilthuizen**	Molluscan evolution	Naturalis / UMS/ Leiden Univ.	Daily guidance
Dr. S. Chiba	Snail chirality	Tohoku Univ., Japan	Collaborator
Dr. J. Koene	Snail sexual selection	VU Univ. / Naturalis	Collaborator
Dr. M. Haase	Reproductive anatomy	Univ. Greifswald, Germany	Advisor
Dr. M. Hosono	Ecology of snail chirality	Kyoto Univ., Japan	Advisor
Dr. A. Davison	Genetics of asymmetry	Nottingham Univ., UK	Advisor
Prof. Dr. H. Spaik	Genomics	Univ. Leiden	Advisor
Prof. Dr. A.R. Palmer	Evolution of chirality	Univ. Alberta, Canada	Advisor
Dr. F. Köhler	Snail phylogenomics	Australian Museum, Sydney	Advisor
Mr. K. Koops	Animal husbandry	Univ. Leiden	Technician
Mr. K. Hendriks	Evolutionary biology	Naturalis / Leiden Univ.	PhD student*

* Kasper Hendriks was closely involved in the writing of the present proposal

** PhD supervisor ("promotor") of the PhD student

2b. Top 5 publications of the applicant and research group related to the proposed research

1. **Schilthuizen, M.**, P.G. Craze, A.S. Cabanban, **A. Davison**, E. Gittenberger, J. Stone & B.J. Scott, 2007. Sexual selection maintains whole-body chiral dimorphism. *Journal of Evolutionary Biology*, 20: 1941-1949.
2. **Hosono, M.**, Y. Kameda, S.-P. Wu, T. Asami, M. Kato & M. Hori, 2010. A speciation gene for left-right reversal in snails results in anti-predator adaptation. *Nature Communications*, 1: e133.
3. **Palmer, A.R.**, 2004. Symmetry breaking and the evolution of development. *Science*, 306: 828-833.
4. **Palmer, A.R.**, 2009. Animal asymmetry. *Current Biology*, 19: R473-R477.
5. **Davison, A., S. Chiba**, N.H. Barton & B. Clarke, 2005. Speciation and gene flow between snails of opposite chirality. *PLoS Biology*, 3: e282.

3a. Scientific summary (max. 250 words)

Please provide the scientific summary (similar as on the fact sheet). Please note that this summary will be used to invite reviewers to assess your proposal, it should therefore have sufficient scientific content.

Asymmetric body forms are a valuable trait for studying character evolution. In vertebrates, the likelihood of situs inversus (reversed heart orientation) has evolved throughout the Tetrapoda. Similarly, coiling direction of snails (dextral or sinistral) displays a particularly dynamic evolution, with certain clades in the gastropod tree characterised by exclusive dextrality, while others display reversals between dextrality and sinistrality, and even dimorphism within species. Because of cross-phylum similarities in the evolutionary dynamics of this simple, binary character system, and because of the evolutionary conservation of certain symmetry breaking developmental genes (e.g., *nodal*, relevant in both Chordata and Mollusca), we believe that multidisciplinary investigation of these patterns will have broad implications throughout the tree of life.

In this project, we investigate the evolutionary dynamism of coiling direction in the land snail family Camaenidae. This family contains genera that are sinistral or dextral, and also genera that contain species of

either coiling direction. Remarkably, the genus *Amphidromus* even contains dextral, sinistral, and dimorphic species. Models predict that under certain conditions, sexual selection may maintain chiral dimorphism, whereas under others, it drives dextral or sinistral fixation.

In one species, we will test for inter-chiral mate choice and enhanced sperm uptake in the lab, and assess the latter also in the field. Second, we aim to resolve the molecular phylogeny of the Camaenidae, using NGS nuclear phylogenomics, and to confront chirality status with reproductive trait, allowing us to test for a signal of sexual selection involved in chiral evolution.

3b. Summary for the broad scientific committee (approximately 250 words)

Please provide a summary aimed at the division-wide committee (Earth and Life sciences). This summary may focus more on background and motivation than the scientific summary.

Asymmetric body forms are a valuable trait for studying character evolution. In vertebrates, the frequency of situs inversus (reversed heart orientation) has evolved throughout the four-legged vertebrates. Similarly, coiling direction of snails (clockwise, dextral or counter-clockwise, sinistral) displays a particularly dynamic evolution, with certain branches in the snail tree characterised by only clockwise species, while other branches display reversals between clockwise and counterclockwise, and even sometimes both forms within a single species. Because some shared developmental genes regulate asymmetry in both mammals and snails, we believe that a multidisciplinary investigation of these patterns will have broad implications throughout the tree of life.

In this project, we investigate the evolution of coiling direction in the land snail family Camaenidae. This family contains species that are clockwise, species that are counter-clockwise, and even species that can be both. In earlier work, we have predicted that under certain conditions, sexual selection can either cause species to become fixed for one coiling morph, whereas under other conditions, sex may cause both morphs to coexist. We will first test these predictions in one camaenid species in the lab and under natural conditions. Then, we will use next-generation sequencing to reconstruct the tree for the whole family Camaenidae, and look for correlations between the evolution of coiling direction and the evolution of reproductive organs.

3c. Summary for the general public (max. 100 words)

Please provide in a title and summary for the general public, preferably in Dutch.

Seksuele selectie en links- of rechtsom gewonden slakken

De meeste dieren hebben een lichaamsbouw met zowel symmetrische als asymmetrische delen. Aangeboren afwijkingen hebben vaak te maken met problemen in de (a)symmetrie (bv. symmetrische organen die zich asymmetrisch ontwikkelen of andersom). Bij slakken is vrijwel het gehele lichaam asymmetrisch, vanwege de links- of rechtsgewonden vorm. Voor een deel wordt die asymmetrie gereguleerd door dezelfde genen als bij zoogdieren. In dit project willen we nagaan hoe omkeringen in de asymmetrie bij slakken in de evolutie hebben plaatsgevonden. Met name willen we weten of seks hierbij een belangrijke rol heeft gespeeld. Resultaten van dit onderzoek zijn extrapol eerbaar naar andere diergroepen.

4. Description of the proposed research

Max. 4 pages (and max. 3600 words); including figures, excluding literature references.

Include details of objectives, innovative aspects, scientific approach, preliminary data, impact, and literature references (include full bibliographical details)

Theoretical Background

Most members of the Bilateria (which include the vast majority of known animals) superficially appear bilaterally symmetric. However, asymmetries commonly disrupt strict bilateral body organisation²⁵. These mostly concern the arrangement of internal organs (e.g., the heart and other visceral organs in mammals²⁹), but often also externally visible parts, such as the head region in flatfish¹¹ and the claws of crustaceans³². Even (nearly) entire-body asymmetry exists, e.g., in the Gastropoda (snails), which are characterised by embryonal torsion, a coiled

visceral mass, an asymmetric brain, and genitalia emerging on one side of the body⁵. As Palmer³¹ argued, asymmetry in body organisation is a suitable character system for studying evolutionary patterns in morphology across a vast range of taxa, allowing phylum- or even kingdom-wide generalisations.

As with all asymmetric three-dimensional shapes²⁸, animal asymmetries are “chiral”, meaning that two non-superimposable mirror-image forms (so-called enantiomorphs, often termed “dextral” and “sinistral”) are possible. At the species level, this leads to two main kinds of asymmetry: antisymmetry, in which both enantiomorphs coexist in roughly equal proportions, and directional asymmetry, where only one of the two possible enantiomorphs exists or predominates⁴⁸.



Fig. 1. A sinistral (on the left) and dextral (on the right) enantiomorph of a snail.

Fig. 1 illustrates antisymmetry in a gastropod. The snail shell on the left coils anticlockwise (sinistral), the one on the right clockwise (dextral). Normally, snail species are directionally asymmetric: only one of the two coiling directions exists in a species. In this case, however, the species (*Amphidromus perversus*) is antisymmetric: both dextral and sinistral enantiomorphs exist side-by-side (see below).

The mechanisms for symmetry breaking in early development vary in some aspects, but are conserved in other aspects. In vertebrates, for example, the developmental cascade surrounding the *Nodal* gene is ubiquitous in determining the left-sided heart, but there is an unexpected diversity in asymmetric signalling upstream of *Nodal* expression²⁶. A precise knowledge of the pathways involved in symmetry breaking throughout vertebrate development is important for understanding the many symmetry-related birth defects in humans^{2,12,28}.

Recently, similar pathways were revealed in chiral development in the Gastropoda. This large and important Mollusca class (>100,000 species of terrestrial, freshwater, and marine snails and slugs) consists entirely of species with early-onset, whole-body asymmetry⁴⁹. The vast majority of these are directionally asymmetric, and usually dextrally coiled; a minority, 5.1% of the species (Palmer et al., unpubl.) are sinistral, and even fewer taxa are dimorphic (antisymmetric) for coiling direction³⁴.

The direction of chirality is determined by a single, yet unknown, chromosomal “master switch” gene with delayed maternal inheritance^{4,34,42}. “Delayed maternal inheritance” means that a snail’s genotype is not expressed on its own body, but on the phenotype of the offspring hatched from the eggs it lays. This means, for example, that, (if the allele for dextral is dominant), a snail with DS genotype, while it may be either dextral or sinistral itself, will lay eggs that hatch as all-dextral broods. As at least four research groups in Europe and Japan (among whom are research team members Spaink and Davison) are homing in on it^{17,18,22,27}, using the model *Lymnaea stagnalis*, the maternal-effect “master-switch” gene is likely to be found very soon. Furthermore, downstream genes have been elucidated in gastropods^{16,18,40,41}, including, surprisingly, an important role for *Nodal*, a gene instrumental in determining direction of asymmetry in mammals, and for the ubiquitous *Dpp/BMP* developmental regulators. This suggests that part of the symmetry-breaking signalling cascade may be conserved across phyla.

In addition, theoretical and empirical studies have provided insight into the population genetics of chirality. Chirality reversals in directionally asymmetric gastropods were found to result in developmental malformations and loss of fitness^{1,15,35}. Moreover, reproduction of reverse-coiled individuals is usually severely impaired because of reversed genital openings and even mirrored courtship behaviour^{1,8,13,21,34,46}. This leads to frequency-dependent selection by the non-reversed part of the population, and should prevent the evolution of reversed species. Yet, theory and observations^{19,47} show that reversals arise regularly, perhaps aided by genetic drift in small populations. Also, the origin of chirally dimorphic species — the ancestral stage through which reversed lineages must have passed — remains mysterious.

Hence, the exact conditions leading to the evolution, diversification, and extinction of left-coiling and dimorphic (left- and right-coiling) lineages remain largely unknown. In this project, we explore a scenario in which, perhaps counter-intuitively (see below), sexual selection is an additional, important force in the evolutionary dynamics of chirality in snails. We investigate whether sexual selection may generate and maintain dimorphic (antisymmetric) species, and may also cause these dimorphic species to become directionally asymmetric again. Although we address these research questions in two subprojects on a chirally dynamic group of land snails, answers to these questions will apply to all gastropods, and have implications for all Bilateria (see below).

Subproject 1. Does sexual selection maintain sinistral/dextral dimorphism in *Amphidromus inversus*?

Amphidromus inversus (Camaenidae) is a tropical treesnail (Fig. 2A): it exists in mixed (antisymmetric) populations of dextral (D) and sinistral (S) individuals. In previous work^{6,33,36-39}, we empirically rejected the hypotheses that antisymmetry is maintained by metapopulation structure, non-heritability of coiling direction, niche differences, sperm chirality, or differential predation. Instead, we found indirect indications that the dimorphism is maintained by sexual selection for D to mate preferentially with S, and vice versa. Such DxS disassortative mating is the opposite of what is normally found: as mentioned above, opposite coiling directions prevent mating in most snails. In *A. inversus*, however, the morphology of genitalia and spermatophores (Fig. 2B) is such that an S spermatophore (which follows the coiling direction of the S individual that produces it) fits better in the oviduct of a D individual and vice-versa. Computer simulations demonstrate that, under this scenario, antisymmetry is maintained under a broad range of parameter values (Fig. 2C). However, direct evidence for increased fitness of DxS mating (compared with DxD or SxS mating) is not yet available. In this subproject, the PhD student will test this hypothesis experimentally, in two ways:

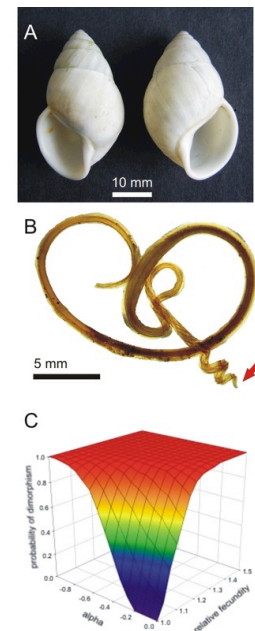


Fig. 2. A, a sinistral and a dextral individual of *A. inversus*. B, the morphology of the spermatophore (the arrow points at the corkscrew-like tip, which presumably has a better fit with the oviduct if the copulating individuals are D and S. C, computer simulations show that sexual selection may maintain antisymmetry.

a) Laboratory experiments.

From our established laboratory colonies, equal numbers of D and S juveniles will be selected and reared to adulthood in isolation. Once the snails are adult (and still virgin), a small pilot study will be set up in which copulations are allowed in 20 DxD, 20 SxS, and 20 DxS couples. After copulation, the individuals are sacrificed at a series of pre-set time intervals of 6 h, and dissected to investigate sperm migration and distribution. We expect to find all stages of sperm transfer from the spermatophore via the oviduct into the spermatheca. Then, a series of choice experiments will be set up (as we did in *Lymnaea* before²¹), in which a (D or S) focal animal will be exposed to a choice between a D and an S target individual. As *Amphidromus* is a hermaphrodite, mating between the target individuals must be prevented by tethering their shells by short strings from the roof of the cage (Y. Nakadera, pers. comm.). This way, only the focal individual will be allowed to roam the arena freely and choose to mate with either target. The interaction will be filmed (to determine how eventual mate choice is affected by courtship) until copulation has been completed. After this, the chosen target individual will be isolated, and, after sufficient time for sperm transfer to the spermatheca (based on the pilot experiments described above), sacrificed. The reproductive system will be dissected³⁸, and counts will be made of the numbers of spermatozoa in the spermatheca³. Since the animals were virgin, and self-fertilization does not occur in this species, these spermatozoa must have come from the focal animal. Morphometric data will be gathered on the form of the recipient's oviduct and the donor's spermatophore. This experiment will be repeated at least 150 times. Finally, we will isolate all focal animals from the mate choice experiments and keep them in isolation for 6 months. The number of offspring produced during that period will serve as a measure of relative fecundity. The experiments described above will result in estimates of mate preference and mate choice, sperm transfer, and relative fecundity in intra-chiral (DxD and SxS) versus inter-chiral (DxS) situations. Based on Ref. 33, we expect that DxS mating will be preferred, and that sperm transfer and relative fecundity will be greater in DxS. The data

obtained on these parameters will be compared with the simulation model results of Ref. 33 to decide whether these indeed are expected to result in stable antisymmetry.

b) Field experiments

To confirm that the patterns found in the laboratory are also relevant in the field, the PhD student will visit the Malaysian island of Kapas (the locality for all our field work on *A. inversus*), and, at the onset of the mating season (the first rains of the monsoon season), collect ca. 500 adult *A. inversus* individuals at our previous field sites 1 and 2 (chiral ratio here is temporally stable at 65% sinistrals: 35% dextrals). All will be stored in 70% ethanol in the field and the reproductive system dissected. Individuals with a single spermatophore in the bursa will be selected (we expect this to be the case in about 40% of the animals³⁸). Based on the coiling direction of the spermatophore (visible until stage 7, as defined in Ref. 38), the chirality of the latest partner can be determined. For all these specimens, the number of sperm in the spermatheca will be determined. Again, even if some of this sperm has already been used to fertilize eggs, our prediction would be that those individuals that have recently experienced an SxD copulation, will retain more sperm in the spermatheca than those having had a recent SxS or DxD copulation.

Subproject 2. Phylogenetic signals of sexual conflict and chirality in the Camaenidae

Despite partial tabulations of the number of independent evolutionary events that have led to sinistrality in certain gastropods^{14,19,20,49}, no complete, global survey has been published yet for all living and fossil gastropod taxa. We have recently (A.R. Palmer & The Gastropod Chirality Consortium, in prep.) compiled such a full overview, by enlisting the help of a large number of taxonomic experts (see <http://news.sciencemag.org>, 20 May 2013). This has led to the identification of at least 111 independent reversals of chirality.

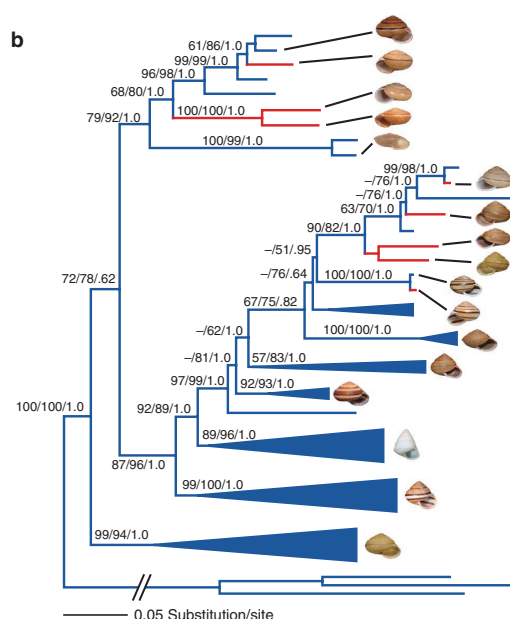


Fig. 3. A phylogenetic reconstruction of the Camaenid genus *Satsuma*, showing (in red) multiple origins of sinistrality²⁰.

In one family (Camaenidae, here interpreted to include the Bradybaenidae), coiling direction was found to be particularly dynamic. This family of large-bodied, mainly (sub)tropical, Australasian land- and tree snails consists of 230 genera and almost 2,000 species. In at least 16 genera, one or more sinistral species are known. Moreover, in the large genus *Amphidromus*, ~40 of ~100 species are antisymmetric^{23,43,44}, with the remaining either dextral or sinistral.

Chiral dimorphism in *Amphidromus*, and more generally, is thought to have been facilitated by the evolution (via sexual selection) of an asymmetric spermatophore tail^{33,38} (see subproject 1).

In subproject 2, we will, in collaboration with two other research groups, reconstruct a molecular phylogeny for the family, with special reference to the genus *Amphidromus*, to investigate the evolution of chirality in the context of sexual selection. Given the species-richness of the family, we will focus on the genera containing sinistral species, taxa geographically and morphologically close to them, and the genus *Amphidromus*. (We will select members of other families in the same

superfamily, Helicoidea, as outgroups to root the phylogenetic reconstruction.) To obtain and select ca. 250-300 target species to be included in the phylogenetic reconstruction, we will collaborate closely with the lab of Chiba who have recently been sampling East-Asian Camaenidae intensively (see Fig. 3), and a similar project at the University of Victoria / Australian Museum, where the focus is on the Australian Camaenidae. To these existing materials, we will add species from (a) the collections of Naturalis and of other research team members, (b) new field work in Southeast Asia, (c) contacts with collectors and (d) existing laboratory cultures. From all the

material, if not yet available, DNA will be extracted, and preparations made of the reproductive systems and the spermatophores.

The following morphological data will be taken: (a) chirality status; (b) lengths of epiphallic caecum, penis, epiphallus, vagina, free oviduct, bursa, diverticulum (if present), dart sac (if present), spermatophore; (c) structure of the spermatophore, especially the spermatophore tip; (d) length and width of each spermatophore tip coil (if present); (e) structure of attachment point of free oviduct to the vagina; (f) body volume (approximated as water replacement of the shell, aperture sealed).

Starting with a subset of six species (of varying relatedness), a randomly distributed representation of the genome will be made using our earlier method^{7,24}. Briefly, extracted DNA will be digested using *MspI*, after which fragments of around 300 bp will be sequenced on an Illumina HiSeq. From this assembly, 100 different contigs (some suited for intragenomic, others for intergeneric resolution) will be selected to serve as phylogenetic markers. Then, for all species available, at least one individual will be sequenced using these new markers. Amplification will be performed using an efficient multiplex PCR and subsequent sequencing in our high-throughput Ion Torrent set-up. Bayesian phylogenetic analysis using BEAST⁹ then forms the backbone for the morphological work. If by then the large set of Australian camaenid marker genes⁴⁵ is available, we will add these to our own loci.

Using the modules available in BayesTraits³⁰, the PhD student will first examine whether the molecular phylogeny provides evidence for evolution of reproductive structures by sexual selection. The expectation would be that correlated evolution exists between traits involved in sperm transfer (the spermatophore itself and spermatophore-producing organs) and in sperm destruction (spermatophore receiving organ). Next, the molecular phylogeny will be used to determine the number and direction of changes of chirality, and correlations of chirality changes with changes in reproductive morphology, the expectation being that antisymmetry evolving from directional asymmetry is associated with increases in the coil complexity of the spermatophore tail, and vice versa.

Integration and broader applications

The results of Subprojects 1 and 2 will tell us (a) whether sexual selection maintains antisymmetry in *Amphidromus*, and (b) whether chiral reversals in the Camaenidae are associated with genital-morphological signals of sexual selection. These results may be generalised throughout the Gastropoda. Today, the Camaenidae, and in particular the genus *Amphidromus*, is one of a small number of snail taxa that display a particularly high degree of chirality reversals and antisymmetry. If our hypothesis is correct that such reversals are a by-product of the rapid evolution of (inherently asymmetric) genital structures, then pockets of chiral dynamism should have come and gone throughout gastropod evolution. This insight may therefore provide an evolutionary and phylogenetic framework for the evolution of the molluscan chirality-determining master-switch gene. The sequence and structure of this gene are expected soon, probably before the end of this 4-year project. If so, our project will open the way for tracking the molecular evolution of this gene in the context of phylogeny and sexual selection in the Camaenidae and other snail families. That, in turn, would open possibilities for further study of the evolution of downstream genes, and hopefully a full evo-devo understanding of gastropod chiral dynamism.

Moreover, our study system can be applied outside of the Mollusca. The evolutionary patterns seen in gastropod coiling direction are mirrored by other body asymmetries, such as insect and mammal genital asymmetry²⁸, claw asymmetry in crustaceans²⁶, body asymmetry in flatfish³² and frequency of heart orientation reversal (situs inversus) in vertebrates³². An approach like ours with snail coiling, that integrates evolutionary ecology, developmental genetics, and phylogenetic character analysis, holds great promise for revealing key insights about the evolution in chirality in these character systems, in particular since some of the symmetry-breaking genes appear to be conserved across widely divergent animal groups²⁶.

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5. Timetable of the project

Please provide the time schedule of the entire project. Specify the activities to be carried out as part of the project.

Literature study	Jan-Feb 2015
Writing Introduction of thesis	Feb-Mar 2015
Pilot study for subproject 1a	Feb-Mar 2015
Field work Malaysia (sampling for subprojects 1b and 2)	Apr 2015
Mate-choice experiments (for subproject 1a)	Apr 2015 – Nov 2015
Dissections (for subproject 1b)	Apr – Jun 2016
Sperm counts (for subprojects 1a and 1b)	Jun – Dec 2015
Data analysis and writing two thesis chapters on subprojects 1a and 1b	Jan – May 2016
Publishing these chapters as a single journal paper	Jun 2016 – Aug 2016
Conference visit—presentation of results	Jun 2016
Field work Southeast Asia (sampling for subproject 2)	Jun – Jul 2016
Anatomy and morphometrics	Aug 2016 – May 2017
Next-generation sequencing	Sep – Oct 2016
De-novo assembly and marker selection	Nov 2016
Multiplex PCR and Ion-Torrent sequencing	Dec 2016 – Dec 2017
Phylogenetic analysis	Oct 2017 – Mar 2018

Writing thesis chapter on camaenid phylogeny	Dec 2017 – Jul 2018
Character evolution analysis	Mar – Jun 2018
Co-organising chirality evolution workshop at Lorentz Centre	May 2018
Writing thesis chapter on character evolution in camaenids	Jun – Sep 2018
Publishing these two chapters as a single journal paper	Sep - Oct 2018
Curating collection material, digitizing data, making data accessible	Nov 2018
Writing Conclusion of thesis—sending thesis to reading committee	Dec 2018

6. Scientific embedding of the proposed research

Affiliation with national and international research programmes, national and international collaborations

This project forms part of the Left-Right Asymmetry research group of Naturalis Biodiversity Center (Naturalis), in collaboration with the Institute Biology Leiden (IBL). Over the past few years, the research programme for this group has been shaped by close interaction with Rich Palmer, who was a KNAW visiting professor in 2010 and 2011, and who co-organised with us a symposium on the evolution of chirality at the ESEB congress in 2011. Although the broader theme of left-right asymmetry (encompassing Naturalis researchers with expertise on various animal and plant taxa) is new and has been in existence only since 2010, Naturalis + IBL have a long history in studying the evolution of coiling direction in snails, founded by the seminal paper by Gittenberger (1988), via series of studies by Van Batenburg & Gittenberger (1996) and culminating in intensive work in the past decade, with numerous studies carried out by Naturalis-researchers Gittenberger (now retired), Uit de Weerd, Schilthuizen (starting while still at Universiti Malaysia Sabah; UMS), Hosono (now at Kyoto Univ.), and others. As is obvious from the research team and co-authorships, close collaborations have been fostered with other research groups locally and worldwide that work on the developmental genetics and evolution of gastropod chirality, such as those at the IBL (Spaink), Tohoku University (Chiba), Kyoto Univ. (Hosono) and the University of Nottingham (Davison). Moreover, K. Shimizu (see references) has applied for a JSPS fellowship to work at Naturalis. All this will ensure that the PhD student will benefit from being “in the loop” where the newest insights are concerned.

For the success of this particular project, other collaborations are relevant. For the histology and micro-anatomy of the *Amphidromus* reproductive tract, and for the interpretation of the results in the context of hermaphrodite sexual selection, the expertise of Joris Koene and his team at VU University, Amsterdam, will be vital (Koene is also research associate at Naturalis). For micro-histology, as well as exploratory soft-body micro-CT-scanning, we will collaborate with the group of Martin Haase at the University of Greifswald, Germany, who has been involved with earlier *Amphidromus* studies of ours.

For field work, and for sourcing samples, close collaboration with the project coordinator’s previous employer, UMS will be particularly useful. The project coordinator has a long-term research permit for Malaysia, and permissions to sample in other countries will be obtained by the PhD student.

For the phylogenetic reconstruction of the Camaenidae, we will link up tightly with two other phylogenetic projects on (parts of) this family, namely the work on using next-generation sequencing for phylogenetic resolution of the Australian Camaenidae (Teasdale et al., 2013) and the molecular phylogenetics on the east-Asian Camaenidae at the labs of Chiba and Hosono.

Finally, close collaborations exist with several teams in The Netherlands, particularly in Leiden, who use and generate next-generation sequencing data for phylogenetics. Worthy of mention are the team of Spaink (IBL; Leiden), the Leiden Genome Technology Centre, and the company BaseClear (with whom we obtained experience in the reduced genome representation technique, published as Lammers et al. [2013], which we will also employ in this project).

In addition to these team members, we engage in lively correspondence with others who have worked on the phylogenetics of the Camaenidae, such as Bronwen Scott (Victoria Univ., Melbourne), Frank Köhler (Australian

Museum), and Chris Wade (University of Nottingham). These collaborators will be able to provide preserved material, and comment on the interpretation of the phylogenetic reconstructions resulting from the present project.

7. Knowledge utilisation (max. 600 words)

Please note that the score for the assessment criterion 'knowledge utilisation' will be determined at the ALW-office based on your answers to the questions per aspect. The referees and the committee do not judge this criterion. Please answer the questions for all 8 aspects:

- 7A: Beneficiaries identified: Potential knowledge users are:

(a) Research groups working on molecular phylogenetics of molluscs: in Subproject 2, we will develop novel markers which will form tools that are likely to be employed by the ca. 50 research groups world-wide that are moving into next-generation molecular phylogenetics of this species-rich phylum.

(b) Research groups working on the embryology, developmental biology, and evo-devo of asymmetry in vertebrates and other taxa that show similar patterns of chirality, and/or that also rely on the same symmetry-breaking genes as mollusks.

- 7B: Stakeholder meetings: The knowledge will be transferred to these stakeholders via publication in novel, open-access, and broad-scope journals, such as *PLoS ONE* and *PeerJ*, as well as conferences, such as the World Congress of Malacology and the ESEB-meetings.

- 7C: Beneficiaries confirmed: N/A.

- 7D: Education: The researcher has already been trained in molecular phylogenetics at the International Molecular Evolution course in the Czech Republic, and in next-generation sequencing via an internship with Peter de Knijff's group at the LUMC. Therefore, the focus of his training during the PhD will be on statistics, data management, and scientific writing, through courses offered by the Research School PE&RC.

- 7E: Data management: Data will be managed via the Google Apps system of Naturalis Biodiversity Center.

- 7F: Data distribution or integration: DNA sequence files will be uploaded to the Sequence Read Archive of Genbank. The experimental results will be uploaded to Dryad. Voucher specimens will be placed in the collections of Naturalis Biodiversity Center, which are currently being fully digitized and made accessible through GBIF.

- 7G: Outreach method identified: The Naturalis research group Character Evolution has been extremely active in outreach activities to the general public, through exhibits, press releases, TV and radio presence and the traditional news press. We aim to do the same with this project, which lends itself eminently for visualisation and public outreach.

- 7H: Outreach time schedule and budget: Naturalis Biodiversity Center is a research institution with a public museum (300,000 actual and >1,500,000 virtual visitors annually). It has very active Education and Science communication departments. We will make ample use of these institute facilities to garner interest for our outreach programs. Specifically, we are planning:

(a) press releases with each publication and with the PhD defense

(b) yearly public talks on snail coiling direction in our "LiveScience" hall for museum visitors

(c) display items on snail coiling direction in the new permanent exhibition, to open in 2017.

8a. Budget

	Year 1	Year 2	Year 3	Year 4
Personnel (mm)	1 PhD stud.	1 PhD stud.	1 PhD stud.	1 PhD stud.
Research costs (k€)				
Equipment	10250			
Consumables*	3000	22500	3000	
Fieldwork/Travel*	3500	1300	6275	

* The sums requested for consumables and fieldwork/travel expenses combined should not exceed 50,000 euro for the entire grant period.

Specification of the requested funds:

Equipment:

digital camera ¹	€ 750
field notebook computer (Toughbook) ¹	€ 2500
high-end GPS ²	€ 600
videography set-up (series of parallel cameras) ³	€ 6400

Consumables:

field consumables (vials, boxes, bags, alcohol)	€ 1500
lab experimentation consumables (boxes, trays, etc.)	€ 1500
HiSeq sequencing costs + bioinformatics	€ 10000
Ion Torrent sequencing costs	€ 12500
histology and electron microscopy consumables	€ 3000

Fieldwork/Travel:

1. field work in Malaysia (1 month)	
flights ⁴ :	€ 2000
land transportation costs:	€ 350
lodging costs:	€ 1150
2. conference visit	
flight:	€ 800
lodging costs:	€ 500
3. field work elsewhere in Southeast Asia (6 weeks)	
flights ⁵ :	€ 3000
land transportation costs ⁶ :	€ 2150
lodging costs ⁷ :	€ 1125

Justifications:

1. *necessary for documenting copulation behaviour in the field and in the lab.*
2. *essential for recording the collection localities in areas with dense tree-cover.*
3. *required for continuous time-lapse videotaping of mating experiments*
4. *one return ticket Amsterdam – Kuala Lumpur, and domestic legs as follows: Kuala Lumpur – Kota Kinabalu v.v.; Kuala Lumpur – Kuala Terengganu v.v., incl. carbon credits.*
5. *one return ticket Amsterdam – Kuala Lumpur, and from there legs as follows: Kuala Lumpur – Taipei; Taipei – Sendai; Sendai – Kota Kinabalu; Kota Kinabalu – Manila; Manila – Jakarta; Jakarta – Kuala Lumpur, incl. carbon credits.*
6. *4-wheel drive vehicle rent: € 245 / week; fuel: € 800*
7. *€ 25 / day*

8b. Financial assistance from other sources

The PhD student will make use of the courses available via the research school PE & RC, for which Naturalis Biodiversity Center donates a contribution. In addition, all microscope, culturing, morphology, and molecular biology facilities, as well as the routine, small-scale running costs for them, will be borne by Naturalis and IBL.

9. Statements by the applicant

N/A	I endorse and follow the Code Openness Animal Experiments (if applicable).
N/A	I endorse and follow the Code Biosecurity (if applicable).
YES	By submitting this document I declare that I satisfy the nationally and internationally accepted standards for scientific conduct as stated in the Netherlands Code of Conduct for Scientific Practice 2012 (Association of Universities in the Netherlands (VSNU)).

YES I have completed this form truthfully.

YOUR DETAILS:

Name: Menno Schilthuis

Place: Leiden

Date: 31 January 2014

Please submit the application to NWO in electronic form (pdf format is required) using NWO's electronic application system, which can be accessed via the NWO website. The application must be submitted from the account of the main applicant. For any technical questions regarding submission, please contact the helpdesk (iris@nwo.nl).
