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TriPTIC

Trichogramma for plant protection: Pangenomics, Traits, and establIshment Capacities

Programme CE18 2014

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A IDENTIFICATION

Acronyme du projet	TRIPTIC
Titre du projet	*Trichogramma* for plant protection: Pangenomics, Traits, and establIshment Capacities
Coordinateur du projet (société/organisme)	Jean-Yves Rasplus (INRAE)
Période du projet (date de début – date de fin)	1 octobre 2014 30 septembre 2019
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B RESUME CONSOLIDE PUBLIC

B.1 RESUME CONSOLIDE PUBLIC EN FRANÇAIS

Vers un usage efficace des trichogrammes en lutte biologique contre les ravageurs

Améliorer notre connaissance des processus écologiques et évolutifs sous-jacents, pour répondre au challenge sociétal du développement de la lutte biologique

Le plan Ecophyto souhaite mettre en place des méthodes de contrôle biologique des ravageurs comme une alternative à l'usage des pesticides. Or, la mise en place d'un contrôle biologique efficace et sûr dépend d'une : 1) caractérisation génétique et phénotypique des souches d'ennemis naturels relâchés ; 2) bonne connaissance de leur biologie, stratégie d'exploitation de l'hôte et dynamique populationnelle ; 3) meilleure compréhension des mécanismes déterminant leur établissement. Le projet TRIPTIC cible un groupe de micro guêpes parasitoïdes, les trichogrammes, un des insectes les plus utilisés en contrôle biologique des ravageurs des cultures pour 1) mieux définir les espèces et inférer leur relations évolutives ; 2) explorer leur microbiome afin de mieux comprendre son influence sur leur biologie ; 3) phénotyper des traits biologiques importants pour le contrôle des hôtes ; 4) prédire le succès d'établissement des souches relâchées et analyser comment les dynamiques locales et spatiales structurent la dynamique des guêpes relâchées. Nous voulions aussi implémenter des bases de données sur les trichogrammes et une collection de souches vivantes utilisées par le projet.

Développer des méthodes haut-débit et acquérir des données génomiques et phénomiques massives pour un meilleur usage des Trichogrammes

Nous avons conduit, en 2015-16, des collectes importantes de trichogrammes. Nous avons développé des méthodes pour i) séquencer des milliers de marqueurs à partir d'un seul individu (< 1mm), ii) analyser ces séquences. Nous avons barcodé des centaines de guêpes pour mieux caractériser les souches conservées dans le Centre de Ressource Biologique EP-coll d'Antibes et utilisé des approches métagénomiques ou PCR pour caractériser leur microbiome et tester la présence d'endosymbiontes. Plusieurs outils ont été développés pour automatiquement phénotyper ces souches : i) analyse d'image

pour compter les œufs parasités, ii) approches vidéo automatisées pour analyser les comportements et les métriques des mouvements, iii) tunnel de vol pour tracer les déplacements des trichogrammes et iv) arène thermique. Nous avons aussi développé les méthodes informatiques nécessaire à l'analyse des résultats. Enfin, nous avons utilisé des microcosmes dans lesquels nous contrôlions et manipulions les facteurs démographiques, génétiques et environnementaux afin de suivre des populations de trichogrammes sur de nombreuses générations. Le CRB EP-Coll a joué un rôle fondamental en maintenant les souches élevées pour le projet, et en fournissant du matériel mort ou vivant. Nous avons délimité les espèces présentes en utilisant des milliers de marqueurs distribués sur le génome des trichogrammes et mis en place des outils d'identification moléculaire fiables. Les *Wolbachia* sont les principaux endosymbiontes détectés et leur taux d'infestation varient entre espèces, nous étudions la possible intégration de leur génome dans des souches aposymbiotiques. Nous avons mis en évidence des comportements de ponte liés aux strates des plantes et avons démontré que les démographies hétérogènes des souches pouvaient induire des dynamiques démo-génétiques différentes dans le temps et l'espace. Les résultats du projet TRIPTIC ont permis d'obtenir de nouveaux financements (y compris ANR) pour poursuivre nos investigations sur des aspects appliqués et fondamentaux. Six publications dans des revues internationales et douze autres en révision ou sur des archives ouvertes ont été produites, et nos travaux ont été présentés dans 11 congrès nationaux et internationaux. Deux thèses de doctorat ont été soutenues et des publications grand public ont vulgarisé nos résultats. Enfin, le CRB EP-coll s'est durablement installé dans le paysage français des ressources biologiques agronomiques.

T. brassicae which is used to control some Lepidoptera worldwide was one of the species studied during the project.

TRIPTIC est un projet de recherche fondamental à but appliqué dirigé par J.Y. Rasplus avec l'aide de V. Calcagno, A. Cruaud, L. Mouton, N. Ris and E. Vercken. Il implique des équipes de trois UMRs françaises ISA, LBBE et CBGP. Le projet a commencé en 2016 pour une durée de 60 mois mais se continue dans les faits sous d'autres formes. Il a bénéficié d'un financement de 600 000 € de l'ANR pour un coût global de 3 397 220 €.

B.2 RESUME CONSOLIDE PUBLIC EN ANGLAIS

On the way to efficiently use* Trichogramma *wasps for biological control of pests

Refine our global knowledge of evolutionary and ecological processes at play, to address the societal challenge of developing biological control

The Ecophyto plan advocated the use of biological control against pests as alternatives to pesticides. It is acknowledged that successful and safe biological control depends on: 1) accurate genetic and phenotypic characterization of the strains of natural enemies released; 2) strong knowledge of their life-history traits, strategies of host exploitation and population dynamics; 3) good understanding of the processes determining their successful establishment. The TRIPTIC project focussed on a group of tiny parasitoid wasps, the genus *Trichogramma*, one of the most commercialized macro-organisms to control pests. It aimed at: 1) investigating species limits and infer relationships among species; 2) explore microbiome to better understand their influence of life-history traits; 3) phenotype life-history traits of interest for biocontrol; 4) predict establishment success of released strains to investigate how local and spatial dynamics interact and shape the dynamics of released wasps. We also wanted to build a comprehensive database implementing our knowledge on these wasps and a reference collection of living strains of *Trichogramma* that could be used by the project and professionals.

Developing new high-throughput tools to acquire massive genomic and ethomic data and improve the use of* Trichogramma *in biocontrol programs

Large surveys were conducted between 2015 and 2016, to sample *Trichogramma*. We developed methods to i) sequence pangenomic markers from single *Trichogramma*, ii) analysed sequencing data. We also sequenced barcode markers on hundreds of wasps to provide characterized strains to the Biological Resource Centre EP-coll. We used metagenomic approach to characterize bacteria present in *Trichogramma* strains and test the presence of heritable symbionts *(Cardinium,* etc) using PCR. We developed innovative tools to automatically phenotype numerous strains: i) an image analysis method to count numbers of parasitized *Ephestia* eggs, ii) A video-analysis pipeline to compute behavioral and movement metrics, iii) a 6-meter-long tunnel to track *Trichogramma* individuals and iv) a thermo-arena. We also developed pipeline to analyse the data obtained. Finally, we used laboratory microcosms, where demographic, genetic and environmental factors are controlled and manipulated and where populations can be monitored over a large number of generations. The BRC EP-Coll played a key role in maintaining biological material for TRIPTIC partners, dead individuals or living strains.

We used 30,000 pangenomic markers to delimit species and develop molecular tools for fast identification. *Wolbachia* was the only endosymbiont detected and its infection varied among species. Oviposition preference behaviors correlated with strata. We observed *sedentary* vs *explorer* modes in movement behaviour, that may have plastic determinism. We demonstrated heterogeneity in strain demography inducing contrasted demo-genetics dynamics in time and space. A strong dynamic was developed, that led to the granting by ANR of both applied and fundamental projects solicited by stake-holders. Six publications in international journals and twelve are under review or available as preprints. Results were presented to 11 national and international conferences. We wrote publications for the general public, two phD thesis were completed. The tools developed are of general interest for other researchers. The Biological Ressource Centre EP-Coll is well-established in the landscape of agronomic resources.

TRIPTIC is a fundamental research project leaded by J.Y. Rasplus with V. Calcagno, A. Cruaud, L. Mouton, N. Ris and E. Vercken, and involving teams of three French laboratories ISA, LBBE and CBGP. The project started in 2016 for a period of 60 months, but in fact is still going on. It benefits from an ANR grant of 600000 € for a global cost of ca 3 397 220 €

C MEMOIRE SCIENTIFIQUE

Mémoire scientifique confidentiel : non

C.1 RESUME DU MEMOIRE

The Ecophyto plan advocated the use of biological control against pests as alternatives to pesticides. It is acknowledged that successful and safe biological control depends on: 1) accurate genetic and phenotypic characterization of the strains of natural enemies released; 2) strong knowledge of their life-history traits, strategies of host exploitation and population dynamics; 3) good understanding of the processes determining their successful establishment. The TRIPTIC project focussed on a group of tiny parasitoid wasps, the genus *Trichogramma*, one of the most commercialized macro-organisms to control pests. It aimed at: 1) investigating species limits and infer relationships among species; 2) explore microbiome to better understand their influence of life-history traits; 3) phenotype life-history traits of interest for biocontrol; 4) predict establishment success of released strains to investigate how local and spatial dynamics interact and shape the dynamics of released wasps. We also wanted to build a comprehensive database implementing our knowledge on these wasps and a reference collection of living strains of *Trichogramma* that could be used by the project and professionals.

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C.2 ENJEUX ET PROBLEMATIQUE, ETAT DE L'ART

C.2.1. Context and Objectives

Our project started with the Ecophyto plan, which recommended the development of biological control programs against pests as alternatives to pesticides (Herth, 2011). We focussed on *Trichogramma* (Hymenoptera), one of the most commercialized biocontrol organisms in Europe (Consoli et al 2010). Our project aimed at: 1) investigating species limits and infer phylogenetic relationships among species; 2) phenotype traits of interest for biocontrol; 3) predict establishment success of released strains. We also wanted to build a reference living collection for *Trichogramma*.

C.2.2. State of the art and challenges

Sampling, identification and management of biological resources. *Trichogramma* includes ca 40 species in Europe (Pintureau 2008), which is approximated as morphological identification is difficult and taxonomy requires improvement. Although surveys were conducted for applied purposes (e.g. Barnay *et al.* 2001; Herz *et al.* 2007, Romeis et al. 2005), the distribution of species is poorly known. *Trichogramma* is among the best case-study to improve organization of biorepository and increase traceability and quality of research in a framework of macro-organisms used as biocontrol agents.

Pangenomic characterization (Trichogramma and microbiome): Many species complexes of parasitoids are reported (e.g. Derocles et al. 2012) which makes sequencing of single specimens desirable. While new sequencing technologies (NTS) open avenues for the sequencing of thousands of genetic markers or the exhaustive characterization of the microbiome, the small size of single *Trichogramma* (< 1mm) makes sequencing challenging. In addition, reference databases for symbionts are still scarce. Finally, accurate analysis of the huge amount of data produced by NTS, while considering biological and methodological bias remains challenging (Kumar et al. 2012).

Phenotyping: Phenotypical assays of life-history traits are time-consuming and tedious. Hence, phenotyping is usually done with idiosyncratic methods and experimental effort, as well as little standardization/sharing. This concerns phenology or mortality curves, but also complex phenotypes such as spatial orientation, exploration and host-seeking behaviors that are unexploited. New ethomics methods are emerging but the minute size of *Trichogramma* constrains their use.

Establishment capacities: Demographical traits are likely to be good predictors of establishment success, yet they are rarely measured because they require high and long-time sampling effort. In addition, introductions of potentially invasive species raise ethical issues, which prevents experiments in the field and constrains them at lab. Natural populations are influenced by many factors, often hard to identify, which makes the comparison of these populations challenging. Consequently, strategies need to be implemented to better control experimental conditions, while still allowing the apparition of population-level emerging properties that derive from individual characters.

C.3 APPROCHE SCIENTIFIQUE ET TECHNIQUE

The project was organized in four work packages (WP1-4) completed by a coordination WP (WP0) and a transversal WP (WP5) in charge of sampling; strain maintenance and knowledge transfer towards stakeholders. More details for each WP are provided in the ANNEXES.

WP1: From genome to species: pangenomic characterization. Coord P1. A. Cruaud.

We developed methods to i) sequence pangenomic markers from single *Trichogramma* wasps ii) analyse sequencing data. Then, we used these methods on specimens collected by WP5 and compared results with Sanger sequencing of 5 markers as well as morphology to provide characterized strains to the EP-coll BRC. Two different protocols were developed: 1) sequencing of DNA sequences associated with restriction sites (RAD-seq); 2) capture of Ultra-Conserved Elements (UCEs) with hybrid probes. RAD-seq requiring a large amount of DNA, we first tested the accuracy of whole genome amplification (WGA) by comparing the number of loci /alleles obtained in RAD libraries produced from i) WGA of a single diploid female and ii) a pool of its haploid sons. We also set up a complete protocol for the capture of hundreds of UCEs from undetectable amount of DNA. Bioinformatic pipelines were developed for the accurate analysis of RAD-seq and UCE data sets.

WP2: From microbiome to phenotype: massive screening of symbionts. Coord P3. L. Mouton

Illumina sequencing of amplicons of the 16S ribosomal RNA (rRNA) gene was performed for a global characterisation of the microbiome. In addition, we used specific PCR to detect heritable symbionts *(Cardinium, Rickettsia, Hamiltonella, Arsenophonus, Spiroplasma, Wolbachia*). We performed a thorough analysis of the presence of *Wolbachia* with real-time quantitative PCR and Multi-Locus Sequenced Typing (MLST) of five genes to identify diversity and variability of strains.

WP3: From individual phenotype to species: high-throughput phenotyping. Coord P2. V. Calcagno

We developed high-throughput methods to characterize traits of interest in *Trichogramma*. Four tasks were pursued: 1) automatic counting of parasitism rates on host eggs; 2) measurement of cold tolerance;

3) characterization of movements and interactions in groups of wasps; 4) characterization of spatial spread, at medium spatial and temporal scales. Tasks were achieved thanks to (i) acquisition of novel imaging equipment; (ii) development of novel phenotyping devices and experimental protocols; (iii) development of new computer codes, algorithms and scripts to analyze data.

WP4: From phenotype to population dynamics establishment success and community performance. Coord P2. E. Vercken

We used an original experimental set-up for mimicking introductions of *Trichogramma* in a meta-population context, which proved its relevance to address questions related to the ecology of early establishment and expansion of introduced populations. We quantified population-scale functional traits (growth rate, presence of positive density-dependence, demographic stochasticity, etc.) and their variation among strains as well as development time and sex-ratio. We also characterized spatial dynamics (local extinction rate, colonisation rate, individual heterogeneity in dispersal phenotypes) and its variation among strains. Finally, we investigated how the presence of positive density-dependence (in growth or dispersal) could affect expansion dynamics.

WP5: From collection to transfer: The Biological Resource Center (Leader N. Ris, ISA)

Sampling was conducted in 2015-2016 on 92 sites. We also sampled along an altitudinal gradient including Meso-Mediterranean and the Supra-Mediterranean. We mainly relied on the exposure of sentinel eggs of *Ephestia kuehniella* but we also sampled natural egg clutches and performed sweeping/trapping. Additionally, samples were provided by colleagues (FREDON Martinique, Algeria, Benin, Iran, Romania, Serbia, Turkey, Ukraine). Integrative characterization of strains was performed with: (i) Sanger sequencing of part of the mitochondrial (mtDNA) gene *COI*, (ii) Sanger sequencing of complementary markers (*Cyt*b, *Wg*, *Ef1-α*, *Rpl27a*, *RpS4*), (iii) crossing experiments, (iv) mounting of male genitalia, antennae and wing for durable storage and acquisition of a high-resolution iconography.

C.4 RESULTATS OBTENUS

All strains/species were kept in the BRC EP-Coll, that was certified ISO9001:2015 by AFNOR in Fall 2018, certification confirmed in Fall 2019. The BRC played a central role by maintaining material for all partners, dead individuals (for WP1 and WP2) or living strains (WP3 and WP4). We sequenced partial *COI* gene for +2000 individuals representing 600 *Trichogramma* samples (Warot 2018). This unprecedented dataset completed by sequencing of 5 other genes (nuclear and mtDNA) enabled to identify *ca* 120 haplotypes, classified into 23 clusters with only 11 associated with a single species name (Annexe, Figure 12). The three largest clusters were associated with different names and considered as species complexes. Crossing experiments confirmed reproductive isolation between differentiated clusters. However, no clear reproductive barriers but variability in reproductive compatibilities were highlighted in the "*brassicae–euproctidis*" complex. The protocols to sequence and analyse (software RADIS; Cruaud et al 2016) hundreds of makers from single wasps with (RAD-seq; Cruaud et al 2018) and without (UCEs; Cruaud et al 2019) amplification of its genome were used to better characterize strains. In addition, 170 slides and photo plates were assembled for more than 20 *Trichogramma* strains (Annexe, Figure 13). By comparing Sanger and RAD-seq results while considering morphology, we started to revisit the systematics of European *Trichogramma*. The RAD-seq tree is presented in Figure 5 of the annexes. Interestingly, there is agreement between *COI* barcode fragment used by WP5 and the RAD tags with the exception of some complexes (e.g. *T. daumalae*).

Aside of this in-depth characterization of species/strains, we showed that the microbiome of analysed *Trichogramma* was simple, apparently only composed of *Wolbachia* with variability of prevalence between species (Table 1 of the annexes). Infections did not appear to be correlated with *Trichogramma* phylogeny, as exemplified by the "*cacoeciae – embyophagum*" complex, which contains 1) aposymbiotic thelytokous strains within *T. cacoeciae s.s.* and 2) *Wolbachia* infections and presence of males (arrhenotoky) in several strains of *T. embryophagum s.s.* (Annexes Figure 7). This last result could suggest that part of the genome of *Wolbachia* has been integrated within the genome of *T. cacoeciae*; an hypothesis we are testing with NTS approaches (analyses under progress see annexes).

Innovative tools were developed for phenotyping: 1) CODICOUNT, to automatically count the number of parasitized eggs (Perez et al. 2017); 2) A video-analysis pipeline to summarize activity levels and trajectories of individuals; 3) A 6-meter-long tunnel to track individuals; 4) a device and associated software to study thermal biology (see annexes for details). Oviposition preference behaviors (20 strains in five species) seemed to correlate with sampling strata. Significant variation was found in geotactic (mostly negative) and phototactic (mostly positive) preferences. Light and gravity preferences were not correlated across strains, but sometimes showed negative interactions and were time dependent. A significant intra-specific variability that was more or less significant according to phenotyped traits (thermal indices, diapause or overwintering) was highlighted between strains sampled in south-eastern France and along the altitudinal gradient, which may suggest adaptive differentiation. Indeed, patterns were consistent with differences in severity of winters. Patterns of activity differed across species, partly reflecting their phylogeny. No association of movement strategies was found with sampling strata. For the first time, we identified two movements called 'sedentary' and 'explorer', between which individuals switch over the course of several hours. This heterogeneity is key to explain population spread and distribution of parasitism event: without considering it, predictions on speed of spread or population distribution are incorrect.

Regarding establishment capacities, we showed that development times were significantly different between strains, and we identified a "slow-developing" group (incl. strains from *T. cacoeciae* and *T. principum*) and a "fast-developing" group (incl. strains from *T. brassicae, T. euproctidis, T. evanescens and T. semblidis*). In addition, several populations displayed positive density-dependence and poorer demographic performance on the whole (lower maximum growth rate and population size, higher extinction rate, Vercken and Mailleret 2020). We also highlighted that positive density-dependence induced "pushed" expansion waves, whose speed depended on carrying capacity (Haond et al., 2018). More pushed populations expanded at a slower rate and experienced more frequent local extinctions on the expansion front. Finally, we found that restricted connectivity induced pushed-like expansions, where neutral genetic diversity was conserved longer on the front than in pulled expansions (Dahirel et al. 2020). In contrast with pulled expansions, front populations in pushed-like expansions remain globally similar to core populations.

C.5 Exploitation des resultats

Results were published in 6 publications in international peer-reviewed journals. Further, two manuscripts available as preprints and four have been submitted recently and are still under review. Six are currently in preparation. We were also invited to present our work to four international conferences and seven national conferences and to give lecture in Brazil and China. Several communications and publications were written for the general public. Finally, 2 PhD theses were completed during the course of the project. Aside of these academic results TRIPTIC enabled the development of devices (Thermo-arena, Double spirale) and softwares /scripts (CODICOUNT, Spartacus, RADIS etc.) that are of interest for researchers largely beyond the scope of the project.

C.6 Discussion and conclusion

Globally, we believe that we successfully worked together to meet our goals and fulfil our objectives. Major challenges caused by the small size of *Trichogramma* were overcome and we developed new genomic and ethomic tools. These tools allow the sequencing of thousands of genetic markers throughout the genome of minute wasps and analyses of complex behavioral traits on hundreds of samples. This is the first time such progress is achieved on tiny insects and this opens new avenues for a better use of *Trichogramma* as biological control agents, biocontrol in general and other areas of research (e.g. minute arthoropods vectors of human diseases). A real dynamic has been started thanks to the project, and we have been solicited by stake-holders to set up more applied projects. Of course, the ambitions of TRIPTIC were high and hard to fulfil within the course of the project. Development of new tools and sampling on the field is time consuming and there are still many works under progress (see annexes). Protocols, pipelines, data and results have been accumulated by all WPs but we are in the

process of taking a step back to integrate all data in a single framework. This is why we have launched several initiatives, that largely relied on TRIPTIC results and developments:

- Successful funding of the ANR Ecophyto Maturation project BIDIME (2020-2023; coordinator P2: N. RIS), (https://ecophytopic.fr/proteger/bidime-biodiversite-des-trichogrammes-diversification-des-produits-de-biocontrole-et) in which, strains obtained during the TRIPTIC project will be evaluated as candidate biocontrol agents of lepidopteran pests in ornamental and vegetable greenhouses. Links between phenotypes and genotypes will be also thoroughly analysed.
- Successful funding of an ANR on pushed vs pulled expansions and their consequence on demographic and evolutionary processes (ANR JCJC "PushToiDeLa", 2018-2022, PR: E. Vercken).
- Spin-off projects such as ODORAMMA (2016-2020), PHENOMENE (2017-2019) or SCALUP (2020-2021) have also been obtained to sustain new ethomics developments (PR: V. Calcagno)
- New surveys of *Trichogramma* biodiversity, in Department of Ain (collaboration with Fondation Pierre Vérots: http://cwww.fondation-pierre-verots.com/www.fondation-pierre-verots.com/index.html) and in Corsica (project 2018-2021 Protect'Agrumes).

Finally, thanks to TRIPTIC, the BRC EP-Coll - one of the first biodepository for biocontrol macro-organisms - is now well-established within the national landscape of agronomic resources. EP-Coll is becoming one of the main actor in the Pilar "Environment" in RARe:, https://www.agrobrc-rare.org/).

C.7 REFERENCES

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D LISTE DES LIVRABLES

Quand le projet en comporte, reproduire ici le tableau des livrables fourni au début du projet. Mentionner l'ensemble des livrables, y compris les éventuels livrables abandonnés, et ceux non prévus dans la liste initiale.

Date de livraison	N°	Titre	Nature (rapport, logiciel, prototype, données, …)	Partenaires (souligner le responsable)	Commentaires
2018	1	establishing the pertinence of WGA methods for reduced representation libraries generation.	Data	P1, A Cruaud	Published in Cruaud et al 2018
2016	2	bio-informatic pipeline for RAD-seq (and similar) data processing	Software	P1, A Cruaud	Published in Cruaud et al 2016
2017	3	easy-to-use identification tools adaptation/invasion potential	Data	P1-P2, A Cruaud, N. Ris	Warot (2018). Used for BRC EP-coll
2016-2020	4	characterisation of strains and gene flow + insights into the evolutionary history of the species complexes	Data	P1-P2, A Cruaud, N Ris	Warot (2018). Publication RADseq in prep. See annexes.

Date de livraison	N°	Titre	Nature (rapport, logiciel, prototype, données, …)	Partenaires (souligner le responsable)	Commentaires
					Communication Cruaud et al. 2016
Postponed	5	towards a precise genetic characterisation of phenotypic traits	Data	P1-P2, A Cruaud, V. Calcagno	Re-planed in the ANR project BIDIME (maturation)
abandoned	6	link between hybridization and presence/absence of symbionts; prediction on hybridization potential of released strains	Data	P1-P3, A Cruaud, L. Mouton	Abandoned not enough data for symbionts
2019	7	Capture of thousands of markers (UCEs) from single *Trichogramma*	Data	P1, A Cruaud	Not initially planned but very promising. Published in Cruaud et al 2019
2019	8	Bioinformatics pipeline for the analysis of UCE data	Data	P1, A Cruaud	Not initially planned but very promising. Published in Cruaud et al 2019
2017		Marchand et al. 2017	Technical paper	ISA	WP5
2018		Release of COI sequences in Genbank	Data	ISA	WP5
2018		Dumbardon et al. 2018	Entomological paper	ISA	WP5
2018		Warot 2018	EPHE thesis ms	CBGP, ISA, LBBE	WP2 & WP5
2018		ISO9001 :2015 (initial audit)	Certification	ISA	WP5
2018		Mougin et al. 2018	Scientific paper	ISA	WP5
2019		Ion Scotta 2019	PhD thesis ms	ISA	WP3 & WP5
2019		ISO9001 :2015 (surveillance audit)	Certification	ISA	WP5
2020		Declaration of invention for 'Thermo-Arena' & 'Spartacus' software	Prototype	ISA	WP3

E IMPACT DU PROJET

E.1 INDICATEURS D'IMPACT

Nombre de publications et de communications (à détailler en E.2)

		Publications multipartenaires	Publications monopartenaires
International	**Revues à comité de lecture**	1 [+1 submitted]	5 [+2 preprints + 3 submitted]
	Ouvrages ou chapitres d'ouvrage		
	Communications (conférence)	2	8
France	**Revues à comité de lecture**		2

	Ouvrages ou chapitres d'ouvrage		2 (thèses)
	Communications (conférence)	1	9
Actions de diffusion	**Articles vulgarisation**		
	Conférences vulgarisation		
	Autres	1 (lecture)	

Autres valorisations scientifiques (à détailler en E.3)

	Nombre, années et commentaires (valorisations avérées ou probables)
Brevets internationaux obtenus	
Brevet internationaux en cours d'obtention	
Brevets nationaux obtenus	
Brevet nationaux en cours d'obtention	
Licences d'exploitation (obtention / cession)	
Créations d'entreprises ou essaimage	
Nouveaux projets collaboratifs	ANR BIDIME (2020-2024)
Colloques scientifiques	
Autres (préciser)	i) Logiciel RADIS (2016) ii) Logiciel CODICOUNT (2017) iii) Prototype Thermo-Arena and Spartacus software (2018) iv) Biological Resource Center EP-Coll that was ISO9001:2015labelled by AFNOR in 2018

E.2 LISTE DES PUBLICATIONS ET COMMUNICATIONS

Publications internationales / Revues à comité de lecture.

Cruaud A, Gautier M, Rossi J-P, Rasplus J-Y, and Gouzy J. 2016. RADIS: Analysis of RAD-seq data for InterSpecific phylogeny *Bioinformatics* 32:3027-3028. 10.1093/bioinformatics/btw352 [WP1 ; P1]

Cruaud A, Groussier G, Genson G, Sauné L, Polaszek A, and Rasplus J-Y. 2018. Pushing the limits of whole genome amplification: successful sequencing of RADseq library from a single microhymenopteran (Chalcidoidea, *Trichogramma*). *Peerj* 6:e5640. 10.7717/peerj.5640 [WP1 ; P1+P2]

Cruaud A, Nidelet S, Arnal P, Weber A, Fusu L, Gumovsky A, Huber J, Polaszek A, and Rasplus J-Y. 2019. Optimised DNA extraction and library preparation for small arthropods: application to target enrichment in chalcid wasps used for biocontrol. *Molecular Ecology Resources* 19:702-710. https://doi.org/10.1111/1755-0998.13006 [WP1, P1]

Dumbardon-Martial T, Lucas P-D, Warot S, Ris N & Groussier G. 2018. Diversité des Trichogrammatidae sur cultures maraîchères à la Martinique. *Bulletin de la Société Entomologique de France*. 123(1):73-75 doi : 10.32475/bsef_2009 [WP5, P2]

Haond M, Morel-Journel T, Lombaert E, Vercken E, Mailleret L, Roques L. 2018. When higher carrying capacities lead to faster propagation. *bioRxiv, 307322, ver. 4 recommended and peer-reviewed by PCI Ecol*. [WP4, P2]

Mougin C, Artige E, Marchand F, Mondy S, Ratié C, Sellier N, Castagnone-Sereno P, Coeur d'Acier A, Esmenjaud D, Faivre-Primot C, Granjon L, Hamelet V, Lange F, Pagès S, Rimet F, Ris N, Sallé G (2018) BRC4Env, a network of Biological Resource Centres for research in environmental and

agricultural sciences. *Environmental Science and Pollution Research*. https://doi.org/10.1007/s11356-018-1973-7. [WP5, P2]

Preprint / HAL internationales

Vercken E, Mailleret L. 2020. The hidden side of Allee effects: correlated demographic traits and extinction risk in experimental populations. *Hal, 02570868*. [WP4, P2]

Dahirel M, Bertin A, Haond M, Blin A, Lombaert E, Fellous S, Calcagno V, Mailleret L, Vercken E. 2020. Shifts from pulled to pushed range expansions caused by reductions in connectedness. *bioRxiv* https://doi.org/10.1101/2020.05.13.092775 [WP4, P2]

Soumises à Publications internationales / Revues à comité de lecture.

Burte V, Prez G, Mailleret L, Calcagno V. 2020. When complex movement yields simple dispersal: a high-throughput analysis of population spread in an egg-parasitoid. Soumise. [WP3, P2]

Ion Scotta M, Magris L, Sellier N; Warot S, Gatti F, Siccardi F, Gibert P, Vercken E, Ris N. 2020. Genetic variability, population differentiation and correlations for thermal tolerance indices in the minute wasp, *Trichogramma cacoeciae*. Soumise à *Evolutionary Applications.* [WP3-5, P2]

Muru D, Marchand A, Calcagno C, Cruaud A, Rasplus J-Y, Ris N, Vercken E, Warot S, Groussier G. 2020. Survey of the diversity of *Trichogramma* species in France and neighbouring areas with information related to their host plants and habitats. Soumise à *Bulletin de la Société Entomologique de France.* [WP5, P1+2]

Warot S, Cruaud A, Groussier G, Malausa T, Martinez-Rodriguez P, Pintureau B, Séguret J, Ris N. 2020. Insights into the molecular diversity and species delineation in the genus *Trichogramma* with a focus on West Palaearctic. Soumise à *PCI Entomology.* [WP1,5, P1+2]

Communications internationales invitées

Cruaud A., Bout G., Genson G. & Rasplus J.-Y. Unjumbling the jumbled trichogrammatids: NGS to the rescue. *Symposium: Evolution and Biology of Chalcidoidea: Integrating Genomics, Fossils, Microbiomes, and Natural History. XXV International Congress of Entomology*, Orlando, USA September 24-30, 2016 [WP1, P1+P2]

Cruaud A., Rasplus J.-Y. 2018. RAD-seq for phylogenies and hybridization tests, methods, analytical pipeline and results. *School of Ecological and Environmental Sciences*, East China University, Shangai, China. October 22 2018. [WP1, P1]

Rasplus J.-Y. 2017. TRIPtic: A project on pangenomics, phenomics and population dynamics of *Trichogramma* to improve their use in biocontrol. 15th *Biological Control Symposium (SICONBIOL)*. 4-8 Juin 2017. Ribeirão Preto, Sao Paulo, Brazil [WP1-5, P1]

Vercken E. When *Trichogramma* ride the wave: evolutionary dynamics along pushed expansion fronts. *International Congress of Biological Control*, Beijing, China, 2018. [WP4, P2]

Vercken E. Phenotypic evolution along pushed expansion fronts. *ESA-ESC-ESBC Joint Annual Meeting*, Vancouver, Canada, 2018. [WP4, P2]

Communications internationales

Burte V., Prez G., Mailleret L., Calcagno V. 2018. Effect of density and host distribution on the spatial diffusion of *Trichogramma cacœciae.* European Congress of Entomology, Naples (Italie). Juin 2018. [WP3, P2]

Calcagno V., Burte V, Perez G, Lamare A, Mailleret L. 2019. A *high-throughput analysis of population spread in Trichogramma parasitoids.* Gordon Research Conference on Movement Ecology of Animals, Lucca (Barga), Italy. [WP3, P2]

Cruaud A., Nidelet S., Sauné L., Genson G., Cruaud P. & Rasplus J.-Y. 2018. Optimized workflow for amplicon, RAD and capture of UCEs from micro-hymenoptera. In: 9th International Congress of Hymenopterists, Matsuyama, Japan, 23-27th july 2018. [WP1, P1]

Haond M. 2018. Propagation in heterogenous landscapes for pushed and pulled populations. European Congress of Entomology, Naples, Italy, 2018. [WP4, P2]

Ris, N., Martinez-Rodriguez, P., Cruaud, A., Malausa, T., Rasplus, J.-Y., Groussier Bout, G., Warot, S., Seguret, J. 2016. Disentangling taxonomic issues and reproductive isolation patterns in complexes of cryptic species in the genus *Trichogramma*. Symposium: Industry-Academia Collaborative Research & Development in Biological Control of Arthropod Pests: Results from Four Years of Marie-Curie Staff Exchange, and Perspectives. XXV International Congress of Entomology, Orlando, USA September 24-30. [WP1, P1 P2]

Publications nationales / Revues à comité de lecture

Marchand A, Sellier N, Warot S, Ion Scotta M, Ris N, Groussier G. 2017. Formalisation d'un Centre de Ressources Biologiques dédiés aux parasitoïdes oophages. *Cahiers Techniques de l'INRA, numéro spécial consacré à l'Entomologie Appliquée* : 49-58. [WP5, P2]

Perez G, Burte V, Baron O & Calcagno V. 2017. Une méthode d'analyse d'image automatique pour quantifier rapidement les nombres d'œufs et les taux de parasitisme chez *Trichogramma sp*. *Cahiers Techniques de l'INRA; numéro spécial consacré à l'Entomologie Appliquée* : 59. [WP3, P2].

Communications nationales invitées

Cruaud A., Gautier M., Sauné L., Galan M., Genson G., Nidelet S., Godefroid M., Kerdelhué C., Rougerie R., Rossi J.-P., Gouzy J. & Rasplus J.-Y. Utilisation du RAD-seq pour la phylogénie de groupes d'insectes : retour d'expérience. *3ème colloque de Génomique Environnementale*, Montpellier 26-28 Octobre 2015 [WP1, P1]

Vercken E. Vagues tirées ou poussées ? D'une dichotomie théorique au continuum empirique. Workshop « PDEs in Biology », Paris-Sud XI, 2019. [WP4, P2]

Vercken E. Dynamiques de propagation dans un espace hétérogène : modélisation et expérimentation. Workshop «Phénomènes de propagation et d'organisation spatiale en biologie », Paris-Dauphine, 2017. [WP4, P2]

Vercken E. Tu tires ou tu pousses ? Dynamiques de propagation dans un système expérimental hote-parasitoide. 40èmes journées des Entomophagistes, Lyon, 2017. [WP4, P2]

Mailleret L. Theoretical and microcosm approaches to biological invasions. Workshop "Physics and Biology of Invasion", Nice, 2016. [WP4, P2]

Haond M. Habitat quality and the velocity of spatial population expansion. Emerging Trends in Applied Mathematics and Mechanics (ETAMM), Perpignan,2016. [WP4, P2]

Calcagno V., Burte V, Perez G, Lamare A, Mailleret L. (2019) Keynote talk, *When complex movement yields simple dispersal: a high-throughput analysis of population spread in an egg-parasitoid.* 41ème journée des entomophagistes. Juan-Les-Pins (France) Mai 2019. [WP3, P2]

Communications nationales

Burte V. Effect of the spatial distribution of resources on the fitness of Trichogramma sp. Colloque de la Société Française d'Ecologie, Marseille, 2016. [WP3, P2]

Haond M. Habitat quality and the velocity of spatial population expansion. International Conference Models in Population Dynamics and Ecology (CMPDE), Marseille, 2016. [WP4, P2]

Haond M. Habitat quality and the velocity of spatial population expansion. Colloque de la Société Française d'Ecologie, Marseille, 2016. [WP4, P2]

Warot S., Cruaud A., Groussier G., Marchand A., Henri H., Mouton L., Rasplus J-Y, Ris N. 2019. Délimitations d'espèces ouest-paléarctiques du genre *Trichogramma*. 41ème journée des entomophagistes. Juan-Les-Pins. Mai 2019. [WP5, P1, P2 & P3]

Lecture /Cours

Cruaud A. Introduction to Next Generation Sequencing for Barcoding, Systematics and Phylogeny (2017) Lecture for the Graduate program in Entomology (1 week). University of São Paulo, Ribeirão Preto, Brazil [WP1, P1]

Diplômes et thèses

Ion Scotta (2019) Distributions des espèces du genre *Trichogramma* le long d'un gradient altitudinal et adaptations locales aux basses températures chez l'espèce *Trichogramma cacoeciae*. PhD Thesis. Univ Côte d'Azur, Nice

Burte (2019) Étude des stratégies de mouvement chez les parasitoïdes du genre Trichogramma : apports des techniques d'analyse d'images automatiques. PhD Thesis. Univ Côte d'Azur, Nice

Warot S. 2018. Caractérisation moléculaire et isolements reproducteurs chez des auxiliaires de lutte biologique. EPHE Thesis. EPHE, Montpellier.

E.3 LISTE DES ELEMENTS DE VALORISATION

Logiciel: RADIS. Analysis of RAD-seq data for InterSpecific phylogeny (Cruaud et al. 2016). Available from http://www1.montpellier.inra.fr/CBGP/software/RADIS/. [WP1, P1]. This software enables the exploration of multiple parameters in combination for the accurate inference of phylogenetic relationships among species.

Logiciel: CODICOUNT which has been implemented as an ImageJ/FIJI plugin, and was made freely available online from the FIJI package manager (Perez et al 2017) [WP3, P2]. A dedicated page and tutorial were created to assist users (see https://www6.paca.inrae.fr/institut-sophia-agrobiotech/CODICOUNT).

Prototype "Thermo-arena" & Software "Spartacus"were developed in collaboration with University of Genova (Italy) to investigate thermal tolerance indices in small-sized ectotherms.

Biological Resource Center EP-Coll (http://www.spe.inra.fr/Toutes-les-actualites/EP-Coll) that was labelled ISO9001:2015 by the AFNOR in 2018. [WP5, P1+P2].

The BRC "Egg Parasitoids Collection" (BRC EP-Coll) hosted by P2 is devoted to "egg (=oophagous) parasitoids" i.e. insects whose pre-imaginal development (before the adult stage) is done inside and to the detriment of a host egg which is killed. The BRC beneficiates from the knowledge, skills, methods and resources developed and acquired during this ANR project.

The two ISO9001: 2015 certified services are :

- the maintain and delivery of various *Trichogramma* strains
- the molecular characterization of *Trichogramma* strains and related taxa

Other potential services include :

- the conservation of private collections for specific uses and users
- the implementation of *Trichogramma* sampling using various methods
- the realization of crossing experiments with regards to reference strains

E.4 BILAN ET SUIVI DES PERSONNELS RECRUTES EN CDD (HORS STAGIAIRES)

Identification				Avant le recrutement sur le projet			Recrutement sur le projet				Après le projet				
Nom et prénom	Sexe H/F	Adresse email (1)	Date des dernières nouvelles	Dernier diplôme obtenu au moment du recrutement	Lieu d'études (France, UE, hors UE)	Expérience prof. Antérieure, y compris post-docs (ans)	Partenaire ayant embauché la personne	Poste dans le projet (2)	Durée missions (mois) (3)	Date de fin de mission sur le projet	Devenir professionnel (4)	Type d'employeur (5)	Type d'emploi (6)	Lien au projet ANR (7)	Valorisation expérience (8)
Muru David	H	david.muru@inrae.fr	20/05/2020	Master 2	France	CDD ingénieur (CIRAD), 2 ans CDD ingénieur (INRA), 2 ans	P2 ISA (E. Vercken coord WP4)	ingénieur	6	30/09/2017	Etudiant	Enseignement et recherche publique	Contrat doctoral	Direct (thèse au sein d'un des partenaires)	Oui
Bertin Aline	F		03/02/2019	PhD	Hors UE (Brésil)	PhD (2012-2016)	P2 ISA (E. Vercken coord WP4)	Post-doc	12	31/08/2017	CDI	PME/TPE	chercheur	Employeur non partenaire	Oui (R&D biocontrôle)
Burte Victor	H		01/02/2019	M2	France	aucune	P2 ISA (V. Calcagno coord WP3)	PhD (2015-2018)	38	31/12/2018			Doctorant		
Laugier Robin	H		30/06/2018	M1	France	aucune	P2 ISA (V. Calcagno coord WP3)	CDD ingénieur	7,5	31/08/2017			stagiaire		
Margris Lucas	H		14/01/2020						2	30/08/2018	Etudiant	_	_	_	Oui
Duraj Camille			28/02/2019	BTS	France	aucune	P2 ISA (N. Ris Coord WP5)		8,5	30/06/2019	Recherche emploi	_	Technicien	_	_
HAOND Marjorie	F		01/02/2020	Master 2	France	PhD (2015-2019)	P2 ISA (E. Vercken coord WP4)	ingénieur	3	28/02/2019	Recherche d'emploi		Post-doc		

ANNEXES.

These annexes provide details about the methods developed, the results obtained, the conclusions drawn and the perspectives opened by each WP.

The project was organized in four work packages (WP1-4) completed by a coordination work package (WP0) and a transversal work package (WP5) in charge of sampling; strain maintenance and knowledge transfer towards stakeholders. Project and WP leaders were all engaged in the Biological Resource Center EP-Coll, whose implementation was achieved during the project.

WP1: From genome to species: pangenomic characterization.
Coord P1. Astrid Cruaud.

As species are difficult to discriminate, and because species complexes may exist, the sequencing of a pool of specimens is hazardous and can lead to wrong conclusions. Thus, we wanted to be able to sequence pangenomic markers from single specimens to provide accurate characterization of strains.

Our first objectives were to develop cutting edge lab protocols and bioinformatic pipelines to i) sequence multiple pangenomic markers from single *Trichogramma* wasps ii) accurately analysed sequencing data. Our second objectives were to use these protocols /pipelines on the specimens collected by WP5 and compared our results with Sanger sequencing of 5 markers as well as morphological observations to provide well identified strains to the BRC EP-coll.

We developed two different protocols for the acquisition of thousands of markers throughout the genome of *Trichogramma* wasps for phylogenetic purposes: 1) sequencing of DNA sequences associated with restriction sites (RAD-seq); 2) capture of Ultra-Conserved Elements (UCEs) with hybrid probes.

RAD-Seq, that is the sequencing of the short fragments of DNA adjacent to each instance of a particular restriction enzyme recognition site (Baird et al 2008), enables the rapid discovery and genotyping of genome-wide Single Nucleotid Polymorphisms (SNPs) that can be used to infer species limit and phylogenetic relationships (Cruaud et al 2014). In addition; RAD markers can be analysed to detect introgression among species (Eaton & Ree, 2013). For *Trichogramma*, we have shown that RAD-seq could allow sequencing of about 120,000 markers throughout the genome of *Trichogramma* (Figure 1; Cruaud et al. 2018).

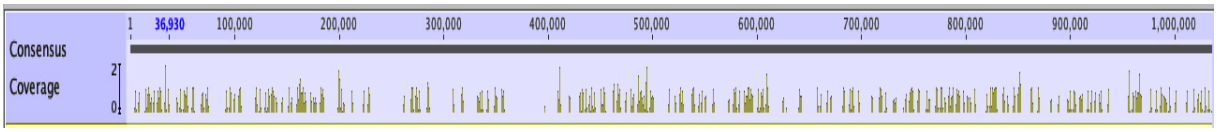

Figure 1: Digestion profile of a part of the genome of *T. pretiosum* (scaffold 9) to show the density of Pst1 RAD tags. (Yellow bars indicate RAD-tags that will be sequenced for each individual).

However, RAD-seq requires a large amount of input DNA (Baird et al. 2008; Cruaud et al. 2014) that is impossible to get from a single tiny specimen of *Trichogramma.* Consequently, we needed to find a method to increase DNA amount. Methods for amplification of total DNA did exist but the amount of DNA that could be extracted from a single *Trichogramma* fall outside of the input DNA amount recommended by manufacturers. Nevertheless, we tested the accuracy of a Whole Genome Amplification (WGA) kit by comparing RAD libraries obtained from the WGA of single specimens (F0 and F1 generation, about 1ng input DNA for the WGA (0.17-2.9 ng)) and a biological amplification of genomic material (the pool of the progeny of the F1 generation –rearing made by WP5) (Figure 2). Globally, we found that 99% of the examined RAD loci (up to 48,189 for one of the crosses, 109 bp each) were compatible with the mode of reproduction of *Trichogramma* (haplodiploidy) and Mendelian inheritance of alleles. The remaining 1% (0.01% of the analysed nucleotides) could represent WGA bias or other experimental /analytical bias. Therefore, we validated a complete lab protocol to get thousands of markers throughout the genome of single wasps. Our results were published in a first peer-reviewed manuscript (Cruaud et al 2018).

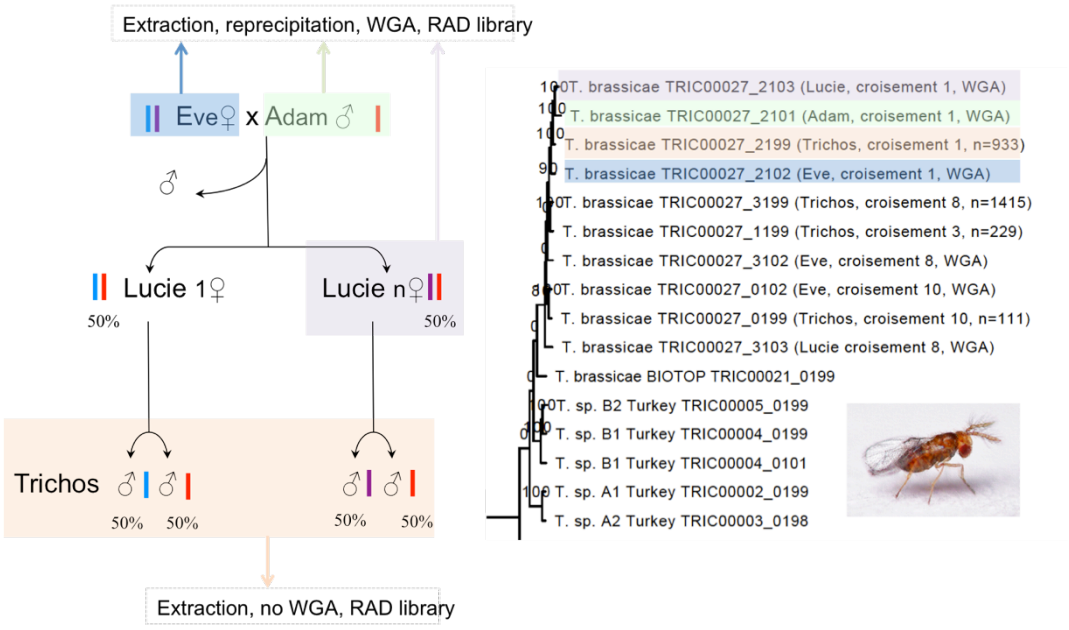

Figure 2. Test of the whole genome amplification protocol to obtain RAD data from a single specimen of *Trichogramma*. Left: summary of the experimental design. Right: Phylogenetic tree resulting from the analysis of 64814 RAD tags (pipeline RADIS Cruaud et al., bioinformatics, 2016). Figure modified from Cruaud et al. 2018.

Analysis of massive sequencing data obtained with New Sequencing Techologies (e.g. Illumina sequencing; up to 2*170M reads produced per run) is not straightforward. It requires taking into account biological and methodological bias, which remains challenging (Kumar et al. 2012). While bias that flaw phylogenetic /species delimitation inferences were more and more reported it was difficult to fully and quickly explore the impact of key analytical parameters on the results (in this case phylogenetic trees) especially with RAD-seq data. In an attempt to make the processing of RAD-seq data easier and allow rapid and automated exploration of parameters/data for phylogenetic inference, we developed the perl pipeline RADIS (Cruaud et al. 2016; Figure 3).
Users of RADIS can let their raw Illumina data be processed up to phylogenetic tree inference, or stop (and restart) the process at some point. Different values for key parameters can be explored in a single analysis (e.g. loci building, sample/loci selection), making possible a thorough exploration of data. RADIS relies on Stacks (Catchen et al. 2013) for demultiplexing of data, removing PCR duplicates and building individual and catalog loci. Scripts have been specifically written for trimming of reads and loci/sample selection. Finally, RAxML (Stamatakis, 2014) is used for phylogenetic inferences, though other software may be utilized. RADIS is written in perl, designed to run on Linux and Unix platforms. The software and its manual are freely available from http://www1.montpellier.inra.fr/CBGP/software/RADIS/. RADIS was published as a second peer-reviewed manuscript (Cruaud et al 2016) and made possible the analysis of thousands of RAD loci sequenced for each strain of *Trichogramma.*

In addition to RAD-seq, we also set up a complete protocol for the capture of hundreds of Ultra Conserved Elements (UCEs, Faircloth et al. 2012) from undetectable amount of DNA extracted from single, poorly conserved specimens. When we started the project, UCEs were still rarely used for phylogenetic inference but appeared promising.

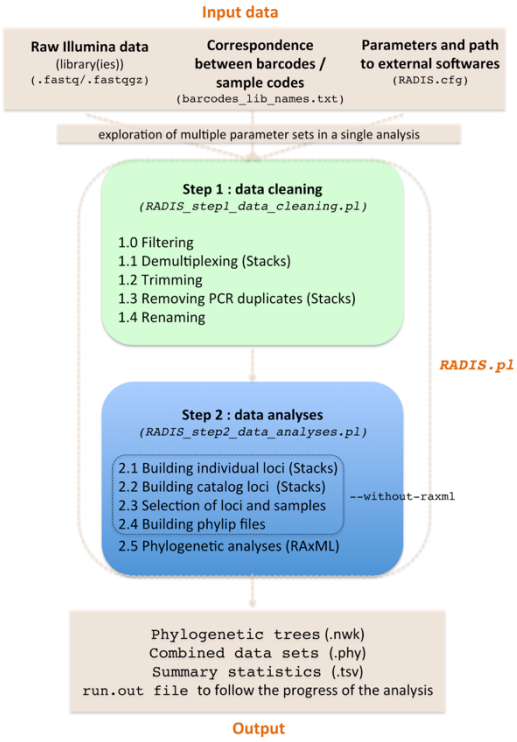

Figure 3. Worflow for processing RADseq data as implemented in the perl program RADIS developed during this project (Cruaud et al., 2016)

An advantage of UCEs over RAD-seq was that it seemed possible to get hundreds of markers from museum specimens for which DNA is too fragmented to enable RAD-seq. In addition, UCEs can be used to resolve deeper relationships that RAD markers. Including museum specimens such as type specimens on which species were described is key in taxonomic studies of difficult groups as it is the only way to validate that identification are correct and new classification are accurate. When we started the project, limitations to routine target enrichment of UCEs were both the complexity of protocols and (again) low input DNA quantity. Therefore, we developed easy to set up optimizations for DNA extraction and library preparation prior to target enrichment (Figure 4).

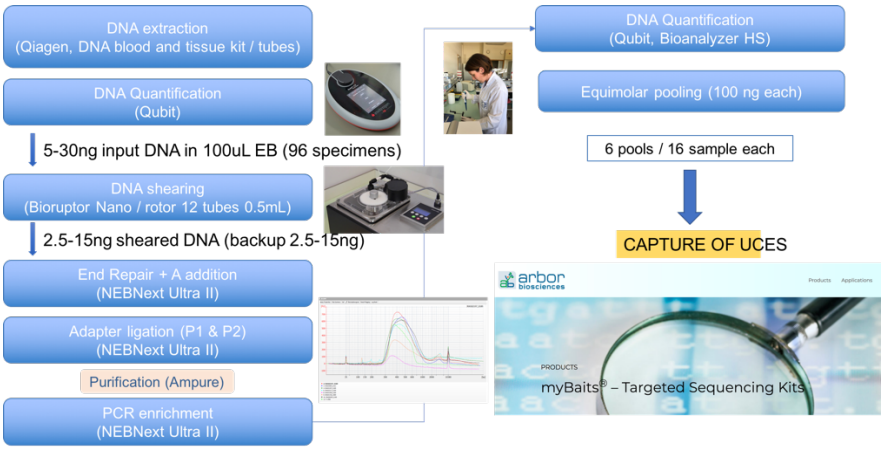

Figure 4. Summary protocol for the capture of > 1000 Ultra Conserved Elements from *Trichogramma* wasps. Protocol and bioinformatic pipeline were published in Cruaud et al., 2019.

Prepared libraries were then used to capture 1,432 UCEs from microhymenoptera used for biocontrol, including *Trichogramma*. Results showed no correlation between input DNA quantities and the number of sequenced UCEs on an Illumina MiSeq.

We also developed a custom pipeline for the analysis and highlighted the potential of UCEs to solve relationships within *Trichogramma*. The protocol (library preparation + target enrichment) allows processing 96 specimens in five working days, by a single person, without requiring the use of expensive

robotic molecular biology platforms, which could help to generalize the use of target enrichment for minute specimens. The protocol and pipeline were published in a third peer-reviewed manuscript (Cruaud et al 2019).

Once these protocols and pipelines were developed and validated, we used them to infer phylogenetic relationships of European *Trichogramma* wasps collected by WP5. Results are not published yet as we need to fully document the diversity of the species but have been presented during 2 international conferences and workshops. A publication is in preparation. The phylogenetic tree (113 samples, 28 species sequenced for about 30,000 RAD tags of 115 bp each throughout the genome) that highlights current issues of identification of strains and taxonomic reassessment based on RAD-seq and morphological observations is presented in Figure 5. When samples were not collected by members of the consortium, identification provided by suppliers are used to annotate tips. Briefly, *T. euproctidis* and *T. embryophagum* as presently identified by stake-holders could be a species complex of at least 2 entities, while what is identified and sold as *T. platneri* and *T. minutum* is indeed the same species. Our sampling revealed potentially undescribed species that need to be further explored (T. spp on tree). This tree highlights the difficulties encountered by non-expert taxonomists to identify species. We indeed recovered (among others) clades in which specimens assigned to *T. brassicae* and *T. cacoeciae* or *T. evanescens* and *T. oleae* were mixed up. Interestingly, there is a global agreement between the classical *COI* barcode fragment that is used by the BRC EP-coll and the RAD tags with the exception of some species complexes (*T. daumalae*).

As conclusion and perspectives, we should mention that:

1- This project made possible the development of new genomic tools for the sequencing of thousands of genetic markers throughout the genome of minute wasps. This is the first time such progress is achieved and this opens new avenues for our understanding of species boundaries within the genus *Trichogramma*. The final RADseq tree (Figure 5) is a first step toward a better understanding of the phylogeny of the genus. Nevertheless, there is still room for improvement. We managed to collect many samples during the course of this project but the dried summers we experienced slowed down our progress. Setting up protocols on this minute wasps also took time. We have now accumulated protocols, pipelines and data in all WPs but still need to take a step back and integrate all data in a single framework to get a better picture of the evolutionary history of the genus. This is why we have submitted a new ANR project BIDIME (which has been granted this year) to complete the ambitious tasks we fixed in the TRIPTIC project. At the end of the TRIPTIC project, we started to implement a 2 step PCR approach protocol we have developed in another project (Cruaud et al 2017) to perform a rapid and massive screening of samples to ensure quality control of strains reared in the BRC. In addition, we have now enough data to start to dig in the genetic determinism of phenotypic key traits for biocontrol.

2- Cutting edge lab protocols and bioinformatics pipelines developed during the course of this project are of a broader interest than only *Trichogramma* as they may help researchers working on minute organisms (most parasitoids; minute arthoropods vectors of human diseases such as sand flies, ticks etc.). We have indeed been invited in several international congresses or to give lecture in Universities on these aspects.

The results from WP1 were published in 3 articles (in peer-reviewed international journals) and were the object of 6 oral presentations in international (5) or national (1) conferences or workshops (of which 5 were invited presentations).

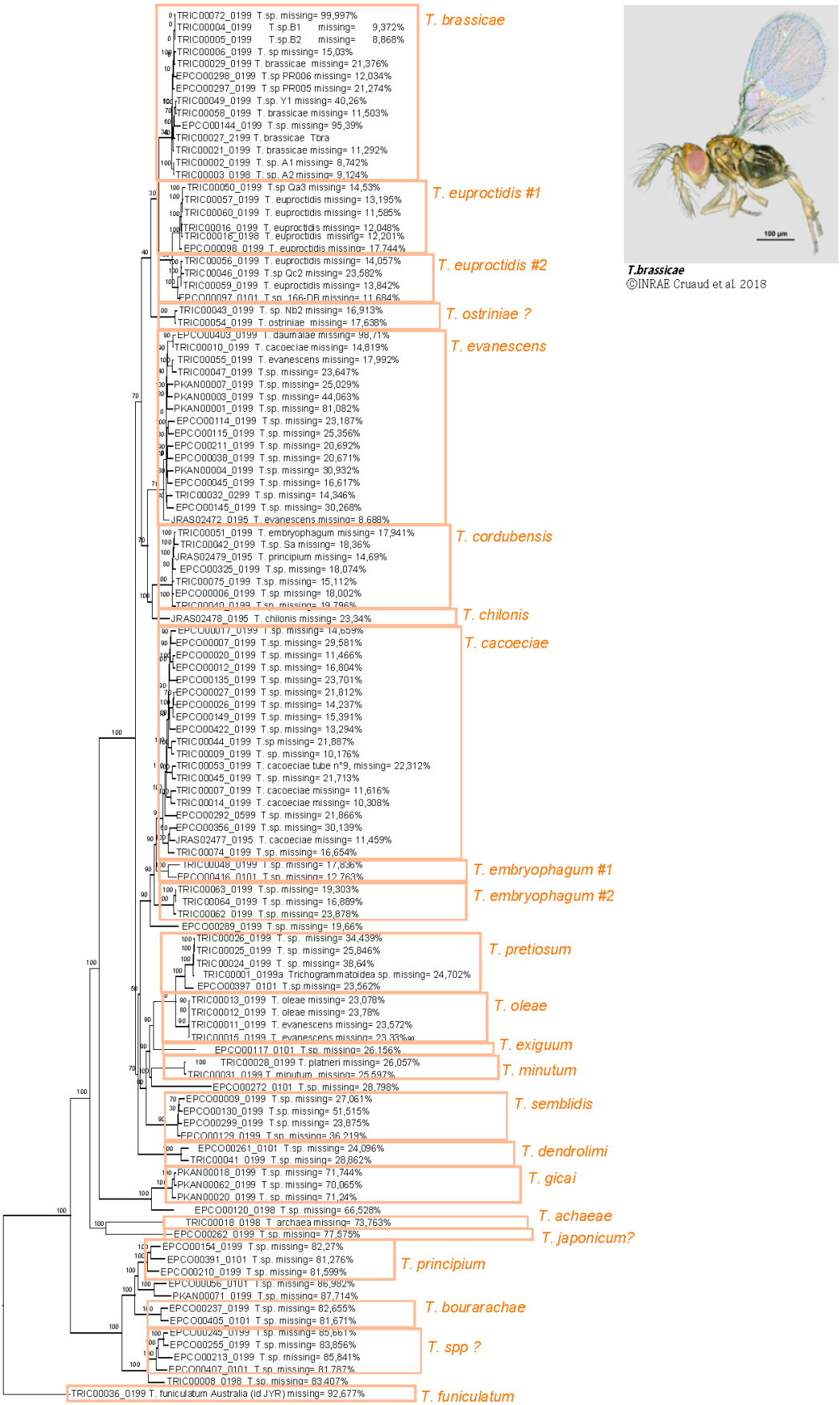

Figure 5. Phylogenetic relationships among 113 samples (ca 28 species) of *Trichogramma* collected during the project. The tree (RAxML; 100 rapid bootstrap replicates) was built from about 30,000 RAD tags of 115 bp each with the pipeline RADIS. When samples were not collected by members of the consortium, identification provided by suppliers are used to annotate tips. Temporary identification based on RAD-seq + 5 Sanger markers (cf WP5) + morphological observation by J.-Y. Rasplus are indicated in orange.

WP2: From microbiome to phenotype: massive screening of symbionts. Coord P3. Laurence Mouton.

Micro-organisms are ubiquitous in animals. They have a variety of repercussions on the host's ecology, physiology and behavior. Besides the cost imposed on the host for maintaining the symbiont population, they can provide fitness advantages to the host or manipulate the host's reproduction. However, despite intense research on insect symbioses these last decades, we still have a limited view of the diversity of symbiotic associations among major groups of insects and of the process of acquisition of microbial communities. The aims of this WP were to realize an exhaustive screening of the microbiota of *Trichogramma sp.*, assess the diversity and the prevalence of the main heritable symbionts, and determine the influence of heritable symbionts on host reproduction.

First, Next-Generation Sequencing (NGS) (without *a priori* approach),was used to characterize the bacteria present in *Trichogramma spp.*. A metagenomic analysis was performed on the 16S ribosomal RNA (rRNA) gene (amplicons of ~450 bp were obtained) which revealed that the microbiome of the analysed species of *Trichogramma* was simple, apparently only composed of *Wolbachia.* The presence of other heritable symbionts that are frequently found in arthropods *(Cardinium, Rickettsia, Hamiltonella, Arsenophonus, Spiroplasma*) was nevertheless investigated using specific PCR on pools of specimens but we did not find any evidence of their presence in *Trichogramma* species. The presence of *Wolbachia* and its prevalence in the different species /strains were also assessed by PCR and real-time quantitative PCR using specific primers. When *Wolbachia* was detected, we performed Multi-Locus Sequenced Typing (MLST) analysis on five *Wolbachia* genes in order to identify diversity and variability of *Wolbachia* strains in *Trichogramma* species.

Prevalence of *Wolbachia* in *Trichogramma* species

Presence of *Wolbachia* has been assessed on single individuals of several strains of 19 *Trichogramma* species through diagnostic detection with species-specific PCR primers. Results indicated a high variability between species: some harbour *Wolbachia* while others don't. *Wolbachia* prevalences highly vary between infected species (Table 1). For species for which a taxonomic affiliation was possible, infection statuses usually match with data obtained previously. Most of the possibly new species or species for which a taxonomic affiliation was not possible were uninfected. Taken as a whole, *Wolbachia* infections do not seem to be correlated with hosts' phylogeny. In particular, discrepancy was observed within the "*cacoeciae – embryophagum*" complex with, on one side, aposymbiotic thelytokous strains (*T. cacoeciae sensus stricto* according to the literature) and, on the other, several strains (including probably *T. embryophagum sensu stricto* … but not only !) where *Wolbachia* infections and some males were observed. This particular point was further investigated (see below).

Diversity of *Wolbachia* strains

MLST analysis was performed on five *Wolbachia* genes (Baldo *et al.*, 2006). However, we managed to obtain sequences in all the *Trichogramma* species studied only for two genes (Cox A and FbpA). Data indicated the presence of two clades of *Wolbachia* : clades A and B (Figure 6), which is consistent with previous studies (Pintureau et al., 2002). Interestingly, two clades of *Wolbachia* were found in the same taxonomic entity (the "*brassicae-embryophagum*" complex), but not in the same individuals. Cues for horizontal transmission were conducted by researching perfect similarities between the *Wolbachia* variants observed in *Trichogramma* and those observed in Lepidoptera. This approach was however not conclusive because of the scarcity of information about the *Wolbachia* infection in Lepidopteran species.

Integration of *Wolbachia* genes in the genome of *T. cacoeciae*?

Routine DNA Barcoding approach as well as multi-locus phylogeny revealed a diversity of closely related taxonomic entity within the so-called "*cacoeciae-embryophagum*" complex (Figure 7). This molecular diversity is associated with striking discrepancy in infection by *Wolbachia*. While the most basal strains B, C and D (Figure 7) are arrhenotokous, *Wolbachia*-infected and geographically and/or ecologically restricted, clade A only includes thelytokous aposymbiotic strains, with some of the

Trichogramma species	Nb. Individuals (Nb. *Trichogramma* strains)	Detection of *Wolbachia*
T. *achaeae*	20 (1 strain)	20 -
T. *bourarachae*	22 (4 strains)	22 -
« brassicae/euproctidis » complex	254 (27 strains)	187 - / 67 +
« cacoeciae_embryophagum » complex	364 (89 strains)	342 - / 22 +
T. chilonis	21 (3 strains)	21 -
T. cordubensis	88 (13 strains)	7- / 81 +
« daumalae_evanescens » complex	132 (19 strains)	123 - /9 +
misA	17 (1 strain)	17 -
misB	45 (9 strains)	40 - / 5 +
T. oleae	53 (9 strains)	4 - / 49 +
T. pretiosum	62 (18 strains)	15 - / 47 +
T. principium	38 (4 strains)	35 - / 3 +
T. semblidis	34 (6 strains)	34 -
swA	5 (1 strain)	5 -
swC	24 (5 strains)	24 -
swD	9 (1 strain)	9 -
swE	3 (2 strains)	3 -
swI	17 (11 strains)	17 -
swJ_ - (putatively *T. gicai*)	16 (11 strains)	16 -

Table 1. Detection of *Wolbachia* in several *Trichogramma* species. Numbers of studied individuals (and strains) are shown. When individuals from the same strain do not have the same status of infection by *Wolbachia* (presence = + / absence = -) details are provided on the relative proportion of individuals that host or not *Wolbachia*. Taxonomic affiliations (1st left column) were based on results obtained in WP1 and WP5.

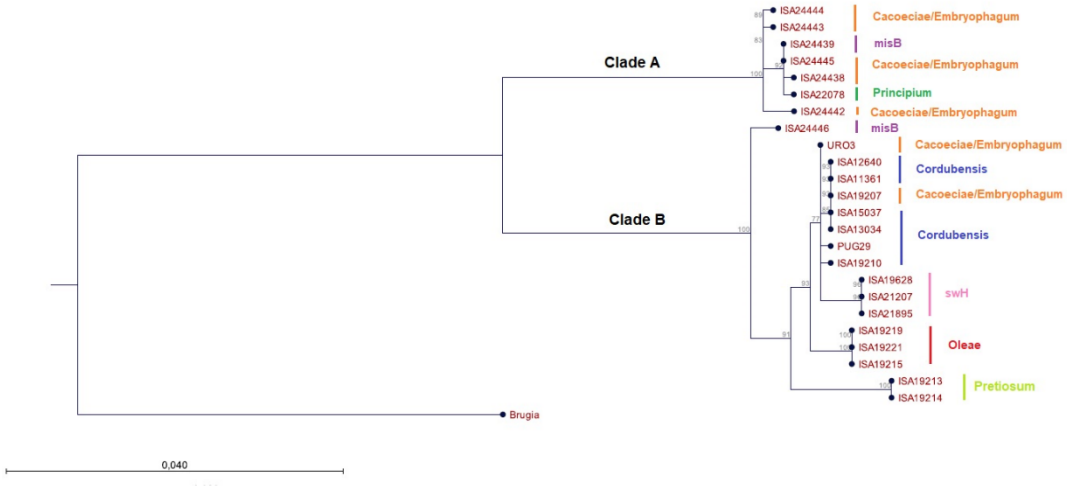

Figure 6. *Wolbachia* phylogeny constructed using maximum-likelihood analyses based on the concatenated sequences of two genes (*CoxA* and *FbpA*). 816bp. Names indicated the *Trichogramma* species from which the *Wolbachia* strain was obtained. *Wolbachia* from the nematode *Brugia malayia* was used as an outgroup.

corresponding haplotypes having a great geographic distribution. One hypothesis would be that part of the *Wolbachia* genome is integrated in the genome of *T. cacoeciae* (*sensu stricto*) and induces thelytoky. Horizontal transfers of *Wolbachia* genes in hosts are widespread (Dunnin Hottop et al., 2007) and it has been recently found that some have evolved as sex ratio distorter (Leclercq et al., 2016). In order to test this hypothesis, we sequenced pools of individuals from "true" *T. cacoeciae* (group A in Figure 7) and

related strains (C and E groups) using Illumina paired end technology (as well as *T. embryophagum* for negative control). We obtained millions of pairs of reads of which 0.13% corresponded to *Wolbachia* sequences (dispatched all along the *Wolbachia* genome) for the representatives of the C and E groups compared to 0.006% for the representative of the A group, which goes well with our hypothesis. However, it can also be explained by a contamination by *Trichogramma* host, *Ephestia sp.*, that also harbours *Wolbachia*. Bioinformatics analysis are still ongoing to disentangle the two possibilities (actual integration *versus* contamination). Anyway, sequences obtained should allow us to obtain a complete genome of the three *Trichogramma* species which constitute important data for refining the phylogeny of *Trichogramma* species (direct link with WP1).

Taken a whole, WP2 confirmed and extended the previous results obtained about heritable symbionts in *Trichogramma* species. We can draw three main conclusions: (i) the community of bacterial reproductive endosymbionts appears quite simple and restricted to *Wolbachia* even when the survey is extended to less known species, (ii) the patterns of *Wolbachia* infections highly vary among species (no infection, partial infection, widespread infection), maybe as a consequence of a variability in the symbiont's acquisition, (iii) the sole manipulation of reproduction observed is the induction of thelytoky.

As a consequence, Task 2.3 dedicated to the study of the phenotypic effects of heritable symbionts on *Trichogramma* species was withdrawn for two reasons:
- Lack of relevance. Indeed, *Wolbachia* is generally well-known to manipulate its host reproduction in variety of ways (Landmann, 2019), which promotes its spread within host populations. Some of these manipulations, such as cytoplasmic incompatibility (CI), cause reproductive isolation (post-mating isolation), which can lead to speciation (Brucker & Bordenstein, 2012). In CI cases, estimating the cost induced by the bacteria is a crucial parameter to understand the *Wolbachia*-hosts dynamics. However, CI was not observed here.
- Technical difficulties. *A posteriori*, the evaluation of the physiological costs induced by *Wolbachia* would have been very interesting in the frame of the "*cacoeciae-embryophagum* context" (see Figure 7). However, B, C, D and E groups (i) were only available through dead individuals, (ii) were not able develop on the routine substitution host *Ephestia kuehniella* or (iii) gave bizarre results after curing *Wolbachia* through antibiotic treatments (Grenier et al., 2002).

Finally, the results obtained in WP2 opened an unexpected question about the possible integration of (some part of) the *Wolbachia* genome in the *Trichogramma*'s one. The results were however obtained quite lately and are still currently analysed.

WP3: From individual phenotype to species: high-throughput phenotyping. Coord P2. Vincent Calcagno.

The aims of WP3 were i) to try and develop new experimental methods and protocols in order to increase the phenotyping throughput we can reach in lab conditions, and ii) to use these methods to characterize traits of interest in several *Trichogramma* strains and species (obtained from WP5).

Four types of phenotyping tasks have been pursued:
1- The automatic counting of parasitism rates by *Trichogramma* on sentinel or rearing host eggs ('egg-counting task').
2- The measurement of the cold tolerance of *Trichogramma* individuals ('thermal biology task')
3- The characterization of movements and interactions in groups of *Trichogramma* individuals, by video tracking at short spatial and temporal scales ('movement task')
4- The characterization of spatial spread by groups of *Trichogramma* at medium spatial and temporal scales ('propagation task')

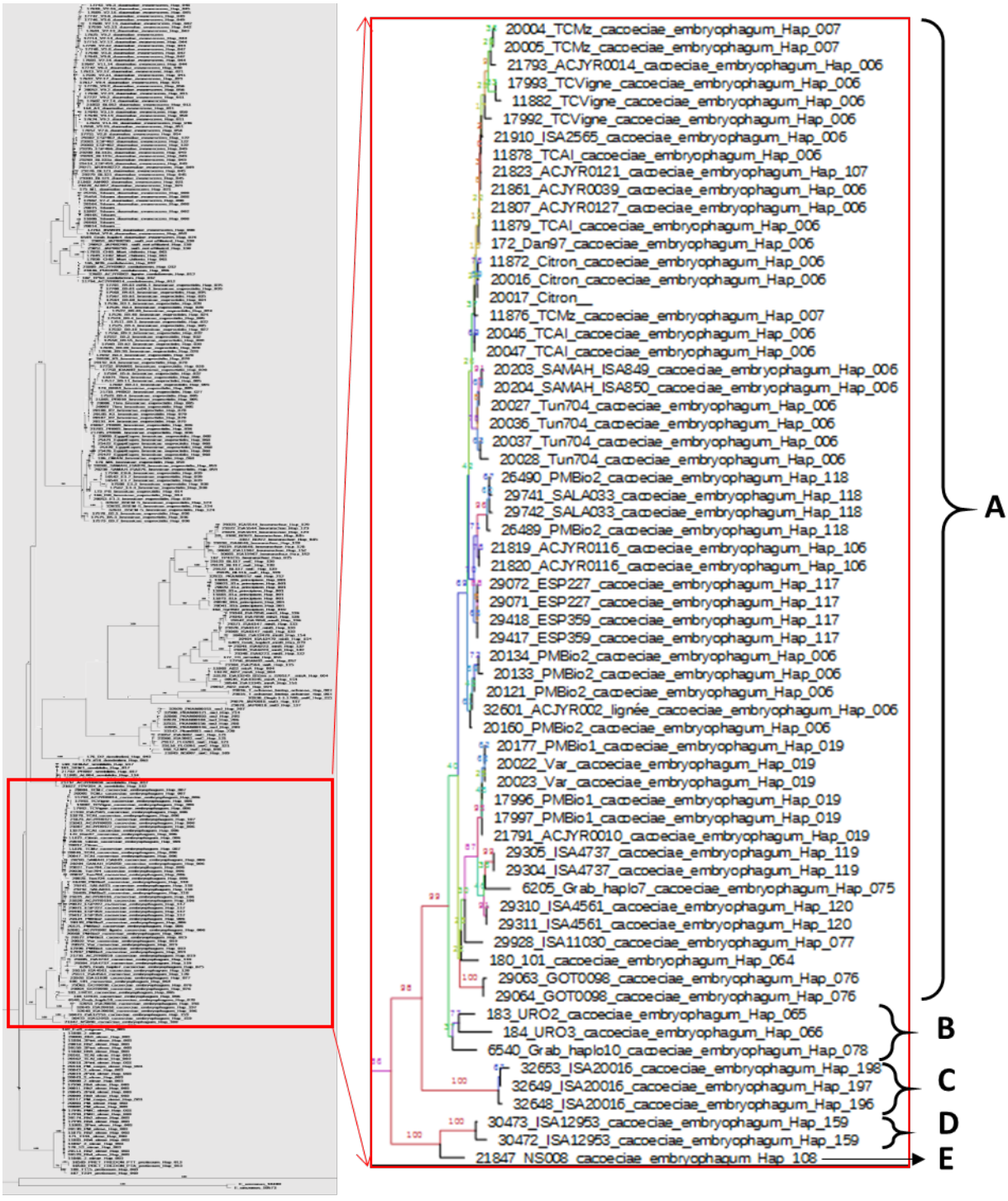

Figure 7. Details about the "cacoeciae-embryophagum" complex (source: Warot S. EPHE Thesis)
This figure was extracted from a Neighbour-Joining tree obtained from part of the mitochondrial coding gene *COI*. The observed molecular clustering was consistent with additional results obtained in multi-locus phylogeny.
Available information for each group is summarized here: A: Thelytokous and aposymbiotic dead individuals and/or living strains, some related haplotypes (e.g. Hap_006) covering a large geographic area; B: Arrhenotokous and *Wolbachia*-infected dead individuals obtained from a strain formerly identified as *T. embryophagum* by B. Pintureau; C. Arrhenotokous and *Wolbachia*-infected dead individuals obtained *Thaumetopoea pityocampa*. Putatively, *T. embryophagum sensu stricto*; D. *Wolbachia*-infected dead individuals; E. Naturally *Wolbachia*-infected and thelytokous living strain but production of both sexes after *Wolbachia*-curing. Pools of Individuals from the group A, C and E were used for Illumina sequencing.

Each of these tasks were conducted by (i) the acquisition of novel imaging equipment; (ii) the development of novel phenotyping devices and experimental protocols; and (iii) the development of new computer codes, algorithms and scripts to analyze the data obtained in a computer-assisted way. The thermal biology of *T. cacoeciae* strains was investigated using different ways. For the investigations on thermal indices, we built a specific device (the "Thermo-arena") and developed a devoted software

("Spartacus") in collaboration with the University of Genova (declarations of invention in progress). Investigations on diapause were realized in collaboration with the private company Bioline Agrosciences. Investigations on overwintering relied on quite simple methodology.

Task 1

This task was started the most early in the project, and was both the least demanding in terms of new technological developments and the most requested by researchers working on *Trichogramma* (Partner 2 + Bioline Agroscience).

We developed an image analysis method designed to automate the counting of number of *Ephestia* eggs on a picture, in the context of standard *Trichogramma* rearing and experiments i.e. *Ephestia* eggs are glued on a paper board and let to be parasitized by adult trichogram females. After 5 days at 25°C, parasitized eggs turn black or dark grey, the proportion of dark eggs *versus* light unparasitized eggs allows to infer the parasitism rate by Trichograms, and an approximate value of the number of expected Trichograms after emergence of parasitized eggs.

We developed a tool that had to take into account the inherent variability in the exact protocol used by different teams or researchers (different colors for the paper board support, different methodologies when obtaining the picture, different levels of heterogeneity across samples…) and across *Trichogramma* species and strains (parasitized eggs darken in slightly different ways in different species) and *Ephestia* eggs (different degrees of freshness and quality, that impact their appearance).

To tackle this issue, we designed a flexible tool that takes in standard color pictures (that users can generate in a number of ways), and automatically learns the specifics of the task at hand through a simple training part: users tell the software what are the different objects to be counted (background, healthy egg, parasitized eggs…) on a training set of pictures. Then, the tool automatically determines the best way, in color space, to recognize the different objects. This is achieved by several sets of linear discriminant analyses in pixel data. Finally, the software applies this strategy to count the number of eggs of both types (parasitized or not) on an entire test of images. It automatically generates an Excel spreadsheet with count results as columns and individual names as rows, allowing batch processing of folders with several hundreds of pictures to count.

The tool, called CODICOUNT, has been implemented as an ImageJ/FIJI plugin, and after tests with different teams in different conditions, was made freely available online from the FIJI package manager. A dedicated page and tutorial were also created to assist users (see https://www6.paca.inrae.fr/institut-sophia-agrobiotech/CODICOUNT). Also, a technical paper has been prepared and published in 2017 (Perez et al. 2017).

The tool has proved very successful, and easy to handle by users after minimal training and assistance. It could count *Trichogramma* parasitization rates and *Ephestia* egg numbers in a variety of situations, with very satisfying accuracy (between 80 and 99% of R2 between automatic counts and reference (manual) counts, depending on settings and image quality). It adds over manual counting the advantage of speed, scalability, repeatability, and smaller variations across days and operators. Furthermore, it also counts healthy *Ephestia* eggs, something manual operators usually renounce to do, because of its difficulty and of time constraints (people usually just count parasitized eggs in labs). This improves quality control and produces more stable estimations.

To date, CODICOUNT and its associated pipeline has become routinely used in Institut Sophia Agrobiotech (Partner 2), by several teams (BPI, RDLB and M2P2) and also in external partner (Bioline). The technician involved in the development of the project (G. Perez) has become a specialist of the method and regularly trains new operators to the tool. As a non-exhaustive list, in the context of TriPTIC, CODICOUNT has been employed to:

1) Study oviposition preference behaviors (with respect to light and gravity) across more than 20 strains in five species (and correlate them with sampling strata (herbs, shrubs or trees; see WP5). Significant

variation across species and strains was found in geotactic and phototactic preferences, though most strains had negative geotaxis and positive phototaxis, in line with expectations. Preferences with respect to light and gravity were not correlated across strains overall, but sometines showed negative interactions (antagonism) and were time dependent (their strength weakened over 4 days). Oviposition syndromes seem to be associated with sampling strata, even though insufficient sampling could be achieved in WP5 to attain strong statistical confidence. Experiments were performed by a PhD student granted by TRIPTIC (V. Burte) and a lab technician (G. Perez) and a manuscript is under preparation.
2) Perform the counting of numbers of parasitized eggs in population dynamical experiments in WP4 (Dahirel et al 2020)
3) Count *Trichogramma* survival rates when exposed to various does of essential oils (spinoff project ODORAMMA 2016-2020; van Oudenhove et al.; manuscript under preparation).

Beyond *Trichogramma*, several teams at ISA (Partner 2) are currently using the tool for their experiments with *Drosophila* or even parasitic nematodes.

Task 2

With regard to "thermal biology of *Trichogramma cacoeciae*", all results are available in a PhD Thesis by a student who was not granted by TRIPTIC but worked on it (Ion Scotta 2019). Taken as whole, we evidenced a significant intra-specific genetic variability between strains originated from south-eastern France or even between strains along a marked altitudinal gradient (from sea level to about 1500m in less than 30km). This intraspecific genetic variability was more or less significant according to the phenotyped traits (thermal indices, diapause or overwintering). It however suggests an adaptive differentiation insofar as significant correlations exist at the strain level between some of these traits and patterns are consistent with differences in climatic conditions (severity of winter conditions). As an example, we detailed below the results obtained for the three thermal indices (CTmin, Chill Coma Temperature, Chill Coma Recovery Activity) (Ion Scotta et al. submitted), this study being, to our knowledge, a rare case where the questions of both the intraspecific variability of thermal indices and their genetic correlations have been addressed.

Task 3

We developed a video-analysis pipeline, starting from the acquisition of videos of *Trichogramma* groups or individuals with high-resolution DSLR digital cameras available (UHD videos; Figure 8). These videos are then analyzed with CTRAX (Branson et al 2008), with adjustments made to fit the tool to Trichograms. Raw CTRAX outputs are then processed by a suite of R scripts for error correction and filtering out of artefacts, and finally analyzed by another set of R scripts to automatically compute behavioral and movement metrics that summarize the activity levels and trajectories of individuals on the video. A methodological publication with the associated scripts in under preparation.

This method has been successfully developed and used in several TriPTIC and follow-up experiments with *Trichogramma*:
1) in the context of TriPTIC, a PhD student (V. Burte) characterized the activity patterns of groups (about 30 individuals) of Trichograms in standardized 10cm x 20 cm trial arenas, for 10 minutes. More than 20 *Trichogramma* strains/species from WP5 and BRC Ep-coll could thus be tested. Activity and movement patterns could be shown to differ marked across species, reflecting to a certain extent species phylogenetic proximity. No association of movement strategies was found with sampling strata, in contrast to the result for geo/phototaxis, which matches ecological expectations. A manuscript on this subject is under preparation.
2) In collaboration with WP4, the pipeline has been used to characterize the movement strategies of initial mixes and over generations in an experimental evolution experiment designed to contrast pushed and pulled invasion waves (Dahirel et al.; manuscript under preparation).
3) The developed pipeline has been used extensively for a follow-up PhD thesis project at Institut Sophia Agrobiotech (S. Lartigue; 2017-2020; funded by the Minister of Agriculture) on the phenotyping of personality traits in inbred strains of *T. evanescens*. This revealed genetic and inter-individual consistent significant movement-related traits in *Trichogramma* (Lartigue et al.; manuscript under preparation).

4) It is currently being used for the analysis of olfactory preferences of *Trichogramma* with respect to plant essential oils used as biopesticides (work in progress; spin-off project ODORAMMA 2016-2020).

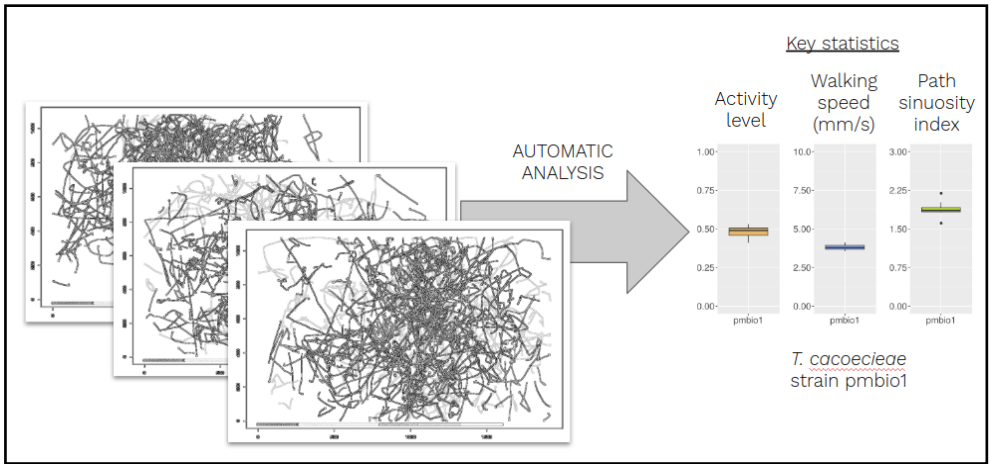

Figure 8: an illustration of tracking methods (Task 3)

Task 4

Task 4 has been the most profoundly developed, in the context of the PhD thesis granted by the project and a follow-up TriPTIC Master project (A. Lamare; 2019). In order to be able to study the spatial spread of groups of trichograms at relevant spatial and temporal scales (several meters, several days), while still being able to track the individuals, we developed an innovative phenotyping protocol, consisting in a long (6 meters) tunnel folded into a double spiral (Figure 9). This allows to have the long tunnel fit into an experimentally manageable area (about 40 x 80 cm). *Trichogramma* individuals are introduced into the enter of the double spiral, and their spatial spread can be tracked for an entire day (8 hours or more). The large surface to be tracked (relative to a *Trichogramma* body size) necessitates an image resolution for tracking that is above the currently existing video systems. Therefore, we developed a high-resolution / low-frequency technique, consisting in timelapses (one image every 30 seconds) of super high resolution pictures (72 Mpx). Using custom-made JAVA, ImageJ and R scripts, these images are then analyzed automatically to determine the location of each individual at each time step, and reconstruct its position regarding the release point, other individuals and the host eggs possibly have been disposed in the device. This system allows the high throughput analysis exploration of the dynamics of population spread, yielding hitherto unmatched data resolution.

The double spiral system was initially developed by the PhD student founded by TRIPTIC (V. Burte) and a lab technician (G. Perez) using styrofoam, and later we have upgraded the system to have double spirals Laser-cut in Plexiglass, allowing fast, cheap and scalable production (collaboration with the FabLab SoFab Eurecom Valley, Sophia Antipolis).

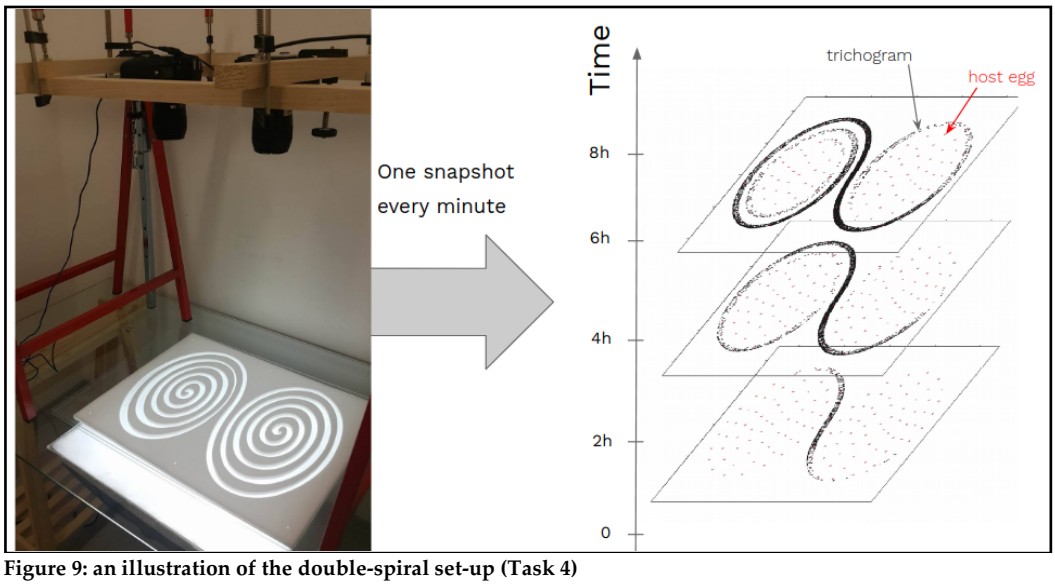

Figure 9: an illustration of the double-spiral set-up (Task 4)

This method has been developed, validated (absence of artefacts, satisfying detection rate, repeatability…) and used by V. Burte in his PhD thesis. He tested the effect of individual density (number of Trichograms released) and host egg distribution (density and level of spatial aggregation) on the rate of spread in *T. cacoeciae*. Results yielded several novel and interesting results. In particular, we identified for the first time in *Trichogramma* the existence of two movement, called 'sedentary' and 'explorer', between which individuals dynamically switch over the course of several hours (Figure 10). This heterogeneity of movement strategies is key to explain the pattern of population spread as well as the resulting dispersal kernel (distribution of parasitism events): with considering this variability, predictions on the speed of spread or population distribution are incorrect. Interestingly, some changes in the dynamics of population spread change after about 4 hours, suggesting that extrapolations of population spread from short term (minutes) behavioral observations (see previous task) is not a straightforward task. The double spiral system shows great promise to fill the gap between small scale behavioral studies and field studies of *Trichogramma*, taking in more biological realism and more meaningful spatio-temporal scales, while preserving experimental manageability and control. These results have been presented at several international conferences and are the object of a research article (Burte et al.; under review).

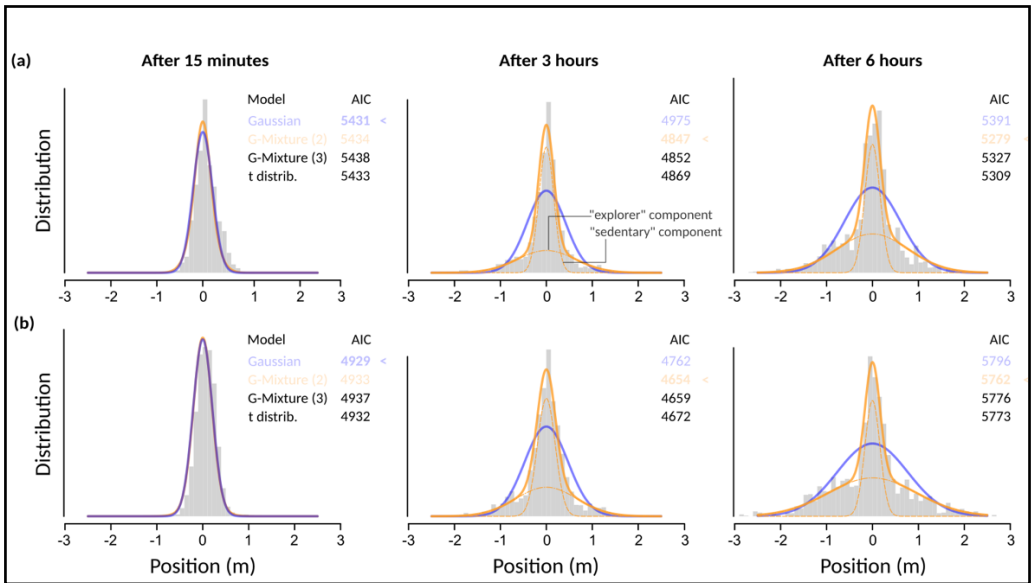

Figure 10: Spatial propagation of groups of *Trichogramma* for their release point (Task 4). After 3 hours or more, individuals belong to two movement modes (explorer/sedentary). This si reflected by the fact that a mixture distribution kernel composed of two Gaussian kernels has betetr fit than either a simple Gaussian kernel or morecomplex mixtures, as determined from AIC values.

Overall, **WP3** has opened a broad range of novel experimental methods for the study of *Trichogramma* and similar insects in the lab. They range from very simple (automatic counting tool) to quite sophisticated and novel (the double spiral system). These methods have already contributed to improving the quality level and throughput of several experiments conducted at ISA (Partner 2), and have made several follow-up projects possible. A real dynamic has been started thanks to the TRIPTIC project, and more and more groups and researchers are interested in and want to deploy 'ethomics-like' methods.

Of course, the ambitions of WP3 were extremely high and were hard to fulfil entirely in just 4 years, and with the support of only one hired PhD student (V. Burte), that was a biologist by training and not a computer scientist, and one research technician (also a biologist). This is why, though promising, several of the methods developed are still to be fully developed and deployed. Similarly, several publications and codes remain to be published. This is why follow-up projects have been pursued. WP3 and its results have been instrumental in obtaining further funding. For instance, ANR project BIDIME (2020-2013) has recently been funded to Partners 1 and 2 (coordinator: Partner 2 N. RIS), and directly builds on methods and results developed in WP3 (Tasks 2 & 3). This project will allow to hire a postdoc with expertise in computer science and machine learning, allowing to complete and refine the methodological developments engaged in TRIPTIC. Spin-off projects such as ODORAMMA (2016-

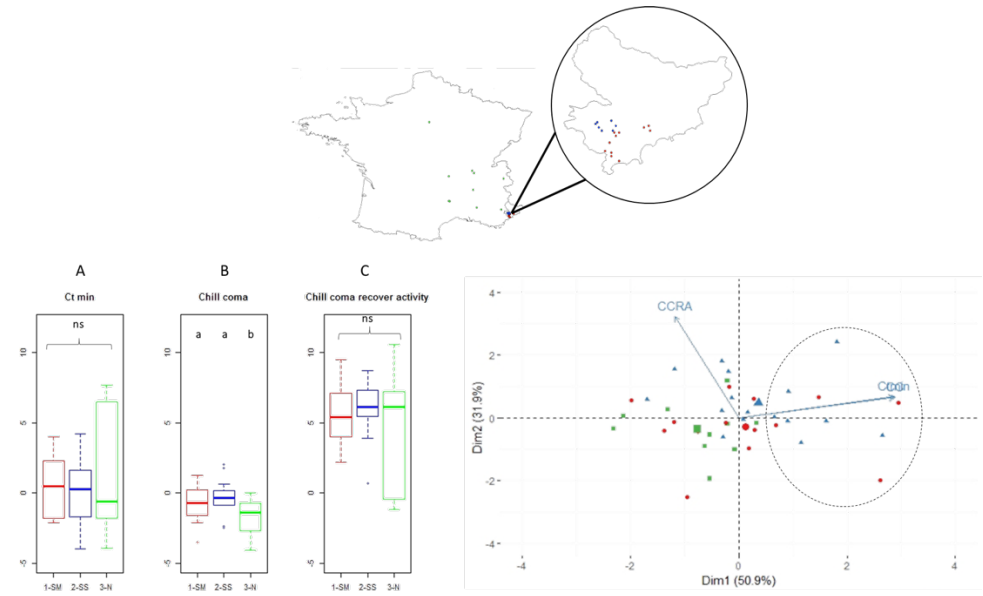

Figure 11 : Intraspecific variability for thermal indices in *Trichogramma cacoeciae* (source: Ion Scotta et al. submitted to Evolutionary Applications)
Three geographic origins were compared within South-eastern France : Northern origin (green), Supra-mediterranean conditions along the altitudinal gradient (blue), Meso-Mediterranean conditions along the altitudinal gradient (red).
Three thermal indices (minimal Critical Threshold, Chill Coma temperature, Chill Coma Activity Recovery) were investigated using the "Thermo-arena" and its devoted software 'Sapatacus'. Top – Center: Geographic mapping of the strains compared with focus on the altitudinal gradient in the department Alpes-Maritimes. Bottom – Left: Trait-by-trait comparisons evidencing a significant differentiation between Northern and more septentrional strains for Chill Coma. Bottom – Right: Principal Component Analysis evidencing, at the strain level, a positive correlation between CTmin and Chill Coma temperature.

2020), PHENOMENE (2017-2019) or SCALUP (2020-2021) have also been obtained to sustain these developments. More generally, Institut Sophia Agrobiotech (Partner 2), at the initiative of V. Calcagno and team M2P2, has started to set up a 'high throughput phenotyping platform' on its site, with new buildings and dedicated devices to be built/purchased next year. Simultaneously, we are trying to hire a permanent research engineer that will be in charge of managing/developing ethomics methods for insects and train people to use them in their research projects.

Beyond these methodological and strategic considerations, WP3 will have been productive on the scientific side too. The two theses of V. Burte (tasks 1,3,4) and M. Ion-Scota (tasks 2) have been defended successfully generated interesting new results on the biology of *Trichogramma*. Although many of them are still awaiting publication, several are published or close to it (Ion Scota et al accepted; Burte et al. under review) and have been presented at international conferences. This is a nice achievement, considering the important methodological developments that WP3 involved, reducing the time available for the actual use of the new methods and subsequent publications.
One salient finding in WP3 has for example been the discovery of 'sedentary' versus 'explorer' modes in *Trichogramma* movement behavior (Task 4), something not documented so far in this group. All evidence indicates that these modes can have a purely behavioral and plastic determinism, existing even in the absence of genetic variation (Burte et al. under review). This is a clear and practical illustration of the importance of taking behavior into account when trying to predict population-level patterns. V. Calcagno has recently obtained a PhD fellowship from INRAE and Universite d'Azur (Trichomove; start date: October 2020) to follow-up on these investigations, as well as a mobility program grant (SCALUP; 2020-2021) to initiate a collaboration with mathematician specialists of movement and scaling. He has further submitted, with his colleague in ISA L. van Oudenhove, a project in collaboration with computer-vision specialists from INRIA Sophia-Antipolis. Therefore, the impetus set-up by TRIPTIC WP3 has proven efficient, and the story can be said not to be over.
The focus on thermal biology was not initially planned. Its development within TRIPTIC made however sense insofar (i) as the grant of Michela ION SCOTTA was supported by another financial support provided, (ii) Part of Michela ION SCOTTA's PhD clearly matched with a TRIPTIC objective (see Task 5.1), (iii) investigations about thermal indices relied on video-phenotyping, an important methodological aspect in WP2.

WP4: From phenotype to population dynamics establishment success and community performance. Coord P2. Elodie Vercken.

Ultimately, biological control requires the successful reproduction and dispersal of an introduced population of a biological control agent against a focus pest. This last stage in the development of a biological control program registers a 30% fail rate, which represents a major barrier for the development of biological control. Therefore, selecting candidate agents beforehand not only on their ability to parasitize/predate the focus pest and their rearing cost, but also on their ability to successfully establish and spread stands as a promising lever for improvement in biological control R&D. Demographical traits, as global descriptors of population performance, are likely to be good predictors of establishment success, yet they are rarely measured in practice because they require higher sampling effort over longer time scales than phenotypic traits. The aim of the WP4 « From phenotype to population dynamics" was to characterize population-level traits related to demography at the local (growth rate, carrying capacity, positive and/or negative density-dependence) and spatial scales (expansion speed, colonization probability), and to analyze how these traits varied or co-varied among populations and species of *Trichogramma*. This part of the TRIPTIC project meant to: (i) document the natural variation in demographic performance among closely related groups ; (ii) uncover the potential trade-offs between growth and establishment at different spatial scales; (iii) explore how evolutionary forces at play during an expansion may modify the spatial structure of genetic and phenotypic variation, and therefore the demographic performance of the population; (iv) test whether the combination of functional diversity at different phenotypic levels improved pest control in a complex spatial setting.

Experimental introductions in laboratory microcosms, where a large range of demographic, genetic and environmental factors can be controlled and manipulated and where populations and meta-populations can be monitored extensively over a large number of generations, provide an excellent tool to investigate how local and spatial dynamics interact and shape the eco-evolutionary dynamics of introduced populations. The predictions obtained from such simplified ecosystems can then be tested and integrated over a larger spatial scale in order to validate the generality of the patterns observed. For this project, we used an original experimental set-up for mimicking introductions of *Trichogramma* in a meta-population context, which proved its relevance as a model system to address questions related to the ecology of early establishment and expansion of introduced populations (Vercken et al. 2013, Morel-Journel et al. 2018, 2019).

The first deliverable for WP4 was the quantification of population-scale functional traits (growth rate, presence of positive density-dependence, demographic stochasticity, stability of population dynamics) and its variation among strains. In accordance with this objective, we characterized development time and sex-ratio in 55 strains. Development times were significantly different between strains, and we identified a "slow-developing" group (incl. strains from *T. cacoeciae* and T. principum) and a "fast-developing" group (incl. strains from *T. brassicae, T. euproctidis, T. evanescens and T. semblidis*). We then characterized local population dynamics on a subset of 20 populations representative of the diversity sampled in the first season. We found that several populations displayed positive density-dependence (i.e., an Allee effect) and that these populations were also characterized by poorer demographic performance on the whole (lower maximum growth rate and population size, higher extinction rate, Vercken and Mailleret 2020).
The second deliverable was the characterization of spatial dynamics (local extinction rate, colonisation rate, individual heterogeneity in dispersal phenotypes) and its variation among strains. We first investigated how the presence of positive density-dependence (in growth or dispersal) could affect expansion dynamics. We showed that positive density-dependence induced "pushed" expansion waves (in contrast with classical "pulled" expansions), and that the speed of pushed expansions depended on carrying capacity (Haond et al., 2018). Then we monitored the expansion over 10 generations of a subset of 15 populations analyzed previously for local population dynamics. We ranked these populations in order of "pushness" based on the strength of the relationship between their expansion speed and carrying capacity, and we found that more pushed populations expanded at a slower rate and experienced more frequent local extinctions on the expansion front (Vercken and Mailleret, in prep).

The third part of the project was the experimental analysis of evolution along expansion fronts: we followed replicates of genetically mixed populations of *T. brassicae* in landscapes characterized by two different levels of structural connectivity (standard/restricted). We found that restricted connectivity induced pushed-like expansions, where neutral genetic diversity was conserved longer on the front than in pulled (or standard) expansions (Dahirel et al. 2020). In contrast with pulled expansions, where dispersal and reproductive potential increased on the front, the phenotype of individuals in pushed-like expansions evolved little across time and space, with front populations remaining globally similar to core populations (Dahirel et al., in prep).
As these developments on the diversity of expansion patterns proved more complex and took more time than expected, and as results from other WPs on individual-level traits were obtained and centralized in the last year of the project, the work on the effect of functional diversity on control efficacy in a realistic agronomical context could not be carried out.

Our results demonstrated a strong heterogeneity in demography among different populations and different species of *Trichogramma* (growth rate, carrying capacity, response to density, dispersal capacities…), which induces contrasted demo-genetics dynamics in time and space. This diversity might prove an excellent tool for experimental approaches aiming at investigating the link between individual and population traits and exploring eco-evolutionary processes at play during spatial expansions. The question of pushed vs pulled expansions and their consequence on demographic and evolutionary processes raised a strong interest in the scientific community, with 5 invitations to present our work in international conferences or workshops, and the successful funding of an ANR starting grant on this topic (ANR JCJC "PushToiDeLa", 2018-2022, PR: E. Vercken).

The results from WP4 were the object of 10 oral presentations in international conferences or workshops (of which 7 were invited presentations), and generated 5 original articles (1 published in 2018, 2 available as preprints at this date, 2 currently in preparation).

WP5: From collection to transfer: The Biological Resource Center. Coord P2. Nicolas Ris.

The main objective of WP5 was to explore the biodiversity of *Trichogramma* and to constitute a collection of well-conserved and well characterized biological material (dead and living organisms). WP5 was thus planned to (i) provide adequate resources for other WPs (WP1-WP4) and (ii) contribute to the creation of a perennial biorepository (the Biological Resource Center "EP-Coll") for both applied and academic purposes, (iii) store relevant side-products (vouchers, extracted DNA, GIS information) and aggregate knowledge from the TRIPTIC expertise. Thus, three different tasks were identified: Task 5.1: the field sampling of *Trichogramma* species; Task 5.2: the integrative characterization of *Trichogramma* strains; Task 5.3: the management of biological resources.

Task 5.1: field sampling of *Trichogramma* species mainly relied on the massive and frequent exposures of sentinel eggs of *Ephestia kuehniella.* Occasionally, two other complementary sampling methods were used, the sampling of natural egg clutches (rare events) or sweeping/trapping (destructive and unselective methods).
Task 5.2: integrative characterization of *Trichogramma* strains mobilized several complementary approaches: (i) the routine Sanger sequencing of part of the mitochondrial gene *COI*, (ii) for a representative subset, the Sanger sequencing of complementary markers (*CytB*, *Wg*, *Ef1-α*, *Rpl27*a, *RpS4*), (iii) standardized crossing experiments (both directions – parental crosses and virgin females as internal controls), (iv) mounting of male genitalia, antennae and wing) for durable storage and acquisition of a high-resolution iconography.
Task 5.3: management of biological resources relied on the implementation and certification of a Quality Management System. The international norm ISO9001:2015 was more precisely targeted

The nature of the results highly differs according to the three tasks :

Task 5.1: the field sampling of Trichogramma species'

A first survey was conducted across France between 2015 and 2016 (from April to September) including a total of 92 sites (Muru *et al.* submitted). This involved a total of 43 collectors including INRAE staff but also volunteers (members of the Société Entomologique de France). *Trichogramma* were sampled by exposing sentinel eggs of *Ephestia kuehniella*, those eggs having been previously sterilized by exposure to ultraviolet radiation. Two sampling methods were used: "Sprays" (direct exposure of the eggs on the substrate -75% of the sampled points) and "Egg cards" (exposure on a piece of cardboard, itself pinned on the substrate - card25%).

A second survey was realised at a very local scale, a marked altitudinal gradient (from sea level to 1500m of elevation in less than 30km) in the framework of a PhD thesis not funded by the project (Ion Scotta et al. submitted). This gradient covers two different bioclimatic levels, the Meso-Mediterranean and the Supra-Mediterranean, the border between these two zones being based on botanic information (CORINE classification). A total of 42 locations were selected in the study area including 6 replicates for each of the 6 "bioclimatic zone x natural habitat" combinations and 6 replicates of cultivated areas within the Meso-Mediterranean. *Trichogramma* were sampled using the "Egg cards" methods. Additionally, *Trichogramma* samples were provided by colleagues (Iran, Serbia, Turkey in particular) through the process R1 (see Task 5.3). Methods were not standardized in these cases.

Taken as a whole, both French surveys evidenced the difficulty of the *Trichogramma*'s sampling, only a few percentages of sprays and/or egg cards being finally parasitized. *Trichogramma* was nevertheless proven pervasive. Along the year, two more favourable periods were observed (May-June and October), the dates being modulated by the local meteorological/climatic conditions. Cultivated areas and Shrublands appears to be the most favourable habitats for *Trichogramma*, at least in South-eastern France. No clear difference was observed between plant species. This time-consuming activity allowed us to renew our living collection and provide adequate biological material for TRIPTIC partners (see Task 5.3 and other WPs).

Task 5.2: the integrative characterization of Trichogramma strains

The routine sequencing of part of the mitochondrial gene *COI* included more than 2000 individuals from more than 600 *Trichogramma* samples (Warot 2018, Warot et al. submitted). This unprecedented dataset allows us to evidence about 120 haplotypes that were at least observed twice. Various analyses were provided on this dataset including the Neighour Joining tree presented below. From these, we revealed 14 different clusters (Latin numeration: I to XIV on Figure 12), each of them being defined as a monophyletic set of at least 2 haplotypes. Generally, each of these clusters was supported by a high value of bootstrap (>80%) except for clusters I, VI and XII that were located in basal positions. Certain haplotypes were not assigned to any of these 14 clusters. Among these, nine haplotypes appeared valid (no variation in their *COI* amino acid sequence) and clearly isolated from each other (Figure 12, Arabic numeration 1 to 9). Finally, 15 very rare (category A) and 1 rare (category B) haplotypes were not affiliated and are likely to be artefacts. Among the 23 clusters / isolated haplotypes, 11 were associated with a single species name: *T. achaeae*, *T. bourarachae* Pintureau & Babault, *T. chilonis* Ishii, *T. cordubensis* Vargas & Cabello, *T. dendrolimi* Matsumura, *T. oleae* Voegelé & Pointel, *T. ostriniae*, *T. nerudai* Pintureau & Gerding, *T. pretiosum* Riley, *T. principium* Sugonjaev et Sorokina and *T. semblidis* (Aurivillius) (Figure 12). Surprisingly, two divergent haplotypes (Hap_009 and Hap_067) were assigned to *T. exiguum* Pinto & Platner (see yellow stars on Figure 12). Each of the three largest clusters (I, VI and XII) included two different species names and were thus considered as species' complexes: "*daumalae–evanescens*", "*brassicae–euproctidis*" and "*cacoeciae–embryophagum*". Finally, seven clusters or isolated haplotypes were not assigned to any available species name. For a representative subset, the molecular characterization was extended to other molecular markers (see § C3) what confirms the clustering observed on *COI* (Warot et al. 2018).

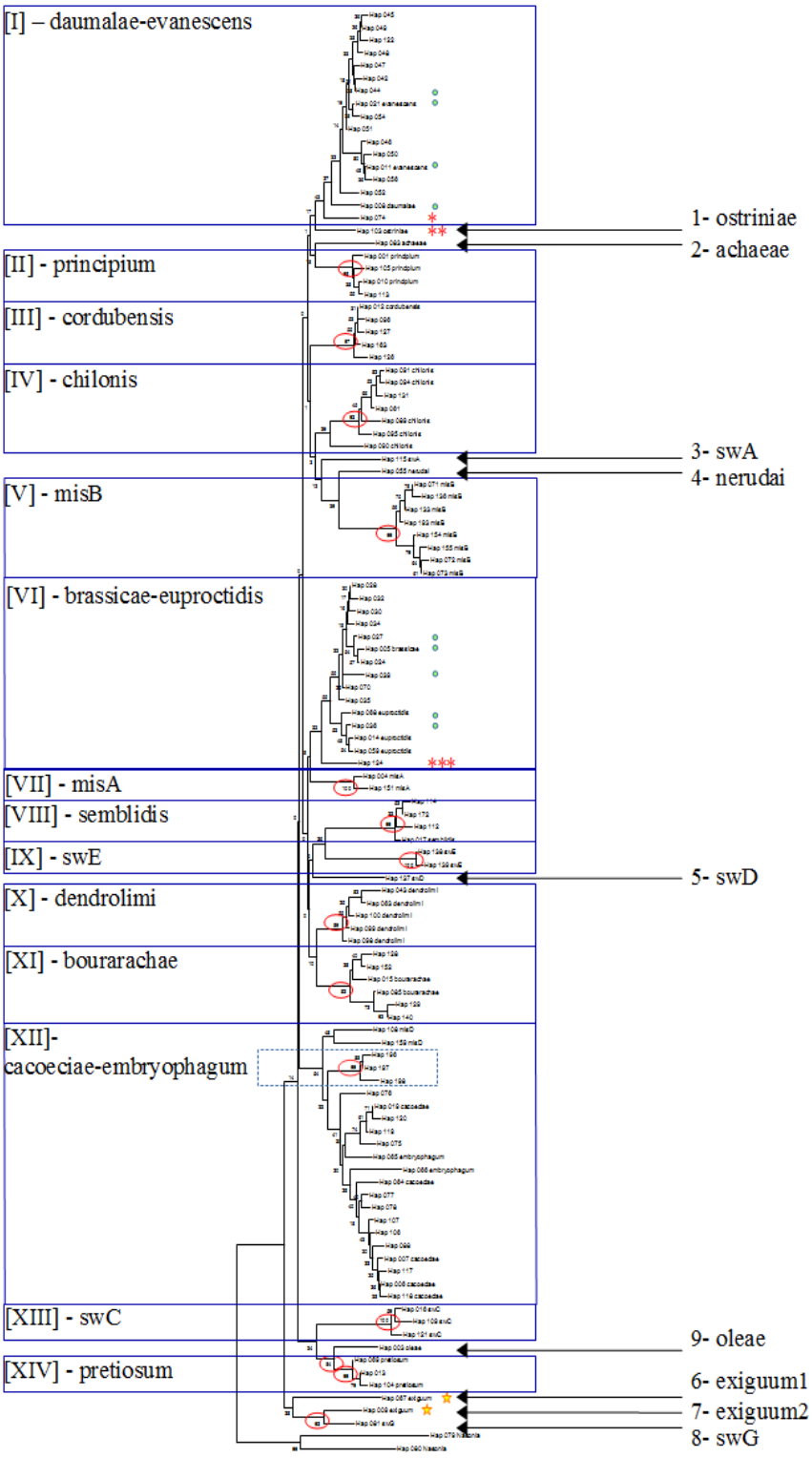

Figure 12: Neighbour-Joining tree obtained from part of the mitochondrial gene *COI*
The tree was obtained using 114 sequences sharing the same amino acid sequence, the Tamura-Nei distance and 500 bootstrap replicates

Crossing experiments were conducted to confirm the reproductive isolation between highly differentiated molecular clusters or haplotypes and to investigate more delicate situation such as the "*brassicae-euproctidis*" complex. *A posteriori*, we evidenced no clear reproductive barriers within this complex but a high variability in reproductive compatibilities, this variability being possibly driven by an isolation by distance (Warot et al. 2018).
To complete the integrative characterization, an important iconography was started about relevant body parts (wings, male genitalia and antennae – see Figure 12) for morphological characterization. Vouchers

were durably mounted (currently: 170 slides - 2 individuals/slide – wing, antennae and genitalia available for each individual - more than 20 *Trichogramma* strains represented). An important stake will be to use this resource to revisit the morphology-based taxonomy of *Trichogramma*, clearly the "missing link" in our integrative approach.

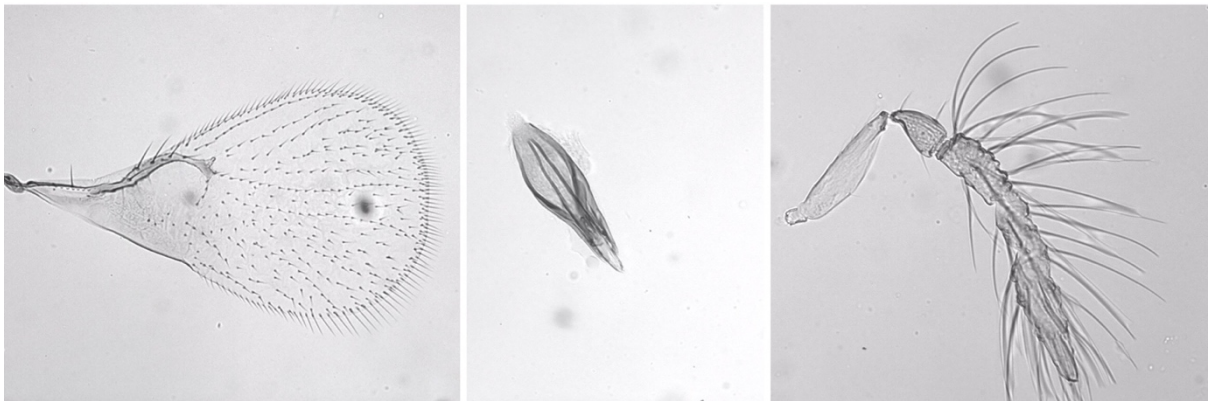

Figure 13 : Example of iconography acquired for morphological characterization (Source : Groussier G.). From left to right: wing, male genitalia and male antennae

Task 5.3: the management of biological resources

Two main types of results were expected from this task: on one side, the certification of the Quality Management System of the BRC EP-Coll (Marchand et al 2017, Mougin et al 2018) and, on the other, the actual use of the BRC EP-Coll by TRIPTIC partners or other colleagues.

With regard to the Quality Management System, the two be certified perimeter included the Management process (M0), three support processes (S1, S2 and S3) and three operational processes (R1, R2 and R3). The three latter were respectively (i) the molecular characterization of *Trichogramma* strains (direct link with Task 5.2), (ii) the maintain of the living collection of *Trichogramma* species and (iii) the distribution of *Trichogramma* (living or dead individuals). During the TRIPTIC project, a very strong investment was realized by several ISA (Partner 2) members (Groussier G, Marchand A, Ris N, Sellier N, Warot S) as well as students in Quality (Gatsinzi G, Lavergne S, Marti L) to improve the organization and satisfy the requirements. The BRC EP-Coll was finally certified ISO9001:2015 by AFNOR in Fall 2018, this initial certification having been confirmed in Fall 2019 (surveillance audit). The 3-years cycle of certification still plans a surveillance audit (Fall 2020) before a full audit in the frame of a new certification (Fall 2021).

With regard to the actual use of the BRC EP-Coll, the BRC EP-Coll played adequately its role by firstly providing and/or maintaining biological material to/for TRIPTIC partners, dead individuals (for WP1 and WP2) or living strains to support experiments of WP3 (in particular, for the PhD Theses of Victor BURTE funded by TRIPTIC and Michela ION SCOTTA) and WP4 (in particular, for the PhD Thesis of Marjorie HAOND and the post-doctoral position of Aline BERTIN funded by TRIPTIC). Meanwhile and outside the TRIPTIC network, the BRC EP-Coll provided and/or stored also biological for the PhD Thesis of Silène LARTIGUE (Collab. Between the Univ. Bourgaogne and ISA), some experiments supervised by Louise Van Oudenhove (ISA) (see internal reports linked to the R3 process). Finally, the BRC EP-Coll also molecularly characterized (*COI* Barcoding) some individuals obtained by other French partners (one cooperative in Guyane, FREDON Martinique, Kan P. & B.) or foreign colleagues (Algeria, Benin, Iran, Romania, Serbia, Turkey) (see internal reports linked to the R1 process). The collaboration with the FREDON Martinique gave the opportunity to publish a paper in an entomological journal (Dumbardon et al. 2018).

The TRIPTIC project was actually a great opportunity to develop three activities (pluri-annual field sampling, massive DNA barcoding, development and certification of Quality Management System) that (i) are very time- and money- consuming, (ii) may appear unrewarding but (iii) were nevertheless necessary to durably provide an expertise and relevant biological material as well as to satisfy international high-quality standards. Most of the technical and methodological objectives were reached, the main pitfall being the delay in the publications of the scientific papers. Thanks to TRIPTIC, the BRC EP-Coll is also now well-anchored within the national landscape about agronomic resource. The BRC

EP-Coll is indeed a main actor in the Pilar "Environment" within RARe:, https://www.agrobrc-rare.org/). In the continuity of TRIPTIC, several initiatives were launched including:

- New surveys of the *Trichogramma* biodiversity, in particular in the Department of Ain (collaboration with Fondation Pierre Vérots: http://cwww.fondation-pierre-verots.com/www.fondation-pierre-verots.com/index.html) and in Corsica (project 2018-2021 Protect'Agrumes). These new *Trichogramma*'s sampling will use at least one other Lepidopteran species as sentinel eggs in order to circumvent potential limits linked to the sole use of *Ephestia kuehniella*.
- The development of new genomic approach in the frame of the starting ANR Ecophyto Maturation project BIDIME (https://ecophytopic.fr/proteger/bidime-biodiversite-des-trichogrammes-diversification-des-produits-de-biocontrole-et) for academic purposes but also applied ones (innovative quality-control).
- The implementation of a Data Management System (BioloMics) for a more reliable and efficient use of information obtained during field surveys, integrative characterization and phenotyping.

Investigations led in the WP5 are likely to produce at least 4 papers in international peer-reviewed journals (two already submitted once). Moreover, the data obtained about the diversity and abundance of *Trichogramma* species and the newly acquired *Trichogramma* strains will be relevant for applied purposes. For instance, the starting ANR Ecophyto Maturation BIDIME (https://ecophytopic.fr/proteger/bidime-biodiversite-des-trichogrammes-diversification-des-produits-de-biocontrole-et) will evaluate some TRIPTIC strains as candidate biocontrol agents for the regulation of lepidopteran species in ornamental or vegetable greenhouses in a Mediterranean context. Finally, both the information and strains acquired could be relevant with regard to regulation issues (importation of exotic strains).

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