Ciliate Mating Types and Pheromones

by
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The story of our knowledge of ciliate mating types and their relevant signaling molecules, originally referred to as mating type substances / factors and today usually described as pheromones (to imply a more general function as chemicals used to communicate between individuals of the same species), is inextricably bound to our understanding about ciliate sexual activity. This activity is commonly manifested as conjugation, autogamy in ciliates, like parthenogenesis in animals, being an additional and much more sporadic alternative. Although conjugation has been described in a variety of organisms, ciliate conjugation is a unique phenomenon of cell-cell interaction and reversible union in mating pairs, that has nothing to do with phenomena of gamete-gamete interaction and irreversible union into a synkaryon. The two ciliates (designated as gamonts) which unite into a conjugal, or mating pair are *de facto* hermaphrodite, vegetatively reproducing cells which will generate two sexually complementary haploid gametic nuclei, one migratory (male) and one stationary (female), only as result of a meiotic process that involves their diploid micronuclei and is triggered by the cell mating union itself.

Ciliate conjugation has long attracted research because it involves a wide variety of nuclear and cellular developmental processes of general interest, and can promptly and massively be induced in laboratory. Obviously, one primary research goal has been to understand the genetic mechanisms responsible for the manifestation of ciliate conjugation.

The first two players, Paramecium aurelia *and* Euplotes patella, *in the study of ciliate mating systems and mating-type substances*

This pursuit was initially centered on *Paramecium*, historically regarded as a paradigmatic experimental model for the study of biology of the entire, yet vast and deeply diversified, ciliate community. A milestone result was obtained by Sonneborn (1937) at the University of Indiana at Bloomington, who demonstrated that mixing different genetic lineages of *Paramecium aurelia* (now *P. primaurelia*) is the experimental condition sufficient and necessary to induce an immediate ciliary cell clumping reaction that soon leads to the formation of conjugal pairs. Based on this demonstration, supplemented with *ad hoc* cross-breeding analyses, Sonneborn reported to have "discovered that there is functional sex diversity" in *Paramecium*, which instead had "long appeared an outstanding example of the absence of sexual differentiation in individuals that nevertheless conjugate"; in addition, he argued that the two cell "sex classes" were genetically determined by a pair of "sex chromosomes". Although the designation of "sex" was later replaced with the more neutral designation of "mating type" (Sonneborn, 1978), the concept of the phenomenological equivalence of ciliate conjugation with a binary event of gamete co-differentiation, interaction, and fertilization was obviously seeded.

On the other hand, only secondary attention was devoted to nearly concurrent studies on conjugation of *Euplotes patella* in which Kimball (1942) reported that "not only can no clear similarity be pointed out between mating

Hausmann/Radek (eds.): Cilia/Flagella – Ciliates/Flagellates

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type determination in *Paramecium* and *Euplotes*, but the method of action of the types in bringing about conjugation appears different" and suggested that the *E. patella* case is "in some respects quite like those of blood groups in man and self sterility in flowering plants". These conclusions were justified by the finding that the *E. patella* mating system had many clearly distinctive traits such as:

(i) the evolution of more than only two mating types,

(ii) multiple alleles of the same gene (annotated as *mt* gene) involved in the mating-type determination,

(iii) the production of mating-type substances freely diffusible into the extracellular environment,

(iv) the capacity of these substances to equally induce the formation of both "heterotypic" and "homotypic" mating pairs (i.e., between genetically different and genetically identical cells, respectively),

(v) the absence of any ciliary clumping reaction antecedent to the mating pair formation, and

(vi) the behavior of cells heterozygous for the mating type as combinations of the two corresponding homozygotes, implying that the *mt* genes in heterozygous combinations are expressed independently from one another and that the differences between the mating-type substances are quantitative as well as qualitative.

A subsequent third player, Blepharisma japonicum

A strong contribution to further credit the bipolar sexual view of ciliate conjugation advanced by Sonneborn was provided by Miyake (visiting researcher in Sonneborn's laboratory in the years 1964–65) through his studies on *Blepharisma japonicum* (reviewed in Miyake, 1978, 1981a, 1981b, 1996). This ciliate is a chronic selfer, like many other heterotrichs; mating pairs spontaneously and pervasively form in its cultures. However, by serially isolating unmated cells from selfing cultures it was eventually possible to obtain temporary non-selfing cell lines capable of forming mating pairs only after being mixed together. These cell lines were designated as mating types I and II, thought of as proxies of a bipolar mating system as in *Paramecium*, and used as "sexually complementary" cell classes in all studies of *B. japonicum* conjugation even though no information was (and still is) available on their genetic determination, and notwithstanding a note by Isquith and Hirshfield (1968) cautioning that these "*Blepharisma* mating types arose in the laboratory and probably would have not survived in nature".

A tryptofan derivative, i.e. a 3-(2′-formylamino-5′-hydroxybenzoil)lactate, was first purified from the culture supernatant of a type-II cell line, designated as "blepharismone" or "gamone 2", and shown to act at nano-gram concentrations as mating inducer of type-I cells (Kubota et al., 1973). Then, a glycoprotein, now known to be formed by a sequence of 272 amino acids plus eight sugars (Sugiura and Harumoto, 2001), was isolated from the culture supernatant of a type-I cell line, designated as "blepharmone" or "gamone 1", and shown to act at pico-gram concentrations as mating inducer of type-II cells (Miyake and Beyer, 1974). In addition to being marked by a striking chemical un-relatedness (Fig. 1), these two molecules were also found to differ in several other significant aspects. While gamone 2 appeared to be very stable, to function as attractant of type-I cells, and to be produced in common among various morphospecies of *Blepharisma*, gamone 1 appeared to be very unstable (its activity needed protection by addition of serum albumin to its preparations), did not attract type-II cells, and was species-specific. Notwithstanding these structural, behavioral and evolutionary divergences, it was assumed that gamone 1 and gamone 2 induce mating pair formation by symmetrically eliciting a positive feedback loop of cell-cell co-stimulation mediated by their binding to hypothetical gamone-receptors expressed only by cells of mating type "sexually complementary" to that of their source cells.

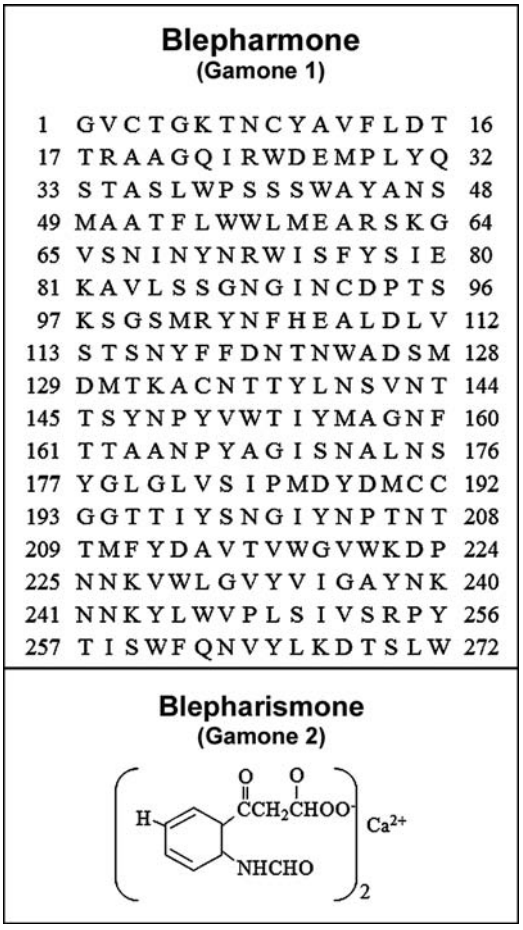

Fig. 1: Gamones of *Blepharisma japonicum*. Amino acid sequence of the glycoprotein gamone 1 (or blepharmone) and chemical structure of the tryptophan derivative gamone 2 (or blepharismone).

This "gamone-receptor hypothesis", based on the applying of the concept of complementarity to cells of interacting mating types (Miyake, 1981a, 1996), has also been generalized to explain the control of mating interactions in all ciliates, including species of *Euplotes*, *Stylonychia* and *Uronychia*, which are characterized by multiple mating systems with undetermined numbers of mating types. This generalization assumed that, (i) "ciliate mating types are produced by differential distribution of gamones and receptors among individuals" each cell expressing a mating type having "none, one or more of different kinds of gamones and none, one or more of different kinds of receptors except the one(s) for its own gamone(s)", and (ii) "when a receptor on the cell surface unites with a gamone specific for the receptor, the cellular mechanism of conjugation is switched on" (Miyake, 1996). Thus, in practice, if a given species has a mating system based on ten interacting mating types (Mt-I to Mt-X), Mt-I cells would have G_1R_2 to R_{10} as "mating-type formula" (where G and R indicate the cell-specific gamones and receptors, respectively, and the numbers indicate the cell mating type) and they would mate, for example, with the "sexually complementary" Mt-X cells having the $G_{10}R_1$ to R_9 formula suitable for the formation of G_1-R_1 and G_{10}-R_{10} complexes responsible for the mating pair formation.

The coming of E. raikovi *and* E. octocarinatus *onto the scene*

For some time immediately preceding the nearly simultaneously inclusion (in 1980) of *E. octocarinatus* and *E. raikovi* into the study of ciliate mating type substances, three other *Euplotes* species, i. e. *E. crassus*, *E. minuta* and *E. vannus* were object of strenuous efforts, at Renzo Nobili's laboratory at the University of Pisa, to identify the *Euplotes* mating type substances. These efforts were fueled by a Akio Miyake's two-year collaboration (1974–75) in Pisa with Renzo Nobili, his friend from the time of their meeting in Sonneborn's laboratory in Bloomington. *Euplotes crassus*, *E. minuta* and *E. vannus* are phylogenetically closely allied with one another (Valbonesi et al., 1988; Petroni et al., 2002), and all are characterized by high-multiple systems of mating types inherited through multiple series of *mat* alleles whose expression has been known, since the pioneer works by Heckmann (1963, 1964) in *E. vannus* and *E. crassus* and Nobili (1966) in *E. minuta*, to be regulated by relationships of serial (or hierarchical) dominance.

Numerous strains of *E. crassus* in particular were used by Miyake and Nobili (1974) for the identification of their mating-type substances, but unlike the behavior of the *E. patella* strains studied by Kimball (1942) all systematically refused to form homotypic mating pairs in response to suspension with cell-free filtrates of cultures of strains expressing other mating types. This lack of supernatant-mediated induction of homotypic mating pairs was thus taken as evidence that the mating-inducing substances of *E. crassus* and its allied species were not freely released into the extracellular environment, but rather were retained firmly bound to the cell body membranes as supposed in *Paramecium* and, as such, difficult to isolate and characterize chemically (Miyake, 1981a). In accord with previous studies by Heckmann and Siegel (1964) in *E. crassus*, it was also presumed that the lack of an immediate ciliary clumping reaction (a lack that distinguishes the mating mixtures of these species and of all other ciliates in general from those of *Paramecium*) was due to under-threshold levels of mating substances present in cells at the time of their mixing. The threshold levels required to obtain successful cell-cell unions in mating pairs would have been reached, during a so-called "waiting period", as result of a positive feedback mechanism triggered by physical interactions between the mixed cells (Miyake, 1981a).

While *E. octocarinatus* monopolized the laboratory of Klaus Heckmann at the University of Münster starting from a few specimens isolated from a freshwater aquarium containing fish and water imported from central America (Möllenbech and Heckmann, 1999), *E. raikovi* monopolized the laboratory of the senior author (P. Luporini) of this article (in the period of his move from the laboratory of Renzo Nobili at the University of Pisa to the University of Camerino) starting from 32 specimens isolated from the same one-liter sample of sea water taken (on the morning of June 13, 1979) from a coarse sandy bottom rich in organic sediment from the beach of Porto Recanati on the Adriatic coast of Italy (Miceli et al., 1981). Fed on the green alga *Dunaliella*, 27 of these specimens survived and generated strains which, due to their unique seven-plus-one array of the fronto-ventral cirri, were all promptly recognized as representative of *E. raikovi*, a species originally described by Agamaliev (1966) from the Caspian sea and then found to be distributed worldwide in temperate seawater, from the coasts of New Hampshire (Washburn and Borror, 1972) to those of China (Jiang et al., 2010).

It was soon evident that *E. raikovi* and *E. octocarinatus* were equally characterized by the evolution of multiple interacting mating types specified by multiple series of alleles co-dominantly expressed at the *mat* locus, a marked propensity to form both hetero- and homotypic mating pairs in their mixtures and, most important, a general readiness to respond with homotypic mating pair formation to suspension with supernatant preparations taken from the cultures of the other mating types (Luporini et al., 1983; Heckmann and Kuhlmann, 1986). In practice, both the *E. raikovi* and *E. octocarinatus* mating systems were found to exactly match the *E. patella* system described by Kimball nearly 40 years earlier and their experimental availability prompted reasonable optimism that finally *Euplotes* mating type substances could be successfully isolated and characterized.

The initial purification and chemical characterization of E. raikovi *and* E. octocarinatus *mating type substances*

The pathway to the purification and characterization of the *E. raikovi* and *E. octocarinatus* mating type substances understandably encountered some initial misinterpretations, essentially due to the limited amounts of material obtained by concentrating culture supernatant preparations through rather time-consuming methods (such as ultra-filtration and hydroxyapatite precipitation) and using not very effective (as understood in retrospect) electrophoretic and chromatographic fractionation procedures (Miceli et al., 1983; Weischer et al., 1985). In particular, there were overestimations of the molecular masses and mating-

inducing activities of the purified products, as well as wrong assumptions that these products were glycoproteins (whereas the carbohydrate component was merely associated, but not covalently bound to the protein component). In addition, in the case of *E. raikovi* the product of the first purification was also reputed to be homogeneous and, in assonance with the *Blepharisma* gamone "blepharismone", specifically named "euplomone *r* 13" (where "euplomone" merges *Euplotes* and gamone/pheromone into a single term, *r* stands for *raikovi* and 13 is the number of the strain used in the purification) (Miceli et al., 1983). However, subsequent Mendelian analysis of the allelic combination at the *mat* locus of strain 13, chosen for the first pheromone purification because of the stronger mating inducing activity measured in the supernatant of its cultures (and deposited at the ATCC center with the catalog # PRA-327), revealed a heterozygous condition for two co-dominantly expressed *mat-1* and *mat-2* genes and, hence, a genetic commitment to co-secrete two distinct proteins. These proteins were soon effectively separated to homogeneity in quantitatively eccentric ratio, shown to be active at nano-molar concentrations, and annotated as E*r*-1 and E*r*-2 thereafter adopting the criterion (equally adopted for the gamones/pheromones of *E. octocarinatus*) of numbering each *Euplotes* pheromone with the same number as the number assigned to its determinant *mat* gene (Concetti et al., 1986; Luporini et al., 1986).

Although *E. raikovi* and *E. octocarinatus* have been studied in parallel because the biology of their mating systems has many matching aspects, the routes to the determination of the structures of their pheromones have significantly diverged, essentially in consequence of the deep quantitative differences in the pheromone secretion that distinguish the two species. No more than 0.5 μg of pure protein could ever be recovered from one liter of supernatant for *E. octocarinatus* pheromones (Schulze-Diekoff et al., 1987) making it difficult to determine their structures by a direct chemical approach. Instead, from 100 to 200 μg/liter of pure protein were in general recovered for *E. raikovi* pheromones (Raffioni et al., 1987), and these high rates of pheromone production were shown to reflect high, cell type-specific degrees of amplification (2.9×10^4 copies were roughly calculated in type-I cells) of the "gene-size" DNA molecules that originate from the micronuclear *mat* genes and drive the pheromone synthesis in the transcriptionally active macronucleus (La Terza et al., 1995). This relative abundance of *E. raikovi* pheromones facilitated the classic automated Edman degradation approach to determine the pheromone amino acid sequences and, more important, was decisive for the successful determination of the pheromone three-dimensional structures via NMR spectroscopy and crystallography. The following two sections summarize the overall knowledge earned through 20 years of research on the *E. octocarinatus* and *E. raikovi* pheromone structures.

Primary structures of Euplotes *pheromones*

Nine pheromone amino acid sequences have been determined in both *E. raikovi* and *E. octocarinatus*, the first two sequences of the *E. raikovi* pheromones, E*r*-1 and E*r*-10, by direct chemical analysis of the purified proteins (Raffioni et al., 1988, 1989) and the other ones (in *E. octocarinatus* in particular) by molecular cloning of the relevant macronuclear coding genes.

They contain 37–51 amino acid residues in *E. raikovi* (Raffioni et al., 1992; Luporini et al., 1995; Vallesi et al., 1996) and 85–109 in *E. octocarinatus* (Meyer et al., 1991, 1992; Tekentrup et al., 1996; Brünen-Nieweler et al., 1998; Möllenbeck and Heckmann, 1999), and all are particularly rich in cysteines and characterized by rather acidic (from 3.25 to 4.08) isoelectric points (Fig. 2 and 3). As it is typical for many secreted protein hormones, also *E. raikovi* and *E. octocarinatus* pheromones are synthesized as immature cytoplasmic precursors (pre-pro-pheromones) organized with the canonical signal-peptide/pro-segment/mature-protein succession, from which the diffusible forms have to be released by proteolytic cleavages of the signal-peptide and pro-segment

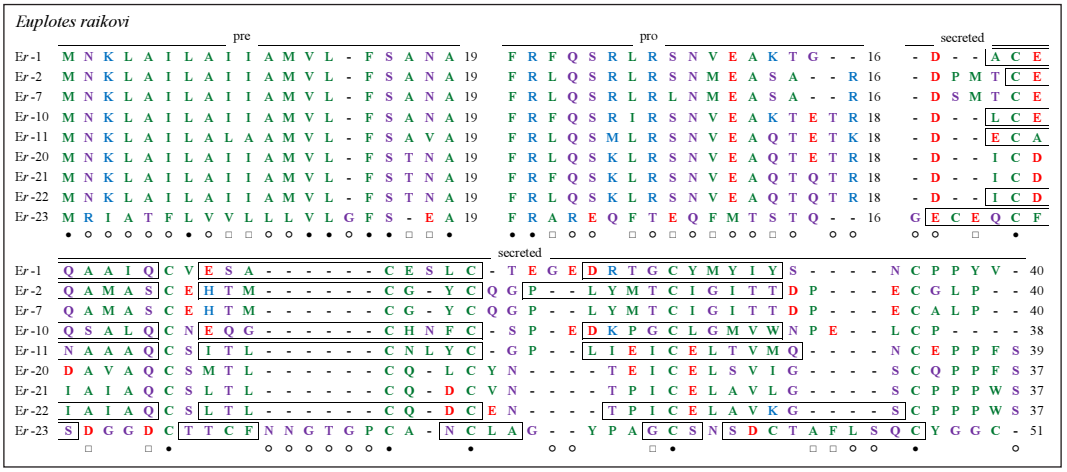

Fig. 2: Multiple amino acid sequence alignment of *E. raikovi* pheromones. Sequences include the signal peptide and pro segment of the pheromone cytoplasmic precursors (pre-pro-pheromones), as deduced from the determination of macronuclear DNA coding sequences. Alignment is based on the Clustal W algorithm and optimized manually by gap insertions. Filled circles, open circles, and open squares mark positions occupied by one, two or three identical amino acids, respectively. Limitedly to the pheromones with known three-dimensional structures, regions involved in the formation of α-helix secondary structures are boxed. Hydrophobic, polar, positively and negatively charged amino acids are color-coded green, violet, blue and red, respectively. The sequence lengths of each pre-pro-pheromone region are reported on the right.

(Miceli et al., 1989, 1991; Meyer et al., 1992). A salient feature of these precursors is the remarkable degree of sequence identity that distinguishes both the signal-peptide (to a major extent) and the pro-segment from the secreted, mature proteins which are, instead, quite variable.

In the signal-peptide sequences of *E. octocarinatus* there is no substitution altogether, and the scattered substitutions that appear in the 19-residue sequences of *E. raikovi* essentially reside in the sequence of E*r*-23 pheromone, which is a somewhat structurally eccentric member of the *E. raikovi* pheromone family (as described below). Between *E. raikovi* and *E. octocarinatus* there is full amino acid conservation in more than half of the positions, as well as a common organization of the basic functional features of any signal peptide. These involve an accumulation of positively charged and hydrophobic residues, and a processing site for signal peptidases which, in good agreement with consensus sequences of known signal peptides (Bendtsen et al., 2004), is represented by the Ala-Phe dipeptide.

With regard to the pro-segment, in *E. octocarinatus* there is only one substitution throughout the first 29 positions (the pheromone Phr4 excluded since its alignment requires the introduction of some gaps to compensate for the shorter sequence of its pro-segment extended for 26 residues against the 36 ones of the other pheromones). Similarly, in *E. raikovi* the degree of sequence identity always exceeds 50% (E*r*-23 excluded). The degree of similarity is also remarkable at the inter-species level and is essentially due to the motif (potentially important for post-translational modifications) Arg/Lys-Xxx-Xxx-Ser (with occasional changes of Arg/Lys with Gln or Met, and Ser with Glu or Thr), that is repeated twice in *E. raikovi* and up to six times in *E. octocarinatus*. On the other hand, variations appear in relation to the pro-segment length, that in *E. octocarinatus* is double that in *E. raikovi* (presumably as a result of an event of gene dupli-

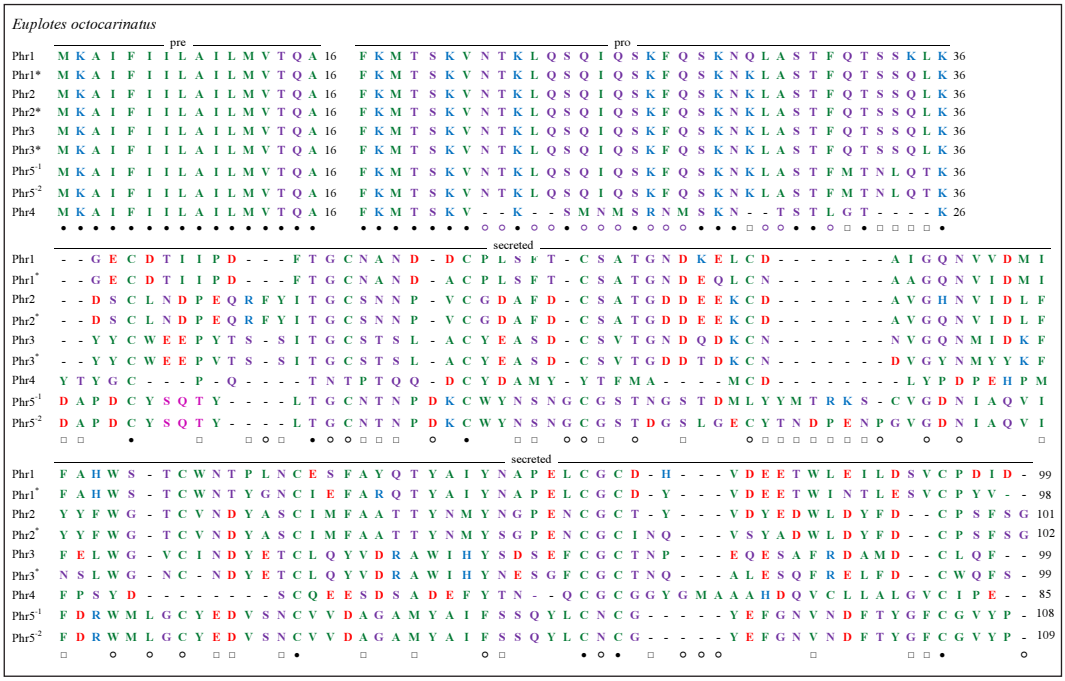

Fig. 3: Multiple amino acid sequence alignment of *E. octocarinatus* pheromones. Sequences include the signal peptide and pro segment of the pheromone cytoplasmic precursors (pre-pro-pheromones), as deduced from the determination of macronuclear DNA coding sequences. Alignment, symbols and other indications as in figure 2.

cation), as well as the processing site for the release of mature pheromone forms that varies from the dipeptide Lys-Asp(Tyr/Gly) in *E. octocarinatus* to the dipeptide Arg(Gln/Gly/Lys)-Asp(Gly) in *E. raikovi*.

Going from the signal-peptide and pro-segment to the secreted proteins, the degree of sequence identity drops drastically at both the intra- and inter-specific levels. Among *E. raikovi* pheromones there are values of sequence identity as low as 25–30%, that are generally regarded as unconvincing for inferring genuine relationships of molecular homology. Nevertheless, the amino terminus of most pheromone sequences carries an Asp residue as recognition site for the proteolytic pro-pheromone processing, and the positions of the Cys residues are fully conserved at intra-specific level. The six Cys residues (designated Cys-I to Cys-VI) of *E. raikovi* pheromones have originally been determined to be paired into three tightly conserved intra-chain disulfide bonds (i. e., I–IV, II–VI and III–V) (Stewart et al., 1992), and four of them find possible counterparts with four of the ten Cys residues of *E. octocarinatus* pheromones, thus implying that the two pheromone families have two disulfide bonds in common.

Three-dimensional structures of E. raikovi *pheromones*

The three-dimensional molecular structures for the *E. raikovi* pheromones E*r*-1, E*r*-2, E*r*-10, E*r*-11, E*r*-22, and E*r*-23 have been determined by homonuclear solution ^{1}H NMR analysis of purified protein preparations (Brown et al., 1993; Mronga et al., 1994; Ottiger et al., 1994; Luginbühl et al., 1994, 1996; Liu et al., 2001; Zahn et al., 2001), and

also by X-ray crystallography in the case of E*r*-1 (Weiss et al., 1995). With the exception of pheromone E*r*-23, these structures closely resemble one another regardless of the deep divergences that may distinguish their amino acid sequences (Fig. 4). The basic architecture is provided by a compact bundle of three nearly parallel helices (designated 1 to 3 starting from the molecule amino terminus), that are connected by two prominent loops of 2 to 4 residues and arranged with an up-down-up topology. The two longer helices 1 and 3 show a regular α-organization, while the shorter helix 2 tends to be less regular and appears as a stretch of distorted 3_{10}-helix turns. The stability of the pheromone architectures is ensured by the strictly conserved disulfide bridges provided by the six Cys residues; two bridges directly fasten the three helices to one another and one bridge ties the carboxyl terminal sequence tail to the loop between helices 1 and 2. The spatial proximity of the two inter-helix disulfide bonds is so close that quantitatively minor conformations of each pheromone may be generated by disulfide isomers derived from all the three possible disulfide combinations between the four Cys residues I, III, IV, and V (Brown et al., 1993; Luginbühl et al., 1994).

On this common basic architecture, every pheromone evolved its own set of local structural tags that are clearly of functional importance to secure its specificity in the binding reactions to the cell receptors. These tags principally involve variations in the conformation of helix 2, the extension of the loop between helices 2 and 3, the amino acid composition of helix 3, and the shape and orientation of a carboxyl terminal tail (entirely lacking in pheromone E*r*-23) downstream of the last Cys residue.

As mentioned above, the pheromone E*r*-23 stands quite apart from the standard molecular architecture shown by the *E. raikovi* pheromone family. This structural eccentricity appears to be the consequence of an event of internal gene sequence duplication that determined an increase (from six to ten) in the number of Cys residues and the insertion (in the middle of the sequence) of a new 11-residue sequence (Di Giuseppe et al., 2002). To accommodate this new sequence stretch, E*r*-23 has markedly modified its molecular architecture into a new five-helix fold model, in which the additional two helices are quite short (involving only three or four residues) and the stabilization is provided a five disulfide bonds (Zahn et al., 2001). Nevertheless, although unique, the E*r*-23 conformation appears to be reconcilable with the standard conformation of all the other *E. raikovi* pheromones. In fact, the helices 1, 2 and 5 of the E*r*-23 structure match the up-down-up arrangement of the three helices of the other *E. raikovi* pheromones, and there is a spatial equivalence of the disulfide bonds tethering the E*r*-23 helices 1, 2 and 5 close to one another with the standard array of three disulfide bonds.

Further evidence that E*r*-23 retained not only structural motifs, but also functional domains in common with the other *E. raikovi* pheromones derives from comparison with the other pheromones for the physico-chemical specificities of the molecular surfaces. As shown in figure 5, E*r*-23 shares with all the other pheromones a quite extended and pronounced surface domain which includes a cluster of ten amino acids and essentially represents the core of the molecular face that is delimited between helices 1 and 2 and involved in the pheromone interactions with their receptors (as described below).

The more recent contribution of E. nobilii *and* E. crassus *to knowledge of pheromone structures*

The study of *E. nobilii* mating and pheromone biology began 20 years later than that of *E. raikovi* and *E. octocarinatus*, and its significance is essentially correlated to the polar habitat that distinguishes *E. nobilii* from the phylogenetically closely allied *E. raikovi* (Jiang et al., 2010). Strains of *E. nobilii* were first collected from the coasts of Terra Nova Bay in Antarctica (Valbonesi and Luporini, 1990) and then from the Svalbard and Greenland Arctic coasts (Di Giuseppe et al., 2011),

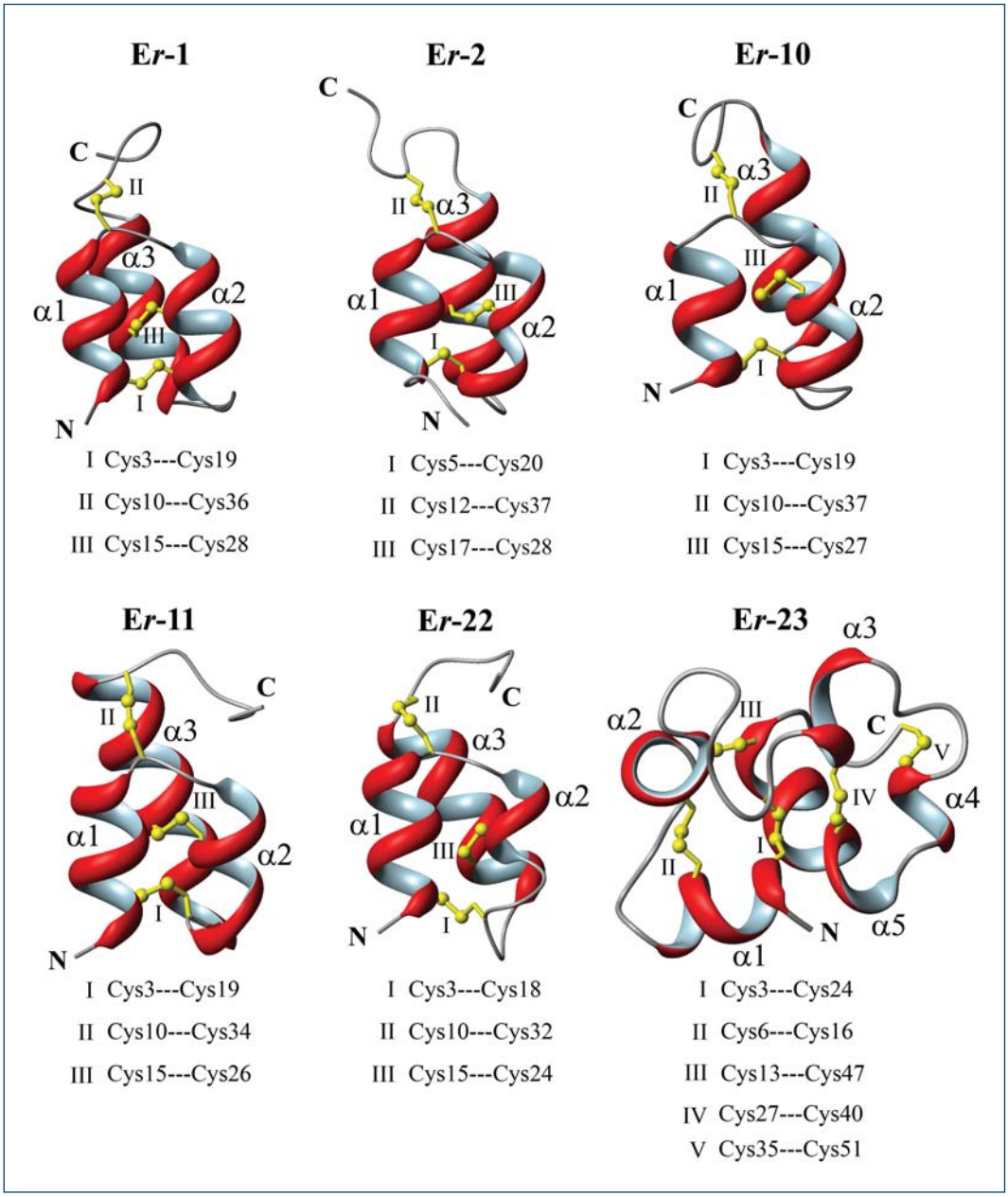

Fig. 4: NMR structures of *E. raikovi* pheromones, as visualized by the program MOLMOL in their ribbon presentations. The α-helices are red and sky-blue colored and identified as α1, α2, α3, α4 and α5. The disulfide bridges and the cysteine sulfur atoms ~~of~~ are highlighted as yellow sticks and spheres, respectively. The positions of the molecule amino and carboxyl termini are marked by N and C, respectively, and the disulfide bond array is indicated for each pheromone structure.

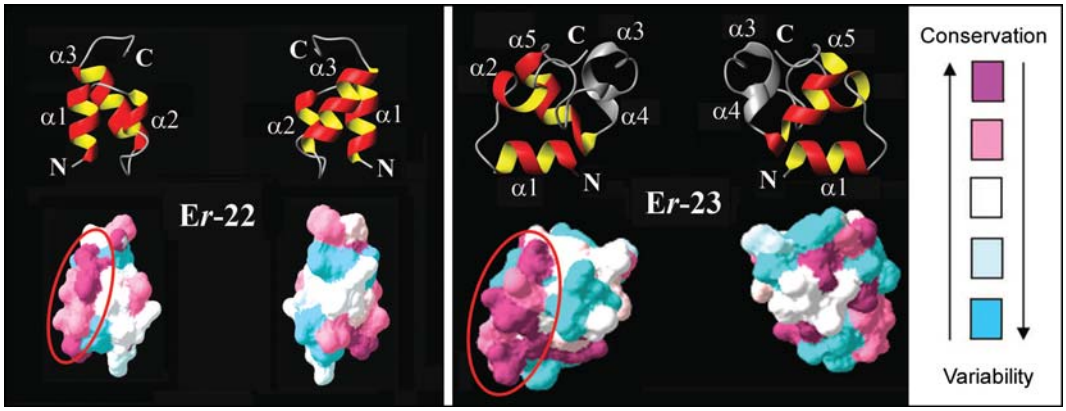

Fig. 5: Molecular surface conservation between the *E. raikovi* pheromone Er-23 (a structural deviant member of the *E. raikovi* pheromone family) and the pheromone Er-22 (taken to represent the other pheromones with known NMR structures). The two molecules are shown in frontal and back views and their α-helices are numbered progressively from the amino terminus (N) to the carboxyl terminus (C); the three helices conserved in both molecules are red-yellow colored, while the two helices unique to the Er-23 structure are grey. The scale of conservation of the molecular surfaces is color-coded dark violet for maximal conservation, white for average conservation and dark turquoise for maximal variability, while the surface patch strictly conserved in both molecules is outlined by red ovals. The scale resulted from the application of the "Conservation Surface-mapping" (ConSurf) and "Rate4Site" programs (Armon et al., 2001; Glaser et al., 2003), which map the evolution rate of the molecular surfaces of homologous proteins with known three-dimensional structures and identify hot spots and patches of a molecular surface that are likely to be in effective contact with other protein domains, nucleic acids and ligands (after Luporini et al., 2006).

and found to possess a mating and pheromone system fully equivalent to those of *E. patella*, *E. raikovi* and *E. octocarinatus* (Felici et al., 1999). The study of *E. nobilii* pheromones thus provided a unique opportunity to probe not only into the structural divergences that distinguish these molecules at inter-species level, but also into the structure-function modifications that adaptive evolution imposes on water-borne proteins that must be active at environmental sub-zero temperatures (Alimenti et al., 2002, 2003a and b).

Seven distinct *E. nobilii* pheromones (collectively designated "E*n*") have been characterized for their amino acid sequences and macronuclear DNA coding sequences (as described below), three (i.e., E*n*-1, E*n*-2 and E*n*-6) specific to Antarctic strains and four (i.e., E*n*-A1, E*n*-A2, E*n*-A3 and E*n*-A4) specific to Arctic strains. For four of them (i.e., E*n*-1, E*n*-2, E*n*-6 and E*n*-A1), which could be purified in more abundance, it was also possible to determine the NMR structures (Pedrini et al., 2007; Placzek et al., 2007; Alimenti et al., 2009; Di Giuseppe et al., 2011).

As in *E. raikovi* and *E. octocarinatus*, in *E. nobilii* pheromones are synthesized as cytoplasmic precursors destined to be proteolytically processed to remove a fully conserved pre-segment and a less strictly conserved pro-segment (Fig. 6). However, while the co-translational cleavage site is similarly provided by a canonical Ala-Phe dipeptide, the post-translational cleavage site is unconventional for known endopeptidases, since it coincides with the combinations Gly-Asn/Asp, Ser-Thr, Ala-Tyr and Glu-Thr/Asp.

The sequences of the secreted pheromones have lengths from 58 to 63 amino acids, intermediate between those of *E. raikovi* and *E. octocarinatus* and, as in *E. raikovi*, they show levels of pair-wise identity that are as low as

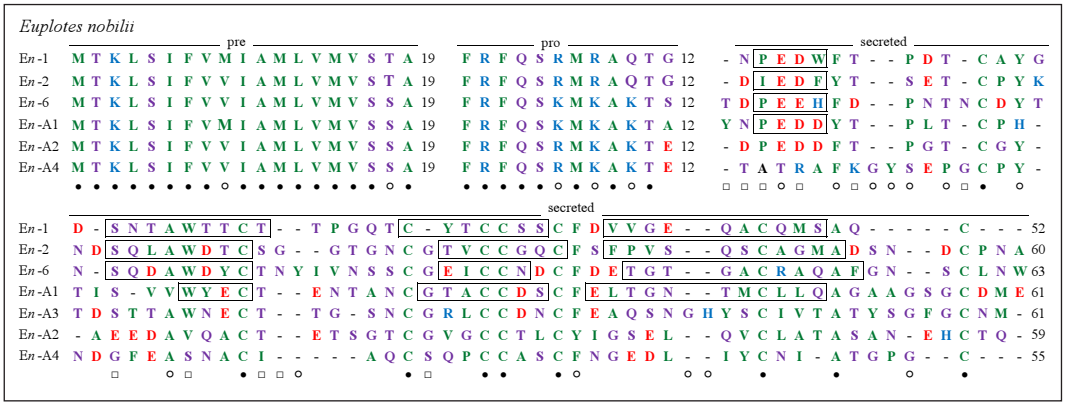

Fig. 6: Multiple amino acid sequence alignment of *E. nobilii* pheromones. Sequences include the signal peptide and pro segment of the pheromone cytoplasmic precursors (pre-pro-pheromones), as deduced from the determination of the macronuclear DNA coding sequences. Alignment, symbols and other indications as in figure 2.

22 %, with full alignment practically limited only to the eight cysteines that are paired in strictly conserved disulfide bonds. Nevertheless, *E. nobilii* pheromones all uniformly show an amino acid composition which clearly reflects their adaptation to freezing polar waters. In comparison with *E. raikovi* pheromones, they are significantly richer in polar and aromatic amino acids (44 % vs. 33 % and 11 % vs. 6 %, respectively), and significantly poorer in the hydrophobic ones (45 % vs. 55 %); only the contents in charged amino acids are equivalent (15 %) (Table 1). Other distinctive features of *E. nobilii* pheromones are lower percent of amino acid residues located in α-helices (46.3 % vs. 65.1 %), higher global hydrophilicity (−0.35 vs. −0.01), lower Aliphatix index (average values of 34.3 vs. 65.5), and lower net charge (−9.5 vs. −7.1).

The three-dimensional structures of *E. nobilii* pheromones manifest unmistakable homology with those of *E. raikovi*, the two protein families having in common an up-down-up three-helix bundle core, a conserved pattern of disulfide bonds, and a structurally disordered carboxy-terminal polypeptide segment (Fig. 7). However, *E. nobilii* pheromones also show unique cold-adaptive structural traits mainly represented by a well-defined long amino-terminal segment of 14 to 17 residues and an extended loop of 5 to 10 residues between helices 1 and 2. These two molecular districts are devoid of regular secondary structures and, hence, may more directly contribute to contrast the negative thermodynamic effects of the polar waters by favouring local enhancements of the polypeptide backbone flexibility. Furthermore, all *E. nobilii* pheromones carry clusters of negatively charged side chains preferentially localized on their long amino-terminal extension where they likely function, as usually observed in psychrophilic proteins (Siddiqui and Cavicchioli, 2006), to weaken the bundle of the molecular structure by widening the solvent-accessible areas and enhancing solvent interactions.

With regard to *E. crassus*, as already described above, the inactivity of cell-free filtrates in inducing homotypic mating pair formation has justified the long-held view that in this species pheromones are represented by cell-bound proteins acting through physical cell-cell interactions (Miyake, 1981a). In addition, based on late position that *E. crassus* takes in the *Euplotes* phylogenetic tree (Petroni et al., 2002; Jiang et al., 2010), it has also been proposed that pheromone secretion in *Euplotes*

Table 1: Cold-adaptation of *E. nobilii* pheromones: amino acid composition comparison with *E. raikovi* pheromones.

	E. nobilii Antarctic[h] (means)	*E. nobilii* Arctic[i] (means)	Arctic + Antarctic (means)	*E. raikovi*[l] (means)
Polar[a]	44.6	43.6	44.1	33.1
Hydrophobic[b]	44.1	47.1	45.6	55.0
Charged[c]	15.7	14.7	15.2	15.9
Aromatic[d]	11.9	10.1	11.0	6.80
Located in α-helices	48.4	44.3	46.3	65.1
Global hydrophobicity[e]	− 0.49	− 0.22	− 0.35	− 0.01
Aliphatic index[f]	25.9	42.8	34.3	65.5
Net charge[g]	−10.1	− 8.9	− 9.5	− 7.1

[a] Asn, Gly, Gln, Ser, Thr, Tyr; [b]Ala, Cys, His, Ile, Leu, Met, Phe, Pro, Tyr, Trp, Val; [c]Arg, Asp, Glu, His, Lys; [d]His, Phe, Tyr, Trp; [e]as determined by the GRAVY index (Schiffer and Dötsch, 1996) which is relative to the ratio between the sum of hydropathy values of all residues and the total numbers of these residues; [f]defined as the relative volume that the amino acid lateral aliphatic chain occupies in a protein (Arnórsdottir et al., 2002); [g]calculated for sea water pH 8.0; [h]pheromones En-1, En-2, En-6; [i]pheromones Er-1, Er-2, Er-10, Er-11, Er-22, Er-23.

should be regarded as a phenomenon distinctive of early branching species such as *E. raikovi* and *E. nobilii*, and destined to be secondarily lost (Vallesi et al., 2008). These views have recently been contradicted by the demonstration that *E. crassus*, like other (presumably all) *Euplotes* species, actually constitutively secretes its pheromones.

In consideration that various *Euplotes* species, including *E. crassus* and its allied species *E. minuta* and *E. vannus*, can easily cross-react with one another most likely in relation to their virtually "open" multiple mating systems (Nobili et al., 1978; Dini and Luporini, 1985; Valbonesi et al., 1992; Kuhlmann and Sato, 1993), a large number of *E. crassus* strains were rather unsystematically screened for identifying cell-culture supernatants able to induce homotypic mating pairs *not* within cultures of other *E. crassus* strains, *but* within cultures of *E. raikovi* strains known to be at the same time able to secrete pheromones and ready to mate in response to suspension with non-self supernatants (Alimenti et al., 2011). This screening resulted in the identification of three interbreeding *E. crassus* strains regularly able to induce *E. raikovi* cells to mate. Cell-free filtrates were prepared from the most reactive one (i. e., the strain L2-D) and found to contain two distinct soluble pheromones, which were purified (in amounts ranging from 15 to 30 µg of protein/liter of filtrate) and both shown to be equally active (at micro-molar concentrations) in inducing effective *E. raikovi* mating and only loose cell-cell ciliary sticking in *E. crassus* (Alimenti et al., 2011).

The structural characterization of these two pheromones (designated E*c*-α and E*c*-1), along with the characterization of their macronuclear DNA coding sequences (described below), revealed that they markedly differ from one another in their secreted sequences and also in their precursor sequences (Fig. 8). There are only six residues fully conserved in their hydrophobic signal-peptide and four in the pro-segment which, in addition, is substantially hydrophilic and extremely basic in the E*c*-α precursor and unusually rich in negatively charged residues of glutamic acid and aspartic acid in the E*c*-1 precursor. At the level of the secreted regions, the comparison between the E*c*-α and E*c*-1 sequences is strongly conditioned by the alignment of the Cys residues, eight in the E*c*-α 56-amino acid

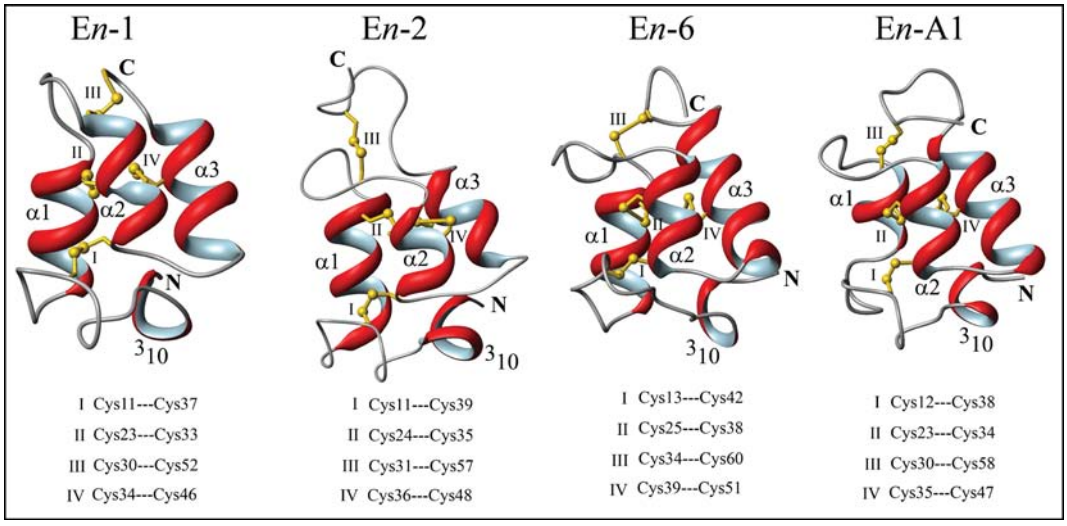

Fig. 7: NMR structures of *E. nobilii* pheromones, as visualized by the program MOLMOL in their ribbon presentations. The three α-helices, which are in common with E. *raikovi* pheromones, and the distinctive 3_{10}-helix are identified as α1, α2, α3 and 3_{10}, respectively. Other indications and representations as in figure 4.

sequence and ten in the E*c*-1 sequence of 45 amino acids. The alignment between the eight Cys residues of the E*c*-α sequence and eight of the ten Cys residues of the E*c*-1 sequence requires an ad hoc insertion of 13 gaps in the E*c*-1 sequence, and it is only through this insertion that it becomes possible to also align eight other residues, six of which (including four closely packed Cys residues) cluster into the unique sequence domain that the E*c*-α and E*c*-1 show in common and presumably use for a common function. This shared cysteine-rich sequence domain excluded, the two pheromone sequences differ markedly in their hydropathy profiles, amino acid composition of their N-terminal regions, and the extension of two inter-cysteine segments located in the sequence core.

The structural characterization of other *E. crassus* pheromones is clearly necessary to understand better the significance of the marked structural differences that exist between the E*c*-α and E*c*-1 pheromones at level of both their amino acid and DNA coding sequences. However, the fact itself that the same L2-D *E. crassus* strain secretes two distinct pheromones clearly conflicts with the consolidated concept that in *E. crassus* (as well as in *E. vannus* and *E. minuta*) the mating types and their associated pheromones are inherited through a multiple series of *mat* alleles regulated by relationships of hierarchical dominance, whereby *mat*-heterozygous cells would synthesize only the pheromone determined by their dominant *mat* allele. Two new and mutually not exclusive explanations have been proposed to account much better for the *E. crassus* genetic mechanism of mating-type and pheromone inheritance (Alimenti et al., 2011). Either the *mat* alleles of *E. crassus* have no mutual relationship of hierarchical dominance, as is the case in the other pheromone-secreting species of *Euplotes*, or the *E. crassus* micronucleus carries two individual *mat* loci resulting from an event of gene duplication.

Macronuclear pheromone-coding genes

The DNA coding sequences of *Euplotes* pheromones have been determined only from the transcriptionally active macronuclear genome, and nothing is yet known about the *mat*-gene

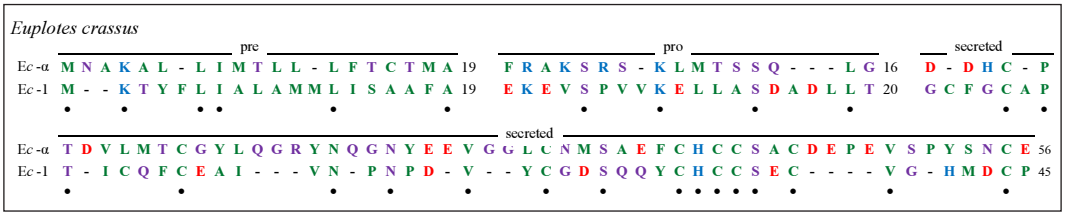

Fig. 8: Amino acid sequence alignment of *E. crassus* pheromones. The two sequences include the signal peptide and pro segment of the pheromone cytoplasmic precursors (pre-pro-pheromones), as deduced from the determination of macronuclear DNA coding sequences. Filled circles mark positions occupied by identical amino acids. Alignment, symbols, and other indications as in figure 2.

sequences that represent their developmental precursors in the transcriptionally inert micronuclear genome. Full-length pheromone genes have been determined in significant numbers only in *E. octocarinatus* and *E. nobilii* (nine and seven sequences, respectively), while in *E. raikovi* only the full-length sequence encoding pheromone E*r*-23 has been reported (Di Giuseppe et al., 2002) (other sequences deduced from cDNA clones lack the non-coding regions) and in *E. crassus* only those of the E*c*-1 and E*c*-α pheromones are known (Alimenti et al., 2011).

In *E. octocarinatus*, the macronuclear pheromone genes extend from 1616 to 1765 base pairs (bp) (telomeres included) and all show the same basic organization characterized by a coding region that starts from position 163 and includes cysteine-encoding TGA codons plus three introns responsible for a subdivision of the coding region into four exons (Brünen-Nieveler et al., 1991, 1998; Meyer et al., 1991, 1992; Teckentrup et al., 1996). The intron sequences are all closely conserved along with their splicing sites provided by the canonical motifs 5′-GAT/TAG-3′. The first intron lies inside the signal-peptide and pro-segment sequences, and is more conserved and much larger (from 371 to 772 bp) than the other two introns (spanning from 45 to 109 bp) that break the pheromone coding sequence. Its G+C content closely matches the exon G+C contents, but no evidence was found that it is involved in coding activities. With regard to the non-coding regions, the 5′ region is practically identical in all genes and includes CAAT and TATA boxes for the transcription initiation, while the 3′-region appears to be a little more variable (although there are identities of up 94%) and contains an AATAAC motif as potential polyadenylation signal.

With respect to the *E. octocarinatus* macronuclear pheromone genes, those of *E. nobilii* have much reduced dimensions (from 950 to 1010 bp) and a coding region, starting from position 362 (361 in the E*n*-6-pheromone coding gene), which is intron-free. Intron sequences have, instead, been identified in the exceptionally extended and strictly conserved 5′-non coding regions, from which they have been supposed to undergo removal for the production of more mRNA species (La Terza et al., 2009; Vallesi et al., 2009, 2012).

Pheromone activity and pheromone receptors

As mentioned above, the conceptual association of ciliate mating types with the duality of sexes, along with the traditional experimental practice of identifying ciliate pheromones on the basis of mating induction assays, have long driven the unique view that ciliate pheromones are “sexual factors” exclusively committed to induce cells to mate. However, the initial study of the biology and physiology of the *E. raikovi* pheromone system soon made evident various aspects challenging this view, strongly supported by theoretical considerations on the obstacles that the Miyake

"gamone-receptor hypothesis" meets in explaining how, in multiple mating systems in particular, pheromones and their cell receptors can harmoniously co-evolve in space and time (Luporini and Miceli, 1986; Beale, 1990). Some general experimental observations clearly indicated that pheromones are molecules that evolved not only for signaling "non-self" by binding in paracrine (or heterologous) fashion uniquely to receptors expressed by other cells than those from which they originate, but also, and most likely primarily, for signaling "self" by binding in autocrine (or homologous) fashion to receptors which are expressed by the same cells from which they are secreted (Luporini and Miceli, 1986). Firstly, cells vary their rates of pheromone secretion in relation to the environmental concentrations of pheromones that they have secreted. Secondly, pheromones are secreted throughout the cell life cycle, also by cells which are in the early developmental stage of "immaturity" that, by definition, is not competent for mating. Thirdly, the induction of homotypic pair formation by the addition of a non-self pheromone may be reversibly inhibited by the addition of the self pheromone.

This concept of pheromone self-recognition functions clearly needed to be corroborated by progressing from initial evidence that *E. raikovi* cells were effectively able to bind competitively their secreted pheromones in specific and reversible manner (Ortenzi et al., 1990; Ortenzi and Luporini, 1995), to the determination of the biological effects generated by the autocrine pheromone binding and of the structure of the pheromone receptors. These effects were identified with the promotion of cell vegetative reproduction (mitogenic proliferation, or growth) and, considering that such autocrine pheromone activity is understandably primary to the paracrine activity directed to target and induce foreign cells to mate, this finding provided an instructive example of how the original function of a molecule may be obscured during evolution by the acquisition of a new one (Vallesi et al., 1995). The way to the structural characterization of the pheromone receptors was opened by further screening the cDNA library constructed for the determination of the macronuclear DNA sequence coding for the *E. raikovi* E*r*-1 pheromone. This screening led to discover the coexistence of E*r*-1-specific cDNA clones with a second species of cDNA clones encoding longer pheromone isoforms of 130 amino acids, which were characterized by the incorporation of the full sequence of 75 residues of the exported pheromone precursor (pre-pro-E*r*-1) and the consequent conversion of the amino-terminal pheromone signal peptide (normally expected to be excised during the translocation process) into a membrane-spanning domain (Miceli et al., 1992). In consideration of their unique organization as transmembrane proteins oriented with the carboxyl terminal region (structurally identical to the secreted pheromone) on the outside of the cell and the apparent propensity of pheromones to interact in solution for associating reversibly into oligomers (Bradshaw et al., 1990), it was assumed that these pheromone isoforms (designated as E*r*-1mem in type-I cells, E*r*-2mem in type-II cells, and so forth) function as effective pheromone-receptors, and argued for a rationalization of the pheromone and pheromone-receptor specificities of *E. raikovi* multiple mating type system as represented in figure 9. Furthermore, this assumption appeared fully in line with the *Euplotes* mechanism of mating type inheritance, strongly implying that, as is the case for the mating-locus in basidiomycete fungi (Kronstad and Staben, 1997; Casselton and Olesnicky, 1998), the complete genetic information for the pheromone and pheromone-receptor synthesis is constrained within the same DNA molecule. Only in this way would any mutation of this coding molecule be symmetrically transmitted to both the molecular entities (the pheromones and their cognate receptors) that are responsible for the evolution of the mating type specificities.

In consideration of the structural equivalence that the membrane-bound pheromone isoforms show with their partner soluble pheromones at the level of their extracellular ligand-binding domains, the determination of the packing of the E*r*-1 molecules into the crystal

mat-Genotypes	Mating types	Pheromones	Pheromone-receptors
mat-1/mat-1	MT-I	E*r*-1 (▼)	E*r*-1R (♀▼)
mat-2/mat-2	MT-II	E*r*-2 (▼)	E*r*-2R (♀▼)
mat-3/mat-3	MT-III	E*r*-3 (▼)	E*r*-3R (♀▼)
•	•	•	•
•	•	•	•

Fig. 9: Molecular specificities of *E. raikovi* multiple mating types. For each cell type, color indicates the structural identity that the soluble pheromone has with its cell receptor at level the extracellular domain.

structure provided a very insightful molecular model to account for the way the autocrine pheromone/pheromone-receptor interactions might take place on the cell surface (Weiss et al., 1995). In practice, the E*r*-1/E*r*-1 molecular interactions in the x-y plane of the crystal have been assumed to fully mimic the autocrine E*r*-1/E*r*-1mem molecular interactions, with half the molecules partially immobilized on the cell surface and oriented with their carboxyl terminus towards the outside, and half the molecules soluble and oriented with their carboxyl terminus towards the inside (Fig. 10). The basic prediction of this model is that increasingly tight E*r*-1mem/E*r*-1 binding is generated by a cooperative process of protein-protein oligomerization, which disposes the molecules into layers and begins with weak association energies arising from the participation of all three helices of the pheromone structure in the formation of two types of dimers, neither of which can stably develop in solution because of the relatively small molecular surfaces that come to be involved in their formation. Dimers of type 1 involve two molecules symmetrically associated through their helices 1 and 2, while dimers of type 2 involve two molecules associated without symmetrical contacts by stacking their helix 3 in anti-parallel manner. Attempts to produce Er-1/Er-2 "hybrid" crystals mimicking paracrine E*r*-1mem/E*r*-2 interactions were unsuccessful. No model could thus be deduced from hard experimental data to account for the way these interactions might take place on the surface of type-I cells induced to mate by E*r*-2 binding. However, a comparison of the backbone molecular conformations and electrostatic surface potentials among different pheromones makes it evident that these interactions can no

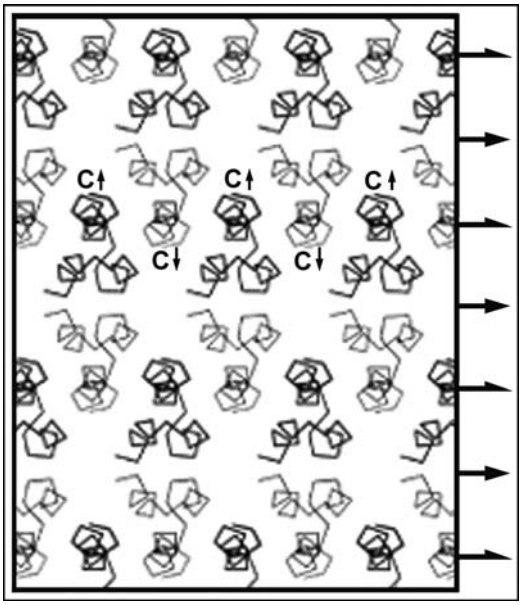

Fig. 10: Crystallographic *xy* plane of the E*r*-1-pheromone crystals (1.6 Å resolution) showing the cooperative molecular interactions generated by the formation of two distinct types of dimers that stabilize the molecular contacts. Position of the twofold rotation axes (→) relating two molecules in the formation of type-1 dimers and the twofold screw axes (→) relating two molecules in type-2 dimers are indicated on the right. In the *xy* plane, half molecules (in bold) orient their carboxyl terminus (C) toward the outside of the plane, while the other half orient their carboxyl terminus toward the inside as indicated by the direction of the C-flanking arrows (after Weiss et al., 1995).

longer take place with a dynamics of cooperative pheromone/pheromone-receptor oligomerization (Vallesi et al., 2005). While dimers of type 1 are likely to be equally formed since no substantial variation in shape and electrostatic potential distinguishes pheromones at the level of the face (delimited between helices 1 and 2) involved in their formation, dimers of type 2 appear to be halted by significant variations that pheromones show in the surface potential of helix 3, more directly responsible for their formation.

Consistently with these observations, it was shown (Vallesi et al., 2005) that the autocrine pheromone/pheromone-receptor (E*r*-1/E*r*-1mem) complexes are continuously internalized via endocytic vesicles (generated in greater number in the zone of the ciliary membranelles particularly rich in parasomal sacs), and that this internalization is inhibited by the induction of paracrine (e.g., E*r*-2/E*r*-1mem) complexes (Fig. 11). These complexes are not internalized, and it has been hypothesized that their persistence on the cell surface serves to directly involve the pheromone-receptors in the formation of the cell-to-cell mating adhesions through mutual binding across the extracellular space. This hypothesis has been prompted by a close look at the energetics of E*r*-1 oligomerization, which indicates that upon paracrine pheromone binding and consequent partial immobilization on the cell surface, E*r*-1mem molecules (and the pheromone-receptors in general) are subject, compared to their soluble counterparts, to a significant loss in solvation energy and rotational/translational entropy and consequent increase in total energy of oligomerization (Weiss et al., 1995). In the case of E*r*-1mem, it has been calculated that this increase in the oligomerization energy is such that five E*r*-1mem/E*r*-1mem interactions would be sufficient to hold together two cells imagined as spheres with a diameter of 50 μm. This additional commitment of the pheromone-receptors in promoting cell-to-cell adhesion finds close analogies with phenomena of juxtacrine cell interactions and adhesion mediated by transmembrane signaling proteins, that were originally documented for membrane-anchored growth factors and are rather diffused in mammalian hematopoietic cell systems (Bosenberg and Massagué, 1993; Singh and Harris, 2005).

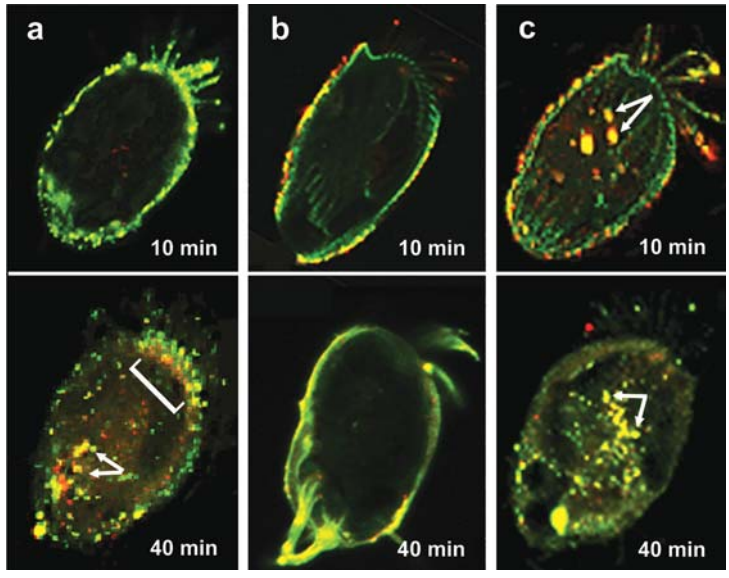

Fig. 11: Confocal images illustrating internalization of autocrine pheromone/pheromone-receptor complexes *versus* persistence of paracrine complexes on the cell surface. **a** Type-I cells suspended with their self pheromone E*r*-1. **b** Type-I cells suspended with the non-self pheromone E*r*-2. **c** Type-I cells incubated with antibodies to the E*r*-1-receptor ectodomain which mimic E*r*-1 binding and induce E*r*-1-receptor clustering and internalization. E*r*-1, E*r*-2 and antibodies to E*r*-1-receptor ectodomain are red fluorescent; antibodies to E*r*-1-receptor endodomain are green fluorescent. Co-localization of the red and green signals is revealed by yellow spots inside the cells (arrows and bracket) in **a** and **c**, and on the cell surface in **b** (after Vallesi et al., 2005).

About the signal-transduction activity of the cytoplasmic domain of the membrane-bound pheromone isoforms, much less is known with respect to the ligand-binding activity of the extracellular domain. Although this cytoplasmic domain includes potential phosphorylation sites for protein kinases A and C as well as sequence stretches potentially responsible for a reversible association with a guanine nucleotide-binding protein (Ortenzi et al., 2000), it remains to be established whether and how it enzymatically interacts with a mitogen-activated-protein kinase (designated as *Er*-MAPK1) that has been found to be specifically phosphorylated in growing cells in association with the autocrine pheromone loop and subject to undergo rapid dephosphorylation in mating cells exposed to interact with a non-self pheromone capable of inhibiting this loop (Vallesi et al., 2010). Also, it remains to be clarified how this *Er*-MAPK1 phosphorylation/dephosphorylation activity correlates with the sharp increases in cytoplasmic cAMP concentrations that follow any interruption of the mitogenic autocrine pheromone loop (Apone et al., 2003).

Cell aging, spontaneous intra-clonal selfing, and pheromone oxidation

In aging *Euplotes* cell cultures, a quite recurrent (although often overlooked) phenomenon is spontaneous selfing, i.e., an unpredicted appearance of intra-clonal, homotypic mating pairs. When nothing was yet known about the *Euplotes* pheromone structure and biology, this phenomenon in *E. crassus* was thought to be the effect of an age-dependent inversion of the relationships of dominance that in *mat*-heterozygous cells regulate the expression of the two *mat* alleles (Heckmann, 1967). In consequence of this inversion, a culture of *mat*-heterozygous cells that homogeneously express their dominant *mat* allele would become a heterogeneous culture in which cells expressing the dominant *mat* allele start coexisting with cells that, instead, express the no-longer recessive *mat* allele; in practice, the culture would become a mixture of cells of two different and compatible mating types fully able to interact for mating. However, intra-clonal selfing in *Euplotes* has also frequently been observed in cultures of *mat*-homozygous cells (Kosaka, 1990; Kuhlmann and Sato, 1993), and these observations clearly imply that it also depends on other causes than just the age-dependent inversion of dominance between *mat* alleles.

An additional and probably more general cause has been shown to reside in phenomena of cell aging-induced oxidation of Met residues that pheromones carry exposed on their molecular surfaces (Alimenti et al., 2012). In the case of the *E. raikovi* pheromone E*r*-1, the oxidized pheromone form was shown to arise from the binding of one atom of oxygen to the Met residue exposed on the third helix. It was initially detected in minimal concentrations in cultures of about 300 cell fissions of age (roughly equivalent to a temporal age of one year), and during the next $2-3 \times 10^3$ cell fissions (corresponding to four-five years of age) these concentrations continuously increased to the point of nearly matching those of the non-oxidized pheromone. Oxidized E*r*-1 was purified and its interactions with the source type-I cells were shown to be no longer of autocrine type directed to promote cell growth, but changed to interactions of paracrine type (in all mimicking those of a non-self pheromone) directed to induce the formation of selfing (homotypic) mating pairs.

Selfing involves breeding between genetically identical cells and in normally outbreeding populations (such as those of *Euplotes* in which conjugation between random mates is likely greatly favored by the evolution of multiple mating systems) it is usually thought to be cause of a reduced fitness (inbreeding depression). However, as originally argued by Heckmann (1967), selfing in *Euplotes* has to be considered anything but deleterious. Like autogamy (Dini, 1984), it tends to maintain pre-existing heterozygous gene combinations (the two gamete-nuclei that merge into the synkaryon being derived from different meiotic products of the cell germinal micronucleus) (Dini et al., 1999), and by usually gener-

ating viable offspring it determines (like any sexual event in ciliates) the re-initiation of the cell life cycle. In the light of these concepts, it thus appears that pheromone oxidation, although it is an obvious sign of cell aging, turns out to be, paradoxically, beneficial to the same aging cells: by inducing them to mate, it practically induces them to terminate a declining life cycle and reinitiate a new one.

Pheromone structural relationships and cross-reactions with other signal proteins

Reliable structural relationships have been revealed by the *E. raikovi* pheromone family with another family of water-borne helical proteins, designated as "attractins", that several species of the marine mollusk, *Aplysia*, release in association with their cordon egg laying and use in the signaling mechanism of mate attraction to eggs (Schein et al., 2001; Painter et al., 2004). The five species-specific attractins that have been structurally characterized all contain 56–58 amino acid residues, six conserved Cys residues, and the common sequence Ile_{30}-Glu-Glu-Cys-Lys-Thr-Ser_{36} (designated as "IEECKTS motif") arranged to form (with the addition of two Met[Leu] and Val[Gln] residues) one of two anti-parallel α-helices that compound the compact folded organization of these molecules. It is just this helical IEECKTS motif that NMR analyses have revealed to have a close counterpart in the helix 3 of *E. raikovi* pheromones (with regard to its backbone conformation and the orientation of conserved side chains) (Garimella et al., 2003). This similarity is also likely to have a functional significance, since recombinant attractins lose their attraction activity when the surface-exposed Glu_{31}, Glu_{32} and Lys_{34} residues of the IEECKTS motif are mutated to Gln residues (Painter et al., 2004). More puzzling, yet of strong interest for practical applications, were the findings that *E. raikovi* pheromones, E*r*-1 in particular, cross-react with mammalian signalling systems. It was initially observed that E*r*-1 binds (with an affinity of the order of 10^{-6} M) to the α and β subunits of the trimeric receptor of mammalian interleukin-2 (IL-2) as well as to IL-2 itself, and that IL-2 is in turn a strong inhibitor of E*r*-1 binding to its cell receptors (Ortenzi et al., 1990; Vallesi et al., 1998). Possible causes of these cross-reactions were thought to reside in the physico-chemical features of two sequence stretches that pheromone E*r*-1 shows in common with IL-2. One sequence of five residues spans between the E*r*-1 helices 1 and 2 while the second sequence of seven residues lies centrally in helix 3; in IL-2, both these sequences are surface-exposed and maintained spatially close to each another by a disulfide bond that is essential for the IL-2 activity (Rickert et al., 2005).

In human lymphoid cells cultured at low serum concentrations, it has recently been shown that pheromone E*r*-1 increases the proliferation and modulates the expression of T-cell factors (such as interferon-γ, tumor necrosis factor-α, interleukin-1β, IL-2, and IL-13), induces the down-regulation of the IL-2-receptor α-subunit by reversible and time-dependent endocytosis, and increases the levels of phosphorylation of the extracellular signal-regulated kinases (ERK) which implies that the E*r*-1 immuno-enhancing activity on T-cells involves effective Er-1 binding the IL-2-receptor and ERK activation (Cervia et al., 2013).

Conclusions and Perspectives

The study of various structures and gene sequences of pheromones of *E. raikovi*, *E. octocarinatus*, *E. nobilii* and *E. crassus* has decisively broadened our knowledge of the biology of these ancient, cell-type specific signal proteins. In particular, the determination of the three-dimensional conformations of a significant number of these pheromones isolated from the supernatant of *E. raikovi* and *E. nobilii* cultures of different mating types has definitively documented that these mating-type signals form species-specific families of structurally homologous proteins, in full accord with their genetic determination through multiple series of single-locus *mat*

alleles. Because of the compounding into the same molecular architecture of structural motifs that are in part common to other family members and in part unique to each family member, it can now be well appreciated how *Euplotes* pheromones can effectively compete with one another for binding to the same cell receptors and, hence, elicit varied and context-dependent cell responses. Identified and for long time studied only for their paracrine (non-self) activity in mating induction, these molecules have eventually revealed a more universal and presumably primordial autocrine (self) function of stimulation of vegetative (mitotic) growth of the same cells from which they constitutively secreted from the very beginning of the life cycle. Consistently with this new function, intriguing similarities of structure and cross-reactions have been detected between some *Euplotes* pheromones and signal proteins (attractins and cytokines) evolved later in multi-cellular organisms. New similarities and cross-reactions will certainly be revealed by the structural characterization of new pheromones from the enormous number of these signaling molecules that we can safely predict to be synthesized and diffused into the environment not only by the dozens of *Euplotes* species that can easily be collected from any aquatic habitat, but also by many other ciliates that, like *Euplotes* species, are characterized by high-multiple mating type systems. We do not know whether these similarities and cross-reactions reflect genuine molecular relationships via divergent evolution, or represent instances of convergent molecular evolution. Nevertheless, they clearly imply that complex self/non-self signaling networks closely recalling those evolved in fungi (Heitman et al., 2007) and flowering plants (Franklin-Tong, 2008) are already at work in ancient unicellular eukaryotes such as ciliates, and should stimulate new research in structural biology of ciliate pheromones also in view of potential applications of the activities of these molecules in biomedicine and pharmacology.

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