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Ciliate Mating Types and Their Specific Protein Pheromones

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Summary. The determination of a number of pheromone structures from species of *Euplotes* provided direct evidence that these cell type-specific signals are represented by families of homologous proteins, consistently with their genetic control through series of single-locus multiple alleles. Due to their structural homology, unequivocally manifested by the organization of similar three-dimensional topologies, pheromones can thus compete with one another to effectively bind to their cells of origin in autocrine fashion, or to other conspecific cells in paracrine fashion. The cell response to these different pheromone interactions will accordingly vary, reproductive (mitotic proliferation) in the former case, mating (sexual) in the latter.

Key words: chemical signalling, ciliates, growth factors, protein families, protein structures, reproduction, sex.

INTRODUCTION

In the wake of the milestone report by Sonneborn (1937) ("Sex, sex inheritance and sex determination in *Paramecium aurelia*") that conjugation of *P. aurelia* involves the intra-specific differentiation of two genetically distinct mating types, a functional equivalence of mating types of ciliates with sexes of any other (micro)organism has for long been widely accepted. Each mating type would be represented by "a strain of conspecific individuals not able to undergo [mating] fusion with each other, but only with members of a complementary mating type" (e. g., Hausmann *et al.* 2003), and the mating type-distinctive signal molecules would behave like "sex substances" (e. g., Kuhlmann and Heckmann 1989) able "to induce chemical interaction and mutual stimulation between cells complementary for mating union and fertilization" (e. g., Miyake 1981, 1996). These concepts are also the foundation of a model, known as the "gamone-receptor hypothesis" (Miyake 1981, 1996), predictive of the mechanism of action of the first two of these signal molecules, denoted as "Gamone-1" (G-1) and "Gamone-2" (G-2), that have been isolated from *Blepharisma japonicum*. G-1 has been characterized as a glycoprotein with a sequence that has now been determined to be of 272 amino acids plus six sugars (Sugiura and Harumoto 2001); G-2, instead, is a 3-(2'-formylamino-5'-hydroxybenzoyl)lactate, i. e., a possible tryptophan derivative (Jaenicke 1984, Miyake 1996). In spite of the fact that G-1 and G-2 presume quite different genetic determinants – in addition to being chemically unrelated, G-1 is species-specific while G-2 is shared in common by a variety of

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species of *Blepharisma* (Miyake and Bleyman 1976, Miyake 1996) - these molecules have been designated to distinguish cells representing two "complementary" mating types, i. e., Mt-I and Mt-II, and presumed to behave as symmetric inducers of sexual cell pairing: G-1 by binding and activating its cognate receptor on Mt-II cells, and G-2 by binding and activating its cognate receptor on Mt-I cells.

Objective reasons, however, exist, as has been pointed out (Nanney 1980, Luporini and Miceli 1986, Dini and Nyberg 1993), in contradiction with the equivalence between ciliate mating types and sexes; hence, in contradiction to the related "gamone-receptor" interpretation of the activity of the *B. japonicum* G-1 and G-2 signals in the terms only of sexual factors that are synthesized by cells of one cell type to target another cell type. Essentially, these reasons are that: (i) rather than being binary and dimorphic like sex, ciliate mating type systems are usually highly multiple (open?) and polymorphic, like the self-incompatibility systems of flowering plants and some mating type systems of fungi (Casselton 2002, Charlesworth 2002); (ii) effective mating takes place in many ciliates between cells of the *same* mating type (homotypic mating, or selfing), just as it takes place between cells of *different* mating types (heterotypic mating); (iii) every mating cell, independently of whether it represents one or the other mating type, generates one migratory and one stationary gamete nuclei, and it is this behavioral divergence between gamete nuclei that has to be credited with the properties of a sexual, "male"/ "female" differentiation.

To these reasons we can now add the knowledge, that is the object of this review, of various aspects of the biology and structure of the mating type signals that have been studied, under the denomination of "pheromones", in three distinct species of *Euplotes*, i. e., *E. raikovi*, *E. octocarinatus*, and *E. nobilii*, the first two of which are species widely distributed in temperate waters (marine and lacustrine, respectively) and the third one is restricted to the marine costal waters of Antarctica. These *Euplotes* species, more properly than *B. japonicum*, can collectively be regarded as instructive models to search into the evolutionary and functional meanings of the ciliate mating type mechanism. While in *B. japonicum* we are substantially ignorant of the genetic basis of the mating types and of the associated glycoprotein and tryptophan-derivative signals, the genetic basis of *Euplotes* mating types has been determined to be represented by series of alleles that segregate at a single, highly polymorphic Mendelian *mat* (mating-type) locus (e. g., Dini and Luporini 1985, Luporini *et al.* 1986, Heckmann and Kuhlmann 1986, Dini and Nyberg 1993). And consistently with this genetic control, *Euplotes* pheromones all appear to be represented by proteins that are homologous members of species-specific families (Raffioni *et al.* 1992); hence, within each family, characterized by structures that are globally similar, yet distinct from one another due to local structural specificities (Luginbühl *et al.* 1994, Liu *et al.* 2001, Zahn *et al.* 2001). These relationships of structural homology that unequivocally exist between mating type-specific pheromones should be kept as a firm and central notion in any attempt to rationalize the function and mechanism of action of ciliate pheromones and, more in general, the evolutionary meaning of the ciliate mating type systems.

Euplotes pheromone synthesis and secretion

Euplotes cells synthesize and secrete their pheromones constitutively throughout the entire life cycle, regardless of whether they are, or are not in a developmental or physiological stage suitable for undertaking a mating process (Luporini *et al.* 1992, 1996). Pheromone gene transcription and pheromone secretion are already shown by cells that are at the very beginning of their clonal life cycle (Luporini *et al.* 1992), when they are usually regarded as "sexually immature" or "adolescent" because of their incapacity to mate (Kuhlmann and Heckmann 1989), as well as by cells that are growing (reproducing) asexually (mitotically) in the presence of food (Vallesi *et al.* 1995), hence in a physiologically inadequate stage to engage in sex.

The rates of pheromone production, as can be assessed by quantitative analyses of protein purified from the cell supernatant, vary significantly among species as well as, within a given species, among mating types (Luporini *et al.* 1986). Pheromones of *E. octocarinatus* (designated Phr-1, Phr-2, and so forth) are produced in minimal amounts, that vary from 0.36-0.38 µg/l (Phr-3 and Phr-4) to 0.5 µg/l (Phr-1) (Weischer *et al.* 1985, Schulze-Dieckhoff *et al.* 1987), with respect to those of *E. nobilii* (designated E*n*-1, E*n*-2, and so forth), and *E. raikovi* (designated E*r*-1, E*r*-2, and so forth) in which the production is in the range from 50 µg/l (E*n*-2) to 150 µg/l (E*n*-1) (Alimenti *et al.* 2002, 2003a), and from 20 µg/l (E*r*-20) to 330 µg/l (E*r*-1) (Raffioni *et al.* 1987, 1992), respectively.

In *E. raikovi*, quantitative variations in pheromone production between cells of different mating types appear to be closely correlated with, and are presumably

caused by, different degrees of amplification of the pheromone (mating-type) genes in the cell macronucleus. This correlation has been established more precisely for the pheromones E*r*-1, E*r*-10, E*r*-2, and E*r*-20, through the utilization of cultures grown at a density of about 10^4 cells/ml for 4-5 days in the presence of food and then left to enter early starvation for an additional 2 days. Pheromone E*r*-1, that is produced in an amount that is about 2-fold those of E*r*-2 and E*r*-10 (330 µg/l *versus* 140 and 180 µg/l, respectively), and about 10-fold that of E*r*-20 (20 µg/l), is specified by a gene whose macronuclear copy number has been counted to be about 2-fold the copy numbers of the E*r*-2 and E*r*-10 genes ($2.5\text{-}2.9 \times 10^4$ *versus* $1.6\text{-}1.8 \times 10^4$ and $0.9\text{-}1.2 \times 10^4$, respectively) (La Terza *et al.* 1995), and about 10-fold the copy number of the E*r*-20 gene ($2\text{-}3 \times 10^3$) (unreported data). These gene-copy numbers hold independently of whether cells carry a homozygous or heterozygous allelic combination at their *mat* locus, and appear to be established at the beginning of the life cycle, when cells develop their new macronucleus from the products of the synkaryon divisions (La Terza *et al.* 1995).

In a functional context, these inter-mating type quantitative variations in pheromone production are usually regarded to be of secondary relevance. Most attention is in fact focused on the diversification in the pheromone structures that can more directly be associated with the cell capacity, or incapacity, to effectively interact to form mating pairs. Nevertheless, they greatly condition which type of mating pair – heterotypic or homotypic, i. e., between cells of *different* mating types, or of the *same* mating type - will prevalently, or exclusively, be formed in a mating mixture, both pair types being *equally* formed and "fertile" in *Euplotes* as in many other ciliates (Heckmann and Kuhlmann 1986) (only *Blepharisma* homotypic pairs are known to be "sterile" and to remain united for days without switching on any meiotic process). In mating type combinations involving cells characterized by similar rates of pheromone production, heterotypic pairing is preponderant. Instead, the combinations that involve cells with dissimilar rates of pheromone production usually generate a prevalence of homotypic pairs, and the proportions of these pairs relative to each one of the two mating types of the combination closely reflect the inter-mating type differences in pheromone production. In general, it holds that: the lower the amount of pheromone that cells secrete, the higher the proportion of homotypic pairs that they form, and, *vice versa*, the higher the amount of pheromone secreted, the lower the proportion of homotypic pairs formed.

These observations have a close counterpart in the measures of pheromone activity, that traditionally are only referred to the paracrine (sexual) pheromone activity and, hence, based on mating inducing assays carried out (according to a procedure originally devised for *Blepharisma* gamones) by monitoring the minimal concentration required for a given pheromone to induce the formation of at least one mating pair between cells secreting another pheromone (Miyake 1981). However, assays carried out with cells suspended in the (virtual) absence, or in the presence of their secreted pheromone, give rise to values of pheromone activity that may change by even three orders of magnitude, i. e., from a range between 10^{-13} and 10^{-11} M in the former case to a range between 10^{-9} and 10^{-8} M in the latter, that is also the range in which fall the values of pheromone binding affinities assessed on the basis of *in vitro* analysis (Ortenzi and Luporini 1995).

Physicochemical features, cytoplasmic precursors, and primary structures of *Euplotes* pheromones

Twenty unique amino acid sequences of *Euplotes* pheromones have so far been structurally determined, either by direct chemical analysis of purified protein preparations, or molecular cloning of the relevant coding genes: nine of 37-51 residues from *E. raikovi* (Raffioni *et al.* 1992, Luporini *et al.* 1995, Vallesi *et al*. 1996, Di Giuseppe *et al.* 2002), nine of 85-109 residues from *E. octocarinatus* (Brünen-Nieveler *et al.* 1991, 1998; Meyer *et al.* 1991, 1992; Möllenbeck and Heckmann 1999), and two of 52-60 residues from (a single strain of) *E. nobilii* (Alimenti *et al.* 2002, 2003a). While pheromones of *E. octocarinatus* belong to strains showing intra-specific mutual mating compatibility (i. e., cells of a strain can mate with, and their pheromones can induce mating between, cells of any other strain), *E. raikovi* pheromones belong to two distinct groups of strains that are mutually mating incompatible, and denoted "PR" and "GA" (with reference to their collection sites, i. e., Porto Recanati and Gaeta on the Eastern and Western coasts of Italy, respectively). In practice, pheromones (E*r*-1, E*r*-2, E*r*-7, E*r*-10, and E*r*-11) of the PR strains can induce mating between PR cells, not between GA cells (E*r*-11 representing a partial exception); similarly pheromones (E*r*-20, E*r*-21, E*r*-22, and E*r*-23) of the GA strains can induce mating between GA cells, but not between PR cells (cells secreting E*r*-11, again, repre-

senting a partial exception). Because of this behavioral (mating) strain diversification, *E. raikovi* pheromones are also regarded as classifiable into distinct protein subfamilies, particularly apt to reveal the structural specificities that condition their varied spectra of activity (Vallesi *et al.* 1996).

As summarized in Table 1 *Euplotes* pheromones are all rich in cysteines (typically, six in *E. raikovi*, eight in *E. nobilii*, and ten in *E. octocarinatus*), and possess acidic isoelectric points (between 3.25 and 4.08). Only *E. nobilii* pheromones denote appreciable differences in polar and hydrophobic amino acid composition in relation with their cold-adaptation (Alimenti *et al.* 2003b), that usually requires a reduction of the protein hydrophobicity associated with increased solvent accessibility and molecular flexibility (e. g., Marshall 1997). They are in fact significantly more polar and hydrophilic than *E. octocarinatus* and *E. raikovi* pheromones, and show a mean value of the aliphatic index (indicative of the degree of thermostability) that is markedly lower than in the two other species (i. e., 24.1 *versus* 50.3 and 65.4, respectively).

As occurs in many secreted protein hormones, also *Euplotes* pheromones (as well as the glycoprotein G-1 of *B. japonicum*) are synthesized as inactive protein precursors (pre-pro-pheromones), that denote a typical signal-peptide/pro-segment/mature-protein construction from which the active pheromones have to be liberated by proteolytic cleavages of the signal-peptide and pro-segment (Miceli *et al.* 1989, 1991; Meyer *et al.* 1991; Sugiura and Harumoto 2001). A salient feature of these pheromone precursors (not yet known in *E. nobilii*) is a remarkable degree of intra- and inter-specific sequence identity that distinguishes both the signal-peptide (to a major extent) and the pro-segment (to a minor extent) from the secreted pheromone forms that are themselves rather variable. This strict structural conservation of the signal-peptide and pro-segment has presumably functional reasons, correlated with the production of membrane-bound pheromone isoforms that in *E. raikovi* have been identified as the cell effective pheromone binding sites and receptors (Ortenzi *et al.* 2000). These isoforms are co-synthesized together with the secreted forms through a process of differential splicing of the primary transcripts of the pheromone genes and incorporate the entire pheromone precursor at their carboxyl region, while extending their amino terminal region by the addition of a new sequence (Miceli *et al.* 1992, Di Giuseppe *et al.* 2002). Due to this incorporation, the signal-peptide and pro-segment become the isoform (pheromone-receptor) trans-membrane and anchoring domains; it thus appears necessary to impose them unique conformational changes to avoid enzymatic processing and removal.

Upon looking closer at the multiple sequence alignment of the pheromone precursors, shown in Figs 1 and 2, it appears that the signal peptide sequences contain no amino acid substitution at all in *E. octocarinatus* and only a dozen substitutions in *E. raikovi*, furthermore mostly concentrated in pheromone E*r*-23 that represents a somewhat deviant member of the *E. raikovi* pheromone family (as discussed below). At the inter-species level, they present closely comparable lengths (19 residues in *E. raikovi versus* 16 in *E. octocarinatus*) and more than 50% positions that are fully conserved. Functionally, all the canonical signal-peptide features are therein recognizable, including a crowding of positively charged and hydrophobic residues and a processing site for signal peptidases represented by the dipeptide Ala-Phe.

The degree of the pro-segment sequence identity in *E. octocarinatus* is such that there is only one substitution throughout the first 29 positions (the structurally "eccentric" pheromone Phr4 excluded); while in *E. raikovi* (E*r*-23 excluded) it varies from 50 to 94%. At inter-specific level, the variations appear limited to: (i) the double length that the pro-segment acquires in *E. octocarinatus* in respect to *E. raikovi* (basically 36 *versus* 18 residues); (ii) the processing site for the release of mature pheromone forms, that changes from the *E. raikovi* dipeptide Arg-Asp (with three substitutions of Arg with Gln, Gly, or Lys, and one of Asp with Gly) to the *E. octocarinatus* dipeptide Lys-Asp/Tyr/Gly. Nevertheless, in both species there is a repetition (from two times in *E. raikovi* to six in *E. octocarinatus*) of the motif Arg/Lys-Xxx-Xxx-Ser (with occasional changes of Arg/Lys with Gln or Met and of Ser with Glu or Thr), that may be of common functional importance for post-translational modifications.

In the secreted pheromones, the degree of sequence identity drops drastically, even to reach intra-specific values as low as 25-30% that are usually seen as a twilight zone to infer true relationships of sequence homology. It seems likely that at the origin of the pheromone diversification between *E. raikovi* and *E. octocarinatus* there is an event of gene duplication. This is strongly suggested by the fact that, as is also the case of the pro-segment, the pheromone sequences in *E. octocarinatus* are practically twice as large as in *E. raikovi*.

Table 1. Basic physicochemical properties of *Euplotes* pheromones.

Pheromones	Total residues (n)	Cysteine residues (n)	Isoelectric point pI	Charged residues (%)	Polar residues (%)	Hydrophobic residues (%)	Glycine residues (%)	Aliphatic index[1]
E*r*-1	40	6	3.71	20.0	45.0	30.0	5.0	53.75
E*r*-2	40	6	3.83	12.5	42.5	35.0	10.0	44.00
E*r*-7	40	6	3.83	12.5	45.0	35.0	7.5	46.50
E*r*-l0	38	6	4.08	18.4	42.1	31.6	7.9	51.32
E*r*-l1	39	6	3.45	12.8	41.0	43.6	2.6	87.69
E*r*-20	37	6	3.33	13.5	45.9	37.8	2.7	84.32
E*r*-21	37	6	3.37	10.8	40.5	45.9	2.7	108.11
E*r*-22	37	6	3.77	16.2	40.5	40.5	2.7	89.73
E*r*-23	51	10	3.33	9.8	50.9	21.6	17.6	23.14
E*n*-1	52	8	3.25	11.5	59.6	23.0	5.8	18.85
E*n*-2	60	8	3.50	15.0	50.0	25.0	10.0	29.33
Phr1	99	10	3.58	20.2	40.4	34.3	5.1	70.00
Phr1*	98	10	3.56	15.3	44.9	33.7	6.1	64.80
Phr2	101	10	3.52	19.8	42.6	29.7	7.9	43.47
Phr2*	102	10	3.41	17.6	43.1	31.4	7.8	47.84
Phr3	99	10	3.88	21.2	47.5	26.3	5.1	48.28
Phr3*	99	10	3.96	19.2	52.5	22.2	6.1	43.33
Phr4	85	8	3.64	15.3	43.5	34.1	7.1	38.00
Phr5^{-1}	108	10	3.92	13.9	47.2	28.7	10.1	48.70
Phr5^{-2}	109	10	3.49	14.7	44.9	28.4	11.9	48.26

E*r*, E*n*, and Phr pheromones are from *E. raikovi*, *E. nobilii*, *E. octocarinatus*, respectively. [1]Calculated according to Ikai (1980), as relative volume occupied in globular proteins by aliphatic side chains.

Conservation is substantially limited to the amino-terminus usually occupied, in *E. raikovi* in particular, by an Asp residue (that likely serves as recognition for the proteolytic pro-pheromone processing), and to the cysteine positions. The retention of these positions is obviously maximal at the intra-specific level (with the partial exception of *E. octocarinatus*), and less stringent at the inter-specific one. In the case of *E. nobilii* and *E. raikovi*, that are species with close phylogenetic kinship (Di Giuseppe and Dini, personal communication), the six Cys residues (designated I to VI) distinctive of *E. raikovi* pheromones (E*r*-23 with ten cysteines excepted) find their counterparts in six of the eight Cys residues of *E. nobilii* pheromones, thus implying an inter-species substantial retention of the disulfide bond pattern Cys-I/Cys-IV, Cys-II/Cys-VI, and Cys-III/Cys-V established in *E. raikovi* (Stewart *et al.* 1992). Between the pheromone families of *E. raikovi* and *E. octocarinatus*, that are species branching phylogenetically rather distant from each other (Bernhard *et al.* 2001, Petroni *et al.* 2002, Song *et al.* 2004), the adoption of some architectural motifs in common seems to be much more dubious. There is in fact only a convincing match of the disulfide bonded Cys-I, Cys-III, Cys-IV and Cys-V of *E. raikovi* pheromones with four of the six fully conserved Cys residues in *E. octocarinatus* pheromones.

Three-dimensional structures of *E. raikovi* pheromones

The molecular architecture was determined for six *E. raikovi* pheromones (of the nine characterized at the level of their primary sequences), i. e., E*r*-1, E*r*-2, E*r*-10, and E*r*-11 of the PR subfamily, plus E*r*-22 and E*r*-23 of the GA subfamily. These determinations were carried out by NMR spectroscopy (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, in the case of E*r*-1, also by X-ray crystallography (Weiss *et al.* 1995). As illustrated in Fig. 3, these pheromones (except E*r*-23) show constructions that closely mimic one another, *regardless* of the extent of divergence that may

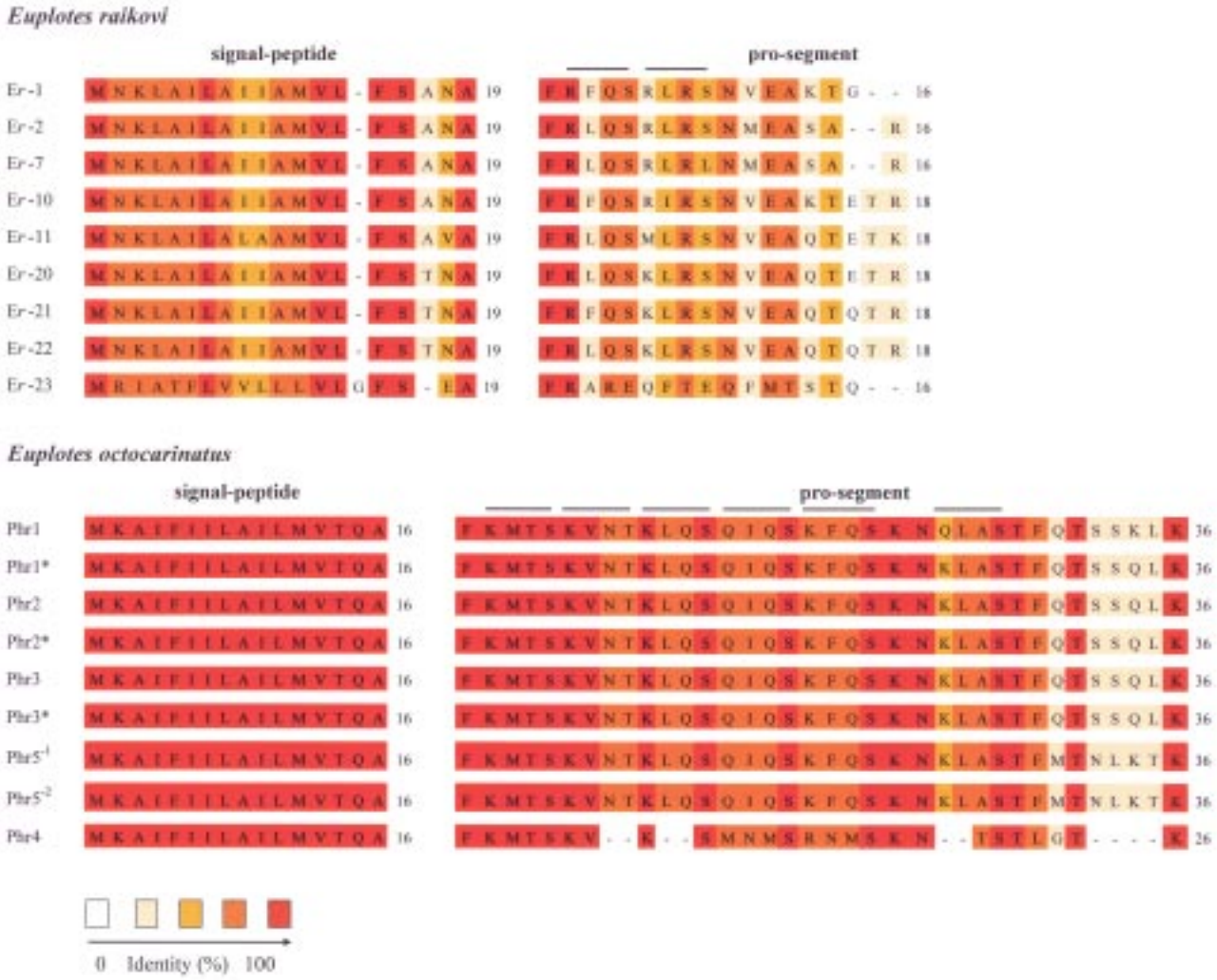
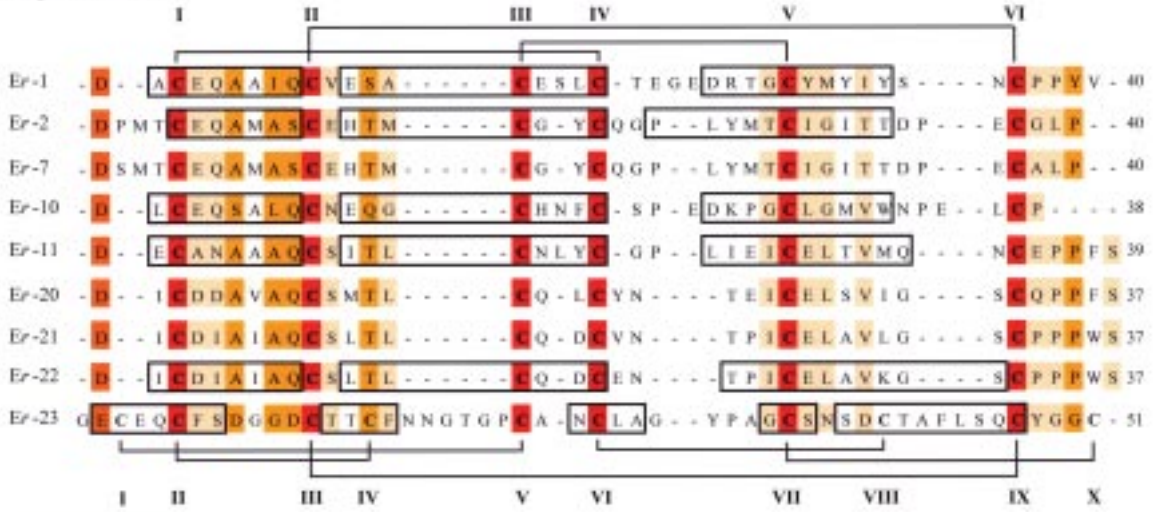


Fig. 1. Multiple sequence alignment of the *E. raikovi* and *E. octocarinatus* pheromone precursors. The alignment is based on the Clustal X program (Jeanmougin *et al.* 1998) and maximized by gap insertions. The numbers of residues of each sequence are reported on the right, and a motif repetition is indicates by lines. Sequence identities are colour-coded using a gradient from white (< 40% identity) to red (100% identity).

Fig. 2. Multiple sequence alignment of *E. raikovi*, *E. nobilii*, and *E. octocarinatus* secreted pheromones. Criteria for arranging sequence alignments and identities are as in Fig. 1. In the *E. raikovi* pheromones, the sequence segments organized in helical structures are enclosed in boxes, while the half-cystine residues are indicated by progressive roman numerals and connected by lines according to their disulfide pairings.

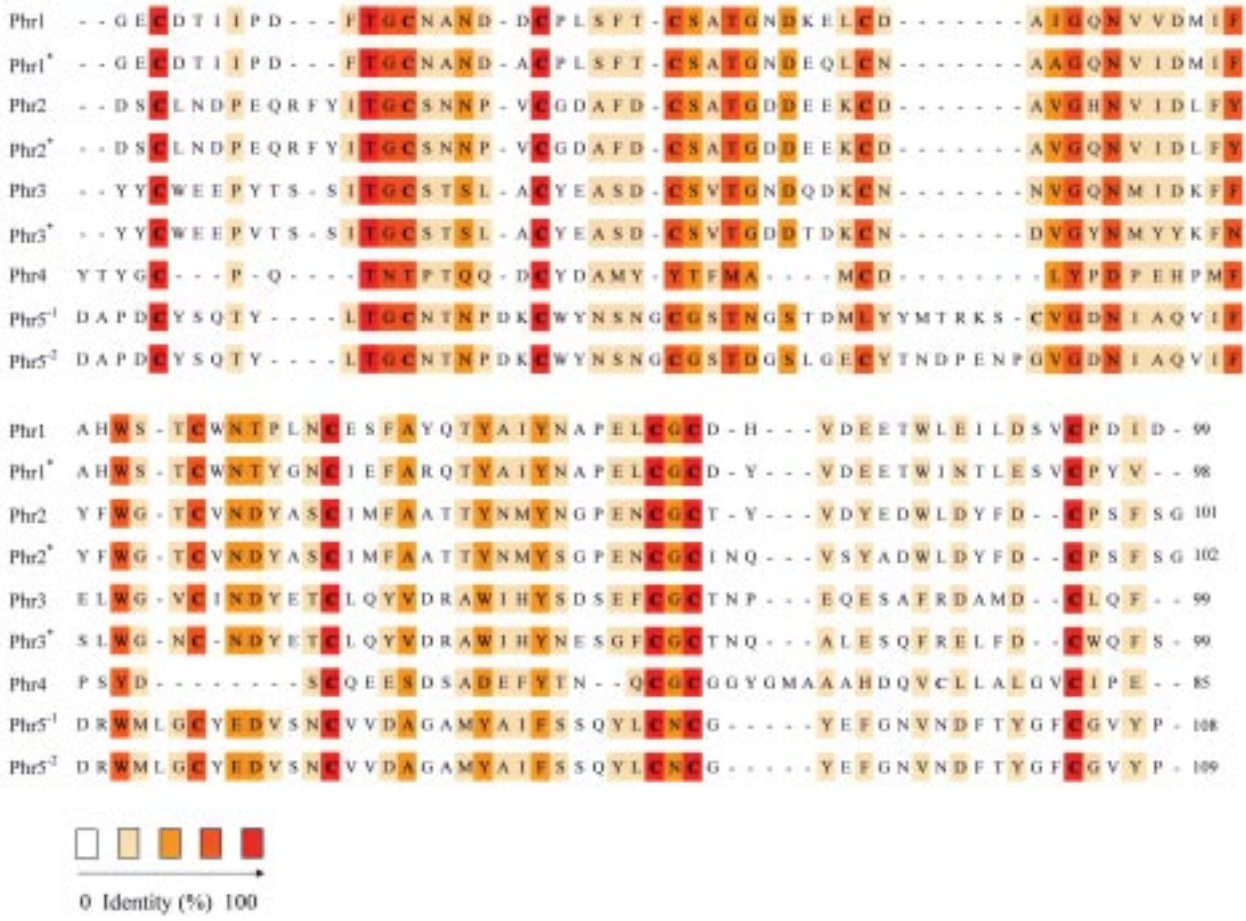
separate their primary sequences. These constructions all conform to a unique model shaped like a pyramid, in which the base is triangular and the vertical edges are provided by three helices, designated 1 to 3 starting from the molecule amino-terminus; they are united by two extended loops of 2-4 residues and show appreciably different lengths and an up-down-up topological orientation. Helices 1 and 3 are uniformly in regular α-organization, while helix 2 tends to be less regular and to appear as a short stretch of distorted 3_{10}-helix turns. Compactness and stability of each pheromone construction are ensured by the three strictly conserved disulfide bridges, two of which (Cys-I/Cys-IV, and Cys-III/Cys-V) connect helix 2 (facilitated in this connection by its

Euplotes raikovi



Euplotes nobilii

Euplotes octocarinatus



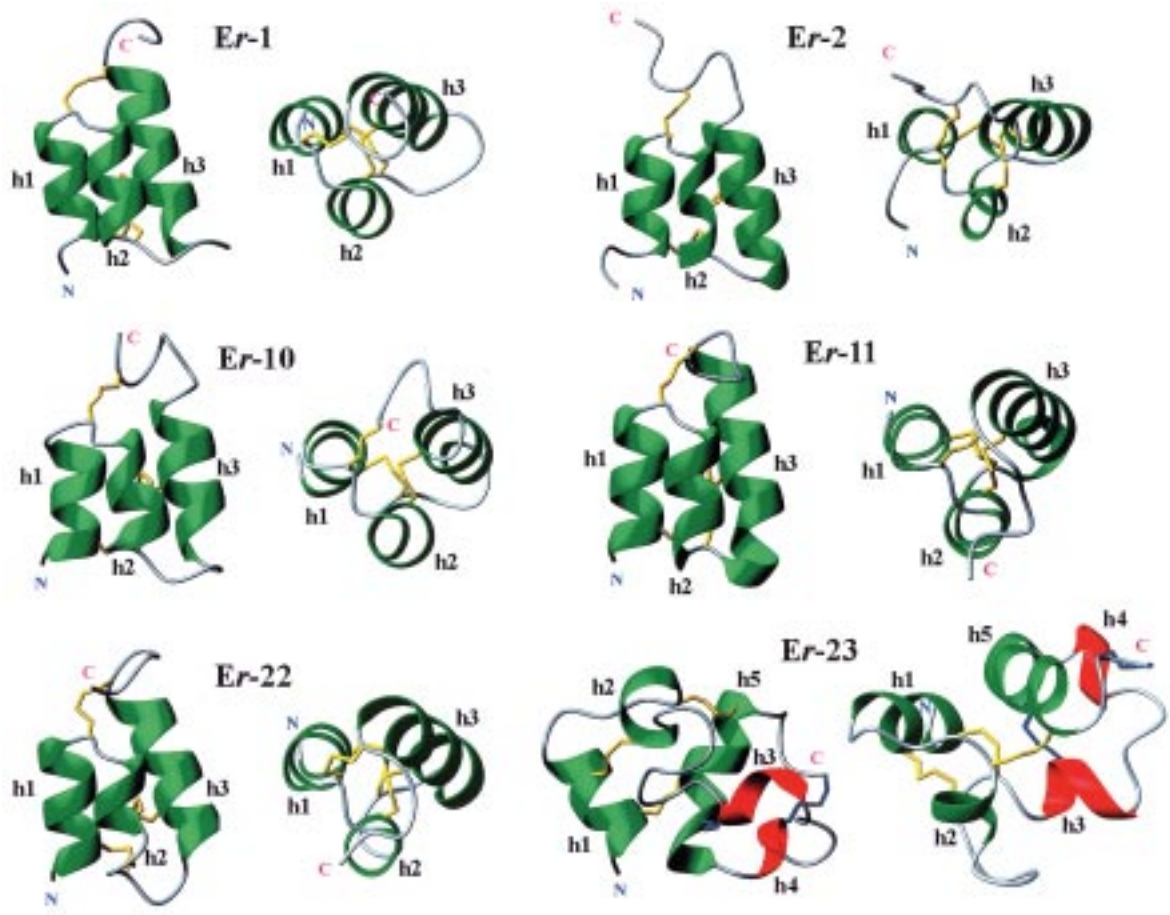


Fig. 3. Comparison of the three-dimensional structures of *E. raikovi* pheromones. Each pheromone is represented in frontal and top views prepared with the program MOLMOL (Koradi *et al.* 1996). The helices (h) are numbered progressively from the molecule amino-terminus (N) to the carboxy-terminus (C). The three helices conserved in all the molecules are coloured green and their three interconnecting disulfide bridges are shown in yellow. The two helices and the two disulfide bridges that are unique to E*r*-23 are coloured red and blue, respectively. The atom coordinates of each pheromone, deposited at the Protein Data Bank (PDB), are to following: E*r*-1, 1ERC; E*r*-2, 1ERD; E*r*-10, 1ERP; E*r*-11, 1ERY; E*r*-22, 1HD6; E*r*-23, 1HA8.

distorted structure that ensures a proper orientation for the disulfide bonds of its Cys III and Cys IV residues) to helices 1 and 3, while the third one (Cys-II/Cys-VI) ties the carboxy-terminal tail to the top of helix 1. The close spatial proximity of the two inter-helix disulfide bonds is such to cause a rather exceptional situation, i. e., in each pheromone the formation of quantitatively minor conformations represented by disulfide isomers generated by all the three possible disulfide pairings between any two of the four Cys residues I, III, IV, and V (Brown *et al.* 1993, Luginbühl *et al.* 1994).

On this common conformational backbone, every pheromone then locally imposes its personal hallmarks to acquire its own architectural specificity (as discussed in great detail by Luginbühl *et al.* 1994, for pheromones E*r*-1, E*r*-2, and E*r*-10). More direct contributions to the construction of these hallmarks, that are likely of great significance also for the pheromone function, appear to be provided by variations in the organization of: (i) helix 2, (ii) the loop connecting helices 2 and 3, and (iii) the carboxy-terminal tail immediately following the last (VI) Cys residue positioned at the end of helix 3. In any case it is this tail (nevertheless absent in E*r*-23, whose sequence ends with a disulfide bonded Cys residue) that more markedly than the other two structures assumes aspects varied enough - in relation to, (i) its length (spanning from one to five residues), (ii) spatial arrangement, and (iii) number of Pro residues (from one to three,

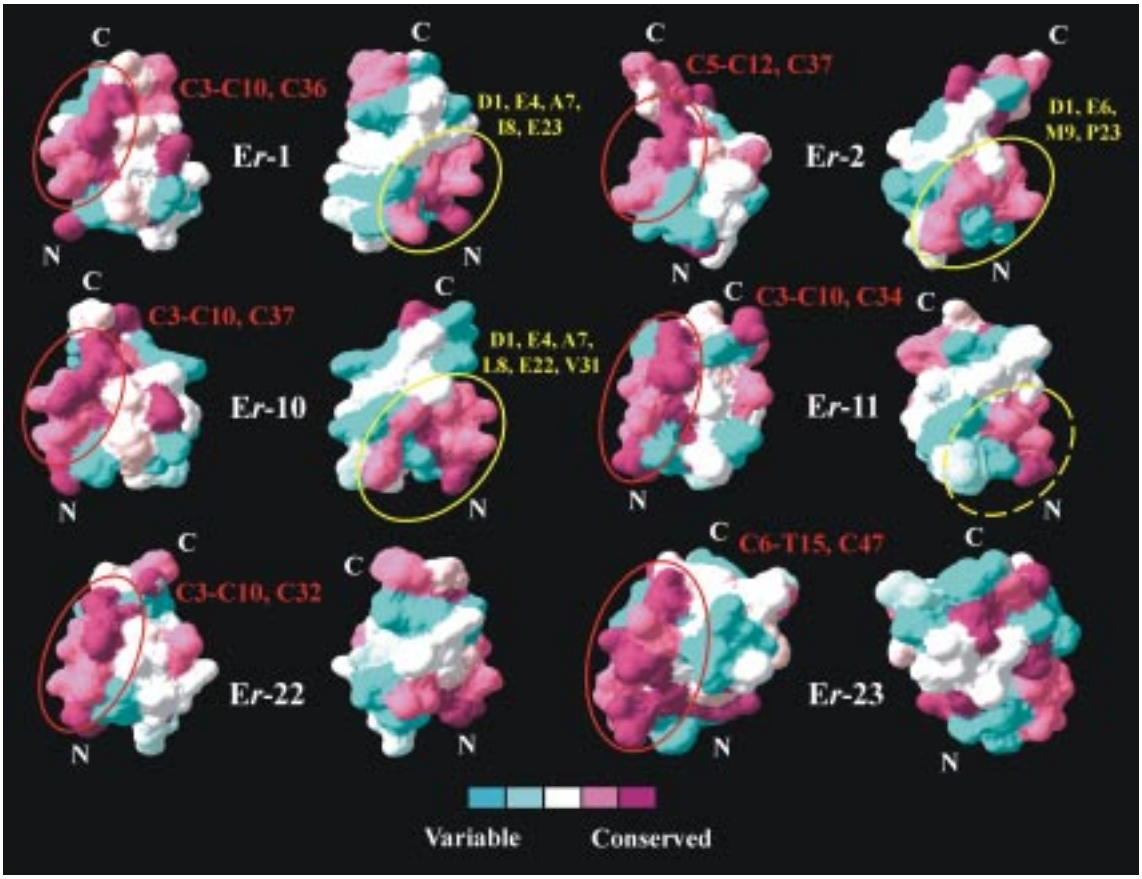


Fig. 4. Comparison of the surface conservation patters of *E. raikovi* pheromones, obtained by using the Rate4Site method (Pupko *et al*. 2002; Glaser *et al*. 2003). The molecular surfaces, prepared with the Swiss-PdbViewer program (Guex and Peitsch 1997), are color-coded as follows: dark violet, maximal conservation; white, average conservation; dark turquoise, maximal variability. The approximate spatial locations of the molecule amino- and carboxy-terminus are indicated by N and C, respectively. Each pheromone is presented in two orientations: in the left one, helices 1 and 2 are in the viewer front, while in the right one there is helix 3. However, in E*r*-1, E*r*-2, E*r*-10, E*r*-11, and E*r*-22, the axis of helix 1 in nearly vertical, while in E*r*-23 this axis is rotated approximately 90° counter-clockwise in the left orientation, and approximately 90° clockwise in the right orientation. The surface patch conserved in all pheromones is delimited by red ovals, while the surface patch conserved in pheromones of the PR subfamily (E*r*-1, E*r*-2, E*r*-10, and E*r*-11) and not conserved in pheromones of the GA subfamily (E*r*-22 and E*r*-23) is delimited by yellow ovals (in E*r*-11, dashed line indicates a less extended conserved patch). The amino acid residues yielding the conserved surfaces are reported jointly with the numbers indicating their positions in the primary structures.

and causing the appearance of conformational isomers containing *cis*- and *trans*-peptide bonds) - to be at the same time distinctive of every single pheromone and discriminative between the PR and GA pheromones. In E*r*-1, E*r*-2 and E*r*-10, that are more typical members of the PR subfamily, the tail terminal residue appears similarly settled over the top of helix 3; instead, in E*r*-22, representative of the GA subfamily, it appears dislocated over the top of helix 2. Intriguingly, an E*r*-22-like dislocation is shown also by E*r*-11 that, although a member of the PR subfamily, still manifests a weak capacity to induce mating between cells of the GA strains.

The E*r*-23 case

Notwithstanding that E*r*-23 is, (i) synthesized by a structurally complete gene regularly amplified to thousands of macronuclear copies, (ii) secreted in amounts (50-60 µg/l) comparable with the other pheromones from the same wild-type strain that co-releases E*r*-22 (hence, a strain heterozygous at its *mat* locus for the two relevant coding genes), and (iii) participates effectively in cell signaling between cells of the GA strains, it is set quite apart from the standard structure of the other *E. raikovi* pheromones because of its sequence elonga-

tion from 37-40 to 51 residues and its increase in the cysteine number from six to 10 (Di Giuseppe *et al.* 2002). Clearly (see Fig. 3), also the molecular architecture of E*r*-23 markedly deviates from the basic fold of the *E. raikovi* pheromone family, as it has to accommodate the addition of the new stretch of 11 residues (Zahn *et al.* 2001). This accommodation essentially occurs at level of the central domain of the E*r*-23 architecture, and determines a new fold based on the presence of five helices (rather varied in extension and organization) that become fastened together by five disulfide bridges provided by the following Cys-residue combinations: I-V, II-IV, III-IX, VI-VIII, and VII-X.

Although the E*r*-23 three-dimensional structure is unique, also this structure appears to be reconcilable with the three-helix bundle topology distinctive of the *E. raikovi* pheromone family (Zahn *et al.* 2001). Its helices 1, 2, and 5 are in fact arranged in an up-down-up fashion just like the three helices distinctive of the family model (see Fig. 3), and their connections are ensured by three disulfide bonds (i. e., Cys-I/Cys-V, Cys-II/Cys-IV, and Cys-III/Cys-IX) that are spatially equivalent with those of the other family members.

Even more convincing support for this structural reconciliation of E*r*-23 with all the other *E. raikovi* pheromones is provided by a comparative analysis of the functionally important regions exposed on the surface of the whole set of the determined pheromone structures. This analysis is based on algorithmic tools, of which the original one is referred to as "Conservation Surface-mapping" (ConSurf) (Armon *et al.* 2001) and an implemented version of it as "Rate4Site" (Pupko *et al.* 2002, Glaser *et al.* 2003), that fit extremely well with the case of the *E. raikovi* pheromone family. They in fact map the rate of evolution of the molecular surface of homologous proteins (better, if this homology is founded on recognized family links) with known three-dimensional structures, and their ultimate target is the identification of surface hot spots and patches that are likely to be in effective contact with other protein domains, nucleic acids, or ligands. Clearly, the rationale underneath these tools is that surface residues of key importance for the protein binding activity should be conserved throughout evolution, just like buried residues that are crucial for the maintenance of the protein fold.

As shown in Fig. 4, the most pronounced conserved surface domain, generated by a cluster of 8-10 residues, appears in common between E*r*-23 and all the other pheromones. In E*r*-23, these residues are provided by the second half of helix 1, helix 2, the helix-1/helix-2 connecting loop, and include Cys-IX; in all the other pheromones, they are provided by helix 1 and include Cys-VI. It appears, in addition, that E*r*-23, like its subfamily member E*r*-22, lacks another principal surface domain that, instead, is conserved in the members of the PR subfamily (significantly, to a major extent in E*r*-1, E*r*-2, and E*r*-10, and to a minor extent in E*r*-11), and is formed by a cluster of residues positioned in the cleft delimited between the internal faces of helices 1 and 2.

Conclusions

The experimental utilization of *Euplotes* has determined decisive progress in our knowledge of the structure and biology of ciliate pheromones, for long limited only to the "gamones" of *Blepharisma*, i. e., a ciliate to be regarded as rather "eccentric" for a number of aspects (first of all, functional and genetic) of its mating types (granted that intra-specific mating type systems did actually evolve in *Blepharisma* and its relative heterotrichs, all of which appear to systematically pursue intra-specific "selfing" in nature). This progress widens our view on the evolutionary significance of ciliate mating type systems, and permits us to better appreciate that the conceptual and functional complexity of these systems is far from the one that is binary and complementary of sex but, rather, appears close to the multiple and polymorphic one of self/non-self recognition systems evolved in multi-cellular life forms.

The characterization of the three-dimensional conformations of a significant number of pheromones from *E. raikovi*, in which mating types have a clearly established genetic basis, has, in particular, made it possible to appreciate visually the major implication that arises from Mendelian analyses of the ciliate mating type systems, i. e., that ciliate pheromones are cell type-specific markers represented by intra-specific families of *structurally homologous* proteins. This structural pheromone homology strongly argues against a model of pheromone activity based on the interaction, uniquely finalized to a cell mating (sexual) purpose, between a pheromone of one cell type and a cognate receptor of it carried by another cell type (Miyake 1981, 1996). Rather, it suggests that pheromones are to be regarded as signal molecules capable of binding, in competition with one another, to each others' cognate receptors; hence, capable of eliciting a variety of context-dependent cell responses, as is the case of cytokine and growth factor networks in more complex multi-cellular organisms.

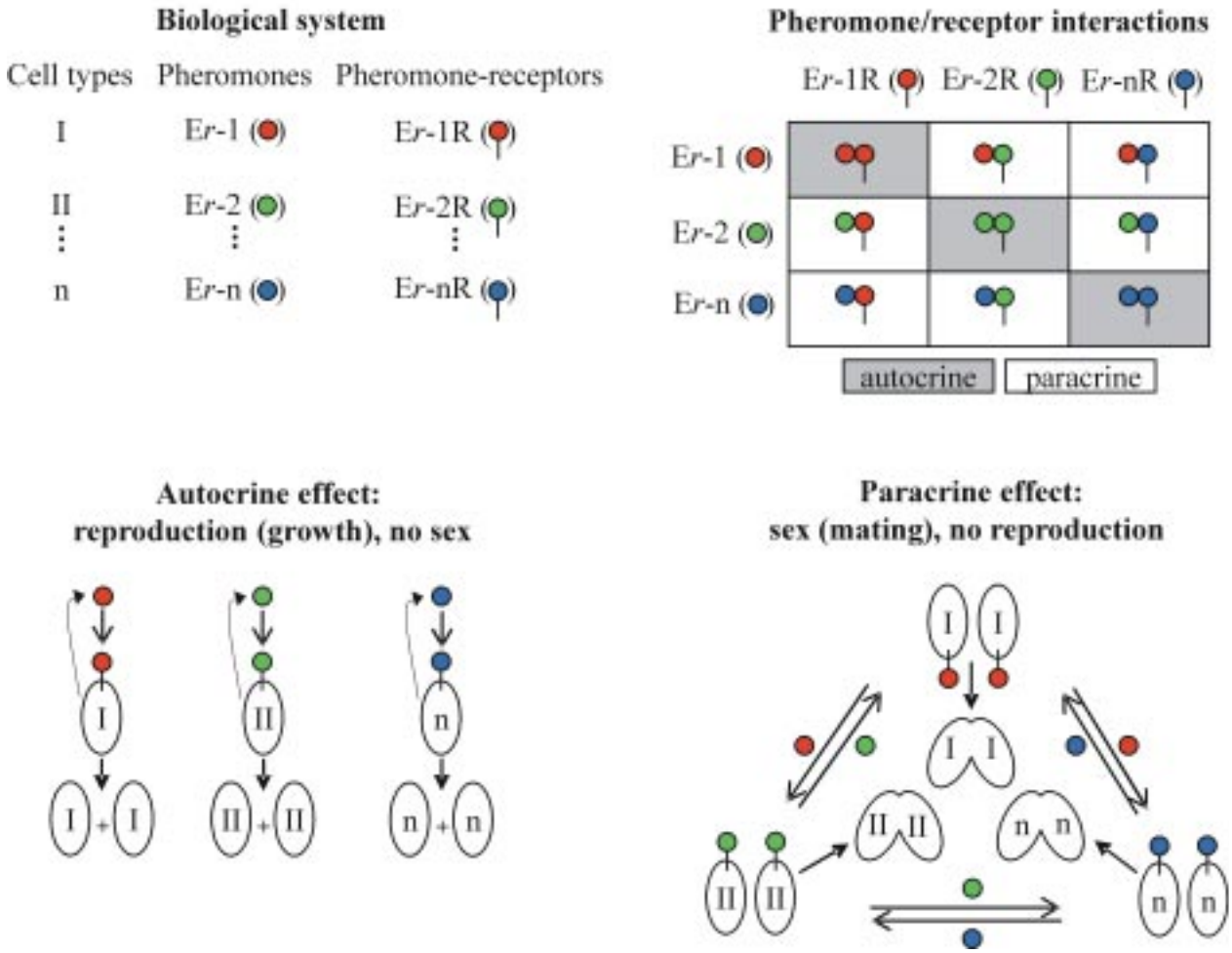


Fig. 5. Summarizing picture of the pheromone biology of *E. raikovi*. The multiple cell (mating) types of the biological system (I, II, … n) are each one represented distinct from the others by its secreted pheromone *and* cognate membrane-bound receptor, whose extracellular domain is structurally equivalent (same color) with the pheromone (both molecules arising from the same gene via alternative splicing). The other panels visualize any possible autocrine (homotypic) and paracrine (heterotypic) pheromome/pheromone-receptor interactions and the relevant effects of these interactions.

In *E. raikovi*, in addition to acting as mating signals (paracrine activity); pheromones have been shown able to promote the vegetative (mitotic) proliferation of the same cells from which they are secreted (Vallesi *et al.* 1995). The pre-requisite to carry out this autocrine mitogenic activity, that we can reasonably regard as being evolutionarily and biologically primary in respect to the paracrine one associated with sex, is that cells secreting a pheromone must be able to synthesize also the receptor for this pheromone. The meeting of *E. raikovi* with this requirement represents, at the same time, an insightful instance of parsimony and elegance, and a unique key to explain how new functional mating types (i. e., cells distinguished by a new pheromone and its cognate receptor) can arise (and also how "old" mating types can disappear) in those mating-type systems which are usually classified, in particular in *Euplotes* and other hypotrichs, as "open" because of the virtually unlimited number of different mating types that they comprise (Ammermann 1982, Valbonesi *et al*. 1992, Dini and Nyberg 1993). By alternatively splicing its primary transcripts, each pheromone gene generates *both* a soluble pheromone (whose release into environment requires proteolytic processing of the cytoplasmic precursor) and its cognate receptor represented by a longer pheromone isoform that utilizes the un-cleaved signal peptide of the cytoplasmic precursor to remain anchored to the cell surface (Miceli *et al.* 1992, Ortenzi *et al.* 2000). The extra-cellular domain of this receptor, in each cell type structurally equivalent to the secreted phero-

mone, functions as ligand (pheromone) binding site (Weiss *et al.* 1995, Ortenzi *et al.* 2000); the structurally unique intra-cellular domain contains sequence motifs that are an object of research interest for enzymatic and transduction activities.

Thus, in conclusion, as diagrammatically summarized in Fig. 5, *Euplotes* (and reasonably other ciliates as well) proves to have very proficiently invested in its pheromone/mating-type systems to control both the basic (quantitative and qualitative) aspects of life, i. e., reproduction and sex. To reproduce (i. e., multiply mitotically), or to mate (i. e., engage in a sexual process with inevitable detriment to reproduction) is an alternative that cells can apparently decide in relation to which type of pheromone-receptor association, i. e., autocrine *versus* paracrine, turns out (in the time and space) to be more effective on their surface. Insightful information on how this association occurs at the molecular level essentially relies on the determination of the E*r*-1 pheromone crystallographic structure (Weiss *et al.* 1995), and functional analyses of E*r*-1 binding to recombinants of the membrane-bound E*r*-1 isoform (receptor) (Ortenzi *et al.* 2000). The challenge for the future is to strengthen this information and, more demanding, shed light on the regulatory molecules and events underlying these varied, i. e., reproductive and sexual, cell responses.

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