Resolving the confounding phenomena of hybridization and facilitated self-fertilization in closely related model earthworm species, *Eisenia andrei* and *E. fetida,* widely used in biomedicine and ecotoxicology

The 'red worms' *Eisenia andrei* (*Ea*) and the 'tiger worm' *E. fetida* (*Ef*) belong to important international workhorses in biomedical sciences due to their sophisticated immune, neural, and circulatory systems, as well as a comprehensive DNA sequencing database. Because the results obtained by sensitive and extremely discriminating genetic tools deployed in such studies can be confounded by the presence of undetected hybrids, the highly complicated breeding systems within and between these species shall be well understood. Composting species belonging to the genus *Eisenia* are mostly cross-fertilizing hermaphrodites, i.e. copulating pairs exchange sperm and each individual stores its partner's sperm in special sacs called spermathecae. After copulation the worms separate and eventually each produces egg capsules ('cocoons') containing its own eggs fertilized by sperm obtained from its partner. Nevertheless, instances of self-fertilization have been reported; in these cases the eggs are fertilized by the own sperm of the cocoon-producing worm. Whether closely related species, *Ea* and *Ef* living alongside each other can mate with each other (i.e. hybridize) is a matter of controversy. Our preliminary findings indicated that *Ea+Ef* pairs produce a lot of mostly sterile cocoons. Crucially, however, a minority of the cocoons produced by the cross-species pairs were fertile and produced hatchlings as consequence of either successful hybridization (yielding first-generation, F1, hybrid offspring designated *EaxEf*) or of self-fertilization (where the genetically 'pure' F1 offspring have the same genetic designation as their single parent) (see Fig. 1). If some of the hybrid worms *EaxEf* are reproductively fertile, they would yield second generation (F2) progeny of mixed provenance, giving both *EaxEf* hybrids and worms of *Ea* and *Ef* species.

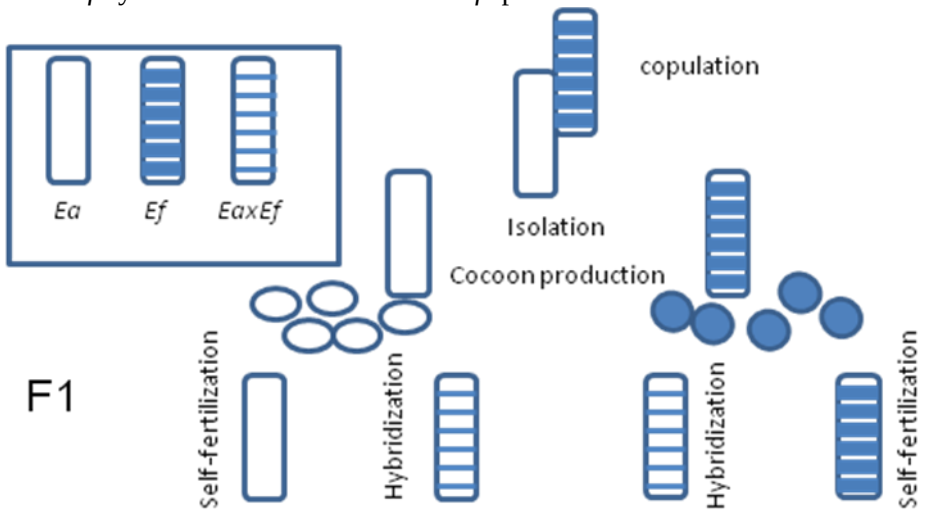

Fig. 1. Scheme of experiments and expected main results

The core aim of the proposed investigation is to discover convenient biomarkers that are able to robustly determine whether individuals in a mixed colony of morphologically almost indistinguishable composting earthworms are genotypically 'pure' progeny of cross-fertilizing parents of the same species, genotypically 'pure' progeny of self-fertilizing single parents of a given species, or cross-species hybrids. A number of minimally invasive approaches to the problem will be adopted: (1) evaluation of pigmentation patterns by photography and image analysis ('red', 'tiger', 'mixed'); (2) spectra of fluorophores in the lymph-like coelomic fluid, especially fluorophore which we refer to as MUG, being a molecular marker of *Ea*; (3) sequencing species-specific haploid DNA of mitochondrial genes (that, alas, cannot discern hybrids); (4) sequencing nuclear gene targets that can reveal whether individual worms are the progeny of parents of the same species (*Ea* or *Ef)* or the hybrid progeny (*EaxEf*) of parents of different species; and (5) the evaluation of nuclear sequence repeats in non-coding DNA ('microsatellites') in order to distinguish the progeny of self-fertilizing uniparental adults. The experimental tools outlined above will be used **to test the hypothesis that incidences of hybridization, 'normal' self-fertilization, or self-fertilization promoted by the presence of a partner from another earthworm species is affected by the origin/age of the parental worms and/or by certain environmental factors such as thermal stress, starvation, soil pollution.**

The sensitive and extremely discriminating genetic tools developed in the project shall be vitally important for proper differentiation of 'pure' species and their hybrids not only among specimens used in scientific laboratories, but also in the field studies on the putative hybrid zones of these species, that might be used as the 'natural laboratories' for monitoring of the ongoing microevolutionary processes.