CO-EXTRA

GM and non-GM supply chains: their CO-EXistence and TRAceability

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WP5 TASK 5.1 Reference systems

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I. Rationale

The aims of this workshop were to open the discussion on the taxon identification issues in function of GMO quantification to specialists from the fields of taxonomy, phylogeny and breeding, to identify the taxonomic/breeding problems for several target crops and to set up an approach how to deal with them. Ideally this workshop should establish a list of crops varieties, related species used or foreseen to be used for introgression, and related taxa not to be detected, to be tested each time a reference systems are to be developed.

II. Conclusions

1. List of terms and definitions

Target taxon: In Rec. 2004/787/EC, the definition is deliberately vague; this implies that different interpretations are possible. The target is either a **species** or other **taxon.**

Taxon:

- should be related to 'ingredient' as legislation does only use 'ingredient' (Reg. 1829/2003/EC, Reg. 1830/2003/EC)
- should be defined for each specific situation (scope per species/ingredient*)
- is the smallest taxonomic/phylogenetic entity* from which the ingredient is obtained.
- can be larger than only one species, depending on definition of reference system
- based on regulation, cultivated species/taxa

**Remark: This entity should be molecularly defined by a reference system; if not achievable at the level of ingredient, then the taxon becomes the smallest entity that is molecularly defined/distinguished by the reference system.*

Species:

- Definition of species depends on the available information (e.g. phylogenetic trees based on morphological or molecular data).
- Therefore the definition of species in function of GMO quantification should be based on molecular data and case-by-case on multiple DNA sequences.

Reference system

- Is an assay (protocol) to quantify the amount of taxon, including target taxon (reference sequence) specific PCR method (primers/probe and reaction conditions)

- within reference system **taxon-specific reference system** and **species-specific reference system** can be defined

Endogenous reference gene/sequence:

- currently mainly genes (coding sequences) are used for design of reference systems

- to solve the specificity problems other alternative genome sequences (e.g. retrotransposons, intergenic elements) could be used to discriminate better between taxa.

Ingredient:

- the term ingredient needs to be clarified in relation to target taxon. Does ingredient cover more than one or exactly one phylogenetic taxon? Does ingredient cover more than one species?

- Legislation terms

    - Related to GMO quantification, 1829/2003/EC mentions: 'ingredients as meant in Article 6 (4) Dir. 2000/13/EC…' plus see official list of ingredients referred to

    - Dir. 2000/13/EC, Article 6:

Article 4. (a) 'Ingredient' shall mean any substance, including additives, used in the manufacture or preparation of a foodstuff and still present in the finished product, even if in altered form.
(b) Where an ingredient of the foodstuff is itself the product of several ingredients, the latter shall be regarded as ingredients of the foodstuff in question. (c) The following shall not be regarded as ingredients: (i) the constituents of an ingredient which have been temporarily separated during the manufacturing process and later reintroduced but not in excess of their original proportions;
(ii) additives:
— whose presence in a given foodstuff is solely due to the fact that they were contained in one or more ingredients of that foodstuff, provided that they serve no technological function in the finished product,
— which are used as processing aids;

(iii) substances used in the quantities strictly necessary as solvents or media for additives or flavouring.
(d) In certain cases Decisions may be taken in accordance with the procedure laid down in Article 20(2) as to whether the conditions described in point (c)(ii) and (iii) are satisfied.

- The scope per species/ingredient has to be clarified (e.g. material defined by its origin (soybean, OSR, …) and/or state of processing of one taxon e.g. soybeans versus pellets/oil/rest material/side products which can be added in the product).

- Necessary to clarify if 'ingredient' include impurities

2. List of crops and varieties

- Specific characteristics to be addressed per crop (through definition by Latin name) include the phylogenetic tree, diversity, interspecific hybrids, F1 hybrid formation, mixture problems.

- List of crops to be addressed at first instance

    - Soybean (*Glycine max* L.*)*
    - Maize (*Zea mays* L.)
    - Oilseed rape (*Brassica napus/Brassica rapa* L.):
    - Rice (*Oryza sativa* L.)
    - Tomato (*Lycopersicon esculentum* L.)
    - Potato (*Solanum tuberosum* L.)
    - Sugar beet, fodder beet (*Beta vulgaris sbsp. vulgaris* L.)
    - Cotton (*Gossypium hirsutum* L.)
    - Eggplant (*Solanum melongena* L.)

3. Reference systems requirements

Reference systems should:

- be specific for taxon of interest

- give consistent assay quantification within taxon (i.e. low variability in quantification among varieties within taxon as opposed to low variability in DNA

sequence). Acceptance level for variability in quantification is now proposed as 1 Ct variability in amplification response between varieties. The whole real time PCR kinetics must be considered as well. It should be expressed as a % variability of the quantification over all reference varieties within the dynamic range of the method.

How many and which varieties to be tested: to be decided.

- be preferably nuclear sequence
- demonstrate low and known copy number or similar copy number to genomic part of GM sequence insert, preferably single copy
- be homozygous

The acceptance levels must be further discussed

4. Specificity/stability testing

- Theoretical and experimental core collections of taxa and varieties to be used for specificity and stability testing have to be established
- The core collections should cover the global genetic variation within marketed products/ingredients (national/public collections; commercial breeding programs of companies; plant variety testing collections)
- Technical aspects for specificity/stability testing that are to be established and formalized:
    - independent validated DNA quantification (concentration measurement) method
    - acceptance criteria/performance requirements for quality of DNA (suitable for real-time PCR): compare PCR efficiency ref system vs. GM system (delta Ct); inhibition; linear range
    - amount of DNA to be added in PCR tests: highest number of copies that does not trigger inhibition to be used in specificity testing
    - type of DNA in PCR specificity tests: pure materials versus mixtures of potentially cross-reacting materials
    - cross-amplification versus aspecific amplification
    - primer specificity: check by melting amplicon (PCR reactions) and/or gel visualisation and/or sequencing

  - consistent/stable amplification in real-time PCR (no absence of amplification)
  - Real-time PCR characteristics/performance

- Technical requirements/criteria for specificity/stability testing to be officially established in collaboration with EuropaBio/ENGL Validation WG/ CRL

5. Executive plan for setting up core collections

Establishment of Co-Extra-funded task force

- Establish a "light" **consortium** or "**task force**" about a **core collection** of varieties and related taxa (to be or not to be detected) for several cultivated taxa for which reference genes are still missing: e.g. ***Triticeae*** (maize, rice), ***Fabaceae*** (soybean), ***Solanaceae*** (potato, tomato, egg-plant), ***Amaranthaceae*** (sugar-beet, fodder beet), ***Brassicaceae*** (rapeseed), ***Malvaceae*** (cotton).

- Establishing a consortium on reference genes by signing a confidentiality agreement between Co-Extra coordinator and WP5 leader and EuropaBio and ISTA members.

- Reallocating a part of **Co-Extra budget** to WP5 for establishing such a core collection.

- Establish a **synthesis** of the available data on reference genes, including all sequences (e.g. Chinese database) phylogenic and taxonomic data, available to all members, all partners involved.

- with **strategies** for establishing new reference sequences (starting from a known sequence such as gene, regulating element or transposons towards defining new sequences by e.g. SCAR or genomic subtraction).

- Growth of seeds and **distribution** of leaves or flour.

- Co-Extra coordinator or WP5 leader receiving **information** from companies and laboratories about the reasons (phylogeny, taxonomy, introgression programs, taxa not to be detected) of using such varieties or related taxa for **avoiding duplicates**.

- Coordinator or WP5 leader of Co-Extra receiving either **seeds**, and conditions of growth in greenhouses for preparing **leaves**, or **flours**.

- **Anonymizing** the varieties and related taxa.

- Distributing the leaves or flours to members of this consortium. Exchanging information on issues and bottlenecks (INRA circulation list) and new techniques to be used such as SCAR or genomic subtraction. Organization of a second meeting.

- Before the end of Co-Extra, collection will be taken in charge by JRC.

- Continuity of the work, of the core collection and diffusion of results through databases.